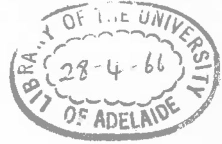

STUDIES OF THE CONTROL OF THYROID FUNCTION AS DISCLOSED BY THE EFFECT OF SALICYLATE

by

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SUMMARY

An examination has been made of the mechanism by which salicylate and related drugs depress thyroid function. Salicylate and 2,4-dinitrophenol produced a depression in plasma PBI in normal rats, confirming previous reports. Sodium γ-resorcylate in sufficient dosage also significantly depressed the plasma PBI of normal rats. Sodium p-hydroxybenzoate was without effect. Similar findings were obtained in thyroidectomized rats maintained on thyroxine, indicating a peripheral action of the drugs in depressing plasma PBI.

Bioassay of TSH in the plasma of normal rats revealed that salicylate, 2,4-dinitrophenol and also γ-resorcylate significantly depressed circulating TSH; p-hydroxybenzoate was without effect. Previous indirect evidence of a depression in TSH release produced by salicylate, 2,4-dinitrophenol and γ-resorcylate was therefore confirmed.

This finding of simultaneous depression in circulating thyroid hormone and TSH is contrary to the concept of the negative feedback regulation of the thyroid-pituitary axis. A depression in circulating thyroid hormone would be expected to stimulate pituitary TSH release. It had been postulated previously that the depression in TSH induced by salicylate and 2,4-dinitrophenol was related to their metabolic

stimulating properties, by an action at the hypothalamic sites controlling pituitary TSH release. However, since γ-resorcylate does not increase metabolic rate, this proposed mechanism for the depression of TSH is excluded.

Using a dialysis procedure, it was shown that the _in vitro_ addition of salicylate and γ-resorcylate to human or rat serum increased the rate of dialysis of radiothyroxine with which the serum was equilibrated; p-hydroxybenzoate produced a smaller effect. An increased rate of dialysis of radiothyroxine is consistent with an increase in free thyroxine. Circulating free thyroxine was elevated two hours after the administration of salicylate and γ-resorcylate to man, whereas p-hydroxybenzoate was ineffective. These _in vivo_ findings were confirmed following more prolonged administration of the drugs to rats; 2,4-dinitrophenol also increased circulating free thyroxine in rats.

Using paper electrophoretic separation of human serum proteins, it was demonstrated _in vitro_ and _in vivo_ that salicylate and γ-resorcylate displaced thyroxine from thyroxine binding prealbumin (TBPA). Although a displacement of thyroxine from TBPA was induced by p-hydroxybenzoate _in vitro_, this drug was ineffective _in vivo_. The separation of rat serum proteins was carried out by starch gel electrophoresis. The addition, _in vitro_, of salicylate and γ-resorcylate to the electrophoretic buffer produced a

large displacement of thyroxine principally from a fast-moving albumin binding site. There was a small displacement of thyroxine by p-hydroxybenzoate *in vitro*. *In vivo*, salicylate and 2,4-dinitrophenol produced a displacement of thyroxine whereas p-hydroxybenzoate and γ-resorcylate were ineffective. The increase in free thyroxine produced by salicylate and related drugs therefore resulted from the displacement of thyroxine from specific binding sites in the serum.

The peripheral action of salicylate and related drugs in depressing plasma PBI is compatible with the displacement of thyroxine into the free state, followed by its disappearance from the circulation.

The depression in TSH release induced by these drugs is also correlated with the increase in circulating free thyroxine. It is concluded that the level of circulating free thyroxine serves as the regulator of the negative feedback system controlling thyroid-pituitary interrelations.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, this thesis does not contain any material previously published or written by another person except when due reference is made to such material in the text.

November, 1964.

PREFACE

This project was undertaken in the Department of Medicine, University of Adelaide, with the aid of a full-time grant from the National Health and Medical Research Council of Australia. The work was carried out under the supervision of Professor B.S. Hetzel, Department of Medicine, to whom the author is indebted for much advice and discussion. The author is also grateful to Mr. N.S. Stenhouse, Division of Mathematical Statistics, C.S.I.R.O. for assistance with the development of the experimental design used for the bioassay of thyroid stimulating hormone and the statistical analyses, and to Miss B.M.Hogg, Department of Medicine, for the preparation of the figures.

CHAPTER I

THE CONTROL OF THE THYROID SECRETION

PART I The Thyroid-Pituitary Interrelationship.

1. The concept of the feedback mechanism.
2. The relation of the thyroid and pituitary to the central nervous system.
3. The integration of feedback control and hypothalamic control of the thyroid-pituitary axis.

PART II Pharmacological Studies of the Control of Thyroid Function.

1. The effect of 2,4-dinitrophenol on thyroid function.
2. The effect of salicylate on thyroid function.

CHAPTER I

THE CONTROL OF THE THYROID SECRETION

INTRODUCTION

This thesis presents studies on the physiological control of the thyroid secretion derived from an examination of the effect of salicylate and related drugs on thyroid function. A historical review of the development of knowledge relating to the control of the thyroid secretion is presented in Part I of this chapter.

A pharmacological approach to the study of the control of the thyroid secretion was made possible by the use of 2,4-dinitrophenol and salicylate. These drugs increase metabolic rate and oxygen consumption in animals, effects which they share with the thyroid hormones. As it does not possess the toxic side effects of 2,4-dinitrophenol, studies of the effect of salicylate on thyroid function were carried out in man as well as in rats. Previous studies of the effects of 2,4-dinitrophenol and salicylate on thyroid function have been reviewed in Part II of this chapter as a preliminary to the experimental studies which form the basis of this thesis.

PART I

THE THYROID-PITUITARY INTERRELATIONSHIP

1. The Concept of the Feedback Mechanism

The possibility of a functional relationship existing between the pituitary and the thyroid was suggested in 1851 by Nièpce who described enlargement of the pituitary in a number of goitrous cretins. Towards the end of the century Stieda (1890) showed experimentally that thyroidectomy caused extensive hypertrophy of the anterior lobe of the pituitary.

However, the first evidence of the regulatory influence of the anterior hypophysis on the structure and function of the thyroid gland was obtained from studies on amphibian metamorphosis. In 1912 Gudernatsch demonstrated that metamorphosis could be induced by the feeding of thyroid tissue. Adler (1914) demonstrated that ablation of the hypophysis of tadpoles caused thyroid atrophy with subsequent failure of metamorphosis. These results stimulated further research, and by 1921 it had been established that the growth and secretion of the amphibian thyroid were influenced by some hormonal factor released from the anterior pituitary (Allen, 1921; Swingle, 1921).

Further elucidation of the pituitary - thyroid relationship followed from the classical studies of Philip Smith. The injection of bovine anterior pituitary extract into hypophysectomized tadpoles led to the restoration of normal function and secretion of the atrophic thyroid glands

indicating that the action of the thyroid stimulating principle was not species-specific (Smith and Smith, 1922, 1923). Smith subsequently demonstrated for the first time in the mammal that atrophy of the thyroid followed hypophysectomy and that restoration of function followed replacement therapy with pituitary extracts (Smith, 1927). Aron (1929, 1930) showed the activating property of extracts of beef pituitary on the guinea pig thyroid.

The thyroid stimulating principle was referred to by various terms such as the "metamorphic principle", the "thyreotropic hormone" or the "thyreoactivator". These terms have been supplanted by the more recent thyrotropin, thyrotrophin, and thyroid stimulating hormone (TSH).

The results of further studies made it apparent that the role of the thyroid gland was not a passive one. It was demonstrated in several species that administration of excess thyroid material to the intact animal led to involution of the thyroid gland which was associated with diminished TSH content in the anterior lobe of the pituitary (Sturm, 1930; Holweg and Junkmann, 1933). Furthermore, the studies of Aron (1930), Loeb, (1932) and Loeser (1934) revealed that activation of the thyroid with exogenous TSH could be partially inhibited by the simultaneous administration of thyroid hormone.

These findings demonstrated the opposing effects of the hormones in the circulation and led to the concept first formulated by Aron in 1931 and upheld by Salter in 1940, who stated, that between the pituitary and the thyroid "there

exists a delicate state of reciprocal stimulation and inhibition which usually results in very delicate balance or homeostasis".

This concept of reciprocity of function between the thyroid and the pituitary was restated by Hoskins (1949) in terms of a servo or feedback mechanism, a principle commonly used in engineering for automatically controlled devices. A servo system is defined as one in which variations in the quantity of output of an apparatus are minimized by "feeding back" a proportion of the output for the control of the system.

Hoskins stated "The pituitary thyroid axis affords an example par excellence of a physiologic servo mechanism. When the titer of circulating thyroxine rises, the anterior pituitary is selectively inhibited and the discharge of thyrotropin is thereby decreased. Contrariwise, episodic or persistent thyroxine deficiency, if sufficient in degree, results in augmented thyrotropin production with resulting tendency for the production of more thyroid hormone."

The availability of radioactive iodine permitted more precise investigations of the thyroid-pituitary interrelationship to be made. Techniques were developed for the estimation of rates of formation and release of thyroid hormone as a reflection of thyrotrophic hormone regulation (Morton et al, 1942).

Application of these techniques has led to greater clarification of the feedback mechanism controlling the pituitary-thyroid interrelationship. Brown Grant et al (1954)

utilized the release rate of I^{131} labelled thyroid hormone from the thyroid of the conscious rabbit as an index of thyroid function. A single injection of a large dose of l-thyroxine resulted in complete inhibition of the release of radioiodine from the thyroid within 2 hours, persisting for 100 hours. After allowing time for the absorption of thyroxine following injection it was found that the pituitary reaction to a raised blood level of thyroxine was far more rapid than had been previously suspected. The injection of TSH into rabbits produced an increase in the release of labelled thyroid hormone after a latent period of about one hour.

Refinements in the assay of TSH were reported which improved the degree of sensitivity and precision enabling quantitative estimations of the circulating hormone to be made (D'Angelo & Gordon, 1950; Gilliland & Strudwick, 1956). An inadequate output of thyroid hormone has been shown to stimulate the secretion of TSH. The TSH content of the blood is raised to a level which is detectable by many assay methods. Quantitative measurements indicated elevated levels of TSH in cretinism, adult myxoedema and in hypothyroidism induced by surgical thyroidectomy.

Although the thyroid activity is reduced following hypophysectomy, the absolute amounts of iodine and thyroxine in the gland remain within normal limits for up to one year, indicating that the low circulating levels of thyroid hormone per se cannot be the principal stimulus to release of hormone from the thyroid gland (Chaikoff and Taurog, 1949).

However, the concept of the feedback mechanism regulating the thyroid-pituitary axis is probably an over simplification of the events which occur. Although TSH is the major regulator of thyroid function, the thyroid still has residual activity in the absence of the pituitary. Iodide trapping is reduced to less than 25 per cent of normal (Vander Laan & Greer, 1950) and hormone release is slowed following hypophysectomy in rats (Wolff, 1951). Organic binding is also diminished but thyroxine formation does occur (Randall and Albert, 1951).

Reference has been made to experiments in which TSH and thyroid hormone were administered simultaneously to the normal rat resulting in a degree of activation of the thyroid less than that found with the same dose of TSH alone (Aron, 1930; Loeb, 1932). Similar findings were obtained using hypophysectomized rats (Cortell and Rawson, 1944) indicating an action of thyroid hormone on TSH not mediated at the hypophyseal level.

The disappearance rate of circulating TSH was shown to be slowed in hypophysectomized and thyroidectomized rats (Loeser, 1934; D'Angelo, 1951). In studies on hypophysectomized rats with metabolic rate elevated by thyroxine, triiodothyronine or 2,4-dinitrophenol, D'Angelo (1955) observed that the disappearance of exogenous TSH was increased by thyroxine and triiodothyronine, but much less so with 2,4-dinitrophenol. Thus a specific type of metabolic alteration induced by the thyroid hormones appears to be implicated in this response.

Nevertheless, the ability to respond to changes in the

level of circulating thyroid hormone appears to be an intrinsic property of the anterior pituitary gland and many of the observed facts can be explained on the basis of a simple negative feedback mechanism.

However, certain stimuli such as cold exposure and emotional and physical stresses exert effects on thyroid activity which cannot be explained in terms of the negative feedback mechanism.

The Effect of Cold

A lowered environmental temperature has been shown to increase thyroid activity. An increased rate of peripheral utilization of thyroid hormone in cold exposed rats was established by Dempsey and Astwood (1943). In 1952, Bondy and Hagewood showed that a significant fall in the circulating thyroid hormone - measured as the serum protein bound iodine (PBI) - occurred within 15 hours of cold exposure in intact rats, and that an even greater fall was observed in thyroidectomized rats maintained on a constant level of thyroxine. In 1952 Rand and co-workers demonstrated that the increased rate of utilization of hormone in cold exposed rats occurred concurrently with a decrease in the PBI at a time when thyroid activation was increased. It was concluded that the increased peripheral utilization of thyroid hormone resulted in a lowered blood level which might act, via the feedback mechanism, to produce increased thyroid activity in the cold.

However, more recent studies of the time relationship of these responses have thrown new light on the reaction to cold.

Del Conte and Stux (1954) described histological changes in the thyroid gland of guinea pigs indicative of increased activity within half an hour of exposure to cold, identical to the response to intravenous injection of TSH. An increased rate of release of I^{131}-labelled hormone occurred after a latent period of four hours of cold exposure in the rabbit (Brown Grant et al, 1954).

If the thyroidal response is secondary to an increased secretion of TSH, then the latent period represents the time taken for the cold stimulus to cause an increased concentration of TSH in the blood plus the time for the thyroid gland to respond to the increased TSH concentration. Using an in vitro assay for TSH, Bottari (1957) found that the TSH levels in the blood of rabbits were increased within thirty minutes of exposure to cold and reached a maximum at three hours. Administration of a large dose of thyroxine did not prevent the increase in TSH or affect the latent period or the speed of reaction. The secretion of I^{131}-labelled hormone from the thyroid of the unanaesthetised rabbit was shown to have a latent period of one-half to two hours following the administration of purified bovine TSH or crude extract of rabbit anterior pituitary (Reichlin and Reid, 1955).

These findings indicated that the short time interval of response to cold stimulus is of the same order as that of direct stimulation of the thyroid gland by TSH. Therefore, mediation by a mechanism such as a rapid nervous reflex must be causative rather than by the feedback system in response

to the fall in the concentration of thyroid hormone in the circulation.

Anatomical connection of the pituitary to the central nervous system has been shown to be necessary for the response to cold stimulus. Von Euler and Holmgren (1956a) demonstrated that the usual increase in the rate of release of thyroidal I^{131} did not occur in rabbits in which the anterior pituitary had been grafted to the eye. This finding was confirmed in similar studies using hamsters with pituitary grafts in the cheek pouch (Knigge and Bierman, 1958). Thus the acute response of the thyroid to cold is dependent upon the presence of a pituitary in normal relationship to the hypothalamus.

The Effect of Stress

Stressful stimuli cause a reduced uptake of I^{131} by the thyroid gland. This effect has been produced in the rat by injection of formalin (Paschkis et al, 1950) by anoxia, starvation, or nephrectomy (Van Middlesworth & Berry, 1951) and tourniquet shock (Hamolsky et al, 1951). In the rabbit physical or systemic stresses such as laparotomy, haemorrhage and intraperitoneal injection of turpentine produced inhibition of thyroid activity within three hours which persisted up to 48 hours. Emotional or nervous stresses such as electric shocks, restraint and sudden changes in environmental lighting produced similar results (Brown Grant et al, 1954). These findings were confirmed by Soderberg (1958) and Campbell and co-workers (1959).

Since the biological half-life of TSH is from 1 to 4 hours

in the rabbit, the inhibitory effect of stress on the secretion of TSH by the anterior pituitary must occur more rapidly than can be accounted for by a purely hormonal reaction. Brown Grant (1957) observed that during the period of thyroid gland inhibition produced by the stress of 48 hour immobilization or laparotomy, the amount of radioactive organically bound iodine in the plasma fell. Therefore, it was not possible that the inhibition produced by these stimuli was mediated by the feed-back system, because an increase in circulating thyroid hormone would be required.

However, observations made only on the thyroidal response to stress may be misleading because it has been shown that such stimuli lead to adrenal cortical activation (Bogoroch and Timiras, 1951; Ingbar, 1953). The thyroid response to stress was considered by Harris (1955) to be the result of a decrease in TSH secretion coincident with the increased secretion of adrenocorticotrophic hormone (ACTH) and probably consequent upon it. However, the thyroidal response was not due to increased adrenal steroid secretion per se, since it occurred in adrenalectomized animals.

The normal anatomical relationship between the hypothalamus and pituitary is required for the thyroidal and adrenal responses to occur in some stress situations. Fortier (1951) showed that the adrenal response to some stressful stimuli was abolished by transplantation of the pituitary and suggested that two types of stress may occur; neurotropic stresses, the response to which is dependent on the normal hypothalamic-pituitary

connections, and systemic stresses which may act on the transplanted pituitary. Thus in rabbits whose pituitary-hypothalamic connections had been cut, it was found that the adrenal and thyroidal responses to surgical trauma were not affected, whereas the responses of these glands to the emotional stress of enforced immobilization were abolished.

Conclusions

Although the thyroid gland maintains a residual activity in the absence of the pituitary, thyroid function is regulated principally by thyroid stimulating hormone. The circulating thyroid hormone level in turn profoundly influences the pituitary release of TSH. The interaction of reciprocal stimulation and inhibition between thyroid and anterior pituitary forming a servo mechanism which regulates thyroid activity does not preclude other mechanisms which may modify this regulation. For example, excess thyroid hormone induces metabolic changes which increase the disappearance of TSH from the circulation so that thyroid stimulation is probably diminished.

However, certain conditions such as cold exposure and emotional stress exert effects on thyroidal activity which are not mediated by the servo mechanism, and may be accounted for by direct nervous stimulation acting on the pituitary which is in anatomical connection to the nervous system. The relationship of the thyroid-pituitary axis to the central nervous system will therefore be discussed in the next section.

2. The Relation of the Thyroid and Pituitary to The Central Nervous System

a. Anatomical Considerations

The anatomical connections of the pituitary to the hypothalamus have been the subject of intensive study for many years and the understanding of these interrelations have been greatly clarified by G.W. Harris and his co-workers (Harris, 1955).

The pituitary gland is subdivided into three parts. The anterior lobe or pars distalis is a glandular portion derived embryologically from the anterior wall of Rathke's pouch. The posterior lobe comprises both the pars intermedia (derived from the posterior wall of Rathke's pouch), and the neural lobe which is a direct downgrowth of neural tissue (the infundibular process). The third portion, the hypophysial stalk consists of the infundibular stem covered by a sheath of the pars tuberalis. The pituitary is connected to the hypothalamus by the hypophysial stalk.

The median eminence forms the central portion of the base of the hypothalamus and is co-extensive with the pars tuberalis, an upward extension which envelops the median eminence and neural stalk as a highly vascular mantle.

The findings of Green (1951) and Harris (1955) indicate that innervation of the anterior lobe plays no significant role in the control of the secretion of thyrotrophic hormone.

The Blood Supply to the Anterior Lobe of the Pituitary.

Harris (1955) stated that the blood supply of the anterior pituitary may be compared in a general way with that of the liver. Both organs have a systemic arterial supply, a portal blood supply and a systemic venous drainage. The vascular supply of the anterior lobe is separate from that of the neural lobe; in sections this is clearly seen, since these two vascular regions are separated by the relatively avascular pars intermedia.

The systemic arterial supply of the anterior lobe consists of one or more arteries derived from the internal carotid artery or the posterior communicating artery. The pattern of these arterial branches varies from species to species. In some, the vessels are absent and the whole blood supply to the anterior lobe is derived from the hypophysial portal system (Wislocki, 1938; Harris, 1947; McConnell, 1953).

The venous drainage of the anterior lobe is by means of short wide veins draining into the venous sinuses of the gland or inferiorly in the sphenoid bone (Harris, 1955).

The hypophysial portal system was first described by Popa and Fielding (1930, 1933) in studies of the human pituitary. Green and Harris (1947) reinvestigated this region and showed that in mammals arterial twigs from the systemic arteries supply a vascular plexus in the pars tuberalis. From this plexus arise capillary loops which penetrate into the median eminence of the hypothalamus where they make intimate contact with the nerve fibres of various nerve tracts, but are removed

from any compact group of nerve cells such as the supra-optic nucleus. These capillary loops were designated the primary plexus of the portal system. Blood from the primary plexus drains down large portal trunks which lie on the surface of the pituitary stalk. The portal trunks distribute their blood into the sinusoids of the anterior lobe.

Although earlier a controversial matter, the direction of the blood flow has been settled beyond doubt by direct microscopic observation of the flow of blood from the primary plexus to the anterior lobe (Green and Harris, 1949).

b. Effect of Hypothalamic Lesions on Thyroid Function

The earlier studies of the effect of hypothalamic lesions on thyroid function relied on histological and metabolic measurements for assessing thyroid activity. Lesions in the infundibular region produced histological signs of both increased and decreased thyroid activity (Cahane & Cahane, 1938). It was postulated from this study that there were two centres in the hypothalamus, one exciting TSH secretion and the other inhibiting secretion of the hormone.

Greer (1951, 1952) studied the effect of diencephalic lesions on thyroid function in rats using direct measurement of thyroidal uptake of radioiodine (T/S ratio), thyroid weight and histological responses. The effect of chronic treatment with propylthiouracil (PTU) on these animals was determined. It was found that animals with hypothalamic lesions responded to PTU with slight atrophy of the thyroid instead of hypertrophy, and that microscopically the glands appeared inert.

However, a rise in T/S ratio occurred. These findings were confirmed by Bogdanove and Halmi (1953).

Reichlin (1957) stimulated increased secretion of TSH in rats by partial thyroidectomy. After removal of three-quarters of the thyroid from normal rats the remnant underwent hypertrophy and showed an increase in T/S ratio. In animals with effective lesions, complete or partial loss of compensatory hypertrophy after thyroidectomy occurred, although the T/S ratio was elevated. These results indicated that thyroid size and iodide trapping ability may vary independently. Findings of thyroid inactivation following hypothalamic lesions has also been observed by Ganong and co-workers (1954, 1955) and Greer (1957). Greer (1957) also noted that lesions which interfered with thyroidal iodine metabolism, appeared to be posterior to those which inhibited the thyroid growth response.

That the changes in function of the thyroid gland observed after hypothalamic lesions are the result of disturbance of TSH secretion has been directly confirmed by bioassay of TSH. D'Angelo and Traum (1956) found that anterior hypothalamic lesions resulted in a diminished concentration of TSH in the blood. However, assay of pituitary TSH content from animals with lesions showed that PTU treatment did stimulate some release of TSH from these animals.

c. The Effect of Hypothalamic Stimulation on Thyroid Function

Early attempts to show changes in thyroid function by electrical stimulation were equivocal owing to the limitations

of the metabolic rate methods used to assess changes in thyroid function. Using histological methods Colfer (1949) and Del Conte, Ravello and Stux (1955) claimed that thyroidal activation occurred following electrical stimulation of the hypothalamus or following diffuse electric shocks applied through the cranium of small laboratory animals. No specific site in the hypothalamus was found but stimulation of the thalamus or corpus callosum proved to be ineffective.

Refinements of technique were made by Harris and Woods (1958). In experiments on rabbits, a wire coil connected to electrodes in the hypothalamus was implanted beneath the skin. Stimulation of conscious unrestrained animals was effected by induction of an electric current from a large primary coil surrounding the animal's cage. Increase in the rate of release of thyroidal I^{131} was produced in association with a rise in the plasma level of labelled hormone, when the electrodes were placed in the supra-optico-hypophysial tract of the anterior hypothalamus.

When the electrode tip was placed in a posterior position in the hypothalamus, in the region where stimulation produces ACTH release, negative results or even inhibition of I^{131} release were obtained. After adrenalectomy more than one third of the animals, in which the electrodes were shown to be in the posterior position, showed a positive thyroid response to stimulation.

An overlap of areas in which stimulation caused an increase in TSH and ACTH secretion was postulated, with the

ACTH response suppressing the thyroid response in animals with intact adrenal glands. This was confirmed by Harris in 1959. An increase in thyroidal activity was observed within 30 minutes following stimulation indicating that the response to hypothalamic stimulation is very rapid and of the same order as the intravenous injection of TSH.

d. <u>The Effect of Separation of the Pituitary from the Hypothalamus</u>.

This procedure may be carried out by section of the pituitary stalk with precautions to prevent the regeneration of the portal vessels, or by transplanting the pituitary to a site in the body remote from the median eminence such as the anterior chamber of the eye or the capsule of the kidney. After such operations the activity of the thyroid gland is reduced, but not to the level seen following hypophysectomy. This has been observed after section of the pituitary stalk of the rabbit, rat and ferret, and after transplantation of the pituitary in the rat, rabbit, mouse, guinea pig and hamster (Brown-Grant et al, 1957; Knigge and Bierman, 1958).

The possibility that the changes observed might be due to non-specific damage resulting from ischaemia was shown not to be the case by Harris and Jacobsohn (1952). When pituitary tissue was transplanted beneath the median eminence it maintained normal thyroid weight and histology, whereas equally well vascularized transplants beneath the temporal lobe of the brain failed to do so.

More recently, Nikitovitch-Winer and Everett (1958) have shown that when the pituitary of the rat was transplanted to the kidney capsule, the expected low ACTH and TSH activity was found, but on retransplantation to the temporal lobe or beneath the median eminence, only those in the latter position showed restoration of normal thyroid and adrenal function when revascularised by the hypophysial portal vessels. Thus the source of the blood supply appears to be the critical factor in conditioning pituitary function.

Thyroid activity is therefore reduced when the normal vascular relationship of the anterior pituitary to the hypothalamus is interrupted, although not to the level seen following hypophysectomy, indicating that the autonomous pituitary retains a limited capacity for TSH secretion.

e. <u>Evidence for Neurohumoral control of TSH Secretion</u>

The means by which the nervous system exerts its control over the anterior pituitary gland must be considered. From the above indications it is apparent that the hypophysial portal system has a specific effect in activating the anterior lobe. Harris (1955) has reviewed the evidence relating to a portal vessel effect or a direct nervous effect. He concluded that there was no evidence to support a direct nervous regulation of the anterior pituitary gland. He stated "The results are best explained on the view that nerve fibres of the hypothalamus liberate some hormonal substance into the primary plexus of the hypophysial portal vessels and that these vessels transmit the substance to the adenohypophysis

where it exerts an activating effect on the gland cells".

Evidence supporting the concept of the neurohumoral control of the hypothalamus over the pituitary was obtained by Guillemin and Rosenberg in 1955. It was demonstrated that hormone synthesis declined when pituitary tissue was grown in tissue culture. This failure could be attributed to inadequacy of the nutrients in the synthetic media, however, it was shown that addition of extracts of the hypothalamus to such tissue cultures enabled the explants to sustain ACTH synthesis and release into the medium whereas similar extracts from other parts of the brain lacked this effect.

Identification of a thyrotrophin releasing factor (TRF) in dog hypothalamic extracts has been claimed by Shibusawa et al, (1959) but this could not be confirmed (Reichlin et al, 1963). The most convincing evidence for a TRF has been obtained by Guillemin and co-workers who have separated hypothalamic extracts by Sephadex gel filtration and isolated a component which activated the thyroid gland of normal but not hypophysectomized rats (Guillemin et al, 1962). Enhanced TSH release from rabbit pituitary tissue has been shown when incubated in the presence of rabbit hypothalamus (Odell, 1963).

A substantial body of evidence has accumulated in support of the concept of the neurohumoral control by the hypothalamus of the anterior pituitary, but there is as yet little knowledge of the manner in which these neurohumors exert their effects.

3. The Integration of Feedback Control and Hypothalamic Control of the Pituitary-Thyroid Axis.

Thyroid activity is reduced when the normal vascular connection of the anterior pituitary to the hypothalamus is interrupted, although not to the level seen following hypophysectomy, indicating that the autonomous pituitary retains a limited capacity for TSH secretion.

However, although thyroid activity is reduced under these circumstances the pituitary is still responsive to changes in the concentration of thyroid hormone in the blood. Khazin and Reichlin (1961) in experiments using hypophysectomized rats bearing intraocular pituitary transplants, showed that hemithyroidectomy was followed by increased TSH release indicated by increased release and uptake of I^{131} and thyroid enlargement. Thyroxine injection led to thyroid inhibition in both pituitary graft bearing hypophysectomized and normal animals but not in ungrafted hypophysectomized animals. Similar results were obtained in rats with hypothalamic lesions showing reduced thyroid activity (Averill et al, 1961). Local injection of thyroxine into the pituitary in quantities ineffective when injected systemically caused almost immediate reduction in TSH secretion rate, showing that the pituitary is itself sensitive to small local increases in thyroxine concentration. (Von Euler and Holmgren 1956; Yamada and Greer, 1959).

Therefore anatomical connection of the pituitary to the hypothalamus is not necessary for the usual feedback response

between pituitary and thyroid gland. These studies indicate that the rate of TSH secretion is directly regulated by the circulating thyroxine over a wide range of thyroid activity, whether normal or lowered due to pituitary transplantation.

Thus the pituitary, whether autonomous or intact, possesses intrinsic capacity to alter the TSH secretion rate in response to changes in blood thyroxine concentration. However, it is apparent that the "setting" of thyroid activity is quite different in these situations. In the normal state the hypothalamus appears to maintain a stimulating effect on the pituitary to maintain "normal" base line pituitary-thyroid function, upon which the response to elevated or depressed levels of thyroid hormone is superimposed.

However, the normal feedback control of thyrotrophic hormone secretion can be overridden by neural factors. Electrical stimulation of the hypothalamus increases thyroid activity. Physiological stresses such as cold exposure which result in thyroid activation, and emotional stresses such as forced immobilization which produce thyroid inhibition, have been shown to have a time course indicative of a direct neural effect on TSH release.

Thus integration of the two major homeostatic mechanisms of the body, the nervous system and the endocrine system appears to be effected at the hypothalamic centres of the brain, enabling the organism to adjust to the constantly changing environment both internal and external.

PART II

PHARMACOLOGICAL STUDIES OF THE CONTROL OF THYROID FUNCTION

Introduction

2,4-Dinitrophenol (DNP) has been used for many years as an intermediate in the manufacture of dyestuffs and explosives. The compound is highly toxic and during the first World War deaths among workmen exposed to dust or vapours of the chemical were reported. (Barral and Martin, 1916; Warthen, 1918). The toxicology of the compound was studied, but little significance was attached to the fever reported to accompany the clinical symptoms of poisoning. (Lutz & Baume, 1917).

Subsequently, Tainter and Cutting (1933) reported that the administration of DNP to both men and animals caused a rapid increase in metabolic rate. Its potential therapeutic value as a drug to replace thyroxine and adrenalin in the treatment of disease states such as hypothyroidism, obesity and asthma was realised. The pharmacology of 2,4-dinitrophenol was therefore examined.

It was demonstrated that although DNP increased the metabolic rate, it did not relieve the manifestations of myxoedema (Cutting et al, 1934), nor did it promote metamorphosis in the tadpole (Cutting and Tainter, 1933). However, it was observed that the calorigenic response of animals to the dinitrophenols was influenced by the state of thyroid activity. The metabolic response of the rat to DNP was increased when hyperthyroidism was induced by the

administration of thyroxine, and diminished in goitrogen induced hypothyroidism (Barker, 1946). Pharmacological studies of the effect of DNP on thyroid function were therefore suggested.

Because of the similarity of salicylate to DNP in its ability to increase metabolic rate and oxygen consumption, the effect of salicylate on thyroid function was also studied. These studies of the effect of 2,4-dinitrophenol and salicylate on thyroid function will now be reviewed as a preliminary to the experimental studies which form the basis of this thesis.

1. <u>The Effect of 2,4-Dinitrophenol on Thyroid Function</u>

The first report of the effect of 2,4-dinitrophenol on thyroid function was that of Wolff, Rubin and Chaikoff in 1950. The administration of DNP to rats for periods of one to twenty-nine days caused a decrease in the concentration of plasma protein bound iodine (PBI) to half that of control animals. This depression of PBI in rats by DNP was confirmed by Goldberg and Chaikoff (1951) and Goldberg and co-workers (1955). A decrease in the PBI following the administration of DNP to man was demonstrated by Castor and Beierwaltes in 1956.

Although earlier studies revealed no effect of DNP on the histology or function of the thyroid gland of the rat (Wolff, Rubin and Chaikoff, 1950; Goldberg and Chaikoff, 1951) it was subsequently demonstrated that when the drug was given for longer periods, a depression of thyroid gland function was produced (Goldberg et al, 1955). There was a decrease in

thyroidal iodine content; a decrease in the thyroidal iodide concentrating capacity; a depression of the conversion rate of the gland; and a slowing of the release of the hormone into the circulation of rats treated with DNP. These findings indicated a diminished stimulation of the thyroid gland by TSH. However, the decrease in iodide concentrating capacity of the thyroid was not as great as that produced by hypophysectomy, suggesting that TSH output was not completely suppressed.

Evidence of peripheral factors also operating to produce the depression in PBI was obtained. Studies were made of the rate of disappearance of I^{131}-labelled thyroxine from the plasma following DNP administration to rats in which thyroid function had been blocked by treatment with propylthiouracil. One group of rats was left hypothyroid and the other maintained euthyroid by daily injection of thyroxine. The half-time for disappearance of radiothyroxine was approximately halved by DNP in both groups. Whether this increased peripheral disposal of thyroxine was caused by increased peripheral metabolism or utilization or increased rate of excretion was not determined (Goldberg, Wolff and Greep, 1955).

These findings of simultaneously depressed circulating thyroid hormone and TSH output were contrary to the concept of the negative feedback mechanism controlling the thyroid-pituitary axis. An increased TSH output would be expected in response to the lowered circulating thyroid hormone.

The results of a further study of the action of DNP on the thyroid-pituitary system were published by Goldberg,

Wolff and Greep in 1957. The histology of the thyroid and pituitary glands of the rat was studied following treatment with DNP, with the antithyroid drug propylthiouracil (PTU) and with DNP and PTU given simultaneously.

Following treatment with DNP alone, the thyroids were small and inactive, whereas following PTU they were enlarged with tall columnar cells, increased colloid and highly vascular. When the drugs were given simultaneously, the thyroids were small and histologically similar to those following DNP treatment or following hypophysectomy.

The cytological changes indicative of augmented TSH production by the anterior pituitary beta cells in PTU treated rats were inhibited by DNP. When DNP was given alone, the pituitaries appeared normal although the beta cells were small and resembled those seen in rats treated with thyroxine. These findings suggested that DNP depressed the release of TSH from the pituitary.

It was considered unlikely that the TSH inhibiting effects of DNP were the result of a nonspecific interference with the synthesis of trophic hormone since, firstly, normally granulated beta cells were found and, secondly, normal responses occurred in other cells of the anterior pituitary following treatment with DNP, as shown by adrenal hypertrophy and the normal response of the gonadotrophic cells to the stimulus of orchiectomy.

The administration of DNP to man or animals causes an increase in the basal metabolic rate (Cutting et al, 1934;

Simkins, 1937). Tissue oxygen consumption is stimulated in tissue slices (Goldberg et al, 1957) and oxidative phosphorylation is uncoupled in isolated mitochondrial preparations (Lardy and Wellman, 1952).

Goldberg and colleagues also demonstrated that other agents which possess pyrogenic and metabolic rate stimulating activity in common with DNP and thyroid hormones also depressed plasma PBI with evidence of a depression in TSH release.

It was concluded therefore that it was possible that inhibition of TSH release was an effect of agents which mimic the thyroid hormone in certain respects. Such effects might be transmitted to the pituitary via the central nervous system. However, it was not clear at which point in the homeostatic mechanism this inhibition was effected. Under certain conditions, a low level of circulating thyroid hormone need not stimulate TSH output and indeed TSH output can be blocked by agents which maintain the body temperature and metabolic rate at supernormal levels. Therefore, rather than the actual level of circulating thyroid hormone, it may be one or more of its peripheral actions which is the important factor in regulation of pituitary TSH output.

Reichlin (1960) examined the effect of DNP on rats with hypothalamic lesions to determine whether the hypothalamus is essential for the mechanism by which DNP depresses TSH secretion from the pituitary. DNP was found to inhibit thyroid gland activity in all control animals. This characteristic response was significantly reduced by hypothalamic lesions, which varied

from localised lesions to almost complete hypothalamic destruction. In contrast to thyroxine administration which was equally effective in normal and brain damaged rats, DNP caused a smaller mean change in thyroid function in 51 out of 56 rats with hypothalamic lesions. No correlation was apparent between the site or extent of damage and the degree of inhibition produced by DNP.

Reichlin considered that a possible explanation of this finding of modified DNP effects in rats with lesions, was that baseline thyroid function was abnormally low, so that even with maximum inhibition, the proportional change in I^{131} release might be less than in controls. However, this was not so, because in one of the experiments eight operated rats had I^{131} release rates in the normal range, and in these animals inhibition was significantly less than normal. Thyroxine administration also produced an inhibition in thyroidal release rate in animals which had a significantly reduced response to DNP.

It was concluded therefore that DNP reduces thyroid activity in part, at least, through an action on a hypothalamic mechanism which influences pituitary thyrotropic function and in part through an action at the pituitary level. Reichlin considered that the finding of an action of DNP at both hypothalamic and pituitary sites could be integrated with the established facts of neural-pituitary-thyroid interrelations.

The pituitary is autonomously responsive to changes in local thyroid hormone concentration regardless of the level

of activity at which it is operating. The hypothalamus, on the other hand, is important in establishing the baseline level of thyrotropic function. Reichlin (1960 a) demonstrated that hypothalamic lesions, in areas which overlap the site controlling thyroid function, disrupt the normal heat regulation of the body, and postulated that in the normal animal, heat regulating and thyroid regulating function may be integrated at the hypothalamic level.

It was therefore considered possible that the hypothalamic control of pituitary-thyroid activity is responsive to changes in overall body heat or oxygen consumption. DNP increases metabolic rate and oxygen consumption. Thus DNP may exert its effects on thyroid function by acting at hypothalamic and pituitary sites via intracellular effects shared by thyroxine.

The influence of DNP on the peripheral metabolism of thyroxine has been studied by Morreale de Escobar and Escobar del Rey (1960, 1961 a). They investigated the effect of DNP on the I^{131} distribution in rats which had reached isotopic equilibration with iodide I^{131} after about 20 days. The animals were sacrificed at different time intervals up to four days following DNP treatment and I^{131} distribution determined. The animals were thoroughly perfused at necropsy to remove blood from the tissues. The trichloroacetic acid (TCA) precipitable I^{131} (a measure of organically bound iodine) of the serum was significantly lower in all treated groups than in control animals.

It was demonstrated that the peripheral tissue concentrations of TCA precipitable I^{131} following DNP treatment did not differ from the controls. Thus following DNP administration to rats, the peripheral tissue levels of thyroid hormone remained unchanged while the serum thyroid hormone levels fell.

The decrease in the serum level of iodinated compounds was shown (Escobar del Rey and Morreale de Escobar, 1958 a, b) to be accounted for by the simultaneous increase of their biliary secretion and, ultimately, their faecal excretion.

It was also shown that the radioactivity in the red blood cells was increased above the control values for some hours in DNP treated rats in spite of the decreased serum level; this finding was confirmed in *in vitro* studies. Increased uptake of radiothyroxine by the diaphragms of DNP treated rats was also observed and confirmed in studies of the *in vitro* addition of the drug to the system (Morreale de Escobar and Escobar del Rey, 1961 b). It was considered likely, therefore, that the maintenance of normal concentrations of iodine containing compounds in most peripheral tissues resulted from the alteration induced by DNP in the normal partition of thyroid hormone between plasma and tissues in favour of the latter.

These findings indicated that following DNP treatment the circulating thyroid hormone no longer reflects the tissue levels. If the circulating thyroid hormone is alone considered to be the regulator of the thyroid-pituitary axis, the situation

would be one of apparent disruption of the thyroid-pituitary servo mechanism. The Escobars have interpreted the findings of Goldberg and co-workers (1955, 1957) to mean that there was no increase in TSH release from the pituitary following DNP administration to rats. They concluded that the behaviour of the pituitary, in not increasing TSH output, was better correlated with some intra-cellular parameter related to the concentration of thyroid hormone in the tissues rather than the level of circulating thyroid hormone.

However, this conclusion is not valid since evidence of a depression in TSH release following DNP administration was obtained by Goldberg and his colleagues (1955, 1957).

2. <u>The Effect of Salicylate on Thyroid Function</u>

In 1946 Denis and Means first observed that salicylate administration increased oxygen consumption in man. This finding has been confirmed both in man (Cochran, 1952, 1953; Hetzel et al, 1959) and in experimental animals (Meade, 1954; Reid, 1957). Oxygen consumption was also increased in tissues removed from salicylate treated animals (Brody, 1956) and oxidative phosphorylation uncoupled in isolated mitochondrial preparations (Smith and Jeffrey, 1956; Charnock, et al 1962).

Because of these similarities to DNP and the fact that the toxicity of DNP precluded a thorough study of its effect in man, Austen et al (1958) studied the effect of salicylate on thyroid function in man.

Following chronic salicylate therapy to normal human subjects oxygen consumption was increased and the serum PBI

was significantly depressed.

No change in the peripheral utilization of thyroid hormone during salicylate treatment was observed. The half-life of radiothyroxine fell during salicylate treatment and returned to normal after withdrawal of the drug. As a consequence, the turnover rate rose. Since the PBI concentration fell, the extrathyroidal organic iodine pool (EOI pool) was reduced. The degradation rate being the product of the smaller EOI pool and the faster turnover rate, was found to be the same during salicylate administration as during the control period. Increased peripheral utilization could not therefore be invoked as an explanation to account for the fall in PBI.

The major portion of thyroxine is normally bound in the plasma to specific thyroxine binding proteins (Robbins and Rall, 1957). A decrease in the amount of these proteins, a change in the association constant for thyroxine, or competition by salicylate for binding sites would reduce the quantity of thyroxine bound and possibly change thyroxine metabolism.

However, there was no demonstrable effect of salicylate on the concentration of thyroxine binding sites using the reverse flow paper electrophoretic method of Robbins (1956) in veronal buffer.

It was concluded therefore that a change in the binding of thyroxine to the specific thyroxine-binding proteins was not the explanation for the increased fractional rate of

disappearance of thyroxine produced by salicylate.

Decreased production of thyroid hormone was indicated by the reduced thyroidal uptake of radioiodide (I^{131}) during salicylate treatment. Increased renal clearance of I^{131} could produce an apparent depression of thyroidal I^{131} uptake but renal clearance was shown to be unchanged by salicylate. Thyroid clearance of I^{131} was also reduced by salicylate. Reduction in uptake and clearance of I^{131} could result from either inhibition of the thyroidal iodide concentrating mechanism or interference with the utilization of iodide for hormone synthesis. However, there was no effect of salicylate on the ability of the rat thyroid to concentrate iodide. At high levels of salicylate there was no interference with the organification of iodide by rat thyroid slices. It was concluded therefore that there was no direct effect of salicylate on the thyroid gland but that the action of salicylate in depressing thyroid function resulted from decreased TSH stimulation of the thyroid.

Wolff and Austen (1958) described a significant slowing, by salicylate, of the secretion rate of hormone from the thyroid in normal subjects. Reduction in uptake became obvious 36 hours after commencing the salicylate treatment.

This inhibition of secretion rate was readily overcome by exogenous TSH administration during salicylate treatment. Moreover, the acceleration of I^{131} release by exogenous TSH was not prevented by salicylate. If the TSH available to the thyroid was diminished by salicylate, this drug would be

expected to interfere with goitre formation in PTU treated rats. It was shown that although salicylate caused a reduction in goitre formation in PTU treated rats, goitre prevention was not complete and therefore the suppression of TSH was only partial.

A comparison of the effect of release of I^{131} from the rat thyroid by salicylate with some of its congeners was made in an attempt to study the mechanism of inhibition. Gentisic acid (2,5-dihydroxybenzoic acid), α-resorcylic acid (3,5-dihydroxybenzoic acid) and γ-resorcylic acid (2,6-dihydroxybenzoic acid) depressed the release of thyroidal I^{131}. The dihydroxybenzoic acids were less active on a weight basis than salicylate as blood levels were difficult to maintain. Meta- and para-hydroxy benzoates caused no change in release rate. The chelation properties of the orthophenolic benzoic acids could not be invoked as an explanation because 3,5-dihydroxybenzoic acid (α-resorcylic acid) which lacks the ability to chelate also caused inhibition of release.

Whereas DNP and salicylate increase oxygen consumption and uncouple oxidative phosphorylation neither α-, γ-resorcylic nor gentisic acids exert these effects. Fever production causes thyroid inhibition but could not account for the action of salicylate, as body temperature was not elevated by salicylate. The possibility of adrenal mediation was not likely as massive doses of cortisone, 50 mg. per day, are required to depress the I^{131} release rate.

Wolff and his co-workers concluded that the inhibition of thyroid function caused by salicylate resulted from a reduction in circulating TSH but the mechanism by which TSH was affected was not resolved.

In 1959 Christensen, using a dialysis technique for the determination of free thyroxine, demonstrated that both salicylate and 2,4-dinitrophenol when added _in vitro_ to the serum caused a significant increase in the amount of radioactive thyroxine passing through the dialysis membrane. The increase in the amount of radiothyroxine passing through the membrane would result from an increase in free thyroxine. (A detailed discussion of the dialysis technique of Christensen appears in Chapter IV).

Christensen considered that the thyroxine-releasing effect of salicylate and 2,4-dinitrophenol was probably a result of the binding of these drugs to some of the thyroxine binding sites of the plasma proteins, although the particular thyroxine-binding protein affected could not be determined by this study. It was further postulated that the increased level of free thyroxine following the administration of these drugs to man and animals might account for their rapid calorigenic action, although a direct effect of these drugs on the uncoupling of oxidative phosphorylation could not be excluded. It was concluded that the increase in free thyroxine would explain the increased fractional rate of disappearance of injected radio-thyroxine and the fall in PBI following salicylate administration.

As was mentioned earlier Austen and co-workers (1958) demonstrated that salicylate did not affect the binding of thyroxine to the serum proteins when examined by paper electrophoresis in veronal buffer.

However Ingbar (1960) using paper electrophoresis in trismaleate buffer demonstrated a selective effect by salicylate, when added to the buffer, of displacement of thyroxine from the thyroxine-binding prealbumin fraction. (The physiological role of the thyroxine-binding proteins is reviewed in Chapter IV). This finding confirmed the _in vitro_ evidence of Christensen of a thyroxine releasing effect of salicylate.

It may be questioned whether the effect of salicylate on TSH output is a direct one, or secondary to the peripheral effects of the drug. It is possible that the inhibition of pituitary TSH release results from a direct pharmacological blockade of the gland. Alternatively, the central effect of salicylate may result indirectly from the increased peripheral metabolism induced by the drug, mediated via the hypothalamus as was suggested for DNP by Reichlin (1960).

The third possibility is that the _in vitro_ effect of salicylate in displacing thyroxine into the free state may also occur _in vivo_ following the administration of the drug. A physiological role of free thyroxine rather than the bound or total thyroxine has been postulated by Robbins and Rall (1960). It is possible that free thyroxine rather than the bound thyroxine is the controlling factor in the feedback

regulation of the pituitary-thyroid axis. If this were the case, an elevated free thyroxine level following the administration of salicylate would be expected to inhibit the release of TSH from the pituitary.

In summary, salicylate causes a fall in plasma PBI level in man. This fall has been shown to have a peripheral and a central component. The mechanism acting to produce these component effects is not understood, but may be related to a direct action of salicylate on the pituitary, to the metabolic stimulating property of the drug, or to a displacement of thyroid hormone into the free state from the specific thyroxine-binding proteins.

Studies have therefore been carried out on both the peripheral and central components of the effect of salicylate on thyroid function.

These investigations have been made in experiments using rats and wherever possible in experiments on human subjects in an attempt to gain further understanding of the mechanism of action of salicylate. The results of these studies are presented in the following chapters of this thesis.

CHAPTER II

THE EFFECT OF SALICYLATE AND CHEMICALLY RELATED DRUGS ON THE PLASMA PROTEIN BOUND IODINE LEVEL

PART I The Effect of Salicylate and Related Drugs on the Plasma PBI in Rats.

1. Studies in normal rats.
2. Studies in thyroidectomized rats maintained on thyroxine.

PART II Conclusions.

CHAPTER II

THE EFFECT OF SALICYLATE AND CHEMICALLY RELATED DRUGS ON THE PLASMA PROTEIN BOUND IODINE LEVEL

INTRODUCTION

As a consequence of the report of Austen and co-workers (1958) that the administration of salicylate to normal human subjects produced a depression in thyroid function, a systematic investigation of the effect of salicylate on the plasma PBI was carried out in man in various states of thyroid function. These results have been published (Hetzel et al, 1962).

Salicylate was administered in a dosage of 6 g. daily for four days as the calcium acetyl salt. In a group of six normal subjects salicylate produced a depression in plasma PBI from a mean level of 6.0 µg. I per 100 ml. (µg. I%) to 4.5 µg. I% after four days. However, the fall reached statistical significance ($P < .001$) after 2 days. The mean level of plasma salicylate was 25 mg. per 100 ml. (mg. %) on the fourth day.

Salicylate was also given in the same dosage, to a group of six subjects maintained on desiccated thyroid extract in whom myxoedema had developed following thyroidectomy. There was a fall in plasma PBI in this group from a mean of 5.6 to 5.1 µg. I% after four days. The fall reached statistical significance on the third day ($P < 0.05$). The mean level of plasma salicylate on the fourth day was 24 mg. %). Comparison

of the PBI values observed in the normal group and the group maintained on desiccated thyroid revealed a significant difference after two days ($P < 0.05$).

The depression in plasma PBI in the normal group was significantly greater than that observed in the group maintained on desiccated thyroid. However, the consistent fall produced by salicylate in the subjects in the latter group indicated the operation of peripheral factors, that is, effects on peripheral metabolism or distribution of thyroid hormone, causing the fall in plasma PBI. Initial plasma levels of PBI were similar in both the normal group and the group maintained on desiccated thyroid. The thyroxine turnover rate would have returned to normal in the maintained group in view of the length of time replacement therapy had been given (Ingbar, 1960a). Hence the greater fall in plasma PBI observed in the normal group would not be expected to result from differences in turnover rate.

It would appear therefore that the difference between the percentage fall in PBI in the two groups (24% in the normal group compared to 11% in the maintained group) resulted from a depression of thyroid secretion in the normal group. Taken in conjunction with the absence of any direct effect on hormone synthesis (Austen et al, 1958), this difference indicated that salicylate produced a decrease in TSH output in the normal group. It was concluded therefore, that salicylate exerts both a central effect (by inhibiting the

pituitary release of TSH) and a peripheral effect in producing the depression in plasma PBI in man.

In another study, the effect of salicylate on the plasma PBI of thyroidectomised rats maintained on thyroxine was determined (Good, Wetzel and Opit, 1960). It was demonstrated that salicylate in a total dosage of 30 mg./100 g. body weight/day caused a fall in the plasma PBI from a mean level of 3.0 µg. I% before treatment to 1.4 µg. I% after 24 hours, to 1.1 µg. I% after 48 hours and to 0.9 µg. I% after 72 hours.

These findings indicated a greater peripheral effect of salicylate in the rat, than that occurring in previously hypothyroid human subjects maintained on desiccated thyroid extract.

It was decided therefore to carry out an investigation of the effect of salicylate and related drugs on plasma PBI in the rat as a first step in more detailed studies of the mechanisms involved in the depression of thyroid function.

PART I

THE EFFECT OF SALICYLATE AND RELATED DRUGS ON THE PLASMA PBI IN RATS

1. STUDIES IN NORMAL RATS

Experiment 1

Materials and Methods

Male black and white rats from the University colony were used in these experiments. They were fed on a diet of commercial rat cubes *ad libitum* and weighed daily. The animals were of the same age and approximately 350 g. in weight. They were numbered and divided into groups by random selection. Prior to treatment approximately 4.0 ml. of blood was collected by cardiac puncture from each rat while under light ether anaesthesia. A period of seven days was allowed for the animals to recover from the effects of the initial blood sampling before the treatments were initiated.

The treatments were administered by gastric gavage twice daily. Sodium salicylate was administered in a dosage of 30 mg/100 g. body weight per day.

As a control for the sodium ion and the experimental manipulation, sodium lactate was administered (24 mg/100 g. body weight/day) to give the same sodium ion concentration as that in the dose of sodium salicylate. Sodium p-hydroxybenzoate, the para-isomer of sodium salicylate, was administered to another group as a control for the salicylate ion in a dosage of 30 mg/100 g. body weight/day.

A fourth group was treated with sodium γ-resorcylate (sodium 2,6 dihydroxy benzoate) in a similar dosage (30 mg/100 g. body weight/day). Wolff and Austen (1958) had shown that thyroidal secretion rate was decreased following subcutaneous administration of γ-resorcylate to rats. It was decided, therefore, to examine the possible effect of this drug on the plasma PBI.

The drugs were given for 48 hours (5 doses). Four hours after the last dose the rats were again anaesthetized and blood samples obtained by cardiac puncture. Blood was collected in heparinized iodine-free syringes, transferred to iodine-free tubes and centrifuged to separate the plasma. The plasma samples were stored in the frozen state at -20°C until required.

Haematocrit determinations were carried out on the blood samples both before and after treatment using a micro-method (Hicks 1963).

Salicylate and γ-resorcylate estimations were made using the method of Trinder (1954). The method did not give a colour reaction with p-hydroxybenzoate. Standard curves were prepared with each batch of determinations and were shown to be highly reproducible.

The Estimation of Plasma Protein Bound Iodine

The term "protein bound iodine" (PBI) has been defined as "that fraction of the blood iodine which is non-dialysable and precipitable with the serum or plasma proteins". (Trevorrow, 1939). In the normal human subject approximately 85 to 90%

of the serum PBI consists of l-thyroxine, the remainder being 3,5,3' triiodothyronine and small quantities of mono- and di-iodotyrosines (Pitt-Rivers and Tata, 1959). Similar findings have been reported for the rat (Pitt-Rivers and Hall, 1961).

Plasma PBI may be estimated by two methods, both of which utilize the same colorimetric reaction for the estimation of iodine. Because of the minute quantity of iodine in normal plasma (4.0 to 8.0 µg. I per 100 ml.) use is made of the micro-method of Sandell and Kolthoff (1934) for the estimation of iodine. This method is based on the catalysis by microgram quantities of iodine, of the reduction of ceric ions to cerous ions by arsenite, the rate of reduction being followed by the rate of decolorization of the yellow ceric salt.

PBI methods differ in the manner in which the hormonal iodine is converted to the inorganic state for subsequent estimation. The first method, developed by Chaney, (1940) was a distillation technique which depended upon the acid digestion of the precipitated protein, followed by distillation into caustic soda of the elemental iodine released. This technique is difficult because of the technical problems involved in controlling the distillation to prevent the loss of iodine.

For this reason an alkaline incineration method was developed by Salter and McKay (1944) in which the proteins precipitated with trichloroacetic acid (TCA) were mixed with strong alkali, dried, and ashed in a muffle furnace to convert the organic iodine into the inorganic state. Subsequently

Barker, Humphrey and Soley (1951) substituted the Somogyi (1930) zinc hydroxide method instead of TCA for precipitation of the proteins. Because of controversy over the efficiency of certain steps in the technique, Acland (1957) made a comprehensive investigation of the analysis.

Acland demonstrated that the results obtained using TCA precipitation were erratic under all conditions of incineration time and temperature. However, zinc hydroxide precipitation and incineration for 3½ hours at 600°C revealed no significant difference between batches of determinations.

When added sodium-l-thyroxine was precipitated with the serum proteins using zinc hydroxide, the recovery of iodine in a series of 5 experiments was 92.9% (89.1 to 96.6%). These results compared more than favourably with those of other workers using either the acid-distillation or alkaline-incineration methods.

A critical examination of Acland's procedure has been made as it seemed best suited for the determination of plasma PBI.

A pool of normal plasma was obtained and in every batch of analyses carried out, duplicate samples of this pool were analysed. This provided an internal check for contamination or loss of iodine. Statistical analysis of a series of 24 duplicate pool plasma samples revealed a standard error of the mean of two duplicates of 0.12 µg. I%.

Recovery of Added Thyroxine:

Sodium-l-thyroxine was added at levels respectively of

2.5, 5 and 10 µg. T4 per 1.0 ml. plasma and incubated at 37°C for 2 hours before precipitation of the proteins.

In a series of experiments made over a period of six months, 22 additions of thyroxine were made to samples of the same plasma in 10 batches of PBI determinations. The mean thyroxine recovery was 90.4% with a range of 84.8% to 95.8%.

Recovery of Added Radioactive Thyroxine:

3 µl of I^{131}-labelled thyroxine (Abbotts: approx. 200 microcuries/ml.) was added to 8.0 ml. of normal human plasma and incubated at 37°C for 2 hours. 1.0 ml. aliquots of this serum were measured into counting tubes and 2.0 ml. water added and stirred. The radioactivity in each sample was measured in a well-type scintillation counter (Ekco Type N597). All samples (except one which was kept as a reference standard for I^{131} decay rate) were precipitated and allowed to stand for 1 hour (samples 1, 2 and 3) or three hours (samples 5 and 6). The supernatants were decanted, the precipitate made up to 3 ml. with water (to standardize on the geometry for counting), mixed, and counted. The protein precipitates were washed three times by the usual procedure, made up to 3 ml. mixed and again counted. The precipitates were made alkaline, dried and ashed. The radioactivity present in the ashed material was measured. Finally after elution, 3 ml. aliquots of the eluates were counted and the total radioactivity in the sample was calculated.

The results, shown in Table 1, expressed as the percentage of the total radioactivity remaining, were calculated by

TABLE 1

RECOVERY OF RADIOTHYROXINE AT VARIOUS STEPS IN THE ESTIMATION OF PROTEIN BOUND IODINE

Sample	Time of Standing Following Precipitation	% Total Radioactivity Remaining in Sample After			
		Initial Precip-itation	Three Washes	Inciner-ation	Elution
1	1 hour	97.1	96.4	90.6	91.2
2	1 hour	97.4	98.2	89.1	89.6
3	1 hour	97.3	98.2	91.7	91.1
4	3 hours	96.4	96.7	89.6	88.4
5	3 hours	96.7	98.7	90.0	89.1
Mean		97.0	97.6	90.2	89.9

comparing the count rate of the sample with that of the standard counted at the same time.

A mean value of 97.0% of the I^{131}-labelled thyroxine was recovered following the initial precipitation. As the radiothyroxine was one week old when used, there was a small percentage of iodide I^{131} present due to deiodination of the thyroxine. There was no change from this recovery after three washes.

90.2% of the added I^{131}-thyroxine was recovered following the incineration of the samples, and no significant loss occurred during elution. Thus, assuming that all the I^{131} labelled thyroxine present in the samples was precipitated, the mean percentage recovery after all steps of the determination would be 92.7%. This value is in good agreement with the recoveries using stable thyroxine.

There was no apparent difference in the results due to the time of standing following the initial precipitation of the plasma proteins. These results indicate that a degree of accuracy was obtained with the method comparable with that achieved by Acland (1957).

Results

Effect on Haematocrit

The values are shown in Table 2. The mean haematocrit value for the pretreatment blood samples was 41%. After treatment (10 days later) the mean haematocrit was 40%. There was no significant change in any of the groups following treatment.

TABLE 2

EFFECT OF EXPERIMENTAL PROCEDURE ON HAEMATOCRIT IN NORMAL RATS

Rat No.	Treatment	Haematocrit		p compared to Control Value
		Before Treatment	10 days Following Treatment	
24 23 7 8 10	Sodium lactate 24 mg./100 g./ day	42 40 38 43 39	41 40 33 46 40	
Group Mean		41	40	N.S.
2 6 15 11 18	Sodium p-hydroxy benzoate 30 mg./100 g./ day	42 42 40 43 38	38 41 39 40 41	
Group Mean		41	40	N.S.
4 5 20 16 17	Sodium salicylate 30 mg./100 g./ day	35 39 44 42 38	36 34 41 39 42	
Group Mean		40	38	N.S.
19 22 3 9 14	Sodium γ-resorcylate 30 mg./100 g./ day	42 44 41 46 40	41 42 41 43 41	
Group Mean		43	42	N.S.
Mean		41	40	

Effect on Plasma PBI

The results are given in Table 3. There was no significant effect of sodium lactate or sodium p-hydroxybenzoate on the plasma PBI in the normal rats in this experiment.

A depression in plasma PBI was obtained following sodium salicylate. The PBI level fell to 1.3 μg. I% from a pre-treatment level of 4.0 μg. I%. This fall was significant ($P < 0.01$). There was a slight fall in plasma PBI following sodium γ-resorcylate from a mean level of 3.2 μg. I% before treatment to 2.8 μg. I% after 52 hours treatment. However, this fall was not significant. The mean plasma level of γ-resorcylate was only 5.3 mg.% compared to a mean plasma level of 44.7 mg.% following salicylate.

Effect on Body Weight

There was no decrease in body weight following treatment with either lactate, p-hydroxybenzoate or γ-resorcylate. The body weight decreased by a mean of 5 g. in the animals treated with salicylate (Table 3).

Discussion

The significant depression in plasma PBI produced by sodium salicylate in normal rats in this experiment confirms the similar finding obtained in previous studies using thyroidectomized rats maintained on thyroxine (Good et al, 1960).

As there was no significant change in plasma PBI following sodium lactate and sodium p-hydroxybenzoate it is

TABLE 3

EFFECT OF SALICYLATE AND RELATED DRUGS ON PLASMA PBI IN NORMAL RATS

Rat No.	Treatment	PBI µg. I %		p*	% decrease in PBI	Weight (g.)		Plasma Level mg. %
		Before Treatment	Treated 52 hrs.			Before Treatment	Treated 52 hrs.	
24 23 7 8 10	Sodium lactate 24 mg./ 100 g./ day	3.5 3.4 4.3 3.2 3.0	3.7 4.3 3.7 3.5 2.8			361 484 385 292 280	366 480 386 292 284	
Mean		3.5	3.6	N.S.		360	362	
2 6 15 11 18	Sodium p-hydroxy benzoate 30 mg./ 100 g./day	4.7 4.8 4.1 3.3 2.8	4.7 3.9 3.5 4.5 3.7			460 410 307 251 232	466 404 308 245 236	
Mean		3.9	4.1	N.S.		332	332	
19 22 3 9 14	Sodium γ-resorcylate 30 mg./ 100 g./day	3.1 3.3 4.9 2.6 2.2	2.7 2.3 4.2 2.5 2.5			400 420 350 230 270	400 413 358 235 271	5.4 6.8 5.0 4.8 4.5
Mean		3.2	2.8	N.S.	12.5	334	335	5.3
4 5 20 16 17	Sodium salicylate 30 mg./ 100 g./ day	4.0 4.7 4.1 3.4 3.7	1.3 1.0 0.7 1.6 2.0			398 406 406 276 278	387 409 404 270 270	49.2 54.0 43.0 40.0 37.2
Mean		4.0	1.3	<0.01	68	353	348	44.7

*P - compared to control data by "t" test.

concluded that the sodium ion concentration and the experimental manipulation were without effect on the results.

The slight depression in plasma PBI produced by sodium γ-resorcylate was not significant. However, the level of γ-resorcylate in the plasma was low when compared with that following salicylate. Wolff and Austen (1958) have referred to the difficulty in maintaining elevated blood levels of the dihydroxy benzoic acids. It was decided, therefore, to examine the effect of a higher dose of γ-resorcylate in an attempt to induce a higher concentration of the drug in the plasma. Such an increase in plasma level might cause a significant depression in the plasma PBI.

Since there was no significant difference in haematocrit between the initial blood samples and those obtained after treatment it is concluded that the seven day recovery period was adequate for the restoration of the blood composition to the pretreatment state. This procedure has been followed in all subsequent experiments.

The loss in body weight following salicylate is consistent with the increased metabolic rate and uncoupling of oxidative phosphorylation produced by this drug.

Experiment 2

Using the same experimental procedure as described in Experiment 1, the effects of sodium γ-resorcylate at a higher dose level and also 2,4-dinitrophenol were assessed in normal rats. Sodium lactate was again used as the control for the experimental procedure.

2,4-dinitrophenol was administered in a dosage of 2.5 mg/100 g. body weight/day, a dose level which was shown by Wolff et al (1950) to depress the PBI level in rats without causing severe toxicity.

The dose level of sodium γ-resorcylate was increased three-fold from the previous experiment, 90 mg/100 g. body weight/day being administered in two divided doses.

Results

The results are shown in Table 4.

A depression in PBI was observed following γ-resorcylate at a dose level of 90 mg/100 g. body weight/day. The PBI was depressed from 4.2 to 1.6 μg. I% which was highly significant ($P < .001$). The mean plasma level of γ-resorcylate was 22.2 mg.%. There was also a fall in PBI from an initial level of 3.8 μg. I% to 1.6 μg. I% following treatment with 2,4-dinitrophenol. This decrease was significant ($P < 0.01$). In this experiment there was a depression in the PBI level following sodium lactate. This fall was significant at the 5% level.

Discussion

The finding of a significant depression in the level of PBI in rats following 2,4-dinitrophenol in this experiment confirms the similar findings of others (Wolff et al, 1950; Escobar del Rey and Morreale de Escobar, 1958).

It was demonstrated by Wolff and Austen (1958) that the release rate of I^{131} from the thyroid was depressed when a maximum blood level of 18 mg.% γ-resorcylate was attained.

TABLE 4

EFFECT OF γ-RESORCYLATE AND 2-4 DINITROPHENOL ON PLASMA PBI IN NORMAL RATS

Rat No.	Treatment	PBI μg. I %		p^{*}	% decrease in PBI	Weight (g.)		Plasma Level mg. %
		Before Treatment	Treated 52 hrs.			Before Treatment	Treated 52 hrs.	
4 6 7 8 19	Sodium lactate 24 mg./ 100 g./ day	3.8 3.5 3.0 3.5 3.6	3.1 3.0 3.0 3.1 2.9			322 267 302 260 230	311 274 298 254 237	
Mean		3.5	3.0	<0.05	14	276	275	
1 11 12 16 18	Sodium γ-resorcylate 90 mg./ 100 g./ day	4.8 4.2 4.2 4.3 3.6	1.5 1.5 1.7 1.4 1.7			293 320 204 349 240	298 318 217 349 253	20.6 23.5 24.5 25.5 17.0
Mean		4.2	1.6	<.001	62	281	287	22.2
3 5 9 13 17	2-4 dinitrophenol 2.5 mg./ 100 g./ day	4.7 4.1 4.1 3.7 2.6	1.8 1.9 1.0 1.7 1.6			312 327 301 280 260	310 317 302 270 263	
Mean		3.8	1.6	<0.01	58	296	292	

*p = compared to control day by "t" test.

The finding of a highly significant depression of the circulating PBI at a blood level of 22.2 mg.% is similar to that obtained following salicylate. This finding indicates that at elevated blood levels both drugs have a similar action on thyroid function in both decreasing the I^{131} release rate from the thyroid, and depressing the circulating thyroid hormone. Unlike salicylate, γ-resorcylate does not produce increased oxygen consumption in rats (Meade 1954) nor uncouple oxidative phosphorylation in isolated mitochondrial preparations (Packer, 1958; Opit, 1964). This finding of a dissociation of metabolic activity from the effect on thyroid function of these drugs has been studied further.

2. STUDIES IN THYROIDECTOMIZED RATS MAINTAINED ON THYROXINE

Previous studies had revealed that salicylate depressed the plasma PBI in thyroidectomized thyroxine-maintained rats after 48 hours' treatment with salicylate (Good et al, 1960). Owing to the disruption of the thyroid-pituitary system in these animals, the effect of salicylate on PBI was due entirely to its peripheral action. Similar findings have been documented for 2,4-dinitrophenol (Escobar del Rey and Morreale de Escobar, 1958). It was decided therefore to compare the effects of salicylate and related drugs on PBI in thyroxine-maintained thyroidectomized rats.

Materials and Methods

Young male rats of the same age and weighing approximately 175 g. were surgically thyroidectomized under ether anaesthesia. Replacement therapy with sodium-l-thyroxine (2.0 μg./100 g.

body weight/day) was started on the following day. There was a consistent loss in body weight following surgery, but this loss was slowly regained over the succeeding ten days. Three weeks after thyroidectomy blood samples were collected by cardiac puncture. Seven days later the treatments were commenced. The drugs were administered by gastric gavage.

Sodium p-hydroxybenzoate, sodium salicylate and sodium γ-resorcylate were administered at 30 mg./100 g./day in two equal doses. Sodium γ-resorcylate was also administered to another group at the higher dose rate used previously (Experiment 2) 90 mg./100 g. body weight/day in two equal doses. 2,4-dinitrophenol was given at the rate of 2.5 mg./100 g./day in the same manner.

The maintenance dosage of thyroxine was injected at the same time each morning. In order to preserve the same circulating hormone level, the same time interval between thyroxine administration and removal of blood samples was maintained on both occasions, before and following treatment.

Post mortem examinations were carried out to assess the effectiveness of thyroidectomy and the possibility of thyroid regeneration. Plasma PBI and salicylate determinations were carried out by the described procedures.

Results

These are given in Table 5.

The mean pre-treatment PBI value for all rats was 7.2 µg. I%. This value was much higher than the value found for normal rats which was approximately 3.5 µg. I%.

Nevertheless, falls in plasma PBI of the same order occurred following salicylate and γ-resorcylate (at the higher dose level of 90 mg./100 g. body weight/day) which were both significant ($P < 0.02$).

There was no significant change in plasma PBI following p-hydroxybenzoate or γ-resorcylate (at the lower dose level of 30 mg./100 g. body weight/day). The mean blood levels of γ-resorcylate were only 5.7 mg.% following the smaller dose whereas 16.9 mg.% was achieved when 90 mg./day were given. There was a significant fall in PBI ($P < 0.05$) when the rats were treated with 2,4-dinitrophenol. Because small numbers of animals were used in each group, the levels of significance were not as high as achieved in previous experiments.

Discussion

Although these animals were maintained with thyroxine at a higher than normal PBI level, the results indicate a similar depression of PBI following salicylate, 2,4-dinitrophenol and also γ-resorcylate when the blood levels approached 20 mg.%. Post mortem examination revealed that there was no thyroidal regeneration or residual thyroid tissue following thyroidectomy, hence the depression in plasma PBI in these animals represents the peripheral effect of the drugs on thyroxine metabolism.

TABLE 5

EFFECT OF SALICYLATE AND RELATED DRUGS ON PLASMA PBI IN THYROIDECTOMIZED THYROXINE-MAINTAINED RATS

Rat No.	Treatment	PBI µg. I %		p*	% decrease in PBI	Weight (g.)		Plasma Level mg.%
		Before Treatment	Treated 52 hrs.			Before Treatment	Treated 52 hrs.	
11 21 13	Sodium p-hydroxy benzoate 30 mg./100g./day	8.4 7.2 5.5	7.9 8.2 7.8			185 200 202	197 202 218	
Mean		7.0	8.0	N.S.		196	206	
2 16 4	Sodium salicylate 30 mg./100g./day	8.2 4.9 7.9	2.8 1.3 2.3			219 268 158	207 262 158	29.0 28.5 28.0
Mean		7.0	2.1	<0.02	70	215	209	28.5
25 7 15	Sodium γ-resorcylate 30 mg./100g./day	7.3 4.5 6.8	6.4 5.7 7.0			203 167 188	199 182 194	6.0 6.0 5.0
Mean		6.2	6.4	N.S.		186	192	5.7
8 14 9	Sodium γ-resorcylate 90 mg./100g./day	8.2 6.8 9.5	3.5 3.0 3.3			164 200 180	159 199 176	14.3 19.6 16.8
Mean		8.2	3.3	<0.02	60	181	178	16.9
10 26 12	2-4 dinitrophenol 2.5 mg./100g./day	7.5 7.2 8.3	4.5 3.6 3.4			177 201 198	185 208 199	
Mean		7.7	3.8	<0.05	51	192	197	

*p - compared to control day by "t" test.

PART II

CONCLUSIONS

The demonstration of a significant depression in plasma PBI in normal rats following treatment with both 2,4-dinitrophenol and salicylate confirms the similar findings reported in man (Castor and Beierwaltes, 1956; Austen et al, 1958; Hetzel et al, 1962). However, the magnitude of this depression was greater in rats than in man. In normal rats the percentage decrease in plasma PBI from the pre-treatment value was 68% (Table 3), whereas in normal human subjects this was only 24% (Hetzel et al, 1962). The depression of PBI (70%) in thyroidectomized thyroxine-maintained rats following salicylate was of the same order as in normal rats (Good et al, 1960). Although the PBI value for the pretreatment samples from the thyroxine-maintained thyroidectomized rat experiment, described in this chapter, was approximately twice the normal level, the percentage decrease following salicylate was again shown to be 70% (Table 5).

As the depression of PBI in thyroidectomized thyroxine-maintained rats was due entirely to a peripheral action of salicylate, it is apparent that the effect on PBI in normal rats is also due largely to a peripheral effect of the drug. A similar conclusion is indicated following treatment with 2,4-dinitrophenol and also γ-resorcylate at a dose level of 90 mg./100 g. body weight/day. Again, although the pre-treatment values for PBI for the thyroidectomized thyroxine-

maintained rats were twice the normal level, the fall following 2,4-dinitrophenol was 58% which is similar to the percentage decrease occurring in normal rats (50%). The percentage fall following γ-resorcylate was 60% in both normal and thyroxine-maintained thyroidectomized rats.

Although salicylate has been shown to exert a peripheral effect in depressing the plasma PBI, a central component mediated via a suppression of TSH release was also evident from studies in man (Wetzel et al, 1962). In secretion rate studies in man Wolff and Austen (1958) have demonstrated a depression of TSH release following salicylate administration.

However, in rats the peripheral effect of salicylate, γ-resorcylate and 2,4-dinitrophenol appears to account almost entirely for the action of the drugs in depressing the plasma PBI. Nevertheless there is also evidence from studies on rats that salicylate and γ-resorcylate inhibit TSH release in this species also. In secretion rate studies Wolff and Austen (1958) demonstrated that both salicylate and also γ-resorcylate at adequate blood concentration cause a slowing of release of hormone from the rat thyroid. Similar findings were reported for 2,4-dinitrophenol, as well as histological evidence of depressed pituitary TSH synthesis (Goldberg et al, 1955).

It has been postulated that the metabolic stimulating action of both salicylate and 2,4-dinitrophenol produces the depression of TSH release from the pituitary, either by a direct effect on the hypothalamus or by a secondary effect mediated via changes in the peripheral cellular metabolism.

(Goldberg et al, 1957; Reichlin, 1960). This possibility may now be questioned since similar changes in PBI by γ-resorcylate have been demonstrated in these studies. Sodium γ-resorcylate does not produce increased oxygen consumption in rats (Meade 1954) nor does it uncouple oxidative phosphorylation in isolated mitochondrial preparations (Packer, 1958; Opit, 1964). The effect of this drug in depressing the circulating thyroid hormone therefore appears not to be associated with metabolic stimulating properties.

Although the studies with these drugs indicate a depression of TSH output, they are however only indirect assessments of pituitary TSH status. It was therefore resolved, prior to a further examination of their mechanism of action, to determine the effects of these drugs on circulating TSH level by direct bioassay.

The results of these studies appear in the next chapter.

CHAPTER III

THE EFFECT OF SALICYLATE AND RELATED DRUGS ON THE CIRCULATING LEVEL OF THYROID STIMULATING HORMONE

PART I The Bioassay of TSH.

1. The action of TSH on the thyroid.
2. The standardisation of the unit of TSH.
3. Definition of criteria used to assess the reliability of bioassays.
4. The design of bioassays.
5. Methods of bioassay of TSH.
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Conclusions.

PART II The Bioassay of TSH by a Modification of the Method of McKenzie.

PART III Studies of the Effect of Salicylate and Related Drugs on Circulating TSH in Rats.

1. The effect of salicylate on circulating TSH in thyroidectomized rats.
2. The effect of sodium-l-thyroxine on circulating TSH in normal rats.
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PART IV The Estimation of TSH in Normal Human Serum.

PART V Conclusions.

CHAPTER III

THE EFFECT OF SALICYLATE AND RELATED DRUGS ON THE CIRCULATING LEVEL OF THYROID STIMULATING HORMONE

INTRODUCTION

In previous studies from this laboratory (Hetzel et al, 1962), it was demonstrated that the depression in plasma PBI produced by salicylate in normal human subjects comprised both a central component, produced by a depression in the release of TSH from the pituitary, and a peripheral component. This finding of a central component in the depression in plasma PBI confirmed the earlier evidence, obtained from secretion rate studies in man, that salicylate depressed the release of TSH from the pituitary (Wolff and Austen, 1958).

Studies of the depression of plasma PBI in rats produced by salicylate, γ-resorcylate and 2,4-dinitrophenol, reported in Chapter II, revealed that there was no difference in the percentage fall in either normal or thyroidectomised rats maintained on thyroxine, indicating only a peripheral action of the drugs. Nevertheless, a slowing of the thyroidal secretion rate has been observed in rats following treatment with salicylate, γ-resorcylate and 2,4-dinitrophenol. These findings suggested a depression of TSH release (Wolff and Austen, 1958).

It was decided that confirmation of this evidence of a depression of TSH release from the pituitary following the administration of these drugs should be sought by direct assay of circulating TSH in controlled experiments. Because the protein structure of TSH has not been characterized, bioassay methods must be used for the estimation of this hormone.

PART I

THE BIOASSAY OF TSH

1. The Action of TSH on the Thyroid

It is generally agreed that TSH stimulates two phases of the metabolism of iodine by the thyroid gland independently. In the first of these, the effect of TSH on the release of preformed thyroid hormone stored in the form of colloid is immediate so that, in small animals, an elevation of the thyroxine content of the blood is evident within 30 minutes, reaching a maximum after about 3 hours.

In the second phase, the action of TSH in increasing the iodide trapping activity of the thyroid is subject to a distinct lag by comparison with its prompt action in accelerating the rate of discharge of the hormone. The trapping of iodide in the rat thyroid is increased by a single injection of TSH, with a latent period of about 6 hours reaching a maximum between 24 and 48 hours.

Continuous or repeated stimulation by TSH results in a progressive depletion of the colloid content of the gland which is evident on histological examination. Prolonged stimulation also causes hypertrophy of the cells, such that the normal flat-celled epithelium assumes a cuboidal or columnar condition. With mild stimulation the increase in the mass of cells is balanced by the loss of colloid resulting in a small net increase in the weight of the thyroid. However, stronger stimulation produces an increase in the number of cells by mitotic division and the combined effects of hyperplasia and cellular hypertrophy result in increased weight.

2. <u>The Standardisation of the Unit of TSH</u>

The sensitivities of the earlier assays of TSH were expressed in terms of animal units. The Junkmann-Schoeller unit (JSU) was defined in 1932 as "that amount of thyrotrophin extract required to produce definite signs of histological stimulation in one out of two guinea pigs after three daily injections" (Junkmann and Schoeller, 1932). However, this assessment of potency is highly subjective, and variations from laboratory to laboratory were inevitable because of differences in technique and in the strains of animals used.

The International Standard for Thyrotrophin was established in 1954 (Mussett and Perry, 1955). The International Unit (I.U.) is 13.5 mg. of the standard substance and was so defined to make it equipotent with the United States Pharmacopeia (U.S.P.) Unit which is 20 mg. of the USP 'Thyrotropin Reference Substance'. Both preparations consist of a mixture of one part of ox anterior lobe extract and 19 parts of lactose.

In the following sections the potency of TSH in blood has been expressed in terms of International milliunits (ImU) per millilitre.

3. <u>Definition of the Criteria Used to Assess the Reliability of Bioassays</u>

According to Borth (1952) there are four such criteria - precision, specificity, sensitivity and accuracy which are of equal importance in assessing the value of a given assay procedure.

Precision

The estimate of precision of a chemical assay can be obtained by carrying out multiple determinations of the same specimen. Precision is expressed as the standard deviation of replicate determinations. In biological assays the labour involved in conducting replicate determinations would be considerable so this method is not practicable.

One of the most convenient methods for bioassays is to express the errors of the various tests in terms of the index of precision (λ). This term was introduced by Gaddum (1933) and is an estimate of the standard deviation of the individual effective doses. In bioassays based on measured effects (such as those for TSH) the index is calculated by dividing the standard deviation (s) by the slope of the regression line of response on the logarithm of the dose (b), i.e. $\lambda = \frac{s}{b}$.

Loraine (1958) concluded that assays in which the index of precision is 0.2 or less, are very precise and suitable for quantitative work, that assays with indices between 0.2 and 0.3 are less precise but may still be used with reasonable confidence and that indices greater than 0.3 indicate assays of low precision unsuitable for quantitative work.

Specificity

In bioassays the term "specificity" refers to the determination of one physiological activity to the exclusion of others. Usually the specificity of the method depends on cumulative evidence that the technique measures what it is supposed to measure and nothing else. One important test of

specificity in bioassays is the parallelism of the dose response lines for the standard material and equally graded doses of the test material.

Sensitivity

This term may be defined as the minimum amount of a substance which can be detected by a particular method.

Accuracy

The accuracy of a quantitative chemical method can be studied by means of "recovery experiments" in which the sample is analysed before and after the addition of a known amount of the substance under investigation. Results are expressed in terms of the percentage of the added compound recovered. However, in hormone assays depending on biological methods such recovery experiments are usually very laborious and cannot readily be carried out.

4. The Design of Bioassays

Gaddum (1953) reviewed the various experimental designs used in bioassay methods and concluded that the most reliable methods are possibly those using either a three point or a four point design.

The Three Point Assay Design

This is the simplest design which is acceptable for routine use. Three groups of animals are used; two groups receive doses of the standard and one the unknown preparation. The dose of the unknown should have an effect intermediate between the two doses of standard. This design provides no information regarding either slope difference (parallelism) or

curvature. Because of their simplicity, three point assays are commonly used in routine studies. According to Loraine (1958) this is justifiable, if at an early stage in the investigation more complex designs have been used to establish that the assay in question satisfies the recognized criteria of validity.

The Four Point Assay Design

Four groups of animals are employed; two groups receive graded doses of the standard material while the other two groups receive equally graded doses of the unknown preparation. Such a design enables the investigator to calculate whether the dose response lines of the standard and unknown preparations differ significantly in slope. If lack of parallelism is demonstrated the assay is invalid.

5. Methods of Bioassay of TSH

The 70 or more bioassay methods for the estimation of TSH reported since 1930 have been reviewed by Brown (1959). Prior to the use of radioactive isotopes by biochemists, bioassay methods were dependent upon either histological or gravimetric responses of the thyroid to TSH. Some methods were based on the increase in metabolic rate produced by the increased thyroid hormone discharged by TSH. These were useful for the estimation of TSH in pituitary extracts, but with a few exceptions (Purves and Greisbach, 1949; D'Angelo and Gordon, 1950) they were not sensitive enough for the detection of TSH in body fluids.

Outstanding among the histometric methods is that of

D'Angelo and Gordon (1950). This is based on the fact that starvation induces metamorphic stasis and thyroid atrophy in the larva of the frog (*Rana pipiens*) at an early hind-limb stage, prior to the eruption of the fore-limbs. The criteria used for estimation of TSH are increase in thyroid acinar cell height and in hind-limb length. In spite of its dependence on subjective interpretation, the method has proved reliable, with a lower limit of sensitivity of 0.1 ImU.

Estimates of the level of TSH in normal human serum were made using this method (D'Angelo et al, 1951). TSH could be detected in some of the sera tested and a range from zero to 0.1 ImU/ml. was reported for normal human subjects. In a series of 10 hypothyroid subjects levels were higher and ranged from zero to 0.5 ImU/ml.

The introduction of radioisotopes of phosphorus and iodine permitted the development of several new techniques for assaying TSH.

Methods Utilising P^{32}

Following earlier reports that increased thyroid activity was associated with increased phosphorus content, Borell (1945) demonstrated that TSH increased the phosphorus content of the thyroid glands of guinea pigs. Subsequently, Borell and Holmgren (1949) developed an assay for TSH based on thyroidal uptake of P^{32}. This method was improved and a degree of sensitivity achieved which enabled the assay to be used for the estimation of TSH in blood and urine (Greenspan et al, 1956). However, further investigation revealed that an extra-pituitary

factor in urine stimulated the thyroidal uptake of P^{32} (Greenspan and Lew, 1959). This lack of specificity severely limited the usefulness of the method.

Methods Utilising I^{131}

These methods are based on the stimulation by TSH of either the thyroidal uptake of I^{131}, or the discharge of radioactive hormone from the thyroid labelled with radioiodide.

1. Assays Based on the Uptake of Radioiodide

The methods using the uptake of I^{131} have not proved sufficiently sensitive to enable measurements of the level of TSH to be made in blood or urine unless these fluids have first been concentrated.

Henry (1951) in a method using guinea pigs, demonstrated a response in thyroidal uptake of I^{131} to 25 ImU of TSH after injections twice daily for 3 days.

A method using the mouse was described by Querido and co-workers (1953) in which the lowest dose of TSH detected was 20 ImU. Subsequently Querido and Lameijer (1956) improved the sensitivity of this method to 8.0 ImU and after appropriate concentration of normal human serum, levels of TSH in the range from 1.0 to 2.0 ImU/ml. were obtained.

2. Assays Based on the Discharge of Radioiodide

Discharge of radioactive hormone from the I^{131} labelled thyroid has proved to be a much more sensitive measure of TSH than any of the other parameters used.

Two groups of workers have developed an *in vivo* technique for measuring the discharge of I^{131} from the thyroid of

day-old chicks. The first group (Gilliland and Strudwick, 1956) used groups of chicks pretreated with thyroxine for three days to suppress endogenous TSH activity. Determination of the radioactivity in the thyroid, by direct counting over the thyroid region, was carried out immediately before and 48 hours after the injection of the test substance. The percentage discharge of I^{131} was used as a measure of TSH activity. The limit of sensitivity of the method was 0.15 ImU but the precision was unsatisfactory. The level of TSH in the serum of several euthyroid subjects was reported to be approximately 0.15 ImU/ml.

Bates and Cornfield (1957) found that a linear relationship existed between the logarithm of doses of TSH and the degree of thyroidal radioiodide depletion over a range from 1.5 to 15.0 ImU. Using this method Bates and co-workers (1959) estimated a level of 0.5 ImU/ml. in concentrated normal human serum.

Assays using the same function of discharge of radio-iodide by thyrotrophin, but measuring the response as an increase in radioactive iodine compounds in the blood, have proved even more sensitive.

Application of this principle to the bioassay of TSH was first made by Adams and Purves (1953; 1955) using guinea pigs. The animals were injected with radioiodide to label the thyroid glands. Endogenous TSH secretion was suppressed by treatment with thyroxine. A blood sample was taken prior to the intra-venous injection of TSH or test material and a second sample

after an interval of three hours, at which time the increase in concentration of released radioactive compounds in the blood was shown to be maximal. The response was expressed as the percentage increase in blood radioactivity.

The design of the assay was such that each animal acted as its own control. Injection of TSH and measurement of the response could be repeated in each animal on at least six consecutive days. A Latin square arrangement of treatments given to six animals on six consecutive days enabled animal variation to be eliminated during statistical evaluation of the results. The dose response was linear over the range 0.1 to 15.0 ImU. The lower limit of sensitivity was such that estimations of TSH could be made on unconcentrated serum. However, Adams and Purves (1957 a, b) were unable to detect TSH in the serum of euthyroid subjects. The TSH levels in four cases of hypothyroidism ranged from 1.0 to 2.5 ImU/ml.

McKenzie (1958) described a modification of the method of Adams and Purves using mice instead of guinea pigs. The response to an intravenous injection of 0.5 ml. of standard thyrotrophin or test substance was measured as the percentage increase in blood I^{131}. The method had a sensitivity of 0.05 ImU/ml. McKenzie (1958) detected TSH in the serum of six patients with myxoedema with a range from 0.12 to 0.64 ImU/ml. From a four point assay of concentrated normal human serum a figure of approximately 0.2 ImU/ml. of TSH was reported.

Using this bioassay technique Yamazaki and co-workers (1961) reported a similar sensitivity of 0.04 ImU/ml.

TSH detected in the sera of 16 out of 20 euthyroid subjects ranged from 0.08 to 0.18 ImU/ml.

The most sensitive of all the I^{131} discharge methods is that of Bottari and Donovan (1958) who carried out measurements with an in vitro preparation of thyroid slices from guinea pigs and obtained linear response to the logarithm of the dose over a very wide range, from 0.001 to 10 ImU. The assay had a sensitivity of 0.01 ImU for the assay of serum. The mean level of TSH in the serum of 120 euthyroid men was found to be 0.22 ImU/ml. In women during reproductive life, the level was higher, a mean value of 0.37 ImU/ml. being obtained. Further improvements in the design of the assay were reported by El Kabir in 1962.

An in vitro method without an obvious in vivo counterpart was developed by Bakke and colleagues (1957). In this procedure beef thyroid slices, incubated in Krebs-Ringer phosphate buffer, responded to the addition of thyrotrophin by an increase in weight. This weight increase was not reflected in an increase of the dry weight of the gland and was thought to be due to a proteolytic process leading to imbibition of water by the slices. Although the method had a sensitivity of 0.01 ImU it was unsatisfactory for the assay of whole serum. However, extracts of serum have been analysed successfully with the technique (Bakke et al, 1961). By this method the potency of normal rat serum was found to be 0.02 ImU/ml. and that of normal human serum only 0.002 ImU/ml. In hypothyroid rats and patients the TSH levels were elevated.

6. Estimates of Level of TSH in Normal Human Serum.

With the exception of the stasis tadpole method of D'Angelo, only those methods based on the discharge of I^{131} by TSH have sufficient sensitivity to detect the TSH activity of normal human serum, without prior concentration of the serum. These methods (D'Angelo et al, 1951; Gilliland and Strudwick, 1956; Yamazaki et al, 1961 - using the method of McKenzie, 1958 - and Bottari, 1958) all give estimates of TSH in normal human serum within a range of approximately 0.1 to 0.4 ImU/ml. In a review of the subject McKenzie (1960) concluded that the average concentration of TSH in normal human adults is of the order of 0.2 ImU/ml.

However, both Purves and Adams (1960) and Bakke and co-workers (1961) have concluded, on the basis of indirect evidence, that the level of TSH in normal human serum is much lower. Bakke and co-workers cited the evidence of Greer and Shull (1957) and Einhorn and Larsson (1959) that the human pituitary secretes between 200 and 600 ImU of TSH per day. By combining this value with the estimate of 35 minutes for the half-life of TSH in the blood of normal human subjects (Bakke et al, 1960), it was calculated that the level of TSH in the plasma of normal human subjects was from 0.003 to 0.008 ImU/ml. The value of 0.012 ImU/ml. derived by Purves and Adams (1960) by similar reasoning, was of the same order.

Neither Adams and Purves (1957 a, b) nor McKenzie (1958) using the methods they had developed which had sensitivities of 0.10 and 0.05 ImU/ml. respectively, could detect TSH in

normal human serum. Bakke and co-workers (1961) stated that several laboratories in the U.S.A., using the McKenzie or Bottari methods, have failed to detect thyrotrophic activity in normal human serum.

It is probable, therefore, that the level of TSH in normal human serum is less than 0.10 ImU/ml. and may be as low as 0.01 ImU/ml.

7. Estimates of the Level of TSH in the Serum of Rats

There have been few estimates of the level of TSH in the serum of normal rats and these vary considerably. Del Conte and Stux (1955) using their assay based on cytological changes in the guinea pig thyroid reported values ranging from 0.004 to 0.012 ImU/ml. However, D'Angelo, using the stasis tadpole method, claimed concentrations of 0.6 to 1.0 ImU/ml. (D'Angelo, 1955, 1960; D'Angelo and Traum, 1958). This was regarded as being too high by Granner, Curtis and Halmi (1961) who demonstrated that the T/S ratio of hypophysectomised rats was not increased by the injection of 3 ml. of normal rat plasma, whereas it did respond to the injection of 1.5 ImU of TSH; this amount of TSH should be present in 3 ml. of normal rat plasma if D'Angelo's value was correct.

Neither Adams and Purves (1955) nor Jagiello and McKenzie (1960) were able to elicit a response to 0.5 ml. of rat serum in their assays which were sensitive to 0.1 and 0.05 ImU TSH respectively. In several determinations of fractionated pooled normal rat serum Bakke and co-workers (1961) derived an average potency of 0.02 ImU per ml. Therefore it would appear that

the level of TSH in normal rat serum is less than 0.1 ImU/ml.

Estimates of the level of TSH in the serum of rats made hypothyroid, either by a low iodine diet or by propylthiouracil treatment have been reported by several workers. Adams and Purves (1955) reported a value of 0.4 ImU/ml. for normal rats maintained on a low iodine diet for six months. Values of approximately 1.3 to 2.4 ImU/ml. have been reported following propylthiouracil treatment (D'Angelo, 1955, 1960). Similar levels were reported by Bakke et al (1961).

Levels of TSH as high as 5.5 ImU/ml. in the serum of thyroidectomised rats have been reported by Adams and Purves (1955) and Adams (1958).

Conclusions

In order to confirm the indirect evidence of the depression in circulating TSH produced by the administration of salicylate and related drugs, it was necessary to choose a sensitive and precise bioassay method, capable of estimating TSH in normal human or rat plasma. It is apparent from the preceding description of the many methods available, that few of them met these criteria. Moreover, the level of TSH in normal man and rats is still a controversial matter.

However, certain of the assays were capable of estimating the elevated levels of TSH in the plasma of thyroidectomised rats. The method of McKenzie (1958) was suitable for this purpose since it was sensitive to 0.05 ImU/ml. and had a degree of precision sufficient for its use in quantitative

work. The method had the further advantages of being relatively simple and rapid.

It was decided therefore to investigate the bioassay method of McKenzie (1958) as a preliminary to using it in the examination of the effect of salicylate and related drugs on circulating TSH in thyroidectomized rats.

PART II

THE BIOASSAY OF TSH BY A MODIFICATION OF THE METHOD OF McKENZIE

Materials and Methods

Preparation of the Mice.

Albino mice were bred in the laboratory specifically for use in the assay. After weaning at four weeks of age they were fed on a diet of commercial dog biscuit of low iodine content (100 µg. I per Kg.) until they were used experimentally at approximately ten weeks of age at a weight of 18 to 24 g.

Radioactive iodide (KI^{131}), 5 to 6 microcuries per mouse, was injected intraperitoneally. This quantity of I^{131} was found to produce a maximal specific activity within the thyroid gland without causing radiation damage within the period of the assay.

Endogenous secretion of thyrotrophin was suppressed by the subcutaneous injection of 10 µg. of l-thyroxine immediately after the radioiodide injection, and by the addition of desiccated thyroid extract (0.1% W/V) to the drinking water for the period of the assay.

The mice were numbered and distributed by random selection into groups. These groups were then allotted the chosen treatments at random.

The animals were used in the assay procedure four days later by which time they had reached equilibrium with the injected radioiodide, by thyroidal uptake and excretion of the excess.

Standard TSH Solutions.

The standard solutions of TSH were freshly prepared immediately before use each day. The requisite weight of International Standard Thyrotrophin was dissolved in a 1% solution of human serum in normal saline. The appropriate dilutions were made with 1% human serum in normal saline. The denaturation of the dilute solutions of TSH by surface activation of the glassware was prevented by the presence of the serum proteins. All glassware used for preparing the standard solutions was kept separate from the laboratory stocks and washed thoroughly before use.

Injection of the Test Materials into the Mice.

The doses of standard TSH and test samples were injected into the tail veins dilated by heating the mice at 30°C for 30 minutes.

Removal of Blood Samples from the Mice.

In the preliminary experiments blood was removed by puncture of the dilated tail veins with a 25 gauge hypodermic needle. The blood which welled out onto the skin surface was collected with a small pipette.

In the later experiments blood taking was greatly simplified by application of the technique of eye puncture. A Pasteur pipette drawn out into a fine capillary was inserted into the retro-orbital venous sinus. On puncture of the sinus, blood flowed rapidly into the pipette and was transferred to a small container.

Plating of the Blood Samples.

0.10 ml. samples of whole blood were plated onto aluminium planchettes for counting. Uniform distribution of the blood sample was achieved by the use of a lens paper disc cut to fit the planchette accurately, and a surface active agent, sodium lauryl sulphate, 0.05 ml. of which was used to saturate the paper disc. The blood sample, pipetted on to the lens paper, spread rapidly and evenly over the surface; this was then dried slowly under an infra red lamp.

Counting of the Blood Samples.

The radioactivity content of the samples was measured using an open window gas-flow counter (Nuclear Chicago Model D-47) with automatic sample changer and printing-timer attached and the time to record 1000 counts was measured for each sample, ensuring a constant probable error of counting of 3%.

Preliminary Experiments

In the preliminary experiments blood was obtained by puncture of the tail veins. This procedure was tedious and so time consuming that it restricted the number of animals which could be treated in each trial. As the tail veins were used for the injection of the test substance and also for the removal of the blood samples both before and after treatment, they often became damaged and constricted making it difficult to use the mice on more than one day. Responses were therefore obtained from single day trials using groups of five mice for each treatment.

The dose response line was obtained by plotting the percentage increase in the count rate after 3 hours for each treatment against the logarithm of the dose. In a series of such trials using a range of dosage from 0.10 to 1.0 ImU TSH, the dose response lines were shown to be highly variable. The standard errors of the individual doses were large and overlapped considerably, due to the variation in responsiveness from animal to animal, making a precise estimate of TSH impossible. Other than by using large numbers of animals to achieve sufficient accuracy it became necessary to measure a succession of responses from each animal on consecutive days in order to eliminate the animal variation.

By employing the technique of eye puncture for blood sampling it became possible to use the mice on three consecutive days, the tail veins then being used only for the injection of the test materials. This permitted a latin square arrangement of treatments.

Each group of six mice received daily doses of three concentrations of standard thyrotrophin on three consecutive days. It was found that the response of the mice on the second and third days to a given dose was affected by the magnitude of the dose given on the previous day or days; the larger the dose on the first day, the smaller was the response on the second day to the same dose. Thus removal of the animal variation revealed the presence of a residual effect to previous treatment, which modified the response. However, the latin square design was incomplete for all the possible

combinations of the residual effect and so could not be analysed for this component.

Although reasonable results could be obtained from this design it was considered that if the factors of animal variation and residual effect could be fully eliminated in a design a more precise assay would result.

A design balanced for residual effects was therefore used.

Modification of the Experimental Design

A two day trial using a design completely balanced for residual effects was used.

Day 1. n treatments were applied to n^2 animals divided at random into n groups with n animals in each. The responses were measured.

Day 2. The n animals which received the same treatment, say Treatment 1, on day 1 received the n different treatments allotted at random, on day 2. See Table 6 for a specific example of the design.

Let

m be the mean response

t_i be the deviation from the mean due to the ith treatment

$i = 1, 2 \ldots\ldots n, \ \Sigma t_i = 0$

a_j be the deviation from the mean due to the jth animal,

$j = 1, 2 \ldots\ldots n^2, \ \Sigma a_j = 0$

d_k be the deviation from the mean due to the kth day,

$k = 1, 2, \ \Sigma d_k = 0$

r_i be the deviation from the mean due to the residual effect of the ith treatment

$\sum_{i=1}^{n} r_i = 0$ and this applies to day 2 only.

So the model for the first day, t_i applied to a_j, is

$$y_{ij1} = m + t_i + a_j + d_1 + e_{ij1}$$

where the errors e_{ijk} are normally and independently distributed $(0, \sigma^2)$,

and for the second day, t_l applied to a_j, is

$$y_{lj2} = m + t_l + a_j + r_i + d_2 + e_{lj2}$$

and the difference is

$$D_{il} = (y_{ij1} - y_{lj2}) = t_i - t_l - r_i + d_1 - d_2 + e_{il}$$

Summing over the n animals which had t_i on day 1

$$\sum_{l=1}^{n} D_{il} = n(t_i - r_i) + n(d_1 - d_2) + \Sigma e$$

Summing over the n animals which had t_l on day 2

$$\sum_{l=1}^{n} D_{il} = -n\,t_l + n(d_1 - d_2) + \Sigma e$$

$$= -n\,t_l + 2\,nd_1 + \Sigma e \qquad \text{since } d_1 = -\,d_2$$

whence may be obtained unbiased estimates of the treatment effects free of residual effects. See Table 7.

1

TABLE 6

BIOASSAY DESIGN BALANCED FOR RESIDUAL EFFECTS OF TREATMENTS

Mouse No.	Day 1 Treatment ImU of TSH	Day 2 Treatment ImU of TSH
14	0.10	0.10
15	0.10	0.20
2	0.10	0.40
9	0.10	0.80
3	0.20	0.10
11	0.20	0.20
16	0.20	0.40
13	0.20	0.80
8	0.40	0.10
12	0.40	0.20
5	0.40	0.40
4	0.40	0.80
10	0.80	0.10
1	0.80	0.20
7	0.80	0.40
6	0.80	0.80

TABLE 7

MODEL OF EXPERIMENTAL DESIGN

The response of a mouse on Day 1 to treatment (i) is compounded of the following factors:

$$m + a_j + t_i + d_1 + e_{ij1}$$

The response of the same mouse on Day 2 to treatment (1) is compounded of the following factors:

$$m + a_j + t_1 + r_i + d_2 + e_{1j2}$$

Thus if the difference of a mouse's responses on Day 1 and Day 2 is denoted by D_{i1} then:

$$D_{i1} = t_i - t_1 - r_1 + 2d_1 + e_{i1}$$

				Row Totals
D_{11}	D_{21}	D_{31}	D_{41}	$-4t_1+8d_1$
D_{12}	D_{22}	D_{32}	D_{42}	$-4t_2+8d_1$
D_{13}	D_{23}	D_{33}	D_{43}	$-4t_3+8d_1$
D_{14}	D_{24}	D_{34}	D_{44}	$-4t_4+8d_1$
Column Totals				
$4t_1-4r_1+8d_1$	$4t_2-4r_2+8d_1$	$4t_3-4r_3+8d_1$	$4t_4-4r_4+8d_1$	$32d_1$

Hence after removal of the day effect, unbiased estimates of the treatment effects are obtained from the row totals.

Results

The analysis of the data obtained from an assay in which five doses of TSH (0.05 to 0.80 ImU) were used and from which a dose-response curve was derived will now be presented as a typical example of the application of the model.

On the first day 25 mice, prepared as described in the methods section, were distributed at random into 5 groups which were allotted treatments of 0.05, 0.10, 0.20, 0.40 and 0.80 ImU TSH at random. The time was recorded when the TSH was injected into these animals and three hours later, blood samples were removed by eye-puncture.

On the second day the mice from each group were allotted the five treatments at random and following injection of the standard doses, blood samples were again removed at three hours. The radioactive iodide content of the blood samples was measured.

The three-hour blood count rates obtained are set out in Table 8. The logarithmic transformation of the count rate was made and the differences in the logarithms of the three-hour count rates (Day 1 - Day 2) for each mouse determined.

The differences between the logarithms of the three-hour blood count rates for each group were tabulated as presented in Table 9 and the analysis of variance in Table 10. The variation due to treatments, unadjusted residual effects (confounded with treatment effects) and error was determined. The analysis revealed that the treatment effects were highly significant ($P < .001$).

TABLE 8

APPLICATION OF ASSAY DESIGN TO THE DETERMINATION OF A STANDARD DOSE RESPONSE CURVE OVER THE RANGE 0.05 TO 0.80 ImU TSH

Mouse No.	DAY 1			DAY 2			Log Difference Day 1-Day 2
	Treatment ImU TSH	3 Hour Count Rate C/300 sec/0.1 ml.	Log 3 Hour Count Rate	Treatment ImU TSH	3 Hour Count Rate C/300 sec/0.1 ml.	Log 3 Hour Count Rate	
Group 1							
17	0.05	1840	3.26482	.05	1160	3.06446	.20036
31	0.05	1100	3.04139	.10	1000	3.00000	.04139
32	0.05	1950	3.29003	.20	2560	3.40824	-.11821
7	0.05	980	2.99123	.40	2180	3.33846	-.34723
16	0.05	2120	3.32634	.80	3840	3.58433	-.25799
Group 2							
26	0.10	1170	3.06819	.05	660	2.81954	.24865
2	0.10	1470	3.16732	.10	1160	3.06446	.10286
4	0.10	2500	3.39794	.20	3400	3.53148	-.13354
12	0.10	1970	3.29447	.40	2730	3.43616	-.14169
10	0.10	2460	3.39094	.80	4760	3.67761	-.28667
Group 3							
15	0.20	1320	3.12057	.05	670	2.82607	.29450
19	0.20	1650	3.21748	.10	1100	3.04139	.17609
37	0.20	2080	3.31806	.20	2010	3.30320	.01486
3	0.20	1460	3.16435	.40	1530	3.18469	-.02034
27	0.20	1850	3.26717	.80	2670	3.42651	-.15934
Group 4							
25	0.40	2320	3.36549	.05	1120	3.04922	.31627
34	0.40	3560	3.55145	.10	2030	3.30750	.24395
38	0.40	2410	3.38202	.20	1870	3.27184	.11018
8	0.40	1880	3.27416	.40	1800	3.25527	.01889
36	0.40	2700	3.43136	.80	3520	3.54654	-.11518
Group 5							
6	0.80	3730	3.57171	.05	2140	3.33041	.24130
39	0.80	3030	3.48144	.10	1900	3.27875	.20269
30	0.80	4020	3.60423	.20	2700	3.43136	.17287
24	0.80	3790	3.57864	.40	3060	3.48572	.09292
29	0.80	1990	3.29885	.80	1630	3.21219	.08666

TABLE 9

ASSESSMENT OF TREATMENT EFFECTS FROM THE DATA OBTAINED FROM TABLE 8

Differences in Log 3 hr. Count Rates					Row Totals
Group 1	Group 2	Group 3	Group 4	Group 5	
.20036	.24865	.29450	.31627	.24130	1.30108= $-5t_1+10d$
.04139	.10286	.17609	.24395	.20269	.76698= $-5t_2+10d$
-.11821	-.13354	.01486	.11018	.17287	.04616= $-5t_3+10d$
-.34723	-.14169	-.02034	.01889	.09292	-.39745= $-5t_4+10d$
-.25799	-.28667	-.15934	-.11518	.08666	-.73252= $-5t_5+10d$
-.48168	-.21039	.30577	.57411	.79644	.98425 50d

since $\Sigma t=0$

Σ Row Totals = Day Variation

TABLE 10

ANALYSIS OF THE SET OF DATA PRESENTED IN TABLE 9

Analysis of Variance

CF = 0.038750

Variation due to:-	d.f.	S.S.	M.S.	Variance Ratio	P
Treatments	4	0.556800	0.139200	25.80	$<.001$
(Regression	1	0.543639	0.543639	100.77	$<.001$)
(Deviations	3	0.013161	0.004387	<1.0	N.S.)
Residuals (unadjusted)	4	0.227989	0.056997	10.56	$<.001$
Error	16	0.086315	0.005395		
Totals	24	0.871104			

$s = 0.07345$

Data from row totals	Estimate after removal of day variation ImU TSH	Estimate from regression ImU TSH
$5t_1 - 10d_1 = -1.30108$	$0.05 = t_1 = -0.22085$	$0.05 = t_1 = -0.20926$
$5t_2 - 10d_1 = -0.76698$	$0.10 = t_2 = -0.11403$	$0.10 = t_2 = -0.10463$
$5t_3 - 10d_1 = -0.04616$	$0.20 = t_3 = 0.03014$	$0.20 = t_3 = 0$
$5t_4 - 10d_1 = 0.39745$	$0.40 = t_4 = 0.11886$	$0.40 = t_4 = 0.10463$
$5t_5 - 10d_1 = 0.73252$	$0.80 = t_5 = 0.18587$	$0.80 = t_5 = 0.20926$
Total $= -0.98425$		
Since $\Sigma t = 0$ Row total=day variation		

$b_{(I)}$ (slope in terms of log dose interval) = 0.34758

$\therefore$ Index of precision $\lambda = \dfrac{s}{b_{(I)}} = \dfrac{.07345}{.34758} = .211$

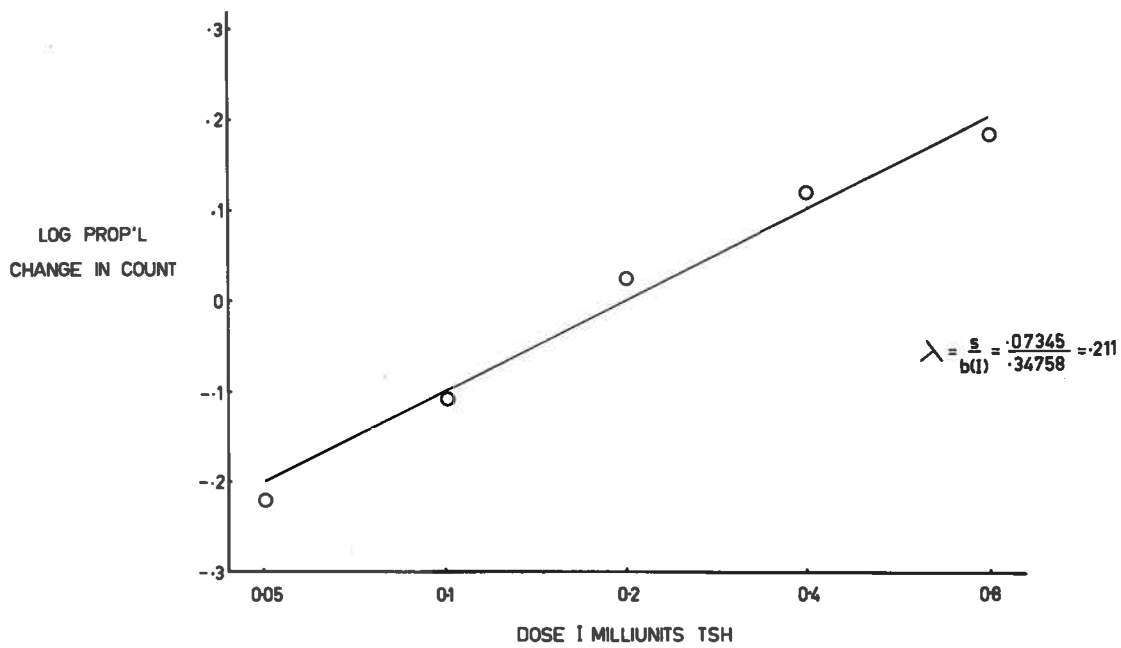

FIGURE 1.

In the description of the model it was shown that each row total comprised both treatment effect and day effect. However, since $\Sigma\ t = 0$, the grand total was equivalent to the sum of the day effects. The data obtained from the row totals were therefore corrected by removal of the day variation and unbiased values for the treatments were obtained (Table 10).

A regression analysis was carried out and estimates of the treatment effects were thus derived. (Table 10)

The dose response curve was obtained by plotting the treatment response expressed as the logarithm of the proportional change in count against the logarithm of the dose in International milliunits (ImU) of TSH. (Figure 1)

Analysis of variance revealed that there was no significant deviation from linearity over the range 0.05 to 0.80 ImU of TSH.

An index of precision $\lambda = \frac{s}{b_{(I)}} = \frac{.07345}{.34758} = .211$ was obtained.

In order to assess the effectiveness of the logarithmic transformation of the three-hour count rate described, a further assay was performed in which both control and three-hour blood samples were collected. The responses from this assay of TSH over the range 0.05 to 0.40 ImU were expressed in four ways:

1. as the difference (Day 1-Day 2) in the percentage increase of the three-hour blood count rate compared to the control blood count rate;
2. as the difference (Day 1-Day 2) in the three-hour count rates;

3. as the difference (Day 1-Day 2) in the square root transformation of the three-hour blood count rates;
4. as the difference (Day 1-Day 2) in the logarithmic transformation of the three-hour blood count rates.

Analyses of variance were carried out on the results from each of these methods of expression of the responses. (Table 11)

The analyses revealed that the highest significance ($P < .001$) for the treatment effect was obtained with transformations of the raw count rate data. The variance ratio was greater with the logarithmic transformation. The treatment effect using the raw three-hour blood count rates was significant ($P < .01$) whereas that obtained with the percentage increase was less significant ($P < .05$). It was proposed therefore, to obtain only three-hour blood samples in all subsequent assays and to express the responses as the difference (Day 1 - Day 2) in the logarithm of the three-hour blood count rates.

Since a high degree of precision was obtained over the range 0.05 to 0.80 ImU of TSH, it was decided to assess the precision of the assay over a lower dose range (0.0125 to 0.20 ImU). The same assay procedure was followed in which five groups of mice were treated with 0.0125, 0.025, 0.05, 0.10 and 0.20 ImU of TSH.

The differences between the logarithms of the three-hour blood count rates obtained from the assay are set out in Table 12. Analysis of variance (Table 13) revealed a high

TABLE 11

ANALYSES OF VARIANCE OF THE DATA FROM A TRIAL USING DIFFERENT TRANSFORMATIONS

Analysis of:	Variation due to:	d.f.	S.S.	M.S.	Variance Ratio	P
Day 1-Day 2 Percentage Increase	Treatments	4	150563.36	37640.84	4.29	< .05
	Residuals (unadjusted)	4	282101.36	70525.34	8.03	<.001
	Error	16	140488.24	8780.52		
	Totals	24	573152.96			
Day 1-Day 2 3 Hour Count rates	Treatments	4	168661.44	42165.36	6.64	<.01
	Residuals (unadjusted)	4	465043.84	116260.96	18.31	<.001
	Error	16	101572.16	6348.26		
	Totals	24	735277.44			
Day 1-Day 2 $\sqrt{\text{3 hour Count rates}}$	Treatments	4	196.7312	49.1828	13.04	<.001
	Residuals (unadjusted)	4	427.5293	106.8823	28.34	<.001
	Error	16	69.3359	3.7710		
	Totals	24	684.5964			
Day 1-Day 2 log 3 hour Count rates	Treatments	4	0.7776	0.1944	16.90	<.001
	Residuals (unadjusted)	4	1.3681	0.3420	29.74	<.001
	Error	16	0.1847	0.0115		
	Totals	24	2.3304			

TABLE 12

THE DETERMINATION OF A STANDARD DOSE RESPONSE CURVE OVER THE RANGE 0.0125 TO 0.20 ImU TSH

Treatment	Differences in Log 3 hr. Count Rates					Row Totals
	Group 1	Group 2	Group 3	Group 4	Group 5	
.0125 mU	-.03386	.20856	.25884	.45092	.66560	1.55006
.025 mU	-.05303	.06695	.28235	.40627	.53737	1.23991
.05 mU	-.25062	.00256	.22140	.27719	.29969	.55022
.10 mU	-.37891	-.00496	.02501	-.00621	.22640	-.13867
.20 mU	-.43739	-.17328	-.10421	-.02110	.18074	-.55524
	-1.15381	.09983	.68339	1.10707	1.90980	2.64628

TABLE 13

ANALYSIS OF THE SET OF DATA PRESENTED IN TABLE 12

Analysis of Variance

Variation due to:	d.f.	S.S.	M.S.	Variance Ratio	P
Treatments	4	.633953	.158488	37.32	< .001
(Regression	1	.624779	.624779	147.1	.001)
(Deviations	3	.009174	.003058	< 1.0	N.S.)
Residuals (unadjusted)	4	1.056129	.264032	62.17	< .001
Error	16	.067947	.004247		
Totals	24	1.758029			

Data from row totals	Estimate of Treatment effect after removal of day variation ImU TSH	Estimate of treatment effect from regression ImU TSH
$5t_1 - 10d_1 = -1.55006$	$.0125 = t_1 = -.20416$	$.0125 = t_1 = -.22357$
$5t_2 - 10d_1 = -1.23991$	$.025 = t_2 = -.14093$	$.025 = t_2 = -.11178$
$5t_3 - 10d_1 = -0.55022$	$.05 = t_3 = -.00419$	$.05 = t_3 = 0$
$5t_4 - 10d_1 = 0.13867$	$.10 = t_4 = +.13359$	$.10 = t_4 = +.11178$
$5t_5 - 10d_1 = 0.55524$	$.20 = t_5 = +.21690$	$.20 = t_5 = +.22357$
Total = -2.64628		
Since $\Sigma t = 0$ Row Total = Day Variation		

$b_{(I)} = .37134$ $S = .06517$ $\lambda = .176$

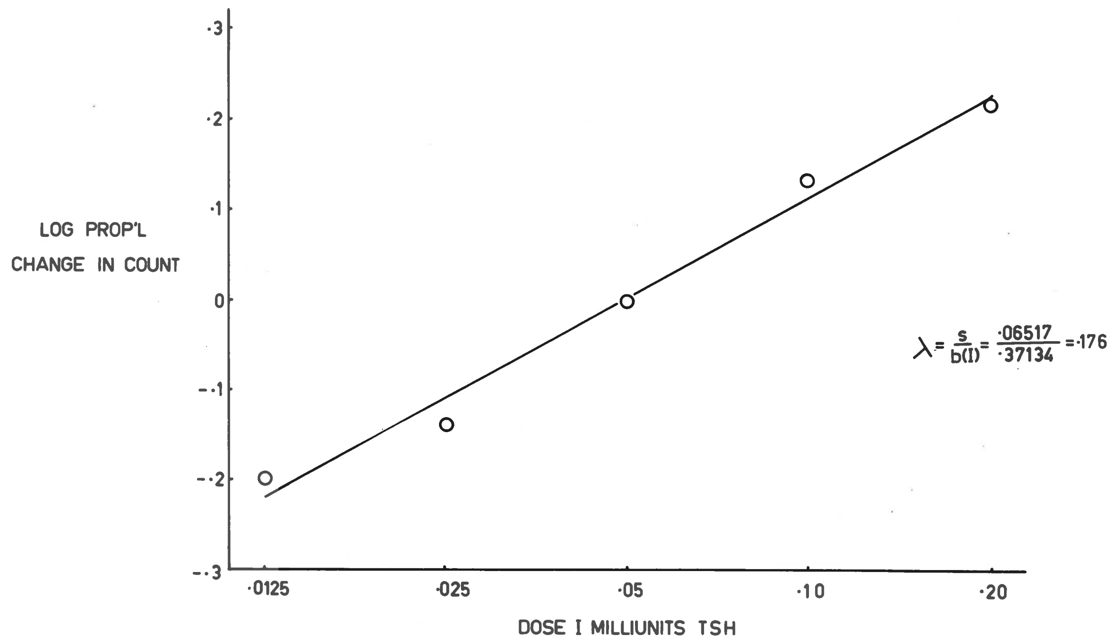

FIGURE 2.

significance for the effects of treatment ($P < .001$).

The data from the row totals were corrected by removal of the day effect and unbiased values for the treatments obtained; regression analysis provided estimates of the treatment effects (Table 13). Analysis of variance showed that there was no significant deviation from linearity over the range 0.0125 to 0.20 ImU.

The dose response curve derived from these data is presented in Figure 2.

An index of precision $\lambda = \frac{s}{b_{(I)}} = \frac{.06547}{.37134} = .176$ was obtained.

These results indicate that the assay design was effective over a wide range of doses with considerable precision and a sensitivity of 0.0125 ImU (0.025 ImU/ml.). Standard dose response curves were obtained at regular intervals throughout the period the assay was used. An average index of precision, $\lambda = .221$, was obtained from nine standard dose response curves over a range of doses of TSH from 0.0125 to 0.80 ImU of TSH. The indices of precision of these assays are listed in Table 14.

<u>Application of the Method to the Estimation of TSH in Rat Plasma</u>

In order to assess the specificity of the assay in estimating TSH in rat plasma compared to International Standard TSH (derived from bovine pituitaries) it was necessary to carry out a four point assay as a check on the parallelism of the dose response lines to standard TSH and rat TSH.

A sample of plasma was obtained from a rat thyroidectomised

TABLE 14

PERFORMANCE OF THE ASSAY OVER A RANGE OF DOSES OF STANDARD TSH FROM 0.0125 TO 0.80 ImU DURING A PERIOD OF 18 MONTHS

Assay No.	s	$b_{(I)}$	$\frac{s}{b_{(I)}} = \lambda$
1	0.0850	.4513	.188
2	0.1072	.3513	.305
3	0.0716	.2971	.241
4	0.1199	.3230	.371
5	0.0735	.3476	.211
6	0.0531	.5278	.101
7	0.0924	.4482	.206
8	0.0922	.4896	.188
9	0.0652	.3713	.176
Mean			.221

Average Index of Precision for 9 Standard Dose Response Curves = .221

eight weeks previously and then left untreated so that the circulating TSH would be increased.

Two aliquots of plasma (A and B) were diluted with saline in the ratios 1:3 (A) and 1:15 (B) such that the ratio of the concentration of TSH in A:B = 4:1. Standard TSH doses of 0.0375 and 0.15 ImU (1:4) were chosen to span the range of TSH expected in the diluted rat plasma.

The results of the assay are presented in Table 15 in which the effects of the treatments were shown to be highly significant ($P < .001$) by analysis of variance. There was no significant difference between the slopes of the response lines for standard TSH and rat plasma, that is, there was no significant deviation from parallelism.

The index of precision for the assay was 0.147.

Estimates of TSH in the two samples were obtained from the results as described in Table 16.

Sample A contained 0.19 ImU TSH, and

Sample B contained 0.06 ImU TSH, giving a ratio of A:B of 3.2:1 which is in good agreement with the expected ratio of 4:1.

Since 0.5 ml. diluted plasma samples were injected into the mice correction factors were applied to convert the estimate into ImU/ml.

The concentration of TSH in the whole serum in the estimate from sample A = .19x2x4 = 1.52 ImU/ml.

and from sample B = .06x2x16 = 1.92 ImU/ml.

TABLE 15

FOUR POINT ASSAY OF TSH IN THE PLASMA OF A THYROIDECTOMIZED RAT DILUTED WITH SALINE

SAMPLE A Rat plasma:saline = 1:3
SAMPLE B Rat plasma:saline = 1:15

Ratio of concentration of plasma A:B = 4:1
Ratio of concentration of standard doses TSH .15:.0375 = 4:1

DATA DERIVED FROM DIFFERENCES (DAY 1 - DAY 2) in LOG 3 HOUR COUNT RATES

.0375 ImU	.30475	.06521	.39425	.10679	.87100
.15 ImU	.13042	-.15415	.12424	-.04588	.05463
A	.11963	-.21971	.12421	-.10750	-.08337
B	.24265	.04806	.19304	.08715	.57090
	.79745	-.26059	.83574	.04056	1.41316

Analysis of Variance

Variation due to:	d.f.	S.S.	M.S.	Variance Ratio	P
Treatments	3	.1488188	.04960396	24.67	<.001
Residuals (unadjusted)	3	.2261721	.07539040	37.50	<.001
Error	9	.01809386	.00201043		
	15	.39307695			

Variation due to:	d.f.	S.S.	M.S.	Variance Ratio	P
Average slope	1	.13517387			
Means	1	.01199573			
Slopes	1	.00164228		< 1.0	N.S.
	3	.1488188			

$b_{(I)}$ = .3054   s = .0448 λ = .147

No significant difference between slopes.

TABLE 16

DETERMINATION OF LEVEL OF TSH IN RAT PLASMA

Sample		Log proportional change in count	
x_1	.0375	-.87100	y_1
x_2	.15	-.05463	y_2
x_3	A	+.08337	y_3
x_4	B	-.57090	y_4

Since $b = \dfrac{x_2 - x_1}{y_2 - y_1}$

$$x_2 = x_1 + b(y_2 - y_1)$$

and $$x_3 = x_1 + b(y_3 - y_1)$$

$$x_4 = x_1 + b(y_4 - y_1)$$

For Sample A

$$b = \frac{\log 0.15 - \log .0375}{-.05463 + .87100} = \frac{.60206}{.81637} = .73748$$

$$x_3 = \log(.0375 \times 1000) + .73748(.08337 + .87100)$$
$$= 1.5740 + .73748 \times .95437$$
$$= 2.2778$$

take antilog

= 190.0 micro units

= .19 milliunits TSH

Sample A = 0.19 ImU TSH

By substitution for x_4

Sample B = 0.06 ImU TSH

Ratio of A:B observed = 3.2:1
Ratio of A:B expected = 4:1
Since injection volume = 0.5 ml. and
A = 1/4 original plasma concentration
B = 1/16 original plasma concentration
Concentration of TSH in whole plasma
A = .19 x 2 x 4 = 1.52 ImU/ml.
B = .06 x 2 x 16 = 1.92 ImU/ml.

This assay indicates that the method can be used for the estimation of TSH in rat plasma with high precision and specificity.

Estimations of TSH in the plasma of three rats thyroidectomized eight weeks previously were also carried out. Since from the previous result a value of 2.0 ImU of TSH could be expected the plasma was diluted with saline in the ratio of 1:2. The three diluted plasma samples were assayed in the same balanced trial with doses of TSH of 0.30 and 0.60 ImU which were expected to span that in the plasma samples.

The results are presented in Table 17.

The analysis of variance revealed that there was no significant effect of treatment. However, as the levels of TSH in the plasma samples were similar to the standard doses this result was to be expected.

The index of precision (λ = .334) for the assay was high and it was therefore considered undesirable to estimate any more than two unknowns with two standard doses in future assays. Nevertheless estimates of TSH were obtained and the correction factors applied for injection volume and dilution.

Thyroidectomized Rat 1 = .51 x 6 = 3.01 ImU/ml.

Thyroidectomized Rat 2 = .42 x 6 = 2.52 ImU/ml.

Thyroidectomized Rat 3 = .42 x 6 = 2.52 ImU/ml.

TABLE 17

ESTIMATION OF TSH IN PLASMA OF THYROIDECTOMIZED RATS

Treatment	Differences in log 3 hour Count Rates					Row Totals
	Group 1	Group 2	Group 3	Group 4	Group 5	
0.30 mU	.05497	.27927	.32943	.01208	.20891	.88466
0.60 mU	.12178	.06989	.04363	.06418	.18350	.48298
Rat 1	.14259	.27394	.09574	-.05679	.11885	.57433
Rat 2	.26549	.19569	-.02770	.04773	.20228	.68349
Rat 3	.16344	.14982	-.02649	.29463	.10474	.68614
	.74827	.96861	.41461	.36183	.81828	3.31160

Analysis of Variance

Variation due to:	d.f.	S.S.	M.S.	Variance Ratio	P
Treatments	4	.018071	.004518	<1.0	N.S.
Residuals (unadjusted)	4	.055436	.013859	1.09	N.S.
Error	16	.202833	.012677		
Total	24				

$b_{(I)}$ = .3373 S = .1126 λ =.334

Estimate of TSH in Rat Plasma

Injection volume 0.5 ml.
2.0 ml. plasma diluted with saline to 6.0 ml.

Thyroidectomized Rat 1 = .51 x 6 = 3.01 ImU/Ml.
Thyroidectomized Rat 2 = .42 x 6 = 2.52 ImU/ml.
Thyroidectomized Rat 3 = .42 x 6 = 2.52 ImU/ml.

Conclusions

Application of these modifications to the design of the assay has resulted in increased precision and sensitivity compared to the method of McKenzie (1958).

McKenzie (1958) reported a sensitivity of .025 with an index of precision of .24. The modified method resulted in a sensitivity of 0.0125 ImU with an index of precision of .221.

Studies of the TSH in the plasma of a small series of rats thyroidectomized eight weeks previously, indicated values of 1.5 to 3.0 ImU/ml; these confirmed the findings of D'Angelo (1955, 1960) and Adams and Purves (1955) that TSH is raised in thyroidectomized rats.

It was considered therefore that the assay was sufficiently precise and sensitive to assess the effect of salicylate and related drugs on the circulating level of thyrotrophin in thyroidectomized rats.

PART III

STUDIES OF THE EFFECT OF SALICYLATE AND RELATED DRUGS ON CIRCULATING TSH IN RATS

1. The Effect of Salicylate on Circulating TSH in Thyroidectomised Rats

Three experiments were performed to assess the effect of sodium salicylate on the circulating TSH in thyroidectomised rats. Two groups of rats were used in each experiment. One group received sodium salicylate; the other was treated with sodium p-hydroxybenzoate as a control for the experimental procedure.

Materials and Methods

The thyroids were excised from male rats weighing approximately 175 g. which were then left without thyroid replacement therapy to allow circulating TSH to rise. The experiments were carried out two months later, by which time the TSH in the plasma was expected to be elevated to about 2.0 ImU/ml.

Since 12.0 ml. of whole blood would be required to determine both plasma PBI and TSH levels, it was necessary to carry out the experiments on blood pooled from groups of 4 rats to obtain sufficient blood. Control blood samples of 3.0 ml. were obtained from the rats by cardiac puncture.

The treatments were given seven days after sampling, when the animals had recovered from the effects of the initial bleeding.

In each experiment, one group of four rats was treated

with sodium salicylate and the other with sodium p-hydroxy-benzoate. The treatments were administered by gastric gavage twice daily with a total of 30 mg./100 g. body weight/day for two days. The rats were exsanguinated four hours after the last dose was given.

Plasma PBI was determined by the method of Acland (1958) and plasma salicylate by the method of Trinder (1954).

Plasma PBI estimations were made on the individual samples from each rat. Because of the larger volume of plasma required for TSH assay the determinations were made on pooled samples obtained by mixing equal volumes of plasma from each of the rats in the group. Pooled samples were prepared from the control plasma and also from the plasma obtained following treatment with each of the drugs.

Plasma TSH was estimated using the bioassay method described in the previous section. Because of the high TSH content expected the pooled samples were diluted with an equal volume of normal saline. The samples relating to each group, both before and after treatment, were assayed together with two standard doses of 0.15 and 0.60 ImU TSH. It was expected that the TSH content of the plasma samples would lie between that of the two standard doses. This design was, in effect, a modified three point assay, with two unknown samples instead of the usual one. The control and treated samples were assayed together in this way so that an analysis of variance could be made to assess whether the estimates of TSH were significantly different.

Results

The results of the three experiments are presented in Table 18. As a result of thyroidectomy without replacement therapy the mean PBI value of 1.8 μg. I% for all control samples was lower than the mean value of 3.5 μg. I% for normal rats obtained in the experiments reported in Chapter I.

There was no change in the mean PBI following sodium p-hydroxybenzoate administration whereas treatment with sodium salicylate depressed the plasma PBI from a mean control value of 1.7 to 1.0 μg. I%.

The TSH estimates from the pooled samples for each experiment are also presented in Table 18. There was a slight fall in TSH from a mean value for the three experiments of 1.63 to 1.41 ImU/ml. following sodium p-hydroxybenzoate. However, a much greater depression in TSH resulted from sodium salicylate administration, the mean control value for the three experiments of 2.19 ImU per ml. was depressed to 1.07 ImU per ml. when the mean plasma salicylate was 51.6 mg. per 100 ml.

Analysis of variance revealed no significant difference between control and treated samples with either sodium p-hydroxybenzoate or sodium salicylate. Paired "t" tests on the results of the three experiments using each drug also failed to show significant depression in TSH following salicylate. This result was not surprising in view of the small number of experiments.

TABLE 18

THE EFFECT OF SALICYLATE ON CIRCULATING TSH IN THYROIDECTOMIZED RATS

Expt.	Treatment	PBI µg. I %		TSH ImU/ml.		P	Index of Precision of Bio-assay	Salicylate mg.%
		Before Treatment	Treated 52 hrs.	Before Treatment	Treated 52 hrs.			
1	Sodium p-hydroxy benzoate 30 mg./100g./day	2.1	2.0	1.68	1.16	NS*	.26	
2		1.7	1.6	1.80	1.68	NS*	.30	
3		1.7	1.7	1.40	1.40	NS*	.21	
Mean		1.8	1.8	1.63	1.41	NS+		
1	Sodium salicylate 30 mg./100g./day	1.7	0.8	2.00	0.81	NS*	.29	58.0
2		1.5	0.9	2.80	1.36	NS*	.36	48.0
3		1.8	1.3	1.76	1.04	NS*	.18	48.8
Mean		1.7	1.0	2.19	1.07	NS+		51.6

* Probability of difference using analysis of variance

+ Probability of difference using paired "t" test

Discussion

Although statistical significance was not attained, it seems likely that the circulating TSH in thyroidectomized rats was depressed following sodium salicylate administration, whereas sodium p-hydroxybenzoate had practically no effect. This interpretation would be consistent with the findings of Wolff and Austen (1958) who demonstrated that although salicylate caused a reduction in goitre formation in PTU treated rats, goitre prevention was not complete and therefore TSH depression was only partial.

However, since the pituitaries of the rats had been maximally stimulated to produce TSH for two months following thyroidectomy, another possibility was that pituitary TSH might be more resistant to depression in these than in normal rats. As the assay, with a sensitivity of 0.0125 ImU (0.025 ImU/ml), was probably capable of detecting TSH in normal rat plasma it was deemed more profitable to assess the effect of salicylate on circulating TSH in normal rats rather than proceed with further experiments on thyroidectomized rats.

2. <u>The Effect of Sodium l-Thyroxine on Circulating TSH in Normal Rats</u>

An experiment was carried out to assess the effect in normal rats of a single dose of sodium l-thyroxine which would be expected to completely suppress pituitary release of TSH.

Bioassay of the plasma of normal rats both before and after treatment with sodium l-thyroxine would indicate, firstly, whether the bioassay method was capable of detecting TSH in normal rat plasma, and secondly, whether statistical significance could be achieved by analysis of variance for the difference in the estimates of the plasma levels of TSH, before and after treatment.

<u>Materials and Methods</u>

A group of eight normal rats weighing approximately 200 g. was used. Control blood samples of 2.0 ml. were obtained from the rats by cardiac puncture and seven days later, the animals were injected intraperitoneally with a single dose of sodium l-thyroxine (10 μg./100 g. body weight). Twenty-four hours later the animals were exsanguinated. TSH determinations were made on pooled plasma samples, both before and after treatment, obtained by mixing equal volumes of plasma from each rat in the group. The pooled plasma samples, before and after treatment, were assayed with two standard doses of 0.0125 and 0.05 ImU of TSH.

Results and Discussion

The results are presented in Table 19. A TSH level of 0.07 ImU/ml. was obtained for the control sample. The injection of sodium l-thyroxine (10 ug/100 g. body weight) depressed this level to 0.02 ImU/ml. Analysis of variance revealed that the difference between the samples was statistically significant ($P < .05$). As a result of these findings it was decided to proceed with an examination of the effect of salicylate and related drugs on circulating TSH in normal rats.

3. The Effect of Salicylate and Related Drugs on Circulating TSH in Normal Rats

A. A Preliminary Study of the Effect of Salicylate

An experiment was carried out to determine whether a depression in circulating TSH could be detected in normal rats following the administration of sodium salicylate. Two groups of rats were selected, one to receive sodium salicylate and the other sodium p-hydroxybenzoate as a control for the experimental procedure.

Materials and Methods

Male rats of the same age and weight (approximately 200 g.) were numbered and distributed at random into two groups of nine rats each. Control blood samples of 2.0 ml. were obtained from each of the rats by cardiac puncture. The treatments were commenced seven days later when the rats had recovered from the initial blood sampling. Sodium p-hydroxybenzoate was administered in the usual dosage of 30 mg/100 g.

TABLE 19

BIOASSAY OF TSH IN THE PLASMA OF A GROUP OF EIGHT NORMAL RATS BEFORE AND 24 HOURS AFTER TREATMENT WITH SODIUM 1-THYROXINE (10 ug./100 g. body weight)

Treatments	Difference in log 3 hr. count rates				Row Totals	
	Group 1	Group 2	Group 3	Group 4		
.0125 mU	.06105	.51009	.17897	-.06541	.68470	T_1
.05 mU	-.16482	.10961	.03237	-.43574	-.45858	T_2
Before Treatment	.03077	.21847	.17234	-.57629	-.15471	T_3
Thyroxine Treated	-.11201	.55612	.37637	.02378	.84426	T_4
	-.18501	1.39429	.76005	-1.05366	.91567	

Analysis of Variance

Variation due to:	d.f.	S.S.	M.S.	Variance Ratio	P
Treatments	3	.301552	.100517	4.84	<.05
Residuals	3	.864134	.288045	13.88	<.01
Error	9	.186759	.020751		
Total	15	1.352445			

$b_{(I)} = .4747$ $s = .1441$ $\lambda = .304$

Estimate of TSH in Plasma

Injection volume 0.5 ml.

Before treatment = .035 x 2 = .07 ImU/ml.
Thyroxine treated = .010 x 2 = .02 ImU/ml.

Difference between samples

$T_3 - T_4 = .99897 = d$

$\frac{d^2}{8} = .12473$

$F = \frac{.12473}{s^2} = \frac{.12473}{.02075} = 6.01$ $P < .05$

Therefore the depression in TSH by thyroxine was significant

body weight/day to one group and the other received sodium salicylate in the same dosage. The drugs were given by gastric gavage twice daily for two days and the rats were sacrificed to obtain blood samples four hours after the last dose. Plasma PBI and plasma salicylate levels were determined on the pooled samples from each group by the usual methods. TSH was assayed in the usual manner on the pool samples, both before and after treatment for each group, with two standard doses of TSH of 0.0125 and 0.05 ImU.

The results of the bioassay of TSH in the samples from the group receiving sodium p-hydroxybenzoate are presented in Table 20 and those from the sodium salicylate treated group in Table 21.

Sodium p-hydroxybenzoate had no significant effect on the level of circulating TSH. A value of 0.06 ImU/ml. was obtained following the drug compared to a control value of 0.07 ImU/ml. The index of precision of the assay (λ) was 0.18. There was a highly significant ($P < 0.001$) depression in TSH following sodium salicylate; the control level of 0.08 ImU/ml. was depressed to 0.01 ImU/ml. The index of precision of the assay (λ) was 0.27.

These results indicate that the pituitary release of TSH was more readily depressed in normal rats by salicylate than in thyroidectomized rats. Sodium p-hydroxybenzoate which acted as a control for the experimental procedure was again without effect.

TABLE 20

BIOASSAY OF THE PLASMA FROM A GROUP OF NORMAL RATS BEFORE AND AFTER TREATMENT WITH SODIUM p-HYDROXY BENZOATE FOR 52 HOURS (30 mg./100 g. body weight/day)

Treatments	Difference in log 3 hr. count rates				Row Totals	
	Group 1	Group 2	Group 3	Group 4		
.0125 mU	.34976	.33870	.34849	.51200	1.54895	T_1
.05 mU	-.20341	.06205	.05774	-.17231	-.25593	T_2
Before treatment	-.05648	.23953	.23493	-.19678	.22120	T_3
p-hydroxy benzoate treated	-.10942	.35671	.20969	-.07081	.38617	T_4
	-.01955	.99699	.85085	.07210	1.90039	

Analysis of Variance

Variation due to:	d.f.	S.S.	M.S.	Variance Ratio	P
Treatments	3	.439983	.146661	7.73	<.01
Residuals (unadjusted)	3	.205161	.068387	3.61	N.S.
Error	9	.170705	.018967		
Total	15	.815849			

$b_{(I)}$ = .74946 s = .13772 λ = .184

Estimate of TSH in Plasma

Injection volume 0.5 ml.

Before treatment = .035 x 2 = .07 ImU/ml.

p-hydroxy benzoate treated = .030 x 2 = .06 ImU/ml.

Difference between samples

$T_3 - T_4 = .16497 = d$

$$\frac{d^2}{8} = \frac{.16497^2}{8} = .00340$$

$$F = \frac{.003402}{s^2} = \frac{.003402}{.018967} = 0.18 \quad \therefore \text{N.S.}$$

Therefore there was no significant effect of p-hydroxy benzoate on TSH

TABLE 21

BIOASSAY OF THE PLASMA FROM A GROUP OF NORMAL RATS BEFORE AND AFTER TREATMENT WITH SODIUM SALICYLATE FOR 52 HOURS (30 mg./100 g. body weight/day)

Treatments	Difference in log 3 hr. count rates				Row Totals	
	Group 1	Group 2	Group 3	Group 4		
.0125 mU	.12077	.19629	.20866	-.02686	.49886	T_1
.05 mU	-.35499	.01461	.14381	-.24149	-.43806	T_2
Before treatment	-.29088	.11889	.16735	-.24541	-.25005	T_3
Salicylate treated	.25703	.43958	.36900	.30943	1.37504	T_4
	-.26807	.76937	.88882	-.20433	1.18579	

Analysis of Variance

Variation due to:	d.f.	S.S.	M.S.	Variance Ratio	P
Treatments	3	.510623	.170208	15.91	<.001
Residuals (unadjusted)	3	.286005	.095335	8.91	<.01
Error	9	.096278	.010698		
Total	15	.892906			

$b_{(I)} = .38905$ $s = .1034$ $\lambda = .266$

Estimate of TSH in plasma

Injected volume 0.5 ml.

Before treatment = .040 x 2 = .08 ImU/ml.
Salicylate treated = .005 x 2 = .01 ImU/ml.

Difference between samples

$T_3 - T_4 = 1.62509 = d$

$\frac{d^2}{8} = .330115$

$F = \frac{.330115}{s^2} = \frac{.330115}{.010698} = 30.86$ $P < .001$

Therefore the depression in TSH by salicylate was highly significant

B. Systematic Studies of the Effect of Salicylate and Related Drugs

Further experiments of the effect of salicylate on circulating TSH in normal rats were therefore carried out and in addition the effect of sodium γ-resorcylate and 2,4-dinitrophenol was examined.

As control blood samples were obtained before treatment, each group acted as its own control and the effect of each drug could be studied as a separate experiment. This simplified the collection of the data since it was desirable to assay the samples as soon as possible after the completion of the experiments, but because of the time involved, only two bioassays could be performed in one week.

Materials and Methods

The same experimental procedure as described for the preliminary experiment, was followed. Sodium salicylate and sodium p-hydroxybenzoate were given in a dosage of 30 mg./100 g. body weight/day; sodium γ-resorcylate at the rate of 90 mg./100 g. body weight/day, (the level which was shown to depress the PBI significantly) and 2,4-dinitrophenol in a dosage of 2.5 mg./100 g. body weight/day.

Estimations of plasma PBI, salicylate and TSH were carried out by the usual procedures on pool samples.

Results

The combined results of all experiments carried out using the four drugs are presented in Table 22 and the results from each treatment have been discussed separately

TABLE 22

EFFECT OF SALICYLATE AND RELATED DRUGS ON CIRCULATING TSH IN NORMAL RATS

Treatment	Expt.	No. of Rats	PBI μg.I %		TSH ImU/ml.		p^+	λ
			Before Treatment	Treated 52 hrs.	Before Treatment	Treated 52 hrs.		
Sodium p-hydroxy benzoate 30 mg./100g./day	1	9	2.5	2.6	0.07	0.06	N.S.	0.18
	2	9	3.5	3.1	0.05	0.04	N.S.	0.30
Mean			3.0	2.9	0.06	0.05		
Sodium salicylate 30 mg./100g./day *	1	9	2.8	0.6	0.08	0.01	<0.001	0.27
	2	9	3.4	1.5	0.10	0.04	<0.01	0.16
	3	9	-	1.8	0.14	0.06	<0.05	0.20
Mean			3.1	1.3	0.11	0.04		
Sodium γ-resorcylate 90 mg./100g./day **	1	8	3.3	1.2	0.08	0.03	<0.01	0.14
	2	6	3.1	1.0	0.06	0.02	<0.05	0.27
	3	6	3.1	1.2	0.09	0.03	<0.001	0.12
Mean			3.2	1.1	0.08	0.03		
2-4 dinitro-phenol 2.5 mg./100g./day	1	8	2.5	1.2	0.10	0.03	<0.01	0.21
	2	8	2.8	1.0	0.06	0.03	<0.05	0.19
Mean			2.7	1.1	0.08	0.03		

p^+ probability of difference using analysis of variance

* mean plasma salicylate level 41 mg.%

** mean plasma γ-resorcylate level 25 mg.%

<u>Sodium p-hydroxybenzoate</u>

Two experiments were carried out to assess the effect of sodium p-hydroxybenzoate. The drug produced no significant change in circulating TSH; mean values of 0.06 and 0.05 ImU/ml. were obtained before and after treatment, respectively. There was no effect of the drug on the plasma PBI.

<u>Sodium salicylate</u>

There was a statistically significant depression in circulating TSH following salicylate administration in each of the three experiments carried out in which the mean value of 0.11 ImU/ml. for the control samples was reduced to 0.04 ImU/ml. following treatment.

The usual reduction in PBI was produced by sodium salicylate, the mean control level of 3.1 μg. I% being depressed to 1.3 μg. I%. The mean plasma salicylate for the three experiments was 41 mg. per 100 ml.

<u>Sodium γ-resorcylate</u>

This drug produced a statistically significant depression in TSH in each of the three experiments, the mean control value being reduced from 0.08 to 0.03 ImU/ml, when the mean γ-resorcylate level in the plasma was 25 mg.%. Moreover, the PBI was depressed to 1.1 μg. I% from a control value of 3.2 μg. I%. The results of experiment 1, as a typical example, are presented in Table 23.

<u>2,4-dinitrophenol</u>

Only two experiments were carried out on the effect of 2,4-dinitrophenol but in each case a statistically significant

TABLE 23

BIOASSAY OF THE PLASMA FROM A GROUP OF NORMAL RATS BEFORE AND AFTER TREATMENT WITH SODIUM γ-RESORCYLATE (90 mg./100 g. body weight/day) FOR 52 HOURS

Treatments	Difference in log 3 hr. count rates				Row Totals	
	Group 1	Group 2	Group 3	Group 4		
.0125 mU	.19642	.35019	.35953	.22772	1.13386	T_1
.05 mU	-.26161	.10543	.11314	-.05385	-.09689	T_2
Before treatment	-.24019	.22991	.20433	-.06630	.12775	T_3
γ-resorcylate treated	.10013	.30733	.33004	.10289	.84057	T_4
	-.20507	.99286	1.00704	.21046	2.00529	

Analysis of Variance

Variation due to:	d.f.	S.S.	M.S.	Variance Ratio	P
Treatments	3	.253152	.084384	17.42	<.001
Residuals (unadjusted)	3	.270238	.090079	18.60	<.001
Error	9	.043597	.004844		
Total	15	.566987			

$b_{(I)}$ = .51106   s = .06960   λ = .136

Estimate of TSH in Plasma

Injection volume 0.5 ml.

Before treatment = .040 x 2 = .08 ImU/ml.
γ-resorcylate treated = .020 x 2 = .04 ImU/ml.

Difference between samples

$T_3 - T_4$ = .71282 = d

$\frac{d^2}{8}$ = .063514

$F = \frac{.063514}{s^2} = \frac{.063514}{.004844} = 13.11$ $P < .01$

Therefore the depression in TSH by γ-resorcylate was significant

depression in TSH resulted. The mean control value of 0.08 ImU/ml. fell to 0.03 ImU/ml. The expected reduction in PBI with this drug was found to occur, the mean control PBI of 2.7 µg. I% being depressed to 1.4 µg. I%. The results of the first experiment with 2,4-dinitrophenol are presented in Table 24.

The average index of precision for the 10 bioassays in this series of experiments was .20, indicating that the assays were very precise. The mean level of TSH in the control samples of all experiments was 0.08 ImU/ml.

Discussion

These experiments demonstrated that sodium salicylate, sodium γ-resorcylate and 2,4-dinitrophenol significantly depress the circulating TSH in normal rats. The findings confirm the indirect evidence of Wolff and Austen (1958) (obtained from secretion rate studies in rats), of a depression in pituitary release of TSH by these drugs. However, the possibility remained that the depression in circulating TSH observed with the drugs in both bioassay and secretion rate studies could be explained by a direct chemical action of the drugs inducing a change in the protein structure of the TSH molecule, thus inhibiting its action on the thyroids of both the assay mice or the rats used in the secretion rate studies. This possibility was tested in the experiments reported in the next section.

TABLE 24

BIOASSAY OF THE PLASMA FROM A GROUP OF NORMAL RATS BEFORE AND AFTER TREATMENT WITH 2-4 DINITROPHENOL FOR 52 HOURS (2.5 mg./100 g. body weight/day)

Treatments	Difference in log 3 hr. count rates				Row Totals	
	Group 1	Group 2	Group 3	Group 4		
.0125 mU	.23308	.61009	.38717	.21241	1.44275	T_1
.05 mU	-.31949	.26718	-.01029	.05830	-.00430	T_2
Before treatment	-.49664	.37430	.06162	.00762	-.05310	T_3
DNP treated	-.12179	.53579	.52469	.33920	1.27789	T_4
	-.70484	1.78736	.96319	.61753	2.66324	

Analysis of Variance

Variation due to:	d.f.	S.S.	M.S.	Variance Ratio	P
Treatments	3	.486039	.162013	10.04	$<.01$
Residuals (unadjusted)	3	.806830	.268943	16.67	$<.001$
Error	9	.145213	.016135		
Total	15	1.438082			

$b_{(I)} = .60087$ $s = .1270$ $\lambda = .211$

Estimate of TSH in Plasma

Injection volume 0.5 ml.

Before treatment = .052 x 2 = .10 ImU/ml.
DNP treated = .015 x 2 = .03 ImU/ml.

Difference between samples

$T_3 - T_4 = 1.33099 = d$

$\frac{d^2}{8} = .221442$

$F = \frac{.221442}{s^2} = \frac{.221442}{.016135} = 13.72 \quad P < .01$

Therefore depression in TSH by 2-4 dinitrophenol was significant

4. The Effect of Administration of Salicylate and Related Drugs to the Assay Mice During Bioassay of Standard TSH

The possibility remained that the depression in circulating TSH in normal rats following the administration of salicylate, γ-resorcylate and 2,4-dinitrophenol was produced by direct chemical interaction of the drugs with TSH resulting in an inhibition of the release of labelled hormone from the thyroids of the assay mice.

This was tested in a four point assay in which two standard doses of TSH were assayed with two standard doses of the same level of TSH immediately followed by the injection of the drug at the usual plasma level detected in rats.

Materials and Methods

A four point assay design was used. Two standard doses of 0.0125 and 0.05 ImU TSH in the usual volume of 0.5 ml. were injected into two groups of mice. The same doses of TSH, but in a volume of 0.25 ml., were injected into the other two groups and followed immediately by the injection of the drug dissolved in normal saline in a volume of 0.25 ml.

The level of salicylate or γ-rescroylate attained in the plasma of rats treated with the drugs did not normally exceed 50 mg. per 100 ml. Since only 0.5 ml. of such plasma was injected into the assay mice, the amount of drug injected was of the order of 0.25 mg. Sodium salicylate and sodium γ-resorcylate were dissolved in normal saline at a concentration of 100 mg./100 ml.

0.25 ml. of the solution of drug (.25 mg.) was injected. The level of 2,4-dinitrophenol in the plasma of rats was not determined. It was considered that the dose of 2,4-dinitrophenol (2.5 mg./100 g. body weight/day) would not produce a level higher than 5 mg. per 100 ml. in the plasma which would be equivalent to 0.025 mg. in an injection volume of 0.5 ml. of plasma. 0.25 ml. of a solution of 2,4-dinitrophenol in saline (10 mg./100 ml.) was injected into the mice to assess the effect of the drug.

Results and Discussion

The results of the four point assays are presented in Tables 25, 26 and 27. Analysis of variance of the treatment effects was carried out for each assay to test for significance differences between the means and between the slopes of the dose response lines. The results indicated that sodium salicylate, sodium γ-resorcylate and 2,4-dinitrophenol were without effect on the response to standard TSH.

These results therefore negate the possibility that depression in circulating TSH produced by salicylate, γ-resorcylate and 2-4 dinitrophenol resulted from an inhibition of the physiological activity of circulating TSH by a chemical action of the drugs.

It was logical to conclude, therefore, that the depression in TSH produced by these drugs resulted from the inhibition of the release of TSH from the pituitaries of the rats.

TABLE 25

THE EFFECT OF ADMINISTRATION OF SALICYLATE (0.25 mg.) TO THE ASSAY MICE DURING THE BIOASSAY OF STANDARD TSH

Treatments	Difference in log 3 hr. count rates				Row Totals
	Group 1	Group 2	Group 3	Group 4	
.0125 mU	.11917	.46948	.20256	.31250	1.10371
.05 mU	-.21160	.08833	-.30421	.10635	-.32113
.0125 + salicylate	.13786	.35612	.08972	.50721	1.09091
.05 + salicylate	-.08655	.08946	-.12228	-.03081	-.15018
	-.04112	1.00339	-.13421	.89525	1.72331

Analysis of Variance

Variation due to:	d.f.	S.S.	M.S.	Variance Ratio	P
Treatments	3	.447873	.149291	19.93	<.001
(Mean slope	1	.444199	.444199	56.59	<.001
(Between means	1	.003674		<1.0	N.S.
(Between slopes	1			<1.0	N.S.
Residuals (unadjusted)	3	.271380	.090460	12.08	<.01
Error	9	.067400	.007489		
Total	15	.786653			

mean $b_{(I)}$ = .5535 s = .08654 mean λ = .156

There was no significant difference between the slopes or means of the dose response lines for either TSH alone or TSH + salicylate.

TABLE 26

THE EFFECT OF ADMINISTRATION OF γ-RESORCYLATE (0.25 mg.) TO THE ASSAY MICE DURING THE BIOASSAY OF STANDARD TSH

Treatments	Difference in log 3 hr. count rates				Row Totals
	Group 1	Group 2	Group 3	Group 4	
.0125 mU	.20216	.28797	.05260	.45193	.99466
.05 mU	-.12710	.15570	-.25436	.20616	-.01960
.0125 + γ-resorcylate	.23293	.44523	.05931	.35035	1.08782
.05 + γ-resorcylate	-.16444	.12117	-.08047	.15705	.03331
	.14355	1.01007	-.22292	1.16549	2.09619

Analysis of Variance

Variation due to:	d.f.	S.S.	M.S.	Variance Ratio	P
Treatments	3	.268293	.089641	16.51	<.001
(Mean slope	1	.267488	.267488	49.27	<.001
(Between Means	1	.001333		<1.0	N.S.
(Between Slopes	1	.000101		<1.0	N.S.
Residuals (unadjusted)	3	.337601	.112534	20.73	<.001
Error	9	.048862	.005429		
Total	15	.655386			

mean $b_{(I)}$ = .4295 s = .07368 mean λ = .171

There was no significant difference between the slopes or means of the dose response lines for either TSH alone or TSH + γ-resorcylate.

TABLE 27

THE EFFECT OF ADMINISTRATION OF 2-4 DINITROPHENOL (0.025 mg.) TO THE ASSAY MICE DURING THE BIOASSAY OF STANDARD TSH

Treatments	Difference in log 3 hr. count rates				Row Totals
	Group 1	Group 2	Group 3	Group 4	
.0125 mU	.06174	.37094	.04258	.36485	.84011
.05 mU	-.28299	.04301	-.30432	.07909	-.46521
.0125 + DNP	.20160	.31562	.00505	.39118	.91345
.05 + DNP	-.24949	.14522	-.21095	.12697	-.18825
	-.26914	.87479	-.46764	.96209	1.10010

Analysis of Variance

Variation due to:	d.f.	S.S.	M.S.	Variance Ratio	P
Treatments	3	.372370	.124123	42.12	<.001
(Average slope	1	.362109	.362109	122.9	<.001)
(Difference between means	1	.007669		2.60	N.S.)
(Difference between slopes	1	.002591		<1.0	N.S.)
Residuals (unadjusted)	3	.419861	.139954	47.49	<.001
Error	9	.026527	.002947		
Total	15	.818758			

mean $b_{(I)}$ = .4998   s = .05428   mean λ = .109

There was no significant difference between slopes or means of the dose response lines for either TSH alone or TSH + 2-4 dinitrophenol.

PART IV

THE ESTIMATION OF TSH IN NORMAL HUMAN SERUM

In order to confirm the depression in circulating TSH induced by salicylate in man it was necessary to overcome the same difficulties which were encountered in the studies in rats. Since the release of pituitary TSH in thyroidectomized rats was shown to be more resistant to depression by salicylate than in normal rats, it was decided not to assess the effect of salicylate on untreated myxoedema patients. Moreover, the onset of myxoedema following thyroid failure in the few subjects available was not readily determined, hence the pituitary in these subjects would probably be more resistant to inhibition by salicylate than the pituitaries of rats in the controlled period of two months following thyroidectomy.

It was decided, therefore, to study normal subjects providing the bioassay was sufficiently sensitive to estimate the level of TSH in normal human plasma. TSH was estimated in the plasma of a small series of normal human subjects.

Each plasma sample was assayed separately with three standard doses of 0.0125, 0.05 and 0.20 ImU TSH. This range of doses was chosen to span the range of TSH concentrations estimated by various workers and discussed in Part I of this chapter.

The results obtained from the four assays carried out were as follows:

Subject	TSH ImU/ml.
A	0.026
B	0.036
C	0.034
D	0.030
Mean	0.031

Since an injection volume of 0.5 ml. was used for the assay, the actual estimates ranged from 0.013 to 0.018 ImU which was close to the sensitivity of the assay (0.0125 ImU). It is acknowledged that this series of estimates is too small to enable any confident statement to be made concerning the level of TSH in normal human plasma. However, for the purposes of the current investigation these estimates permitted the conclusion that the level of TSH in normal human plasma was close to the limits detectable by the assay, so that it would be impossible to detect, with accuracy, any depression in this level resulting from the administration of salicylate or related drugs.

PART V

CONCLUSIONS

A critical examination of the bioassay of TSH by the method of McKenzie (1958) has been carried out. Modifications in the assay design were applied with consequent improvement in both precision and sensitivity of the method. The method was shown to be specific for TSH in the plasma of rats.

The elevated level of TSH in the plasma of thyroidectomized rats was readily detected by the bioassay method. A preliminary examination of the effect of salicylate on the elevated level of TSH in rats, thyroidectomized two months previously, was therefore undertaken. Sodium salicylate was administered in a dosage which had been shown previously to produce a significant depression in plasma PBI. Although salicylate produced an apparent depression in TSH in the plasma of these rats, statistical significance was not reached.

It was subsequently demonstrated that the bioassay method was sufficiently sensitive to estimate TSH in normal rat plasma. The study was therefore extended to an examination of the effect of salicylate and related drugs on circulating TSH in normal rats. The administration of sodium salicylate, sodium γ-resorcylate and 2,4-dinitrophenol produced a significant depression in circulating TSH in normal rats. A simultaneous depression in plasma PBI was demonstrated.

Sodium p-hydroxybenzoate was without effect on the levels of TSH or PBI in normal rats.

The possibility that the depression in TSH was caused by a direct chemical action of the drugs on TSH, inhibiting its physiological activity, was also tested. However, when administered to the assay mice in the same concentrations as those obtained in rat plasma, the drugs did not affect the response to standard doses of TSH. It was concluded therefore, that the depression in circulating TSH produced by the drugs resulted from an inhibition of the release of TSH from the pituitary.

The level of TSH in the plasma of normal human subjects was shown to be lower than that in the plasma of normal rats. As the level of TSH was close to the sensitivity of the assay, it was concluded that it would not be possible to detect, with accuracy, any depression in TSH resulting from the administration of salicylate or related drugs to normal man.

This demonstration of a significant depression in the level of circulating TSH in normal rats, following the administration of either sodium, salicylate, sodium γ-resorcylate or 2,4-dinitrophenol therefore confirms, conclusively the indirect evidence of Wolff and Austen (1958) that the drugs depress the release of TSH from the pituitary.

CHAPTER IV

THE EFFECT OF SALICYLATE AND RELATED DRUGS ON CIRCULATING FREE THYROXINE

PART I The Theoretical Basis of the Concept and Determination of Circulating Free Thyroxine.

1. General equations for the interaction of thyroxine with serum proteins.

2. The theoretical basis for the estimation of free thyroxine.

3. Factors affecting the interaction between thyroxine and the serum proteins.

PART II The Estimation of Free Thyroxine by the Christensen Dialysis Procedure.

PART III The Effect of Salicylate and Related Drugs on Free Thyroxine in Man.

PART IV The Effect of Salicylate and Related Drugs on Free Thyroxine in Rats.

PART V Conclusions.

CHAPTER IV

THE EFFECT OF SALICYLATE AND RELATED DRUGS ON CIRCULATING FREE THYROXINE

INTRODUCTION

It was demonstrated in Chapter II of this thesis that the administration of salicylate and 2,4-dinitrophenol to rats produced a depression in plasma PBI. This finding confirmed earlier reports of such an effect of the drugs in man and rats. (Castor and Beierwaltes, 1956; Goldberg et al, 1957; Austen et al, 1958). In these earlier studies, indirect evidence was obtained that a depression in pituitary release of TSH occurred simultaneously with the depression in plasma PBI produced by these drugs. It has been conclusively demonstrated (Chapter III), by direct bioassay of TSH in the plasma of normal rats, that both salicylate and 2,4-dinitrophenol produced a significant depression in circulating TSH.

This finding of simultaneous depression of circulating thyroid hormone and TSH is contrary to the concept of the negative feedback system regulating the thyroid-pituitary axis. A depression in circulating thyroid hormone would be expected to produce an increase in TSH output. However, the feedback regulation of TSH secretion may be overridden by neural factors affecting the hypothalamus.

Because of the similarity of the metabolic stimulating properties of 2,4-dinitrophenol and thyroxine, Goldberg and

co-workers (1957) proposed a thyromimetic action of 2,4-dinitrophenol to account for its action in depressing pituitary TSH release, by an effect on the hypothalamus, mediated via the increased peripheral metabolism. Evidence that such a mechanism produced the depression in pituitary TSH release by 2,4-dinitrophenol was obtained by Reichlin (1960). 2,4-Dinitrophenol was significantly less effective in causing thyroid inhibition in rats with hypothalamic lesions than in normal rats.

It has been demonstrated in the previous chapters of this thesis that sodium γ-resorcylate also produces a simultaneous depression in PBI and TSH in normal rats similar to that which follows salicylate and 2,4-dinitrophenol administration. However, sodium γ-resorcylate does not stimulate metabolic rate or increase oxygen consumption in rats (Meade 1954). The possibility that the action of salicylate and 2,4-dinitrophenol in depressing pituitary TSH release is dependent upon their metabolic stimulating properties may therefore be excluded.

It is apparent that, of the various suggestions made so far to explain the mechanism of action of salicylate and 2,4- dinitrophenol in disrupting the negative feedback regulation of the thyroid-pituitary axis, none has been confirmed experimentally. The validity of the hypothesis that it is the level of the total circulating thyroid hormone which controls the negative feedback regulation of thyroid-pituitary activity may therefore be questioned.

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Considerable attention has been given to the manner in which interaction with the serum proteins might influence the physiological activity of the thyroid hormones.

In a comprehensive review of the literature relating to the binding of the thyroid hormones to the serum proteins, Robbins and Rall (1960) concluded it was most likely that the circulating free thyroxine was the physiologically active moiety of the total circulating thyroxine and that the bound thyroxine acted as an inactive storage form. It was also postulated that the circulating level of free thyroxine might act as the regulator of the negative feedback system controlling thyroid-pituitary interrelations.

A method for the determination of free thyroxine in serum was developed by Christensen in 1958. Radioactive thyroxine was added in a tracer quantity to serum on one side of a dialysis membrane. The rate of transfer of radio-thyroxine across the membrane was shown to be dependent upon the level of free thyroxine in the serum.

In 1959, using this dialysis technique, Christensen demonstrated that the addition in vitro of either salicylate or 2,4-dinitrophenol to the serum caused a significant increase in the rate of transfer of radiothyroxine across the semi-permeable membrane. Christensen concluded that the thyroxine-releasing effect of salicylate and 2,4-dinitrophenol probably resulted from the binding of the drugs to one of the specific thyroxine-binding proteins, thus displacing bound thyroxine into the free state. It was postulated, therefore,

that _in vivo_ an increase in free thyroxine would account for the increased fractional rate of disappearance of injected radiothyroxine, and the fall in PBI following salicylate administration. The depression in TSH resulting from the administration of these drugs might also be explained by the increase in free thyroxine, if it is the level of free thyroxine which controls the negative feedback regulation of the thyroid-pituitary system.

It was decided to repeat the _in vitro_ studies of the effect of salicylate and 2,4-dinitrophenol in the dialysis system of Christensen. Because of the similar depression in PBI and TSH produced by sodium γ-resorcylate, the studies were extended to an examination of the _in vitro_ effect of γ-resorcylate.

Because of changes in the equilibrium resulting from the depression of the circulating thyroid hormone following administration of salicylate, it is not possible to extrapolate the _in vitro_ results to the _in vivo_ situation. The effect of salicylate and related drugs on circulating free thyroxine was therefore studied following their administration to both man and rats.

An investigation of the Christensen dialysis method for estimating free thyroxine in serum was carried out before its application to these studies.

PART I

THE THEORETICAL BASIS OF THE CONCEPT AND DETERMINATION OF CIRCULATING FREE THYROXINE

1. General Equations for the Interaction of Thyroxine With Serum Proteins

It has been recognized for many years that the thyroid hormone is carried in the blood in a non-dialysable, but chemically dissociable linkage with the plasma proteins (Trevorrow 1939). The specificity of the interaction between the thyroid hormone and plasma proteins was revealed in 1952 when it was demonstrated (following electrophoresis of human serum on filter paper in veronal buffer at pH 8.6) that I^{131}-labelled thyroxine migrated principally with the α-globulins in the zone between α_1 and α_2-globulin. A small proportion, about 10 per cent, migrated with albumin (Gordon et al, 1952; Larson et al, 1952; Winzler and Notrica, 1952). The moiety in the inter α-globulin region with which the thyroxine was associated came to be known as thyroxine binding globulin (TBG).

Thyroxine bound to any one of the sites on the serum proteins has been shown to be readily exchangeable with thyroxine on the other sites or with thyroxine (either stable or radioactive) added to the serum (Deiss et al, 1953; Robbins et al, 1954). In this type of reversible reaction,

the thyroxine bound to the proteins must be in equilibrium with free or unbound thyroxine. The distribution of thyroxine between bound and free forms is dependent upon the total concentration of thyroxine, the concentration of each of the binding sites and the association constant for each of these sites.

It is possible to derive general equations for the equilibrium interaction between thyroxine and a mixture of different types of binding sites. This subject has been reviewed by Robbins and Rall (1960). In the mathematical treatment it has been assumed that there is no interaction between successively bound thyroxine molecules when a protein contains multiple binding sites. In applying these equations to the situation in serum, the additional assumption has been made that each class of thyroxine-binding protein has a site with a single association constant, although the equations are applicable to the more complicated situation of multiple types of sites for each protein. *In vivo*, the additional possibility that equilibrium may not always be attained must also be considered.

Let ( ) = molar concentration

k = the intrinsic association constant

P = unoccupied binding sites

P^t = total binding sites

TP = occupied binding sites = bound thyroxine

T = free (unbound) thyroxine

T^t = total thyroxine.

Subscript $_{1,2\,\ldots n}$ = individual classes of binding sites

subscript $_{i}$ = any class of binding sites.

From the law of mass action

$$k_1 = \frac{(TP_1)}{(T)(P_1)}\ ,\ k_2 = \frac{(TP_2)}{(T)(P_2)}\ ,\ \ldots\ k_n = \frac{(TP_n)}{(T)(P_n)} \qquad (1)$$

Since only one concentration of free thyroxine can exist in the mixture, and $(P_i) = (P_i^t) - (TP_i)$

$$\text{Then} \qquad (T) = \frac{(TP_i)}{k_i\left[(P_i^t)-(TP_i)\right]} \qquad (2)$$

On rearranging

$$\frac{1}{k_i(T)} = \frac{(P_i^t) - (TP_i)}{(TP_i)} = \frac{(P_i^t)}{(TP_i)} - 1 \qquad (3)$$

from equation (2)

$$(T) = \frac{(TP_1)+(TP_2)\ \ldots+(TP_n)}{k_1(P_1)+k_2(P_2)\ \ldots+k_n(P_n)} = \frac{\Sigma(TP_i)}{\Sigma k_i(P_i)} = \frac{\Sigma(TP_i)}{\Sigma k_i\left[(P_i^t)-(TP_i)\right]} \qquad (4)$$

$$(T) = \frac{(T^t) - (T)}{\Sigma k_i(P_i)} \qquad (5)$$

$$\text{and} \qquad (T) = \frac{(T^t)}{\Sigma k_i(P_i) + 1} \qquad (6)$$

From equation 2 it may be seen that the concentration of free thyroxine could be calculated from a knowledge of the concentration of bound thyroxine (TP), the concentration of total binding sites (P^t), and the association constant k, for any one of the binding sites in the mixture.

Robbins and Rall (1960) calculated the association constant for TBG using data obtained from experiments using electrophoresis in veronal buffer. The mass law equations governing the interaction between thyroxine and a single type of site on each of two proteins - TBG and albumin - were employed (the importance of thyroxine binding prealbumin (TBPA) as a third binding site of thyroxine was not appreciated at the time of this study). The theoretical treatment appeared to be justified by the fit of calculated curves to the experimental data but the absolute value was based on a number of assumptions. The value obtained 7.9×10^9 was very large but is consistent with the experimental finding that the small quantity of TBG in serum competes effectively with the much larger amount of albumin for the available thyroxine. Lein (1952) derived a value of 7.9×10^4 for the association constant of bovine serum albumin.

The interaction of thyroxine with the serum proteins is of such intensity that the equilibrium concentration of free thyroxine is extremely low. Robbins and Rall (1957) calculated the free thyroxine level when it was thought that thyroxine was bound to only two types of sites on the serum proteins. This did not affect the result as the concentration

of free thyroxine may be calculated from the data for only one of the binding sites, irrespective of the number of other binding sites interacting with thyroxine (equation 2). It was assumed by Robbins and Rall that the distribution of thyroxine among the binding proteins determined electrophoretically represented the actual distribution *in vivo*.

The concentration of TBG binding sites and the proportion of the total thyroxine bound to TBG were determined. The value previously obtained for the association constant of TBG was used. Using this method, the mean value for free thyroxine in a group of normal adults was 0.62×10^{-10}M. This value for free thyroxine is equivalent to 0.06% of the total thyroxine concentration.

2. The Theoretical Basis for the Estimation of Free Thyroxine

A method for the determination of free thyroxine in serum, based on the rate of transfer of thyroxine across a dialysis membrane, was developed by Christensen in 1959. A full description of this method is presented in Part II of this chapter. In this procedure, identical concentrations of stable thyroxine and serum were placed on both sides of a semi-permeable membrane in identical chambers A and B in a specially constructed dialysis cell provided with adequate stirring. A trace of radiothyroxine in buffer was added to chamber A and buffer alone to chamber B. Equal volumes of serum were removed from both chambers after 6, 18 and 24 hours dialysis. The radioactive protein bound iodine (PBI^{131}) content of the

sample in chamber A was determined and also the PBI^{131} content of the samples from chamber B after 6, 18 and 24 hours. From these results, the percentage of radiothyroxine transferred across the dialysis membrane at the stated times was determined. The experimentally determined rate of transfer of radiothyroxine gave a linear function of time over a 24 hour period.

If a tracer dose of radiothyroxine ($^{*}T$) is added to a sample of serum, the following relationships will be valid after equilibrium is established:

$$\frac{(T)}{(T^{t})} = \frac{(^{*}T)}{(^{*}T^{t})} \qquad (7)$$

If it is assumed that all of the organic iodine in the serum is in the form of thyroxine and is estimated as PBI (which is only approximately true) then:

$$\frac{(T)}{(PBI)} = \frac{(^{*}T)}{(PBI^{131})} \qquad (8)$$

$$\therefore \quad (T) = \frac{(^{*}T)}{(PBI^{131})} \cdot (PBI) \qquad (9)$$

From the law of mass action (equation 1)

$$(T) = \frac{1}{k} \cdot \frac{(TP_1)}{(P_1)} \qquad (10)$$

Assuming the bound thyroxine (TP_1) = PBI then:

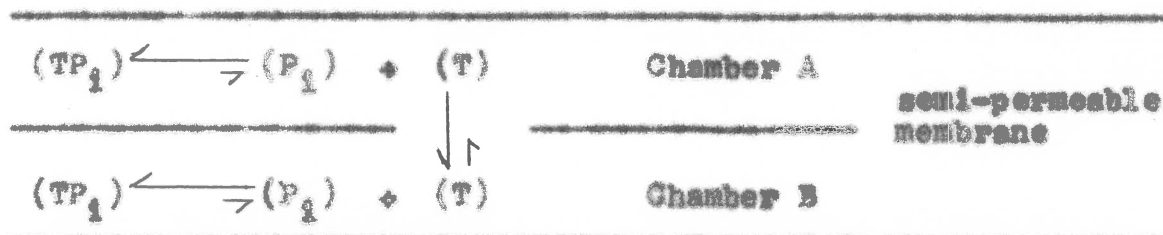
$$(T) = k \cdot \frac{(PBI)}{(P_1)} \qquad (11)$$

A comparison of equations 9 and 11 shows that the ratio of free radiothyroxine to total radiothyroxine is inversely proportional to the residual binding capacity of the serum. This relationship is however only of relative validity since equation 11 presupposes that all binding sites have the same affinity for thyroxine, which is an approximation.

Determination of the ratio of free radiothyroxine to total radiothyroxine would yield a measure of the residual binding capacity of the serum under study and thus permit calculation of the free thyroxine level in the serum, providing the level of PBI is known.

Since it would be difficult to determine the absolute value of the ratio of free radiothyroxine to total radiothyroxine and since only relative values are necessary, this magnitude will be expressed by a quantity that is proportional to it, namely, the percentage of the total amount of radiothyroxine added, that has passed the membrane in 24 hours. The product of this latter value and the PBI value is an expression of free thyroxine in arbitrary units. An absolute value cannot be obtained by this method.

Robbins and Rall (1960) have mathematically analysed such a system of kinetic dialysis. The dialysis method used by Christensen may be schematically described as follows:



It was assumed that the transfer of thyroxine across the membrane is a first order process, and that when a trace quantity of radiothyroxine ($^{*}T$) is added to chamber A, the interchange between $^{*}T$ and T is very fast compared to the other rates involved. Since the radiothyroxine which passes through the membrane is bound to the serum proteins, there is no back diffusion, and chamber B can be treated as an infinite sink. Therefore the fall in concentration of $^{*}T$ in chamber A will be expressed by

$$(^{*}T) = (^{*}T)_{o}\ e^{-Kt}$$

where t = time

$(^{*}T)_{o}$ = molar concentration of $^{*}T$ at t=0

K = proportionality constant (t^{-1})

The value of K will depend on various characteristics of the system such as the nature of the membrane - pore size and charge, the dimensions of the membrane, the volume of the chambers, the temperature, and the pH of the serum. These factors were kept constant.

Thyroxine transfer also depends on the interrelation of the various rate processes:

(1) the rate of mixing in the chambers,

(2) the rate of interchange between stable and radioactive thyroxine,

(3) the rates of association and dissociation of thyroxine from its binding sites, and

(4) the rate of transfer across the membrane.

Rates 1 and 2 would not appear to be limiting rates.
It is assumed that the rate of dissociation is fast relative to the rate of dialysis, but it is conceivable that the former could be rate limiting.
Equation 12 can be rewritten in the form

$$ {}^{*}T_{out} = KV\,({}^{*}T) \qquad (13) $$

where ${}^{*}T_{out}$ = the moles of radiothyroxine leaving chamber A per unit time

V = the volume of chamber A.

Rearranging equation 13

$$ K = \frac{{}^{*}T_{out}}{V({}^{*}T)} \qquad (14) $$

Since labelled thyroxine is expected to be indistinguishable from stable thyroxine, the same relationship must hold for the stable molecule

$$ T_{out} = KV(T) \qquad (15) $$

It can be seen that K defined the rate of transfer of the free thyroxine pool V(T). However, the experimental data was expressed as the fraction of the total thyroxine pool $V(T^{t})$, transferred per unit time. This value is obviously not the same as K. This will be designated the "fractional" transfer rate.

Since the labelled material is identical with the stable molecule, on substituting the value of K from equation 14 into equation 15 -

$$\frac{{}^{*}T_{out}}{V({}^{*}T^{t})} \cdot V(T^{t}) = T_{out} \tag{16}$$

This is essentially the calculation employed by Christensen (equation 9) for relating the "fractional" transfer of radio-thyroxine to the free thyroxine concentration.

By substituting equation 6 into equation 15 -

$$\frac{T_{out}}{V(T^{t})} = \frac{K}{\Sigma k_{i}(P_{i}) + 1} \tag{17}$$

It is evident from this equation that the transfer of T_{out} of chamber A, when expressed as a fraction of the total thyroxine in chamber A, is an inverse function of the residual binding capacity of the serum (P_i).

As previously mentioned the dialysis method of Christensen does not give an absolute value for free thyroxine, but permits only relative assessment of levels of free thyroxine.

Using this method, Christensen (1960) examined the level of free thyroxine in the serum of normal subjects, patients with thyroid disease, and also pregnant women. The mean value of free thyroxine in a series of 30 normal subjects was 3.2 units, with a range from 2.1 to 4.5 units. The mean value in the thyrotoxic group was 9.4 units whereas that from a small series of myxoedema patients was 0.6 units. The values of free thyroxine for pregnant women fell within the normal range, in spite of increased PBI levels.

In order to determine absolute levels of free thyroxine

by a dialysis procedure, it would be necessary to determine accurately the minute amount of thyroxine present in the dialysate that was considered to represent the unbound or diffusible fraction of hormone in serum. Such a procedure has been reported by Sterling and Hegedus (1962).

Using this method they obtained a value of 1.3×10^{-10}M free thyroxine for normal serum. This was twice that (0.6×10^{-10}M) computed by Robbins and Rall (1957). A value of 0.6×10^{-10}M free thyroxine was also calculated by Sterling, Rosen and Tabachnick (1962) from equilibrium dialysis studies using human serum albumin.

These results, derived by several different approaches, all give values for free thyroxine of the same order of magnitude which fit the theoretical considerations derived from the mass laws.

3. Factors Affecting the Interaction Between Thyroxine and the Serum Proteins

Other generalizations may be made from the equations derived from the law of mass action.

If it is assumed that the thyroxine-binding capacity of one of the proteins in the serum (P_1^t) rises but the concentration of free thyroxine, (T), is kept constant, equations 3 and 4 predict that the concentration of thyroxine bound to this site (TP_1) will increase. The concentration of thyroxine bound to the sites (TP_i) other than 1, will remain unchanged and the thyroxine bound to all the binding sites $\Sigma(TP_i)$, will rise, as will the total thyroxine (T^t).

The ratio $\frac{(TP_i)}{(T^t)}$ for sites other than i=1 will fall.

Since $\frac{(T)}{(T^t)} + \frac{\Sigma(TP_i)}{(T^t)} = 1$, the ratio $\frac{(TP_1)}{(T^t)}$ will rise.

Since (TP_1) rises, (P_1) will rise but (P_2) ... (P_n) will not change.

A special case of this is found in human pregnancy and oestrogen administration to men or non-pregnant females. In normal pregnancy the PBI rises in the absence of other signs of hyperthyroidism (Danowski et al, 1950; Freedberg et al, 1957). The thyroxine binding capacity of TBG increases early in pregnancy and remains elevated for several weeks post-partum (Dowling et al, 1956a; Robbins and Nelson, 1958). Both Christensen (1960) and Sterling and Hegedus (1962) demonstrated that the level of free thyroxine in pregnant women was similar to that in normal subjects. Thus pregnant women remain euthyroid presumably because free thyroxine levels are within the normal range.

If on the other hand the thyroxine binding capacity of one of the proteins in the mixture, (P_1^t), falls, but the total thyroxine concentration, (T^t), is kept constant, equation 6 predicts that the concentration of free thyroxine, (T) must rise. Equation 2 then indicates that the concentration of thyroxine bound to the other sites, (TP_2) ... (TP_n), will rise and that the concentration of unoccupied binding sites for these proteins (P_2) ... (P_n) will fall.

It follows from the initial conditions that the concentrations of both occupied and unoccupied binding sites for this protein (TP_1) and (P_1) will fall and the ratio $\frac{(TP_i)}{(T^t)}$ for sites other than 1 will rise.

This would be expected to be the case if a drug which inhibits the binding of thyroxine to one of the serum proteins were added in vitro to normal human serum. Christensen (1960) postulated that salicylate and 2,4-dinitrophenol exerted such an effect on one of the binding proteins in the blood.

The in vivo effect of such a drug is not easily predicted because of the possible decrease in the total thyroxine circulating, caused by excretion of thyroxine released from the specific thyroxine binding proteins.

PART II

THE ESTIMATION OF FREE THYROXINE BY THE CHRISTENSEN DIALYSIS PROCEDURE

Materials and Methods

Two dialysis cells were constructed+ using a modification of the design of Christensen (1959a). Each cell consisted of two chromium-plated brass plates cut out to form straight-sided chambers measuring 4 mm. x 32 mm. x 93 mm. Rubber gaskets were inserted around each chamber. A dialysis membrane was placed between the two halves which were tightly screwed together to form two identical sealed chambers separated by the dialysis membrane. The volume of each chamber was 12 ml. Mixing of the contents of the chamber to which the radiothyroxine was added (chamber A) was effected by means of an impellor sealed through the wall of the chamber, driven externally by a belt drive. The cells were suspended in a water bath maintained at a temperature of 37.5 ± 0.5°C. The serum samples were admitted through a small hole in the top of each chamber from a pipette with a fine bore plastic tube attached. After insertion of the sample, the hole was sealed. Samples were removed in the same manner.

Radio-l-thyroxine was obtained from Abbotts Laboratories (Oak Ridge, Tennessee). The specific activity of the various batches used was about 30 mc./mg. The preparation, purified

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+ Paton Industries, Adelaide.

by the manufacturers, was supplied in solution of 50% propylene glycol. There was a slow release of inorganic iodide I^{131} from the material. The radiothyroxine solution was stored at $4^{o}C$ to inhibit the deiodination and deamination reactions caused by auto-ionization (Tata, 1959). The solution after one, two and three weeks storage at $4^{o}C$ was subjected to paper chromatography in tertiary amyl alcohol saturated with 2N ammonia. Only two areas of radioactivity were revealed which were identified as inorganic iodide and thyroxine. The radiothyroxine was never used for more than three weeks after its preparation.

Phosphate buffer (pH 7.4) was prepared by mixing 90 ml. of 0.5M KH_2PO_4 and 410 ml. of 0.5M Na_2HPO_4.

The dialysis membranes were prepared from 15 cm. lengths of Visking dialysis tubing which were cut open on one side and unfolded to form membranes 6 cms. wide.

Blood samples were collected by venipuncture. The serum was separated and stored in the frozen state until just before use.

Procedure

A volume of 35 µl. of radiothyroxine solution was diluted with phosphate buffer to a concentration of 0.1 µg. thyroxine per ml. 1 ml. of this solution was pipetted into a test tube. Into another test tube was pipetted 1 ml. of the phosphate buffer alone. To each tube was added 0.1 ml. of 5% sodium thiosulphate and then 10 ml. of the serum to be analysed. The contents of each tube were mixed and incubated

at 37°C for 90 minutes. After assembly, the cells were attached to the supporting rack and suspended in the water bath at 37.5°C. On completion of the incubation, 10 ml. of the serum mixture containing the radiothyroxine was pipetted into chamber A and 10 ml. of the non-radioactive serum mixture was pipetted into chamber B. The stirrer was started and the time noted. At 6, 18 and 24 hours samples of 3 ml. were withdrawn from chambers A and B.

The Determination of Free Thyroxine in the Samples

The PBI^{131} was determined in the samples removed from chamber B by the following procedure. The 3 ml. samples were originally pipetted into test tubes measuring 0.5 x 6.0 inches which fitted the well-type crystal used for subsequent radio-activity measurement. 6 ml. of 20% trichloroacetic acid (TCA) were added with stirring to precipitate the serum proteins. After standing for several minutes, the tubes were centrifuged for 20 minutes at 1500 r.p.m. After discarding the super-natants the samples were washed with 10% TCA. Carrier iodide (0.5% solution of KI) was added to assist in the removal of inorganic iodide I^{131} present in the radiothyroxine solution. The effectiveness of this procedure, which was described by Christensen, was verified experimentally. The samples were vigorously stirred twice and centrifuged for 20 minutes at 1500 r.p.m. After washing the protein precipitates three times in this manner, the final supernatants were discarded and the samples were well drained.

The radioactive content of the protein precipitates were

measured using a well-type scintillation crystal (Ekco Type N597). In order to standardize the counting error at a low level, the time for 30,000 counts was recorded for each sample. From the values obtained the count rate for 10 ml. of sample was determined.

The PBI^{131} content in the serum added to chamber A prior to the commencement of the dialysis was determined in a 100 µl. aliquot to which inactive serum was added to a volume of 3 ml. This sample was treated in the same way as the samples from chamber B. From the radioactivity measurement, the count rate for 10 ml. of the sample was calculated.

For calculation of the percentage of the total radiothyroxine dialysed in 6, 18 and 24 hours, the appropriate radioactive decay factor was applied to the count rates from the chamber B samples. The quantity of radioactive thyroxine that had passed the membrane (the PBI^{131} content of chamber B) in a given time, was expressed as a percentage of the total amount of radioactive thyroxine added (the PBI^{131} content of chamber A) at the commencement of the dialysis. When the percentage of the total radiothyroxine dialysed was plotted against time a linear relationship resulted.

As stated above the serum samples were diluted with 1 ml. of phosphate buffer. No correction was introduced for this dilution as the results were expressed in arbitrary units which depended on the dimensions of the apparatus.

Results

To enable the precision and reproducibility of the method to be assessed, six determinations of the same normal serum sample were made. The values obtained ranged from 0.96 1.15 per cent of the total radiothyroxine dialysed in 24 hours. The mean value was 1.01 per cent with a standard deviation from the mean of 0.03 per cent.

During the course of the investigations reported in this chapter a series of results from normal subjects, and untreated thyrotoxic and myxoedema patients was collected.

Normal subjects

Estimates of free thyroxine were made on the serum from 20 normal subjects. The results are shown in Table 28. The values for the percentage of the total radiothyroxine dialysed in 24 hours varied from 0.77 to 1.28 with a mean of 1.08. The PBI values ranged from 4.3 to 7.8 μg. I%. The mean value for free thyroxine calculated from these values was 5.9 units. The lowest value of free thyroxine in the series was 3.9 units, the highest was 8.7 units.

Thyrotoxic subjects

As shown in Table 29, the level of free thyroxine was found to be high in each of the eight patients examined; a mean value of 39.0 units free thyroxine was obtained. The percentage of the total radiothyroxine dialysed in 24 hours for all thyrotoxic subjects was greater than that found in normal subjects, ranging from 1.82 to 4.55 per cent.

TABLE 28

ESTIMATION OF FREE THYROXINE IN THE SERUM OF 20 NORMAL HUMAN SUBJECTS

Subject	% Total Radiothyroxine Dialysed in 24 hours	PBI µg I %	Free Thyroxine (units)
1	0.98	5.2	5.1
2	1.18	5.1	6.0
3	1.12	4.9	5.5
4	1.07	4.6	4.9
5	1.17	5.0	5.9
6	1.04	5.6	5.8
7	1.00	6.3	6.3
8	1.11	7.8	8.7
9	1.09	6.7	7.3
10	1.22	4.9	6.0
11	1.28	4.6	5.9
12	1.23	5.0	6.2
13	1.01	6.4	6.5
14	0.98	4.6	4.5
15	1.10	5.2	5.7
16	1.05	5.9	6.2
17	0.91	4.3	3.9
18	0.98	6.7	6.6
19	0.77	6.9	5.3
20	1.20	5.0	6.0
Mean	1.08	5.5	5.9

Myxoedema Subjects

Only three patients with untreated myxoedema were available during the period of these studies. The free thyroxine values were low; a mean level of 1.1 units of free thyroxine was obtained. (Table 29)

The linear relationship resulting from the plot of the percentage of the total radiothyroxine dialysed against time in hours for typical examples of a normal, a thyrotoxic and a myxoedema subject are shown in Figure 3.

Discussion of the Method

The estimates of free thyroxine in normal, thyrotoxic and myxoedema subjects fall into three well separated groups. Although the number of subjects in these series is small, these findings confirm the similar results of Christensen (1960). The values for the normal range of 3.9 to 8.7 units are approximately twice those reported by Christensen for his normal series (2.1 to 4.5 units), but this discrepancy is accounted for by the modified design of the cells. Christensen pointed out that alterations in the dimensions of the apparatus would yield values different from those which he obtained. The conditions of temperature and pH were the same as those used by Christensen. All dialyses were performed using membranes derived from the same batch of dialysis tubing, identical to that used by Christensen.

It was concluded from these results that the method was precise and reproducible, and could be used for further studies of the effects of salicylate and related drugs on circulating free thyroxine.

TABLE 29

ESTIMATION OF FREE THYROXINE IN THE SERUM OF UNTREATED THYROTOXIC AND MYXOEDEMA PATIENTS

Subject	% Total Radiothyroxine Dialysed in 24 hours	PBI µg I %	Free Thyroxine (Units)
Thyrotoxic			
1	1.96	13.7	26.9
2	2.03	12.1	24.6
3	1.85	16.7	30.9
4	2.82	12.3	34.7
5	4.55	18.7	85.1
6	3.24	15.1	48.9
7	1.82	10.9	19.8
8	3.55	11.7	41.5
Mean	2.73	13.9	39.0
Myxoedema			
1	0.71	1.9	1.3
2	0.57	1.9	1.1
3	0.88	1.1	1.0
Mean	0.69	1.6	1.1

COMPARISON OF THE DIALYSIS RATE OF I^{131} THYROXINE IN VARIOUS STATES OF THYROID FUNCTION

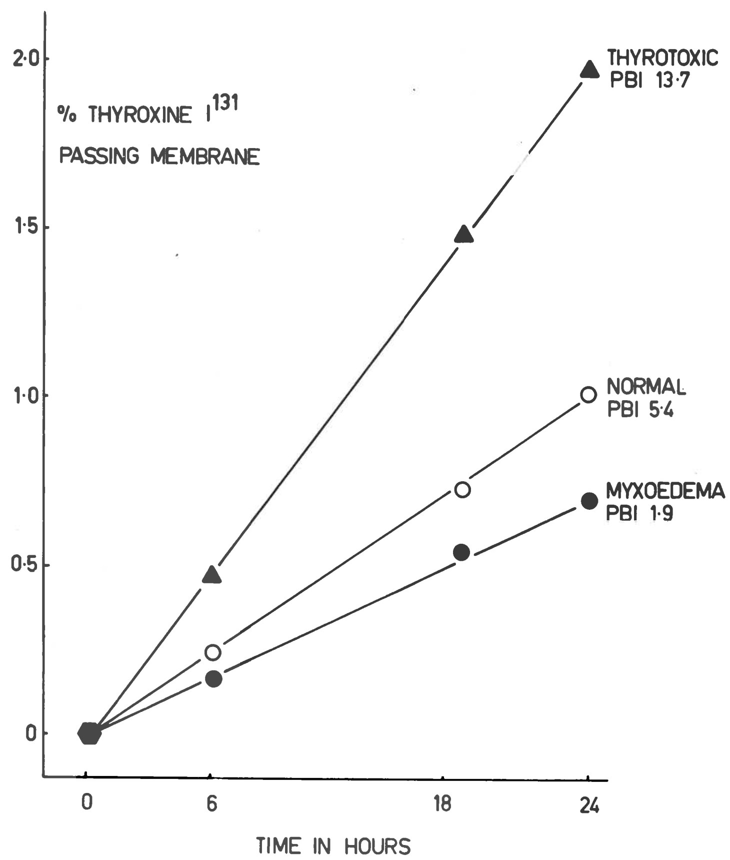


FIGURE 3.

PART III

THE EFFECT OF SALICYLATE AND RELATED DRUGS ON FREE THYROXINE IN MAN

IN VITRO STUDIES

Studies of the *in vitro* effect of salicylate on the dialysis rate of radiothyroxine were repeated using p-hydroxybenzoate as a control for the salicylate ion.

It has been demonstrated in the previous chapters of this thesis that the administration of γ-resorcylate to rats produced depressions in both PBI and TSH similar to those produced by salicylate and 2,4-dinitrophenol. The effect of the *in vitro* addition of γ-resorcylate to human serum, on the dialysis of radiothyroxine was also studied to determine whether this drug produced an increase similar to that produced by salicylate.

Materials and Methods

A large pool of normal human serum was obtained for these studies. The drugs, p-hydroxybenzoic acid, salicylic acid and γ-resorcylic acid were dissolved in the phosphate buffer in quantities such that when the serum was added, final concentrations of 10, 30 and 50 mg. of the drugs/100 ml.serum were obtained. The pH of the phosphate buffer was adjusted to pH 7.4 following the addition of the drugs. The drugs were added to the serum in both chambers of the dialysis cell.

Results

The results of the *in vitro* effects of the drugs are presented in Table 30. When salicylate was added at a level

of 50 mg./100 ml., the value for the percentage of the total radiothyroxine dialysed in 24 hours was 2.9 compared to the control level of 1.17. The effect was less marked at 30 mg./100 ml. and although smaller at 10 mg./100 ml. was still apparent. The results of the _in vitro_ addition of p-hydroxybenzoate on the dialysis of human serum are also shown in Table 30. Although ineffective at 10 mg./100 ml., p-hydroxybenzoate did produce a slight increase in the percentage of the total radiothyroxine dialysed in 24 hours at both 30 and 50 mg./100 ml; at the highest concentration the level was of the same order as that produced by 10 mg./100 ml. salicylate.

When γ-resorcylate was added to normal human serum at 30 mg./100 ml. the percentage of the total radioactivity dialysed in 24 hours was greatly increased. The value of 2.60 per cent for the addition of 30 mg./100 ml. γ-resorcylate was intermediate between the values obtained with salicylate at 30 mg. and 50 mg./100 ml.

The _in vitro_ effects of the three drugs in the dialysis rate of radiothyroxine are compared in Figure 4.

Discussion

The increased rate of dialysis of radiothyroxine observed following the _in vitro_ addition of salicylate to normal human serum confirms the report of Christensen (1959).

There was no increase in the rate of dialysis of radiothyroxine when p-hydroxybenzoate was added at the lowest concentration of 10 mg./100 ml. However, there was a slight

TABLE 30

EFFECT OF IN VITRO ADDITION OF SALICYLATE AND RELATED DRUGS ON THE DIALYSIS OF RADIOTHYROXINE IN NORMAL HUMAN SERUM

Sample	% Total Radiothyroxine Dialysed in 24 hours at concentration of drug added to serum			
	0	10 mg.%	30 mg.%	50 mg.%
Pool Serum	1.17			
+ salicylate		1.58	2.00	2.93
+ p-hydroxy-benzoate		1.14	1.43	1.74
+ γ-resorcylate			2.60	

IN VITRO EFFECT OF SALICYLATE AND RELATED DRUGS ON THE DIALYSIS RATE OF I^{131} THYROXINE IN NORMAL HUMAN SERUM

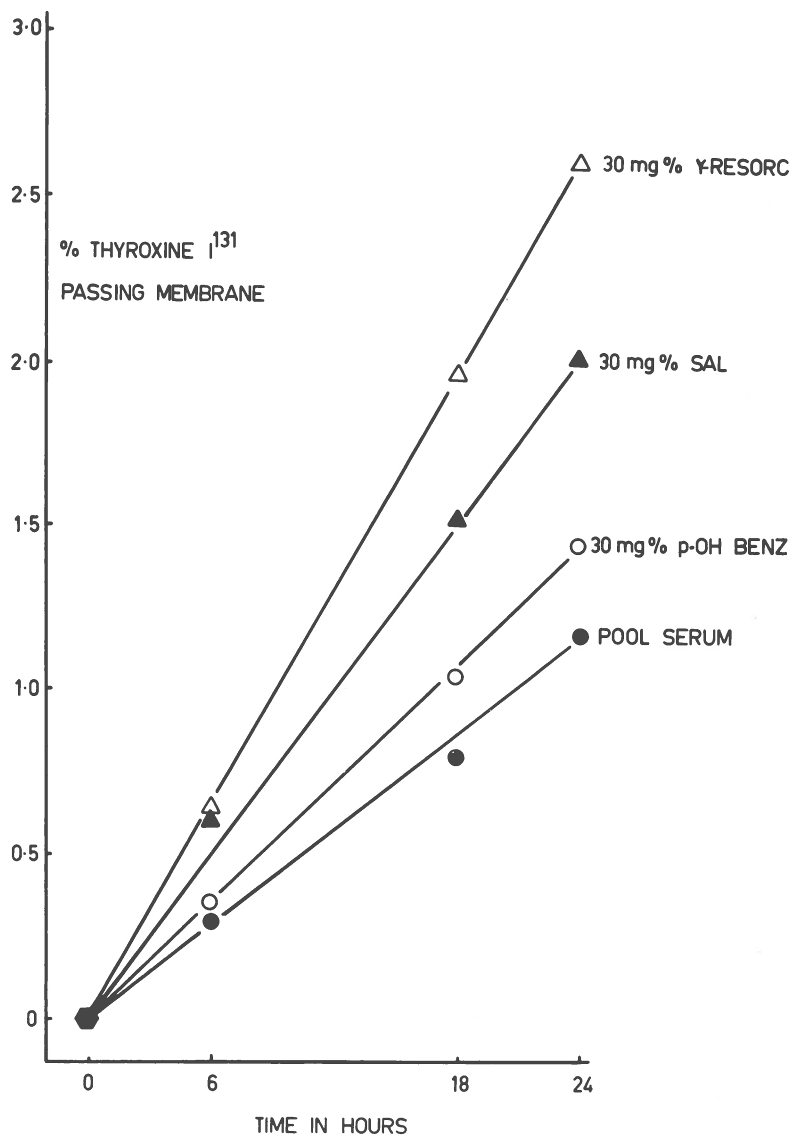


FIGURE 4.

increase when the higher concentrations were used. It was considered likely, therefore, that the increase in dialysis rate produced by salicylate was probably dependent upon its thyroxine releasing effect and not related to non-specific effects such as ionic size.

The *in vitro* addition of γ-resorcylate at 30 mg. per 100 ml. produced an increase in the dialysis rate of radio-thyroxine greater than that produced by an equivalent concentration of salicylate.

However, as mentioned previously it is not possible to predict from these *in vitro* findings whether the circulating free thyroxine would be elevated following the administration of the drugs to man or animals since the equilibrium state is altered by the depression in circulating thyroid hormone. An investigation of the effect of salicylate and related drugs on circulating free thyroxine following their administration to human subjects was therefore carried out.

IN VIVO STUDIES

Two experiments were carried out to examine the effects of the drugs on circulating free thyroxine. Only sodium p-hydroxybenzoate and sodium salicylate were used in the first experiment. In the second, the effect of γ-resorcylate was also studied.

Experiment 1

Materials and Methods

Three healthy normal volunteers in the post-absorbtive state attended the laboratory on two occasions three weeks

apart. A blood sample of 60 ml. was obtained from each subject before the administration of the drugs. The drugs, either sodium salicylate or sodium p-hydroxybenzoate, were given orally in a dose of 5 g. The drugs were administered in random order on the first day. Each subject received the alternative treatment on the second day. Blood samples of 60 ml. were obtained 1 hour and 3 hours after the drugs were given.

Free thyroxine, PBI and salicylate levels were estimated by the described methods.

Results

The results of the administration of sodium p-hydroxybenzoate and sodium salicylate on circulating free thyroxine in the individual subjects are shown in Table 31. The mean values for the three subjects are given in Table 32.

Although there was some variation in the PBI values following p-hydroxybenzoate in the individual subjects, the mean values reveal that there was no consistent effect of the drug on the PBI. The percentage of the total radiothyroxine dialysed in 24 hours was within the range for normal subjects both before and following p-hydroxybenzoate. The mean values for free thyroxine were unchanged following the administration of 5 g. of sodium p-hydroxybenzoate.

Following sodium salicylate there was a consistent fall in the PBI in each subject over the 3 hour period (Table 31). The mean pre-treatment value of 6.1 µg. I% was reduced to 5.4 µg. I%. The mean serum salicylate reached 33.4 mg.%

.4

TABLE 31

EFFECT OF ADMINISTRATION OF SODIUM SALICYLATE AND SODIUM p-HYDROXY-BENZOATE ON CIRCULATING FREE THYROXINE IN NORMAL MAN

Subject	Day	Treatment	Sample	Salicylate mg.%	PBI µg.I %	% Total Radio-thyroxine Dialysed in 24 hrs.	Free Thyroxine (units)
A	2	Sodium p-hydroxy-benzoate (5 g.)	Before treatment		6.1	0.83	5.1
			After 1 hr		6.0	0.88	5.3
			After 3 hrs		5.5	0.94	5.2
C	2		Before treatment		5.3	1.09	5.8
			After 1 hr		4.8	1.19	5.7
			After 3 hrs		5.5	0.96	5.3
D	1		Before treatment		4.9	1.22	6.0
			After 1 hr		5.1	1.30	6.6
			After 3 hrs		5.5	1.18	6.5
A	1	Sodium salicylate (5 g.)	Before treatment	0	6.3	1.00	6.3
			After 1 hr	28.1	6.2	1.53	9.5
			After 3 hrs	31.0	6.0	1.67	10.0
C	1		Before treatment	0	6.7	1.09	7.3
			After 1 hr	33.0	5.6	1.92	10.8
			After 3 hrs	27.8	5.4	1.72	9.3
D	2		Before treatment	0	5.3	0.96	5.1
			After 1 hr	39.0	4.9	1.84	9.0
			After 3 hrs	40.2	4.9	2.08	10.2

Mean values presented in Table 32

TABLE 32

EFFECT OF ADMINISTRATION OF SODIUM SALICYLATE AND SODIUM p-HYDROXY-BENZOATE ON CIRCULATING FREE THYROXINE IN NORMAL MAN

(Mean of 3 subjects from Table 31)

Treatment	Sample	Serum Salicy-late mg.%	PBI µg.I %	% Total Radio-thyroxine Dialysed in 24 hrs	Free Thyroxine (units)
Sodium p-hydroxy-benzoate (5 g.)	Before treatment		5.4	1.05	5.7
	After 1 hr		5.2	1.12	5.8
	After 3 hrs		5.5	1.03	5.7
Sodium salicylate (5 g.)	Before treatment	0	6.1	1.02	6.2
	After 1 hr	33.4	5.6	1.76	9.9
	After 3 hrs	33.0	5.4	1.82	9.8

after 1 hour, and remained almost unchanged at 33.0 mg.% at the third hour. The percentage of the total radiothyroxine dialysed in 24 hours increased from a mean pre-treatment level of 1.02 to 1.76 one hour after sodium salicylate. There was only a slight increase to 1.82 per cent at 3 hours. From Table 32 it may be seen that the free thyroxine rose to a maximum of 9.9 units after 1 hour following salicylate and remained practically unaltered at this level at the third hour.

The rates of dialysis of radiothyroxine for the control and three hour samples following the administration of the drugs (the mean values for the three subjects) are illustrated graphically in Figure 5.

Discussion

It was demonstrated in this experiment that, in spite of the fall in PBI following the administration of salicylate, the circulating free thyroxine is considerably elevated. The free thyroxine level is the product of the percentage of the total radiothyroxine dialysed in 24 hours and the PBI. Therefore, even to maintain an unchanged free thyroxine level in the presence of a fall in PBI, it is necessary that the percentage of the total radiothyroxine dialysed in 24 hours should rise. It was not possible to predict from the *in vitro* studies the level to which this value would rise following the *in vivo* administration of salicylate.

Nevertheless, in spite of the fall in PBI, the free thyroxine level rose from a mean pre-treatment level of 6.2 units to 9.9 units one hour after salicylate, and remained

IN VIVO EFFECT OF SALICYLATE ON THE DIALYSIS RATE OF I^{131} THYROXINE IN NORMAL MAN

(MEAN OF 3 SUBJECTS)

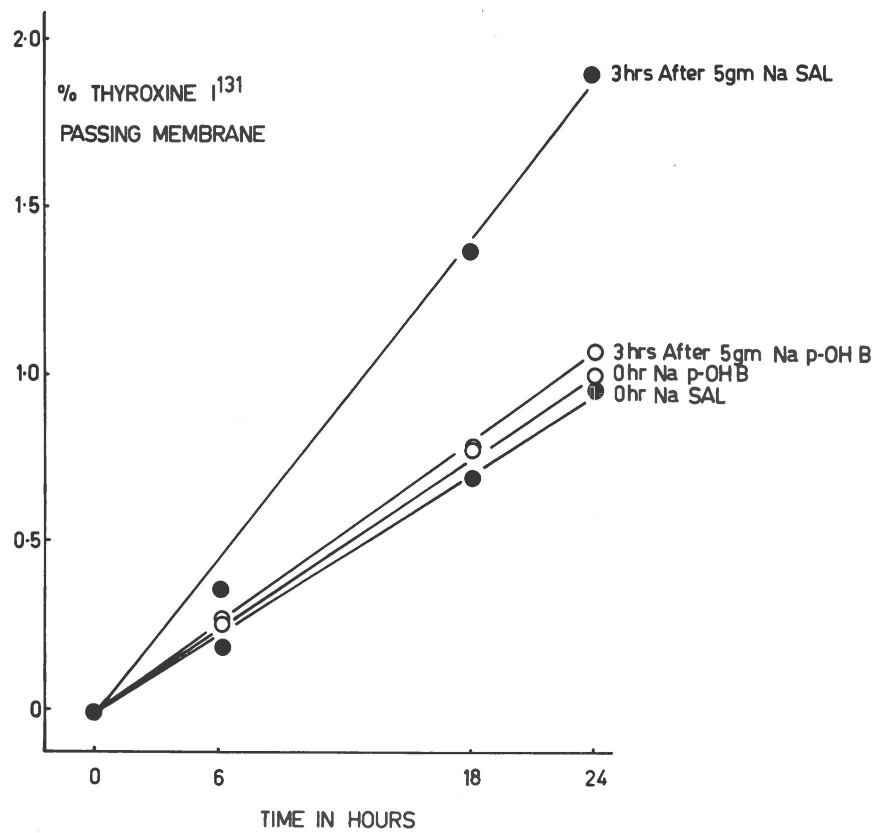


FIGURE 5.

elevated at this level at the third hour.

Although p-hydroxybenzoate <u>in vitro</u> produced a slight increase in the dialysis rate of radiothyroxine, it had no effect on free thyroxine when administered to normal human subjects.

In view of the increased dialysis rate of radiothyroxine induced by γ-resorcylate added <u>in vitro</u> to the dialysis system, it was decided to determine the effect of administration of sodium γ-resorcylate on the circulating free thyroxine in normal human subjects.

<u>Experiment 2</u>

<u>Materials and Methods</u>

This experiment was carried out on five healthy normal voluteers on two occasions three weeks apart. After control blood samples of 60 ml. were obtained, the drugs sodium p-hydroxybenzoate, sodium salicylate and sodium γ-resorcylate were administered.

Sodium p-hydroxy benzoate and sodium salicylate were given in a dose of 5 g. as in experiment 1. In order to attain a blood level comparable to that achieved following salicylate (30 mg./100 ml.), a total dosage of 8 g. of sodium γ-resorcylate was given. The drug was administered as two doses of 4 g. each, the second dose being administered 30 minutes after the first.

The treatments were given to the subjects on the two days of the experiment as follows -

Subject	Treatment	
	Day 1	Day 2
K	Sodium p-hydroxybenzoate	Sodium γ-resorcylate
L	Sodium γ-resorcylate	Sodium p-hydroxybenzoate
M	Sodium salicylate	Sodium γ-resorcylate
N	Sodium γ-resorcylate	Sodium salicylate
O	Sodium p-hydroxybenzoate	Sodium salicylate

It was shown in experiment 1 that there was no difference between the one hour and three hour values for free thyroxine following the administration of salicylate. As the subjects (prisoners from the Yatala Labour Prison) were not available for more than a short time, it was possible to take only a single blood sample two hours after the administration of the drugs. In the case of sodium γ-resorcylate the blood samples were taken two hours after the first dose was ingested.

Results

The results of the effects of the drugs on the PBI and free thyroxine for the individual subjects are presented in Table 33 and the mean values in Table 34.

Results similar to those in experiment 1 were obtained following the administration of sodium p-hydroxybenzoate and sodium salicylate. There was no effect of p-hydroxybenzoate on the PBI or free thyroxine. Sodium salicylate depressed the mean PBI value of 5.5 µg. I% to 5.1 µg. I%. The mean percentage of the total radiothyroxine dialysed in 24 hours

was 1.69 two hours after salicylate, compared to a control level of 1.07. As a result, the free thyroxine was increased from a mean control level of 5.9 units to 8.6 units after 2 hours when the level of salicylate in the serum was 29.0 mg.%.

Following the administration of sodium γ-resorcylate a mean serum level of 37.9 mg.% was obtained. The PBI was depressed from a control value of 5.5 to 3.6 μg.% after two hours. This depression was much greater than that obtained for a similar blood concentration of salicylate. The percentage of the total radiothyroxine dialysed in 24 hours was also greater than that following salicylate. The level, two hours after γ-resorcylate, was 2.55 compared to the control value of 1.09.

The free thyroxine level two hours after γ-resorcylate administration was 9.2 units compared to the control level of 6.0 units. This increase was of the same order as that produced by sodium salicylate.

A comparison of the dialysis rates of radiothyroxine for each of the drugs is presented in Figure 6.

Discussion

The depression of the PBI level by sodium γ-resorcylate in normal human subjects confirms the similar finding in rats. Although the percentage of the total radiothyroxine dialysed in 24 hours was greater following γ-resorcylate than following salicylate, the increase in free thyroxine was of the same order with both drugs because of the lower PBI following γ-resorcylate.

TABLE 33

EFFECT OF ADMINISTRATION OF SALICYLATE AND RELATED DRUGS ON CIRCULATING FREE THYROXINE IN NORMAL MAN

Subject	Treatment	Sample	Salicylate mg.%	PBI µg.I %	% Total Radio-thyroxine dialysed in 24 hrs	Free Thyroxine (units)
K	Sodium p-hydroxybenzoate (5 g.)	Before treatment		5.0	1.23	6.2
		After 2 hrs		5.2	1.07	5.6
L		Before treatment		5.7	0.99	5.6
		After 2 hrs		5.6	1.21	6.8
O		Before treatment		5.9	1.05	6.2
		After 2 hrs		5.8	1.15	6.7
M	Sodium salicylate (5 g.)	Before treatment	0	5.9	0.98	5.8
		After 2 hrs	17.3	5.5	1.46	8.0
N		Before treatment	0	5.2	1.10	5.7
		After 2 hrs	33.6	4.3	1.80	8.1
O		Before treatment	0	5.5	1.12	6.2
		After 2 hrs	36.0	5.2	1.80	9.4
K	Sodium γ-resorcylate (8 g.)	Before treatment	0	4.8	1.15	5.5
		After 2 hrs	46.5	2.9	2.51	7.3
L		Before treatment	0	6.4	1.01	6.5
		After 2 hrs	30.8	4.5	2.53	11.4
M		Before treatment	0	4.6	0.98	4.5
		After 2 hrs	49.0	3.0	2.49	7.5
N		Before treatment	0	6.0	1.23	7.4
		After 2 hrs	25.3	4.0	2.68	10.7

Mean values presented in Table 34

TABLE 34

THE EFFECT OF ADMINISTRATION OF SALICYLATE AND RELATED DRUGS ON CIRCULATING FREE THYROXINE IN NORMAL MAN

(Mean values from Table 33)

Treatment	Sample	Mean Serum Salicylate mg.%	PBI µg.I %	% Total Radio-thyroxine Dialysed in 24 hrs	Free Thyroxine (Units)
* Sodium p-hydroxybenzoate (5 g.)	Before treatment		5.5	1.09	6.0
	After 2 hrs		5.5	1.14	6.3
* Sodium salicylate (5 g.)	Before treatment		5.5	1.07	5.9
	After 2 hrs	29.0	5.1	1.69	8.6
+ Sodium γ-resorcylate (8 g.)	Before treatment		5.5	1.09	6.0
	After 2 hrs	37.9	3.6	2.55	9.2

* Mean of 3 subjects

+ Mean of 4 subjects

IN VIVO EFFECT OF SALICYLATE AND RELATED DRUGS
ON THE DIALYSIS RATE OF I^{131} THYROXINE
IN NORMAL MAN

(MEAN OF 3 SUBJECTS)

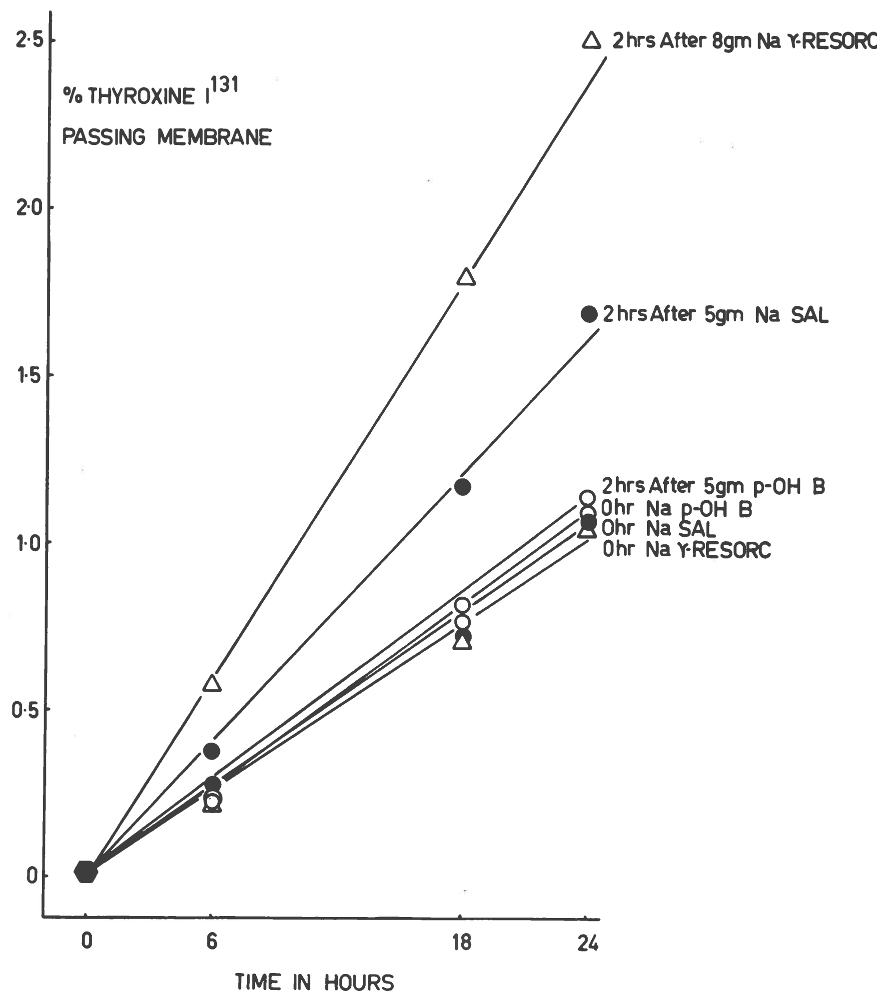


FIGURE 6.

PART IV

THE EFFECT OF SALICYLATE AND RELATED DRUGS ON FREE THYROXINE IN RATS

IN VITRO STUDIES

Materials and Methods

A large pool of normal rat serum was obtained from a group of 36 normal male rats weighing approximately 200 g. The effect of p-hydroxybenzoate, salicylate, and γ-resorcylate on the dialysis rate of radiothyroxine was examined. The drugs were added to the serum in both chambers of the dialysis cell in the same manner as for the human studies at concentrations of 30 mg./100 ml.

Results and Discussion

The results of the *in vitro* additions are given in Table 35.

The control value of 4.2 per cent of the total radiothyroxine dialysed in 24 hours for the pooled sample of normal rat serum was within the range usual for normal rat serum. This level, although much higher than for normal man, is consistent with the greater thyroxine turnover and higher metabolic rate of rats.

As in the case of human serum, the *in vitro* addition of p-hydroxybenzoate to rat serum caused only a small increase in the dialysis rate of radiothyroxine compared with the increase which followed the *in vitro* addition of salicylate and γ-resorcylate. Salicylate and γ-resorcylate produced

TABLE 35

EFFECT OF IN VITRO ADDITION OF SALICYLATE AND RELATED DRUGS ON THE DIALYSIS OF RADIOTHYROXINE IN NORMAL RAT SERUM

Sample	% Total Radiothyroxine dialysed in 24 hours at concentration of drug added to serum	
	0	30 mg.%
Pool Rat Serum	4.2	
+ p-hydroxy-benzoate		6.7
+ salicylate		13.0
+ γ-resorcylate		14.0

IN VITRO EFFECT OF SALICYLATE AND RELATED DRUGS

ON THE DIALYSIS RATE OF I^{131} THYROXINE

IN NORMAL RAT SERUM

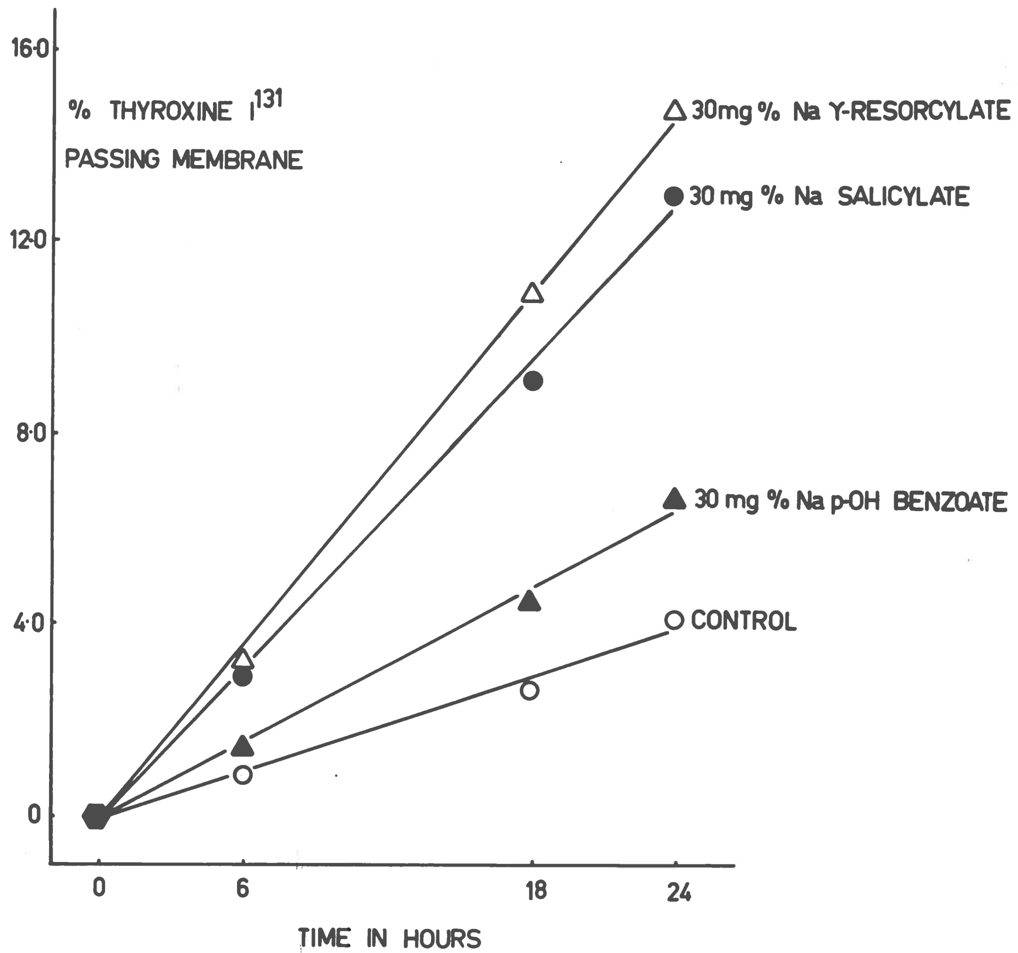


FIGURE 7.

considerable increases in the dialysis rate, values of the percentage of the total radiothyroxine dialysed in 24 hours of 13.0 and 14.8, respectively, being obtained.

Linearity of the dialysis rate over the 24 hour period was demonstrated when the values of the percentage of the total radiothyroxine were plotted against the time in hours. The results are shown in Figure 7.

These large increases in the dialysis rate of radiothyroxine following the _in vitro_ addition of salicylate and of γ-resorcylate confirm the qualitatively similar findings reported for human serum by Christensen (1959) as well as in the previous section of this chapter.

IN VIVO STUDIES

Studies on the administration of the drugs to normal rats were made in two experiments. In the first, the effects of sodium p-hydroxybenzoate, sodium benzoate and sodium salicylate on circulating free thyroxine were investigated. In the second, the effects of sodium γ-resorcylate and 2-4 dinitrophenol on free thyroxine were assessed.

A volume of 20 ml. of serum is required for each estimation of free thyroxine by the Christensen method. Because of the relatively small blood volume of the rat, it was necessary to carry out the experiments on groups of rats. Determination of free thyroxine was made on a pool of serum obtained by mixing equal volumes of serum from each rat in the group. Control blood samples obtained by cardiac puncture were used only for the determination of PBI. It was not

possible to carry out free thyroxine estimations on control samples, because of the small amount of blood obtained. Instead sodium lactate was administered to one group to serve as a control for the experimental procedure. Following treatment with the drugs the rats were exsanguinated. Sufficient serum for the estimation of free thyroxine was obtained from five rats.

Experiment 1

Materials and Methods

20 normal male rats weighing approximately 250 g. were numbered and divided at random into four groups of five rats each. Control blood samples of 3 ml. were obtained by cardiac puncture of the rats while under light ether anaesthesia. After a recovery period of eight days the treatments were commenced.

Sodium lactate, in a dose of 24 mg./100 g. body weight/day was administered to one group. Sodium p-hydroxybenzoate, sodium benzoate, and sodium salicylate were administered in a dosage of 30 mg./100 g. body weight/day, the drugs being given by gastric gavage in equally divided doses every 12 hours for 48 hours. The rats were exsanguinated four hours after the last dose was given.

Results

Effect on Plasma PBI

The effects of the drugs on the PBI are shown in Table 36.

There was no significant effect on the PBI following treatment with sodium lactate, sodium p-hydroxybenzoate or

TABLE 36

EFFECT OF SALICYLATE AND RELATED DRUGS ON PLASMA PBI IN NORMAL RATS

Rat No.	Treatment	PBI µg. I %		p*	Salicylate mg.% Treated 52 hrs.
		Before Treatment	Treated 52 hrs.		
11 27 10 29 13	Sodium lactate 24 mg./100 g./ day	2.8 3.0 3.1 3.5 3.0	3.5 3.0 3.3 3.7 3.5		
Mean		3.1	3.4	N.S.	
19 34 17 15 26	Sodium benzoate 30 mg./100 g./ day	3.0 3.0 2.9 2.7 3.0	3.2 3.4 3.0 3.0 3.2		
Mean		2.9	3.2	N.S.	
24 18 23 28 29	Sodium p-hydroxy-benzoate 30 mg./100 g./ day	3.2 2.9 3.3 2.9 3.1	2.9 2.8 2.8 2.6 3.1		
Mean		3.1	2.8	N.S.	
12 25 14 21 31	Sodium salicylate 30 mg./100 g./ day	3.1 3.4 3.1 2.9 3.0	1.1 1.4 1.3 1.4 1.3		39.6 34.8 40.6 41.2 40.8
Mean		3.1	1.3	$< .001$	39.4

* p = compared to control day by "t" test

sodium benzoate. However, the usual depression following sodium salicylate was obtained. The fall in PBI from the pre-treatment value of 3.1 to 1.3 μg. I% after 52 hours of salicylate treatment was highly significant ($P < 0.001$).

Effect on Free Thyroxine

The results are set out in Table 37.

There was no increase in free thyroxine produced by administration of either sodium p-hydroxybenzoate or sodium benzoate. The free thyroxine values were slightly smaller (10.9 and 11.5 units respectively) than that obtained following the administration of sodium lactate (12.2 units) which was used as the control. However, there was a large increase in the percentage of the total radiothyroxine dialysed in 24 hours following salicylate treatment. This value of 11.8 per cent when multiplied by the low PBI value of 1.3 μg. I per 100 ml. gave a free thyroxine level of 15.3 units. The dialysis rates of radiothyroxine following the administration of the drugs to rats are illustrated in Figure 8.

Experiment 2

Materials and Methods

The same experimental procedure was followed in this study which has already been described in Chapter II (Experiment 2).

Sodium lactate (24 mg./100 g. body weight/day) was again used as a control for the experimental procedure. Sodium γ-resorcylate was administered in a total dose of 90 mg./100 g.

TABLE 37

EFFECT OF ADMINISTRATION OF SALICYLATE AND RELATED DRUGS FOR 52 HOURS ON THE CIRCULATING FREE THYROXINE IN NORMAL RATS

Treatment	PBI µg.I %	% Total Radiothyroxine Dialysed in 24 hours	Free Thyroxine (units)
Experiment 1			
Control Sodium lactate (24 mg./100 g./day)	3.4	3.6	12.2
Sodium p-hydroxy-benzoate (30 mg./100 g./day)	2.8	3.9	10.9
Sodium benzoate (30 mg./100 g./day)	3.2	3.6	11.5
Sodium salicylate (30 mg./100 g./day)	1.3	11.8	15.3
Experiment 2			
Control Sodium lactate (24 mg./100 g./day)	3.0	4.2	12.6
Sodium γ-resorcylate (90 mg./100 g./day)	1.6	10.8	17.3
2-4 dinitrophenol (2.5 mg./100 g./day)	1.6	10.8	17.3

IN VIVO EFFECT OF SALICYLATE AND RELATED DRUGS ON THE DIALYSIS RATE OF I^{131} THYROXINE IN THE NORMAL RAT

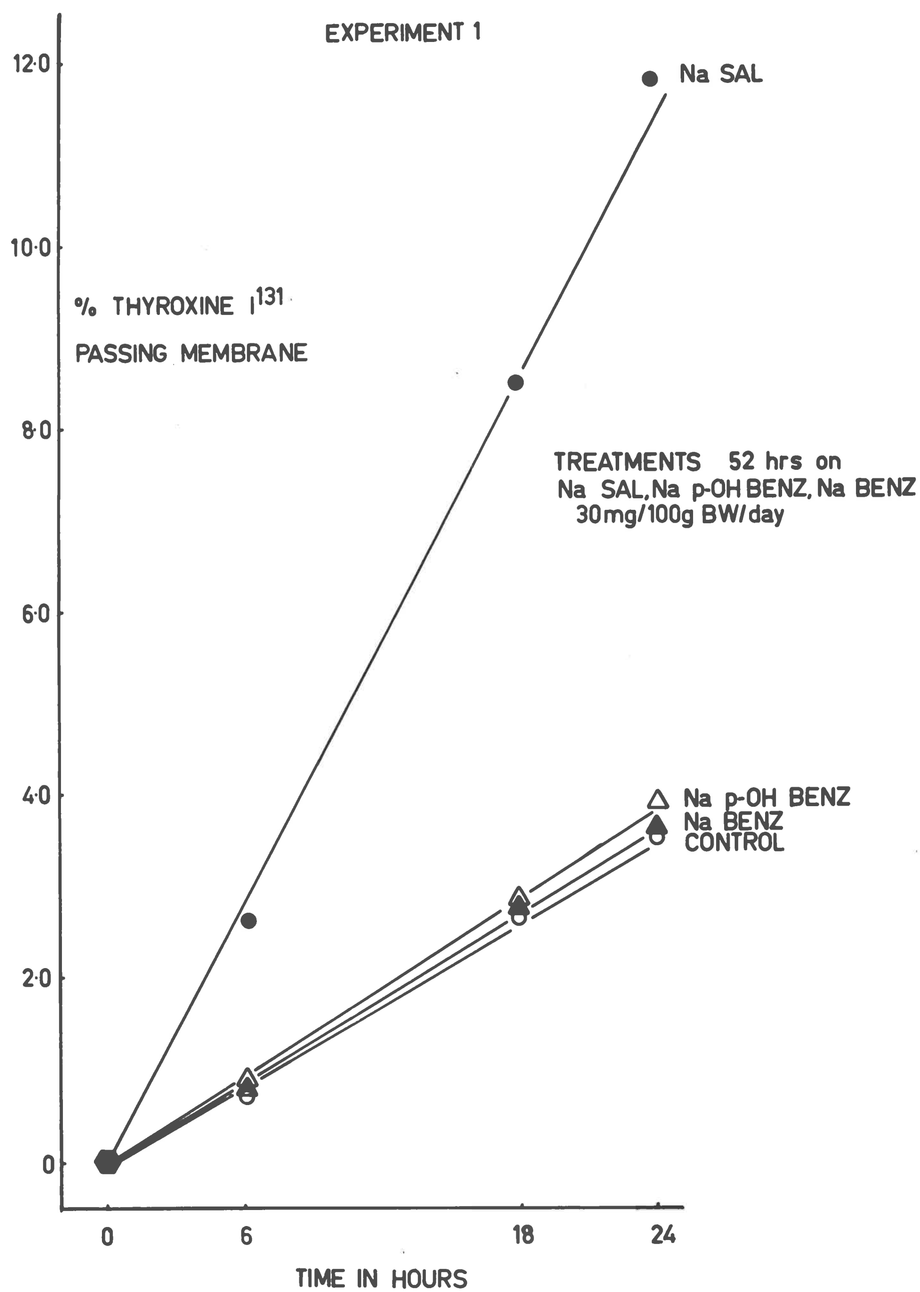


FIGURE 8.

IN VIVO EFFECT OF γ-RESORCYLATE AND 2,4-DINITROPHENOL

ON THE DIALYSIS RATE OF I^{131} THYROXINE

IN THE NORMAL RAT

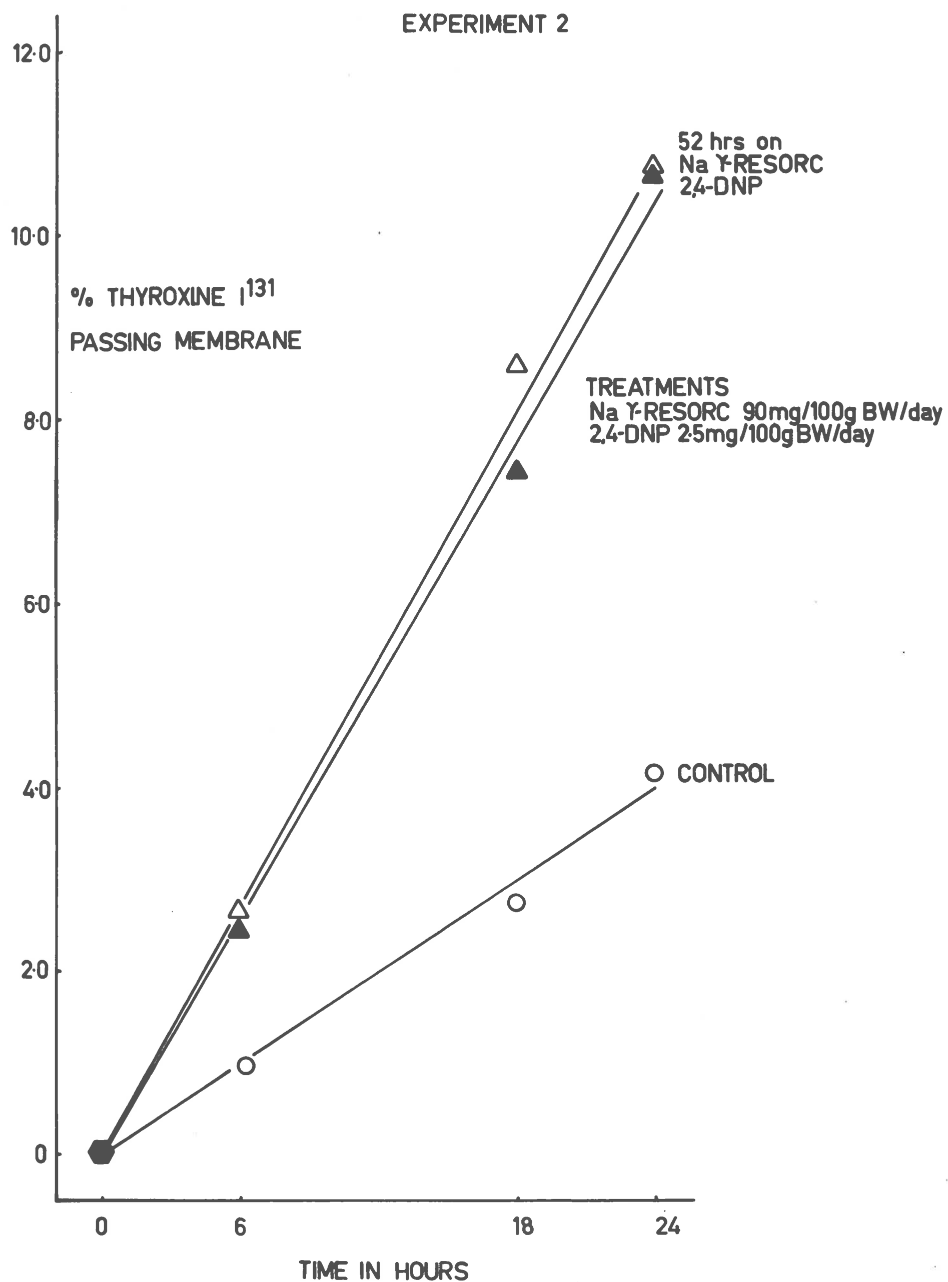


FIGURE 9.

body weight/day. 2,4-Dinitrophenol was given at the rate of 2.5 mg./100 g. body weight/day.

Results

Effect on Plasma PBI

The PBI results are set out in Table 4 (Chapter II). There was a slight fall in PBI following treatment with sodium lactate. Significant depressions in PBI following sodium γ-resorcylate and 2,4-dinitrophenol were reported.

Effect on Free Thyroxine

The results are presented in Table 37.

A value of 12.6 units of free thyroxine was obtained following sodium lactate treatment. A value of 10.8 per cent of the total radiothyroxine dialysed in 24 hours was obtained following both sodium γ-resorcylate and 2,4-dinitrophenol, compared to the control level of 4.2 per cent. The free thyroxine level was elevated to 17.3 units following both drugs. The dialysis rates obtained in this experiment are shown in Figure 9.

Discussion

The demonstration of an increase in free thyroxine following both salicylate and γ-resorcylate administration to normal rats confirms the similar effect of the drugs in normal man. Because of its high toxicity, 2,4-dinitrophenol could not be given to human subjects; when administered to normal rats 2,4-dinitrophenol caused an increase in free thyroxine.

This finding is in accord with the <u>in vitro</u> effect of the drug.

Sodium p-hydroxybenzoate was without effect on the circulating free thyroxine in rats, as was the case in human subjects and did not produce a depression in PBI following its administration to rats. Sodium benzoate was also without effect on the PBI in normal rats, neither did it effect the free thyroxine.

PART V

CONCLUSIONS

The report of Christensen (1959) that the *in vitro* addition of salicylate to human serum caused an increase in the dialysis rate of radiothyroxine has been confirmed in experiments reported in this chapter. These studies were extended to an examination of the effects of drugs chemically related to salicylate.

The *in vitro* addition of p-hydroxybenzoate, in the same concentrations as used with salicylate, produced much smaller increments in the dialysis rate of radiothyroxine than did salicylate. The drug was ineffective when added at a level of 10 mg. per cent, whereas there was an appreciable effect with salicylate at that level. The *in vitro* addition of γ-resorcylate to human serum at a concentration of 30 mg. per 100 ml. produced a greater increase in the dialysis rate of radiothyroxine than the same concentration of salicylate.

From a consideration of the equations derived from the law of mass action relating the interaction between thyroxine and the specific thyroxine-binding sites on the serum proteins, it was established that an increase in the dialysis rate of radiothyroxine must depend upon the release into the free state of thyroxine bound to the serum proteins. A mechanism for the action of salicylate and γ-resorcylate depending on the release of bound thyroxine into the free state, followed by the rapid metabolism or excretion of the free thyroxine,

may therefore be postulated to account for the depression in PBI following the administration of the drugs to man. However, the <u>in vivo</u> effect of administration of the drugs to man cannot be extrapolated directly from the <u>in vitro</u> findings because of the altered equilibrium consequent upon the lowered PBI.

The circulating level of free thyroxine was shown to be elevated within two hours of the administration of a single oral dose of both salicylate and γ-resorcylate to normal human subjects. Thus, although the equilibrium was altered by the lowered total thyroid hormone circulating, the free thyroxine level was elevated. The administration of p-hydroxybenzoate was without effect on the level of free thyroxine.

Large increases in the dialysis rate of radiothyroxine were produced by the <u>in vitro</u> addition of salicylate and γ-resorcylate to normal rat serum, whereas a small increase occurred following the <u>in vitro</u> addition of p-hydroxybenzoate at the same drug concentration (30 mg. per 100 ml.).

The effect of more prolonged administration of the drugs was examined in experiments using normal rats. In spite of the greatly lowered circulating thyroid hormone level following treatment for 52 hours with salicylate, γ-resorcylate and 2,4-dinitrophenol, the free thyroxine was elevated above the control value in each case. There was no significant change in the PBI level nor was the free thyroxine elevated following the administration of sodium p-hydroxybenzoate or

sodium benzoate to other groups of normal rats for 52 hours.

The mechanism producing the acute depression in PBI and acute increase in free thyroxine within two hours of the administration of salicylate and γ-resorcylate to man was therefore still in operation following repeated dosage of the drugs over a much longer period, providing the blood level of the drugs was maintained, as was the case in the rat experiments.

It is apparent from these studies that only those drugs which were capable of producing an increase in the circulating free thyroxine, presumably by displacement of thyroxine from the specific binding sites on the serum proteins, caused a simultaneous depression in the PBI. This was the case with salicylate, γ-resorcylate and 2,4-dinitrophenol. On the other hand, p-hydroxybenzoate and benzoate which did not produce increases in free thyroxine following their administration in either acute or chronic experiments, failed to depress the PBI level.

It was demonstrated in Chapter III that salicylate, 2,4-dinitrophenol and γ-resorcylate significantly depressed the circulating TSH level of normal rats when administered for 52 hours in the same dosage as that used in the experiments on free thyroxine reported in this chapter. Under the same conditions sodium p-hydroxybenzoate did not depress the TSH level in rats.

The finding of lowered PBI levels and depressed TSH release following administration of salicylate, 2,4-dinitrophenol and γ-resorcylate would appear to be correlated with the finding of increased levels of free thyroxine produced by displacement of thyroxine from the thyroxine-binding sites on the serum proteins.

There is an apparent disruption of the feedback mechanism following salicylate, γ-resorcylate and 2,4-dinitrophenol administration, since the lowered thyroid hormone level would be expected to stimulate output of TSH from the pituitary. If, however, the level of free thyroxine rather than the total thyroid hormone is considered to be the biologically effective regulator of the feedback mechanism, this apparent disruption may be explained. Thus, despite the lowered circulating thyroid hormone (measured as PBI), the free thyroxine was elevated following salicylate, γ-resorcylate and 2,4-dinitrophenol treatment. The increased level of free thyroxine would suppress TSH release in its capacity as regulator of the thyroid-pituitary axis.

Thus the action of salicylate, γ-resorcylate and 2,4-dinitrophenol in depressing both PBI and TSH may be related to an ability to displace thyroxine from some of the specific thyroxine-binding sites on the serum proteins.

Studies of the effect of salicylate and related drugs on the binding of thyroxine to the serum proteins are described in the following chapter.

CHAPTER V

THE EFFECT OF SALICYLATE AND RELATED DRUGS ON THE BINDING OF THYROXINE TO THE SERUM PROTEINS

PART I — The Method of Determination of Thyroxine Binding by Paper Electrophoresis.

PART II — The Effect of Salicylate and Related Drugs on Thyroxine Binding in Man.

PART III — The Effect of Salicylate and Related Drugs on Thyroxine Binding in Rats.

1. The method of determining thyroxine binding in serum using starch gel electrophoresis.

2. The effect of salicylate and related drugs on thyroxine binding in rats.

3. The effect of γ-resorcylate on thyroxine binding in human serum determined by starch gel electrophoresis.

PART IV — Conclusions.

CHAPTER V

THE EFFECT OF SALICYLATE AND RELATED DRUGS ON THE BINDING OF THYROXINE TO THE SERUM PROTEINS

INTRODUCTION

It was demonstrated by Gordon and co-workers (1952) that when human serum, equilibrated with I^{131}-labelled thyroxine, was subjected to electrophoresis on filter paper in veronal buffer at pH 8.6, the I^{131}-labelled thyroxine migrated principally with the α-globulins in the zone between α_1- and α_2-globulin. A small proportion, about 10%, migrated with albumin. This finding was confirmed by Wingler and Notrica, (1952) and by Larson, Deiss and Albright, (1952). The inter α-globulin protein with which the thyroxine was associated came to be known as thyroxine binding globulin (TBG).

Deiss, Albright and Larson (1953) demonstrated that I^{131}-labelled thyroxine could be added in vitro to normal human serum in amounts up to 15 µg. per 100 ml. without affecting the distribution of radioactivity localized in the two binding sites. When the serum was enriched with increasing concentrations of stable hormone there was a displacement of labelled hormone from TBG to albumin, so that an increasing percentage of thyroxine was associated with albumin, and a correspondingly smaller percentage with TBG. Calculated values for the total quantity of thyroxine associated with TBG were shown to increase continuously as the thyroid hormone concentration rose (Robbins and Rall, 1955). These workers ascribed the failure to demonstrate a binding maximum for TBG

to the trailing of albumin carrying with it thyroxine across the TBG zone.

Robbins (1956) therefore devised a system of reverse-flow paper electrophoresis in which hydrodynamic flow of the buffer opposes the electrophoretic migration of albumin and therefore prevents the trailing of this component. Employing this technique with veronal buffer, Robbins found an average thyroxine binding capacity of TBG in normal serum of 20 μg. thyroxine per 100 ml. The thyroxine binding capacity of albumin appeared to be unlimited.

An inter α-globulin protein with a high thyroxine binding capacity was isolated by Ingbar in 1958. This protein was homogeneous by electrophoretic and ultracentrifugal criteria. During the isolation, another protein fraction was recovered which also bound thyroxine avidly during paper electrophoresis in veronal buffer. The rapid anodal migration of this component, to a position ahead of albumin, indicated that it was a prealbumin. A similar prealbumin with strong thyroxine binding properties had been isolated in 1956 (Schultze et al, 1956). This was called thyroxine binding prealbumin (TBPA). A mixture of this thyroxine binding prealbumin with an equal concentration of serum albumin was subjected to electrophoresis in veronal buffer. It was demonstrated that thyroxine was bound predominantly by prealbumin over a wide range of concentrations which indicated that the interaction between thyroxine and prealbumin was greater than that between thyroxine and albumin (Ingbar, 1958).

Because it permitted good electrophoretic resolution of the serum proteins, a buffer containing trishydroxy-methylaminomethane (tris) and maleic acid was used to separate normal serum proteins on cellulose columns. TBPA could be consistently demonstrated in normal serum using this buffer. When paper electrophoresis of normal human serum was carried out in trismaleate buffer, TBPA was revealed as a normal component of human serum. Thus TBPA could be regularly demonstrated in human serum following paper electrophoresis in trismaleate buffer but not when veronal buffer was employed.

Ingbar considered that veronal might either interfere with the binding of thyroxine by TBPA, or promote interaction between TBPA and other proteins such that the electrophoretic mobility of TBPA was altered. On the other hand, TBPA could be an artefact induced by electrophoresis in trismaleate buffer.

Tata (1959) demonstrated that during electrophoresis of serum which was enriched with a prealbumin (TBPA) prepared by Schultze, that the TBPA appeared to change mobility and migrate with the α-globulins. By the use of electrophoretic and immunological techniques, Tata claimed that TBG resulted from an interaction between TBPA and another serum protein. He suggested that the greatest portion of TBPA in the circulation was in this combined form. However, it was subsequently shown that the findings were artefactual owing to the contamination of the 'purified' TBPA with TBG.

TBPA has been demonstrated in human serum by electrophoresis not only in trismaleate but also in borate, phosphate and alanine buffers (Ingbar, 1960), and also in ammonium carbonate buffer (Beierwaltes and Robbins, 1959). The thyroxine binding capacity of TBG was shown to be identical in both the veronal and trismaleate buffers (20 µg. thyroxine per 100 ml.).

These findings indicated that veronal ions inhibit the binding of thyroxine to TBPA. Subsequently this was confirmed by Tata, Widnell and Gratzer, (1961) who demonstrated that the binding of thyroxine to TBPA was prevented by the addition of veronal to buffers such as trismaleate and phosphate using paper, cellulose-acetate strip and column electrophoresis.

Tata and co-workers (1961) also showed that TBG and TBPA exhibited differences in their capacity to bind substances related to thyroxine. TBG interacted most intensely with thyroxine but hardly at all with tetraiodothyroacetic acid, whereas the affinity of TBPA for the acetic acid analogue was greater by a hundredfold. It was concluded that each molecule of TBG has a single thyroxine binding site, one of which interacts with thyroxine and is veronal sensitive; the other site which is not affected by veronal does not interact with thyroxine. Both binding sites of TBPA exhibit a high affinity for tetraiodothyroacetic acid but do not bind triiodothyronine. This evidence conclusively proved that both TBG and TBPA are normal components of human serum.

Christensen (1959) demonstrated that the <u>in vitro</u> addition of either 2,4-dinitrophenol or salicylate to serum caused an increase in the rate of dialysis of radiothyroxine. He postulated that this effect was produced by displacement of thyroxine bound to the specific binding sites of the serum proteins. Austen and co-workers (1958) had postulated that this mechanism might operate in depressing the PBI following the administration of salicylate to man. However, in studies of thyroxine binding using paper electrophoresis in veronal buffer at pH 8.6, they were unable to demonstrate any change in the thyroxine binding capacity of the serum from normal human subjects treated with salicylate.

Using the trismaleate buffer system at pH 8.4 for paper electrophoresis, Ingbar (1960) reported that when salicylate was added to the buffer system at a concentration of 50 mg./100 ml., the proportion of thyroxine normally bound to the TBPA site was diminished, and that the amount displaced was bound to the TBG site.

Wolff, Standaert and Rall (1961) made a comprehensive study of the effect of salicylate (and other drugs which depress PBI) on the binding of thyroxine to human serum proteins, using paper electrophoresis in ammonium carbonate buffer at pH 8.4. The drugs were added <u>in vitro</u> either to the buffer system or to the serum prior to electrophoresis, in concentrations up to 50 mg. per 100 ml. When added to either buffer or serum both 2,4-dinitrophenol and salicylate inhibited the binding of thyroxine to the TBPA site and the

displaced thyroxine was bound to TBG instead. However, the effect was greater when the drugs were added to the buffer, as this provided a reservoir of the drug which permitted replacement of any unbound drug which migrated off the paper during electrophoretic separation.

It was reported by Oppenheimer and colleagues (1961) that chronic treatment of patients with diphenylhydantoin caused a depression in the PBI. It was postulated that the depression in the PBI might result from a displacement of thyroxine from one of the thyroxine binding sites of the serum proteins. This was confirmed, since at clinically acceptable concentrations of diphenylhydantoin, there was a significant displacement of thyroxine from TBG to prealbumin.

DL-tetrachlorothyronine was also shown by Wolff and co-workers (loc. cit.) to displace thyroxine from TBG and at high concentrations, also from TBPA. This drug, when administered to both normal and thyroidectomized guinea pigs maintained by thyroxine, produced a depression in PBI similar to the depression produced by salicylate in rats reported in Chapter II.

A decrease in the number of available binding sites either in TBG or TBPA produced by diphenylhydantoin and tetrachlorothyronine in the first case, or by salicylate and 2,4-dinitrophenol in the second, would be expected by mass law relations to increase the free thyroxine at the expense of the fraction bound. Wolff and co-workers postulated that the increase in free thyroxine which followed the administration

of such drugs to man or animals would accelerate thyroxine disappearance from the circulation and ultimately lower the PBI to a new steady state value at which the concentration of free thyroxine might be perfectly normal.

However, the finding (Chapter IV) that an increase in free thyroxine resulted from the chronic administration of salicylate, 2,4-dinitrophenol and γ-resorcylate indicated that a steady state of PBI and free thyroxine was not rapidly reached.

It was decided to examine the mechanism postulated for the action of these drugs in producing an increase in free thyroxine _in vivo_, namely, the displacement of thyroxine from the thyroxine binding sites. Studies of thyroxine binding were therefore made on the serum samples obtained in both the human and rat experiments and used for free thyroxine determination reported in Chapter IV.

Before carrying out these investigations the precision of the method employing paper electrophoresis in ammonium carbonate buffer at pH 8.4 was assessed. Further, the _in vitro_ experiments with salicylate were repeated and extended to an examination of chemically related drugs.

PART I

THE METHOD OF DETERMINATION OF THYROXINE BINDING BY PAPER ELECTROPHORESIS

Materials and Methods

Elzinka, Carr and Beierwaltes (1961) adapted, by a simple modification, a standard Durrum-type electrophoresis cell so that the reverse-flow paper electrophoretic separation of serum proteins described by Robbins (1956) could be performed without the use of a specially constructed apparatus. Results obtained with this simplified apparatus were shown to compare favourably with those obtained using Robbins' apparatus, and therefore this modification was used for these studies.

Apparatus

A Spinco Model R, Paper Electrophoresis Cell (Beckman Instruments Ltd.) was used in conjunction with a Stacpac Regulated Power Supply (Fairey Aviation Co. of Australasia) and Schleicher and Schuell No. 2043 paper strips (3.0 by 30.6 cms.) employed.

Reagents

I^{131}-labelled thyroxine solution:

Radiothyroxine in 50% propylene glycol, 200 microcuries/ml. (specific activity of approximately 30 millicuries/mg.) from Abbott Laboratories, Oak Ridge, Tennessee, U.S.A. On the day the shipment arrived at the laboratory, the radiothyroxine was added in the correct concentration to the serum samples to be analysed. The presence of serum proteins stabilizes the

radiothyroxine against deiodination, and the dilution in serum proteins reduces the effects of autoirradiation (Tata, 1959).

Ammonium Carbonate Buffer:

The pH of 0.12M ammonium carbonate solution was adjusted to 8.4 by addition of either carbon dioxide or ammonia. The various drugs were added and the pH re-adjusted to 8.4 before use.

Bromophenol Blue Solution:

1% (W/V) bromophenol blue and 1% (V/V) glacial acetic acid dissolved in 95% ethanol.

0.5% acetic acid solution:

10 ml. glacial acetic acid diluted to 2 litres in glass distilled water.

Procedure

On receipt of the radiothyroxine, 2 ml. aliquots of the sera to be analysed were enriched to a level of 10 μg. I^{131}-labelled thyroxine per 100 ml. After addition of the radiothyroxine, the samples were incubated for three hours at 37°C and stored in a refrigerator at 4°C until assayed.

The ammonium carbonate buffer was placed in the buffer compartments of the electrophoretic cell so that the anodal compartment contained 525 ml. of buffer, whereas the cathodal side contained only 475 ml. Eight paper strips were placed in the cell and allowed to reach equilibrium with the buffer. A glass rod was then slipped under the strips, 8 cm. from the anodal end, on a line previously marked. For each analysis

10 µl. of serum was applied to duplicate papers on the line stabilized by the glass rod. After application of the samples, the rod was removed and the cell closed and sealed.

Electrophoresis was carried out at 140 volts for 16 hours in a constant temperature room set at 18 ± 1°C. On completion of the electrophoresis, the paper strips were dried in an electric oven at 110°C for 30 minutes to coagulate and fix the proteins. One of each pair of duplicate strips was set aside for radioautography and subsequent dyeing of the protein bands. On the second strip of each duplicate set the radioactivity on each thyroxine binding site was measured.

Radioautography

The paper strips were fixed by tape to Kodak No-Screen X-ray film, numbered and pinned to a board in two places. The X-ray film was developed after an exposure period of at least ten days (or longer depending on the specific activity of the sample).

Dyeing of the Paper Strips

After the radioautographs had been developed, the paper strips were immersed in the 1% bromophenol blue solution for ten minutes. Excess dye was removed from the strip by several washes in 0.5% acetic acid solution over a period of two hours. The papers were then dried at room temperature. The stained strips were realigned on the film by the pin holes, and the specific protein bands containing the radioactive thyroxine located.

Measurement of the Radioactivity on the Strips

The second strip of the duplicate set was divided into 0.5 cm. sections commencing at the anodal end of the paper. The sections were numbered, cut and placed in numbered counting tubes. 1.0 ml. of concentrated sulphuric acid was added to each tube. After the paper had dissolved the contents of the tubes were mixed by gentle shaking. The radioactivity of each section was measured using a well-type scintillation counter (Ekco Type N597). The count rate of each section was plotted as the ordinate, against the section number as the abscissa. Identification of each radioactive peak was made from the radioautograph of the duplicate strip. The total radioactivity on each binding site was calculated by addition of the count rates of each 0.5 cm. section forming the peak. The radioactivity carried on each binding site was then expressed as a percentage of the total radioactivity in the sample.

The three sites of binding of thyroxine in human serum, inter α_1-, α_2-globulin (TBG), albumin, and prealbumin (TBPA) were readily identified and the proportion of total radiothyroxine on each site assessed.

Precision of the Method

Eight replicate determinations of the proportion of radiothyroxine carried on the binding sites of a single sample of normal human serum were carried out. The sample was subjected to electrophoresis in quadruplicate in one batch and subsequently in four different batches. The radioactivity

carried on each binding site was determined and expressed as a percentage of the total radioactivity in the sample.

The results are shown in Table 38.

The mean values of the percentage of the total radioactivity bound on each site with the standard deviation were respectively, TBPA 45.8 ± 1.0, Albumin 19.8 ± 1.8 and TBG 34.4 ± 1.3.

The method was evidently precise and so could be used to determine alterations in the proportion of radiothyroxine bound to the three specific thyroxine binding proteins.

TABLE 38

RESULTS OF REPEATED ESTIMATION OF THYROXINE-BINDING IN THE SAME SAMPLE OF HUMAN SERUM

Electrophoretic Separation Batch Number	% Total Radioactivity on Thyroxine-Binding Sites		
	TBPA	ALB	TBG
1	46.3	19.3	34.4
1	44.9	21.5	33.6
1	46.7	19.8	33.5
1	43.4	22.5	34.1
2	46.5	20.3	33.2
8	46.1	17.4	36.5
11	46.3	17.0	36.7
14	46.1	20.4	33.5
Mean	45.8	19.8	34.4
S.D.	± 1.0	± 1.8	± 1.3

PART II

THE EFFECT OF SALICYLATE AND RELATED DRUGS ON THYROXINE BINDING IN MAN

IN VITRO STUDIES

Materials and Methods

The drugs, p-hydroxybenzoic acid, salicylic acid, γ-resorcylic acid and 2,4-dinitrophenol were dissolved in the ammonium carbonate buffer at a concentration of 50 mg./100 ml. and the pH readjusted to 8.4.

Sera from four normal subjects were subjected to electrophoresis using the standard buffer and the percentage of the total radiothyroxine on each site determined.

The distribution of radiothyroxine in the same sera was then determined following electrophoresis in buffer containing p-hydroxybenzoate. Subsequently the same sera were analysed following the addition of salicylate, γ-resorcylate, and 2,4-dinitrophenol to the buffer in turn.

Results

The results of the *in vitro* additions are presented in Table 39.

Salicylate and γ-resorcylate when added to the buffer at a level of 50 mg./100 ml. produced a large displacement of radioactive thyroxine from TBPA to TBG. The displacement with salicylate (26.0%) was slightly greater than that with 2,4-dinitrophenol (24.6%) and still greater than with γ-resorcylate (21.3%). When p-hydroxybenzoate was added to

TABLE 39

THE IN VITRO EFFECT OF SALICYLATE AND RELATED DRUGS ON THYROXINE BINDING IN NORMAL HUMAN SERUM

Addition to Buffer (50 mg.%)	No. of Expts.	% Total Radioactivity on Thyroxine Binding Sites			% Total Radioactivity displaced from TBPA
		TBPA	ALB	TBG	
Control	4	30.4	15.7	53.9	
p-hydroxy-benzoate	4	19.6	12.6	67.8	10.8
Salicylate	4	4.4	15.7	79.9	26.0
γ-resorcylate	4	9.1	16.5	74.4	21.3
2-4 dinitrophenol	4	5.8	32.6	61.6	24.6

the buffer in the same concentration there was a displacement of only 10.8% of the total radioactivity.

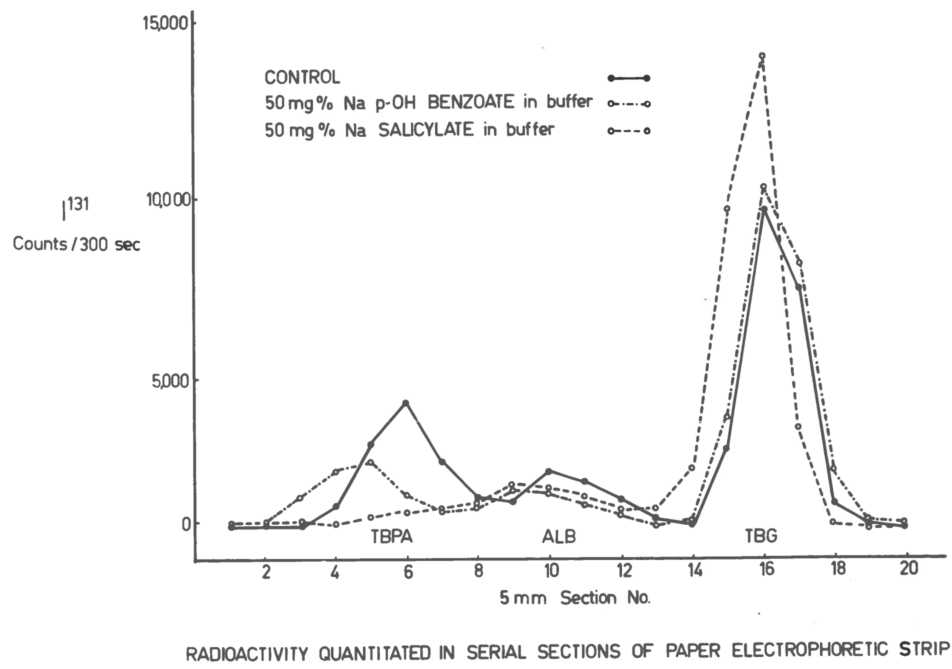
A comparison between the count rates on the serial sections of these strips and the control pattern is represented graphically for salicylate and p-hydroxybenzoate in Figure 10 and for γ-resorcylate in Figure 11.

Discussion

The *in vitro* addition of salicylate and 2,4-dinitrophenol in a concentration of 50 mg./100 ml. to the electrophoretic buffer produced a marked displacement of radioactive thyroxine from TBPA, confirming the findings of Wolff and co-workers (1961). In the case of salicylate the displaced thyroxine was bound to TBG, but with 2,4-dinitrophenol the displaced thyroxine was bound to both TBG and albumin. This latter finding was observed by Wolff and co-workers when 2,4-dinitrophenol was added to the buffer in a concentration of 3×10^{-4}M. The concentration of drug in the buffer in the present study was 2.7×10^{-3}M. However, the result obtained in this study, when all but 5.8 per cent of the radiothyroxine was displaced from TBPA, was similar to the 5.0 per cent displacement found by Wolff and co-workers using the lower drug concentration, indicating that the drug had exerted a maximal effect on the displacement of thyroxine from the binding sites of TBPA at the lower concentration.

The action of γ-resorcylate in displacing radiothyroxine from TBPA to TBG to the same degree as salicylate is consistent with the increased dialysis rate of radiothyroxine produced by

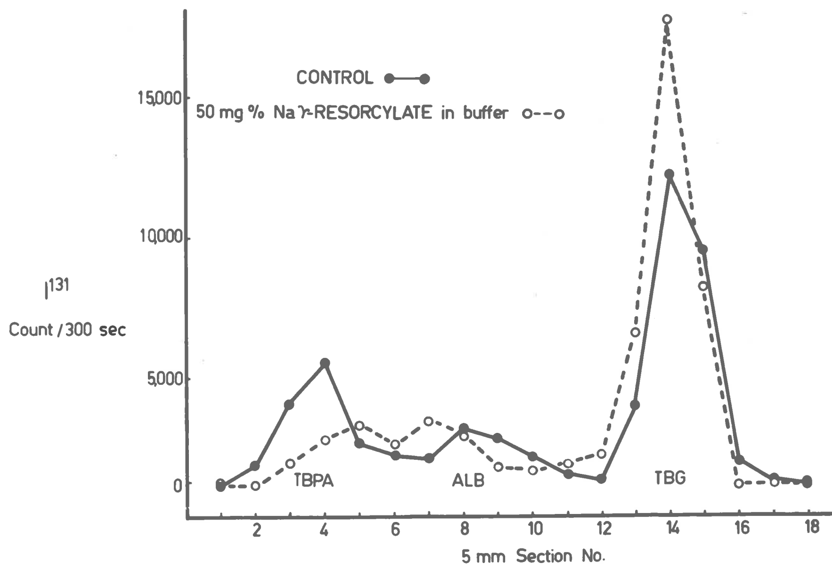
IN VITRO EFFECT OF SALICYLATE AND p-OH BENZOATE ON THYROXINE BINDING IN NORMAL HUMAN SERUM



RADIOACTIVITY QUANTITATED IN SERIAL SECTIONS OF PAPER ELECTROPHORETIC STRIP

FIGURE 10.

IN VITRO EFFECT OF γ-RESORCYLATE ON THYROXINE BINDING IN NORMAL HUMAN SERUM



RADIOACTIVITY QUANTITATED IN SERIAL SECTIONS OF PAPER ELECTROPHORETIC STRIP

FIGURE 11.

the drug which was reported in the previous chapter.
The demonstration of a smaller effect, compared to salicylate, of the addition of p-hydroxybenzoate *in vitro* on the displacement of thyroxine from TBPA substantiates the small increase noted in the dialysis rate of radiothyroxine following the *in vitro* addition of the drug.

THE EFFECT OF ADMINISTRATION OF SALICYLATE AND RELATED DRUGS ON THYROXINE BINDING IN NORMAL HUMAN SUBJECTS

Materials and Methods

Binding studies were carried out on the serum samples obtained from the two experiments which tested the effect of salicylate and related drugs on free thyroxine in normal human subjects. (Chapter IV)

Results

Experiment 1

The results obtained from the measurement of radioactivity on the binding sites of the serum taken before and three hours after treatment of each of the subjects with the drugs are shown in Table 40. The mean values are given in Table 41. The one hour samples were not analysed because after that interval the free thyroxine levels were not appreciably different from the levels at three hours. Following administration of sodium p-hydroxybenzoate the binding of radioactive thyroxine to the prealbumin binding site was not inhibited. However, three hours following sodium salicylate, at a mean serum level of 36.3 mg. salicylate/100 ml., there was an inhibition of binding by TBPA, 9.5 per cent of the added radiothyroxine being displaced to TBG.

Graphs obtained by plotting the count rates of the serial sections of the strips, demonstrating the *in vivo* effect of p-hydroxybenzoate and salicylate at three hours, compared to the control are shown in Figure 12 and Figure 13.

TABLE 40

THE IN VIVO EFFECT OF SALICYLATE AND p-HYDROXY BENZOATE ON THYROXINE BINDING IN NORMAL MAN

Subject	Day	Sample	% Total Radioactivity on Thyroxine Binding Sites			Sample	% Total Radioactivity on Thyroxine Binding Sites		
			TBPA	ALB	TBG		TBPA	ALB	TBG
A	2	Before treatment	26.5	19.2	54.3	3 hrs after sodium p-hydroxy benzoate (5 g.)	25.3	17.4	57.3
C	1		28.1	16.3	55.6		28.0	16.1	55.9
D	1		25.3	11.8	62.9		27.0	14.8	58.2
Mean			26.6	15.8	57.6		26.8	16.1	57.1
A	1	Before treatment	27.9	14.9	57.2	3 hrs after sodium salicylate (5 g.)	19.7	13.7	66.6
C	2		31.4	16.5	52.1		20.0	16.4	63.6
D	2		28.0	16.4	55.6		19.2	15.0	65.8
Mean			29.1	15.9	55.0		19.6	15.0	65.3

Mean values presented in Table 41

5

TABLE 41

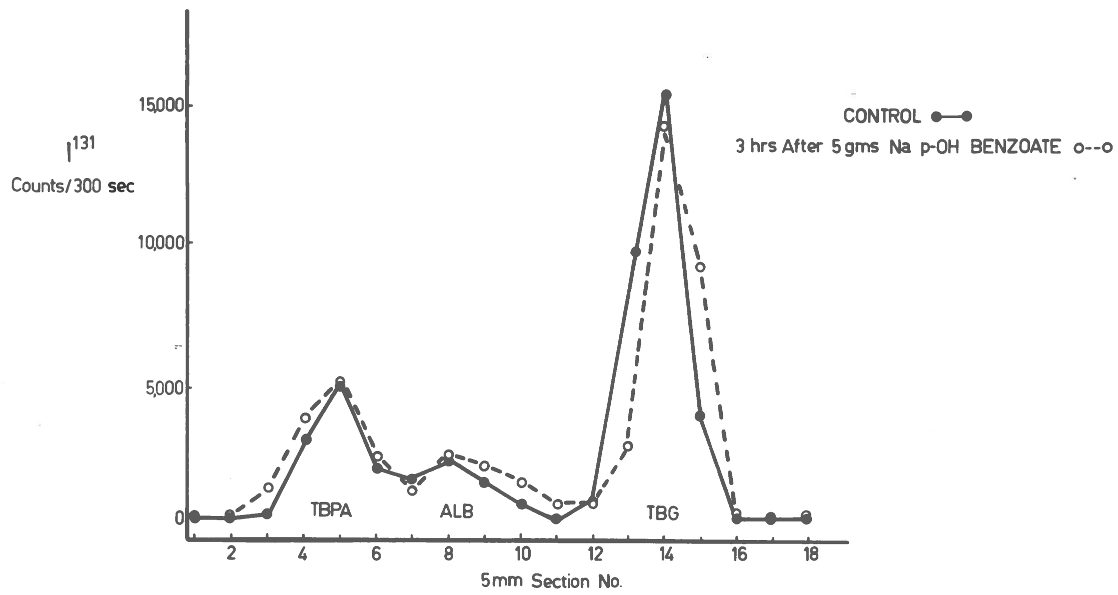
THE IN VIVO EFFECT OF SALICYLATE AND p-HYDROXY BENZOATE ON THYROXINE BINDING IN NORMAL MAN

(Mean of 3 subjects from Table 40)

Treatment	Sample	PBI µg.I %	Serum Salicylate mg.%	% Total Radioactivity on Thyroxine Binding Sites			% Total Radioactivity displaced from TBPA
				TBPA	ALB	TBG	
Sodium p-hydroxy benzoate (5 g.)	Before treatment	5.4		26.6	15.8	57.6	
	After 3 hrs	5.5		26.8	16.1	57.1	N.S.
Sodium salicylate (5 g.)	Before treatment	6.1	0	29.1	15.9	55.0	
	After 3 hrs	5.4	36.3	19.6	15.0	65.3	9.5

Typical radioautographs (Figure 14) reveal the greater displacement of radiothyroxine from TBPA following the *in vitro* addition of salicylate to the buffer compared to the effect seen *in vivo* three hours after administration of the drug.

IN VIVO EFFECT OF p-OH BENZOATE ON THYROXINE BINDING IN NORMAL MAN



RADIOACTIVITY QUANTITATED IN SERIAL SECTIONS OF PAPER ELECTROPHORETIC STRIP

FIGURE 12.

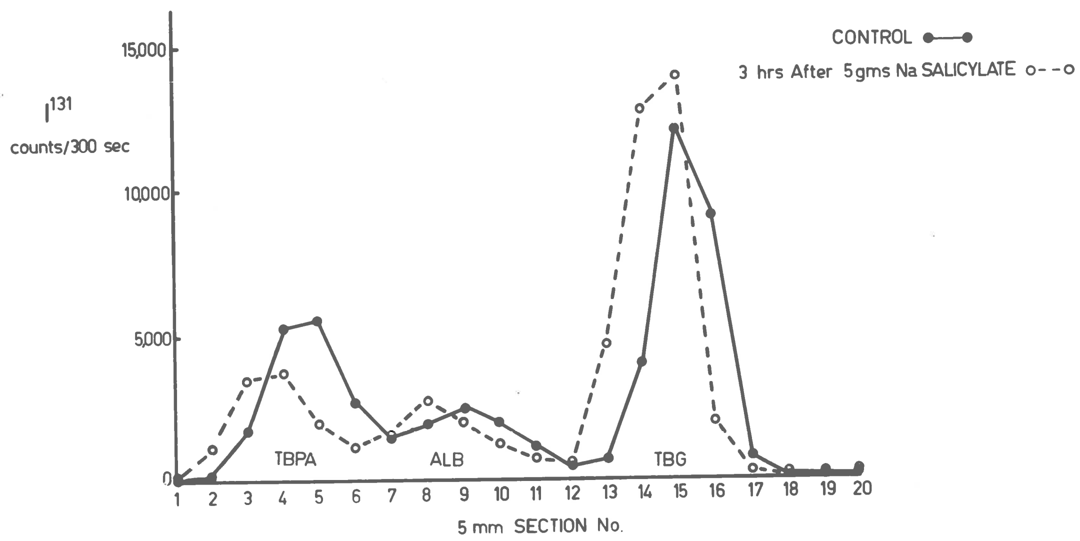
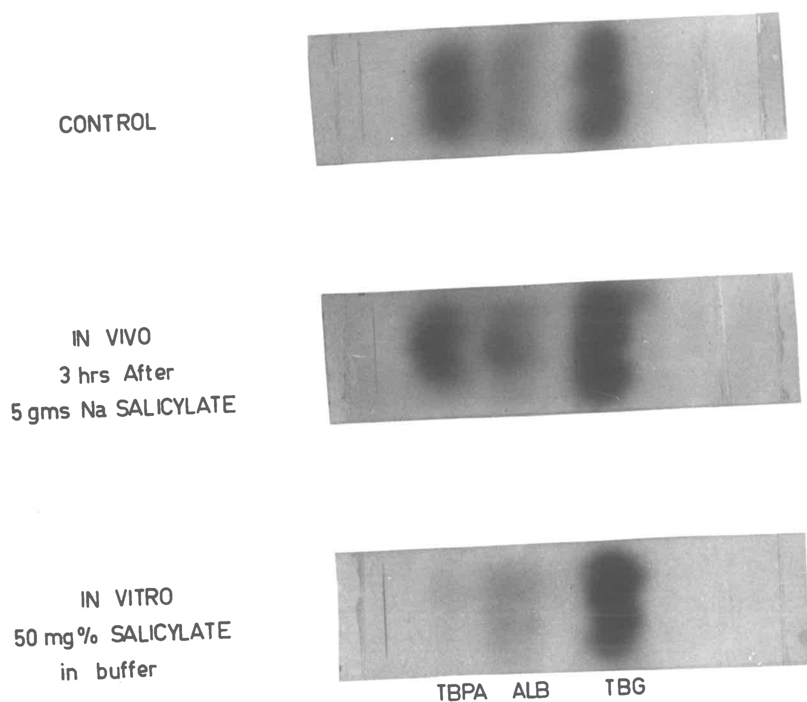
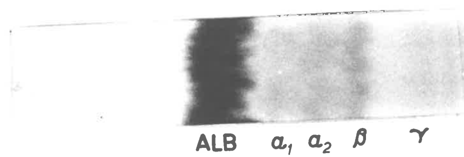


FIGURE 13.

IN VIVO AND IN VITRO EFFECT OF SALICYLATE
ON THYROXINE BINDING IN NORMAL MAN



RADIOAUTOGRAPHS DEMONSTRATING POSITION OF BINDING SITES



STAINED PAPER ELECTROPHORETIC PATTERN

FIGURE 14.

Experiment 2

The effects of the drugs on the individual subjects may be seen in Table 42. The mean results (Table 43) indicate, as in the previous experiment, that there was no effect of p-hydroxybenzoate on thyroxine binding. After two hours, salicylate at a mean serum level of 29.0 mg.%, caused the displacement of 15.1 per cent of the total radioactivity from the TBPA site. However, the displacement following γ-resorcylate, at a mean serum level of 37.9 mg.%, was 24.1% of the total radioactivity.

Typical graphs (Figure 15) demonstrate the displacement of radiothyroxine from TBPA to TBG by γ-resorcylate, in vivo. Radioautographs comparing the in vivo and in vitro effects of γ-resorcylate on thyroxine binding are presented in Figure 16.

Discussion

The inhibition of the binding of added radiothyroxine to TBPA, in the serum samples obtained after the administration of salicylate and of γ-resorcylate to normal human subjects, indicates that both drugs effectively compete with thyroxine for the available binding sites on TBPA, at serum drug levels attained at least two hours after their ingestion. Sodium p-hydroxybenzoate was without effect.

The decrease in the number of binding sites available to thyroxine in the TBPA fraction, produced by salicylate and γ-resorcylate, would be expected to result in a displacement of thyroxine into the free state. These results are therefore consistent with the findings of increased circulating free thyroxine in normal human subjects treated with these drugs.

TABLE 42

THE IN VIVO EFFECT OF SALICYLATE AND RELATED DRUGS ON THYROXINE BINDING IN NORMAL MAN

Subject	Day	Sample	% Total Radio-activity on Thyroxine Binding Sites			Sample	% Total Radio-activity on Thyroxine Binding Sites		
			TBPA	ALB	TBG		TBPA	ALB	TBG
K	1	Before treatment	46.3	19.3	34.4	2 hrs. after sodium p-hydroxy benzoate (5 g.)	46.1	20.4	33.5
L	2		44.6	14.5	41.0		44.1	17.4	38.5
O	1		42.8	17.6	39.6		40.8	16.0	43.3
Mean			44.6	17.1	38.3		43.7	17.9	38.4
M	1	Before treatment	32.8	15.5	51.7	2 hrs. after sodium salicylate (5 g.)	24.4	15.7	59.9
N	2		50.0	15.7	34.3		28.7	15.5	55.8
O	2		40.5	18.6	40.8		24.8	18.8	56.3
Mean			41.1	16.6	42.3		26.0	16.7	57.3
K	2	Before treatment	47.4	19.0	33.5	2 hrs. after sodium γ-resorcylate (5 g.)	21.7	23.4	54.9
L	1		43.1	15.9	41.0		18.8	20.1	61.2
M	2		33.8	15.1	51.1		14.4	15.5	70.1
N	1		44.4	15.0	40.6		17.4	15.9	66.7
Mean			42.2	16.2	41.6		18.1	18.7	63.2

Mean values presented in Table 43

TABLE 43

THE IN VIVO EFFECT OF SALICYLATE AND RELATED DRUGS ON THYROXINE BINDING IN NORMAL MAN

(Mean values from Table 42)

Treatment	Sample	PBI µg.I %	Serum Salicylate mg.%	% Total Radioactivity on Thyroxine Binding Sites			% Total Radioactivity displaced from TBPA
				TBPA	ALB	TBG	
* Sodium p-hydroxy benzoate (5 g.)	Before treatment	5.5		44.6	17.1	38.3	
	After 2 hours	5.5		43.7	17.9	38.4	N.S.
* Sodium salicylate (5 g.)	Before treatment	5.5	0	41.1	16.6	42.3	
	After 2 hours	5.1	29.0	26.0	16.7	57.3	15.1
+ Sodium γ-resorcylate (8 g.)	Before treatment	5.5	0	42.2	16.2	41.6	
	After 2 hours	3.6	37.9	18.1	18.7	63.2	24.1

* Mean of 3 subjects

\+ Mean of 4 subjects

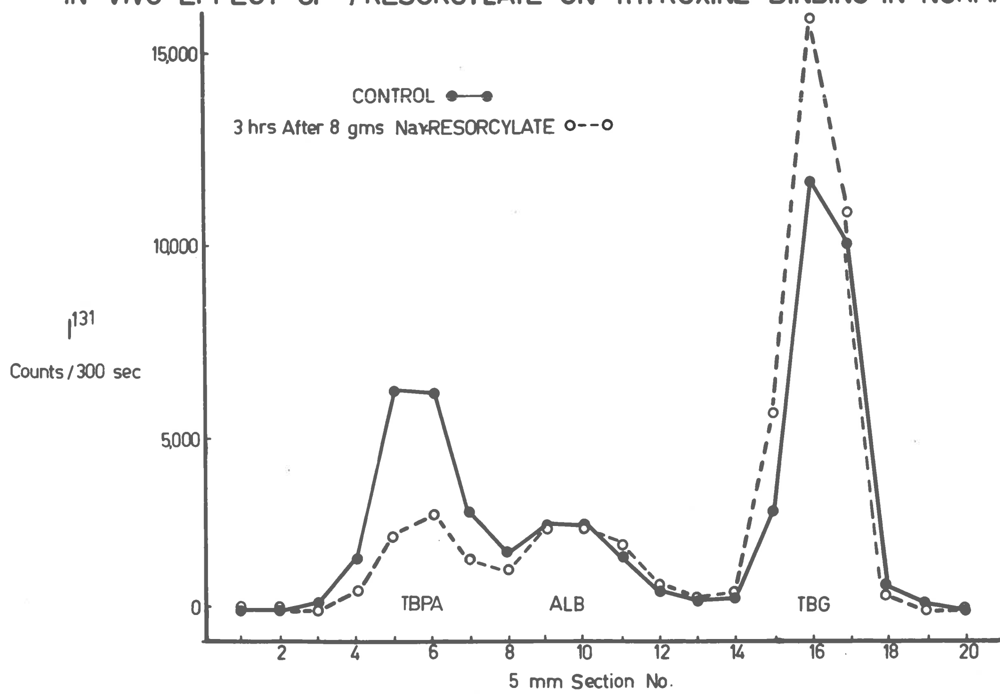
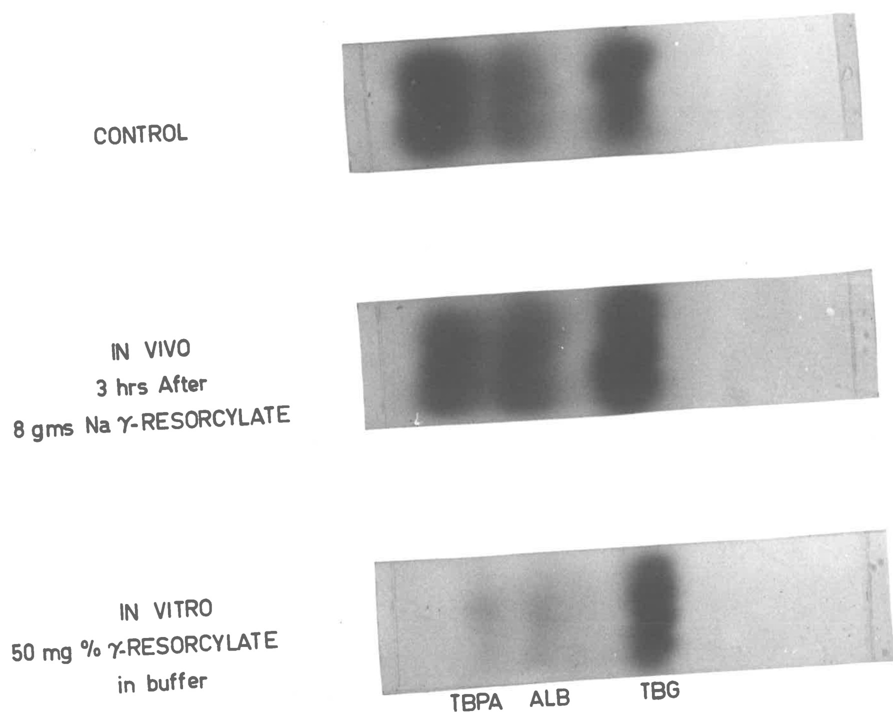
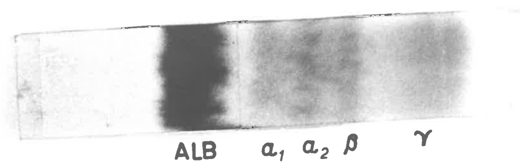


FIGURE 15.

IN VIVO AND IN VITRO EFFECT OF γ-RESORCYLATE ON THYROXINE BINDING IN NORMAL MAN



RADIOAUTOGRAPHS DEMONSTRATING POSITION OF BINDING SITES



STAINED PAPER ELECTROPHORETIC PATTERN

FIGURE 16.

PART III

THE EFFECT OF SALICYLATE AND RELATED DRUGS ON THYROXINE BINDING IN RATS

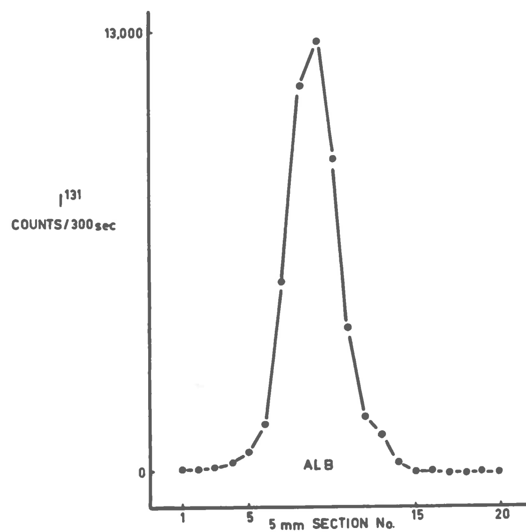
Introduction

The paper electrophoretic separation of normal rat serum proteins was carried out using the same experimental procedure as for human serum. However, it was found that thyroxine was associated only with the albumin band. The single peak on albumin obtained from plotting the radioactivity measured in the serial sections of the paper strip compared to the radio-autograph and stained protein pattern is shown in Figure 17. The *in vitro* addition of salicylate to the buffer, at a concentration of 50 mg./100 ml., did not affect this distribution.

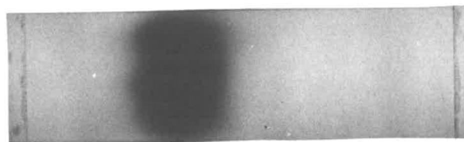
This finding of a single binding site on albumin in rat serum, following electrophoresis in ammonium carbonate buffer at pH 8.4, had been reported by Farer, Robbins, Blumberg and Rall in 1962. Other workers (Van Arsdel and Williams, 1956; Myant, 1957; and Myant and Osorio, 1960) using barbiturate buffer at pH 8.6 and also borate buffer at pH 7.4 described a single binding site on α-globulin. However, Dubowitz, Myant and Osorio (1962) using paper electrophoresis in trismaleate buffer showed that the α-globulin binding site was demonstrable when the pH of the buffer was 8.6, but at pH 7.4 the α-globulin merged into the albumin zone, the thyroxine being carried at the trailing edge of this zone.

The discrepancy between these findings is unexplained.

DEMONSTRATION OF THYROXINE BINDING BY PAPER ELECTROPHORESIS IN NORMAL RAT SERUM

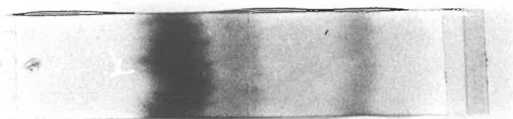


RADIOACTIVITY QUANTITATED FROM SERIAL SECTIONS OF PAPER ELECTROPHORETIC STRIP



ALB

RADIOAUTOGRAPH DEMONSTRATING POSITION OF BINDING SITE



ALB

STAINED PAPER ELECTROPHORETIC PATTERN

FIGURE 17.

It has been considered (Farer et al, 1962) that the differences might be due to an intraspecies variability or more likely to an artefact of the paper electrophoretic technique such that albuminbound thyroxine migrated with the globulins, as had previously been demonstrated by Robbins (1956).

In a comprehensive study of the thyroxine-serum protein complexes of animals representative of the classes of the vertebrates, Farer and colleagues (1962) used both paper and starch gel electrophoresis. Of particular interest to this study was the electrophoresis of normal rat serum. As has previously been mentioned, paper electrophoresis in ammonium carbonate buffer at pH 8.4 revealed only a single binding site on albumin. However, the separation of rat serum by starch gel electrophoresis in borate buffer at pH 8.6, followed by radioautography of the gel revealed three distinct binding sites of radioactive thyroxine. There was a prominent band in the same location as human band 2 (albumin) corresponding to a pale-staining narrow protein zone extending just ahead of the dark-staining broad albumin zone. The second, a diffuse band of radioactivity trailing behind the first was associated with the leading half of albumin. There was a third band, slow moving, faintly visible in the β-globulin region.

Since these binding sites in rat serum were readily distinguishable in the starch gel system, it was decided to examine the effect of salicylate and related drugs on the displacement of thyroxine from one or other of these sites.

1. THE METHOD OF DETERMINING THYROXINE BINDING IN SERUM USING STARCH GEL ELECTROPHORESIS

Starch gel electrophoresis was carried out according to the method of Smithies (1959).+

Materials and Methods

Apparatus

The apparatus was constructed according to the specifications of Smithies (1959). It consisted of a Plexiglas tray with removable end plates into which the gel was poured, backed by a water-cooled jacket. A plastic cover, into which a slot-former was inserted, fitted over the tray. The electric current was delivered to the apparatus from a regulated power supply (Paton Industries, Adelaide).

Reagents

Starch, hydrolysed. Connaught Medical Research Laboratories Toronto.

Borate Buffer pH 8.6 for preparing the starch gel contained 0.03 moles of boric acid and 0.012 moles of sodium hydroxide per litre.

Electrolyte Borate Buffer pH 8.6 containing 0.3 moles of boric acid and 0.06 moles of sodium hydroxide per litre was prepared.

+ The author is indebted to Dr. I.R. Falconer of the Department of Agricultural Chemistry, Waite Agricultural Research Institute, University of Adelaide, for making available the starch gel electrophoresis apparatus.

Procedure

The starch gel was prepared from 80 g. of hydrolysed starch and 700 ml. of the borate buffer. The mixture was gently heated in a 2 litre flask with vigorous shaking until the correct consistency was obtained. The flask was then attached to a vacuum pump to withdraw trapped air from the gel. The electrophoresis tray and cover, with a slot-former for eight slots each 1 cm. wide inserted, were heated to 70°C. The gel was poured into the tray and the cover lowered into position slowly to avoid trapping air bubbles. Weights were placed on the cover to keep it firmly in position and the assembly left for several hours to cool.

The cover was removed slowly to avoid damage to the slots in the gel. The serum samples to be analysed (20 μl.) were run into the slots using micro-pipettes. Petroleum jelly at 50°C was poured over the samples in the slots to seal them in position. The gel was then covered with a polythene sheet. The end plates of the apparatus were then unscrewed, the electrode wicks inserted and the plates screwed back in place.

The apparatus was assembled in the vertical position on a special rack in the cold room at 0°C. The electrode tanks were filled with electrode buffer and the wicks inserted. The electrodes were attached with the anode at the bottom so that the albumin would migrate downwards. Cold water was circulated through the water jacket.

Electrophoresis was carried out for 16 hours at 180 volts (a voltage gradient of 6V/cm.) On completion of the electro-

phoresis, the plastic sheet and petroleum jelly were removed from the surface of the gel. The gel was trimmed square at the ends and turned out onto a tray.

The rat serum samples to be analysed were equilibrated with radiothyroxine added at a concentration of 10 µg. per 100 ml. as previously described for human serum.

<u>Measurement of the Radioactivity on the Thyroxine Binding Sites</u>

The gel was marked off into 5 mm. sections. The individual samples in the gel were then separated. A sample of normal rat serum was included in each run. The gel strip from this sample was stained with nigrosine (0.025% (W/V) nigrosine and 7.5% (V/V) glacial acetic acid dissolved in 50% methanol) to reveal the protein bands. This stained pattern, marked off in 5 mm. sections, was used as a marker to identify the protein areas of the other samples in the run.

The individual gels were then cut up into 5 mm. sections which were placed in numbered counting tubes. The radioactivity of each section was measured in the well-type scintillation counter and the count rate plotted against the section number revealing the peaks of radioactivity. The sum of the radioactivity on each binding site was expressed as a percentage of the total radioactivity in the strip.

The total count rate of the radioactivity in each binding site was required. Since the radioactivity would not be dispersed evenly through the thickness of the gel along its

complete length it was not practicable to split the gel through the smallest dimension to obtain two halves, one for subsequent staining and the other for counting. It was evident that resultsobtained from counting a half gel would not be accurate enough to detect differences produced by displacement.

Owing to the limited time for which the apparatus was available, no radioautographs were obtained.

Three binding sites of thyroxine in normal rat serum were identified in these studies, in the positions recorded by Farer et al, (1962). These sites have been designated A, B and C. Band A which carried between 50 and 60 per cent of the radioactivity was located just ahead of the leading edge of albumin. Band B, which closely followed band A, was associated with the albumin region and carried about 25 per cent of the radiothyroxine. The third, band C, in the region of the β-globulins, was more diffuse and had associated with it approximately 20 per cent of the radioactivity. The effect of _in vitro_ addition of salicylate and related drugs to the electrophoretic buffer on the distribution of thyroxine on these binding sites was therefore determined.

2. THE EFFECT OF SALICYLATE AND RELATED DRUGS ON THYROXINE BINDING IN RATS

IN VITRO STUDIES

Materials and Methods

The drugs, p-hydroxybenzoic acid, salicylic acid and γ-resorcylic acid were added to both the starch gel borate buffer and the electrode buffers in a concentration of 50 mg./100 ml. and the pH of each buffer readjusted to 8.6.

The proportion of radioactivity on each binding site in normal rat serum was assessed from the radioactive count rates of the serial sections of the gel. The same serum samples were analysed following the *in vitro* addition of the drugs to the buffer.

Results

Although only a small number of estimations were carried out consistent findings were obtained with each of the treatments. The results are shown in Table 44.

Following the *in vitro* addition of p-hydroxybenzoate to the buffer there was a mean displacement of 10 per cent of the radiothyroxine from the fast moving albumin component (band A) to both bands B and C, the greater proportion of the shift being to band C (6.7 per cent). A typical graph obtained by plotting the count rates for the serial sections after electrophoresis in normal buffer and following the *in vitro* addition of p-hydroxybenzoate to the buffer is shown in Figure 18.

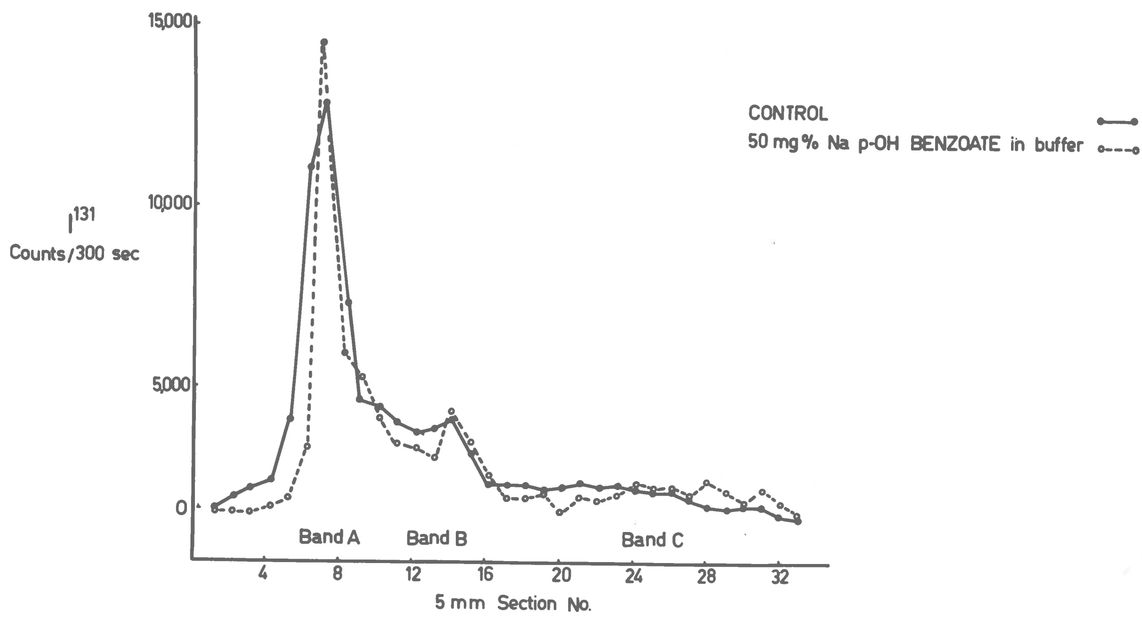
There was a very much greater displacement of thyroxine

TABLE 44

THE EFFECT OF IN VITRO ADDITION TO THE ELECTROPHORETIC BUFFER OF SALICYLATE AND RELATED DRUGS (50 mg%) ON THYROXINE BINDING IN NORMAL RAT SERUM

Rat No.	Sample	% Total Radio-activity on Thyroxine Binding Sites			% Total Radio-Thyroxine displaced from Thyroxine Binding Sites	
		A	B	C	A	B
30	Normal buffer + p-hydroxy benzoate	47.1 36.5	34.8 38.8	18.1 24.7		
29	Normal buffer + p-hydroxy benzoate	66.1 56.7	22.3 24.8	11.6 18.5		
Mean	Normal buffer + p-hydroxy benzoate	56.6 46.6	28.6 31.8	14.8 21.6	10.0	
5	Normal buffer + salicylate	46.8 7.2	31.3 6.0	21.9 86.8		
9	Normal buffer + salicylate	55.9 7.7	25.6 11.1	18.5 81.2		
13	Normal buffer + salicylate	55.7 9.1	26.6 12.8	17.7 78.1		
Mean	Normal buffer + salicylate	52.8 8.0	27.8 10.0	19.4 82.0	44.8	17.8
5	Normal buffer + γ-resorcylate	46.8 -	31.3 -	21.9 100.0		
9	Normal buffer + γ-resorcylate	55.9 -	25.6 -	18.5 100.0		
13	Normal buffer + γ-resorcylate	55.7 -	26.6 -	17.7 100.0		
Mean	Normal buffer + γ-resorcylate	52.8 -	27.8 -	19.4 100.0	52.8	27.8

IN VITRO EFFECT OF p-OH BENZOATE ON THYROXINE BINDING IN THE NORMAL RAT



RADIOACTIVITY QUANTITATED IN SERIAL SECTIONS OF STARCH GEL ELECTROPHORETIC STRIP

FIGURE 18.

from band C amounting to 44.8% of the total radioactivity, when salicylic acid was added to the buffer. Salicylate also displaced a large proportion of the radiothyroxine from band B. The radioactivity displaced from both bands A and B was found on band C in the β-globulin region. The distribution of thyroxine on the binding sites following electrophoresis with salicylate added to the buffer is shown in Figure 19.

The addition of γ-resorcylate to the buffer at a concentration of 50 mg.% caused the displacement of all the radioactivity from bands A and B_2 to band C. This effect is demonstrated in Figure 20.

Discussion

Although only a small number of experiments was carried out the results are conclusive. The degree to which the drugs displaced thyroxine was similar to the findings with human serum after paper electrophoresis; p-hydroxybenzoate exerted the smallest displacement, whereas the effect of salicylate and γ-resorcylate at the same concentration was much greater.

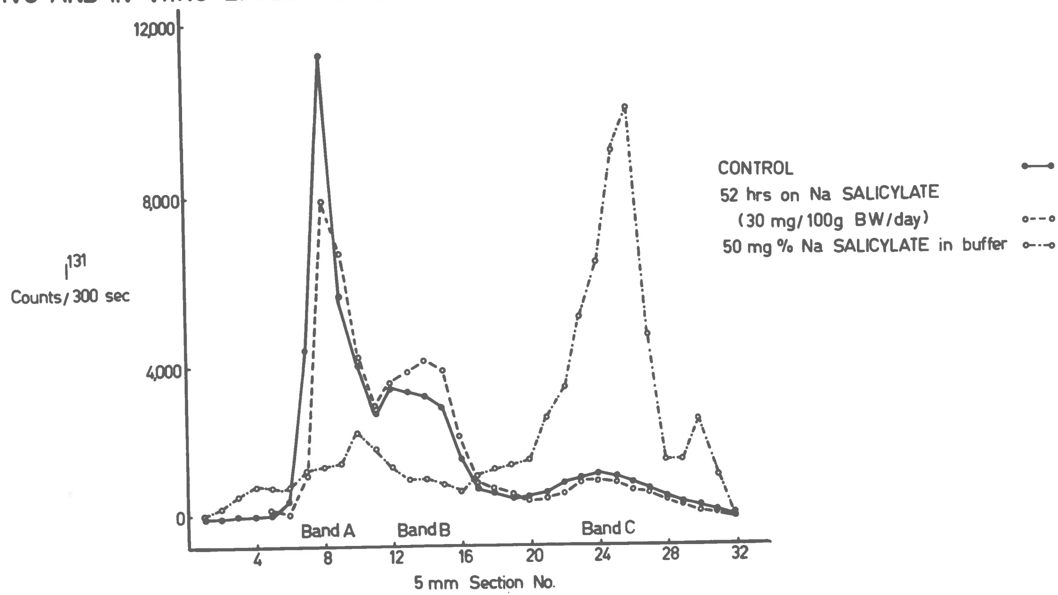
IN VIVO STUDIES

Materials and Methods

Serum samples on which free thyroxine had been determined (in two experiments described in Chapter IV) following the administration of the various drugs to rats were used for these studies on thyroxine binding. Sodium p-hydroxybenzoate and sodium salicylate were each given in a dosage of 30 mg./100 g. body weight/day.

The dosage of sodium γ-resorcylate was 90 mg./100 g.

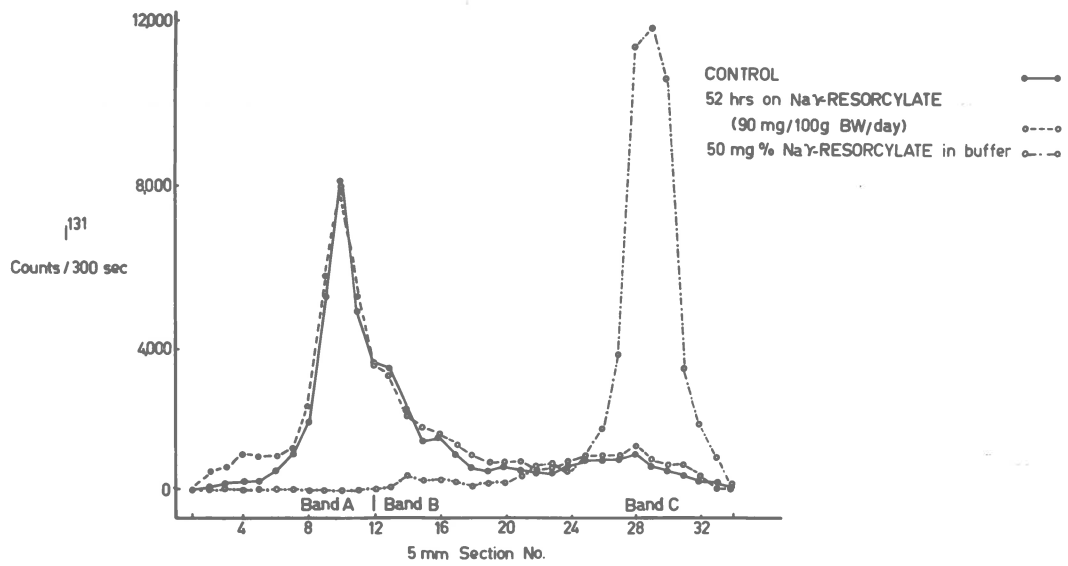
IN VIVO AND IN VITRO EFFECT OF SALICYLATE ON THYROXINE BINDING IN THE NORMAL RAT



RADIOACTIVITY QUANTITATED IN SERIAL SECTIONS OF STARCH GEL ELECTROPHORETIC STRIP

FIGURE 19.

IN VIVO AND IN VITRO EFFECT OF γ-RESORCYLATE ON THYROXINE BINDING IN THE NORMAL RAT



RADIOACTIVITY QUANTITATED IN SERIAL SECTIONS OF STARCH GEL ELECTROPHORETIC STRIP

FIGURE 20.

body weight/day and that of 2,4-dinitrophenol, 2.5 mg./100 g. body weight/day.

Results

Three rats from each group receiving the treatments were employed and serum samples obtained before and after treatment were separated in the same starch gel.

The results are presented in Table 45.

There was no displacement of thyroxine following the administration of sodium p-hydroxybenzoate. Salicylate produced a mean displacement of 14.2 per cent of the total radioactivity from band A, most of the displaced thyroxine being transferred to band B. 2,4-Dinitrophenol produced a similar effect, 12.8 per cent of the radiothyroxine on band A being displaced mainly to band B. There was no displacement of radiothyroxine following the administration of γ-resorcylate.

Typical examples of the in vivo effects of these drugs are shown in Figures 19, 20 and 21 which illustrate the graphs of the radioactivity in the serial sections of the gels.

Discussion

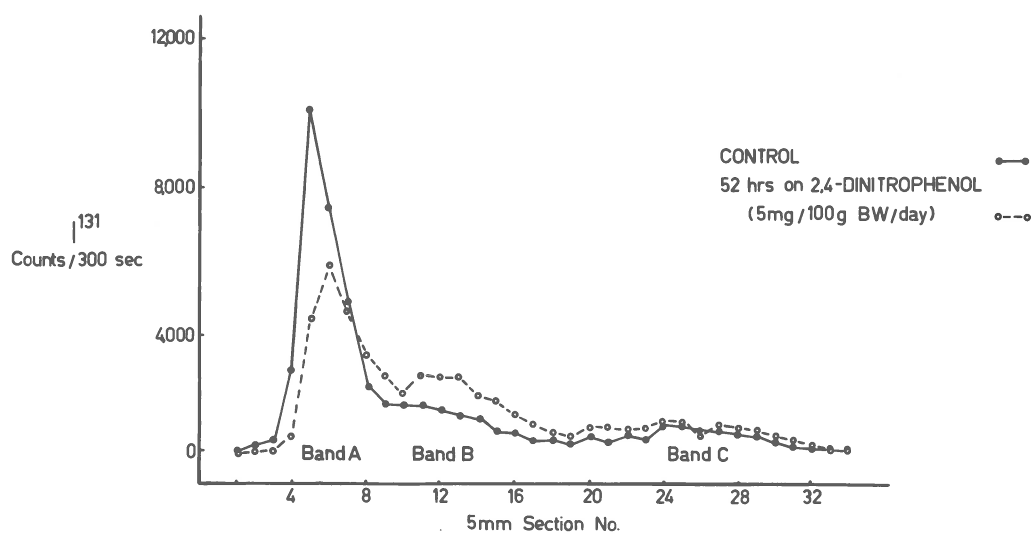
In vivo, the effects on thyroxine binding of sodium p-hydroxybenzoate and sodium salicylate in rats are similar to the effects of the drugs in normal man; p-hydroxybenzoate was without effect whereas considerable displacement of thyroxine from band A of rat serum was produced by sodium salicylate.

TABLE 45

THE IN VIVO EFFECT OF SALICYLATE AND RELATED DRUGS ON THYROXINE BINDING IN NORMAL RATS

Rat No.	Sample	% Total Radioactivity on Thyroxine Binding Sites			Sample	% Total Radioactivity on Thyroxine Binding Sites			% Displacement from Band A
		A	B	C		A	B	C	
Sodium p-hydroxy benzoate (30 mg./100 g./day)									
28 29 24	Control	46.5 66.0 59.1	33.7 22.3 23.2	19.8 11.7 17.7	Treated 52 hrs	49.0 62.6 58.3	30.5 24.2 28.0	20.5 13.2 13.7	
Mean		57.2	26.4	16.4		56.6	27.6	15.8	N.S.
Sodium salicylate (30 mg./100 g./day)									
12 14 21	Control	43.9 59.9 52.5	35.8 17.8 32.5	20.3 22.3 15.0	Treated 52 hrs.	21.6 47.3 44.7	55.4 29.4 41.7	23.0 23.3 13.6	
Mean		52.1	28.7	19.2		37.9	42.1	20.0	14.2
2-4 dinitrophenol (2.5 mg./100 g./day)									
17 5 13	Control	66.9 46.8 55.7	17.5 31.2 26.6	15.6 22.0 17.7	Treated 52 hrs.	42.9 38.8 49.3	36.1 40.2 29.3	21.0 21.0 21.4	
Mean		56.5	25.1	18.4		43.7	35.2	21.1	12.8
Sodium γ-resorcylate (90 mg./100 g./day)									
12 1 11	Control	61.7 63.3 52.8	18.3 17.7 24.0	20.0 19.0 23.2	Treated 52 hrs.	61.5 61.2 53.0	18.2 19.3 21.6	20.3 19.5 25.4	
Mean		59.3	20.0	20.7		58.6	19.7	21.7	N.S.

IN VIVO EFFECT OF 2,4-DINITROPHENOL ON THYROXINE BINDING IN THE NORMAL RAT



RADIOACTIVITY QUANTITATED IN SERIAL SECTIONS OF STARCH GEL ELECTROPHORETIC STRIP

FIGURE 21.

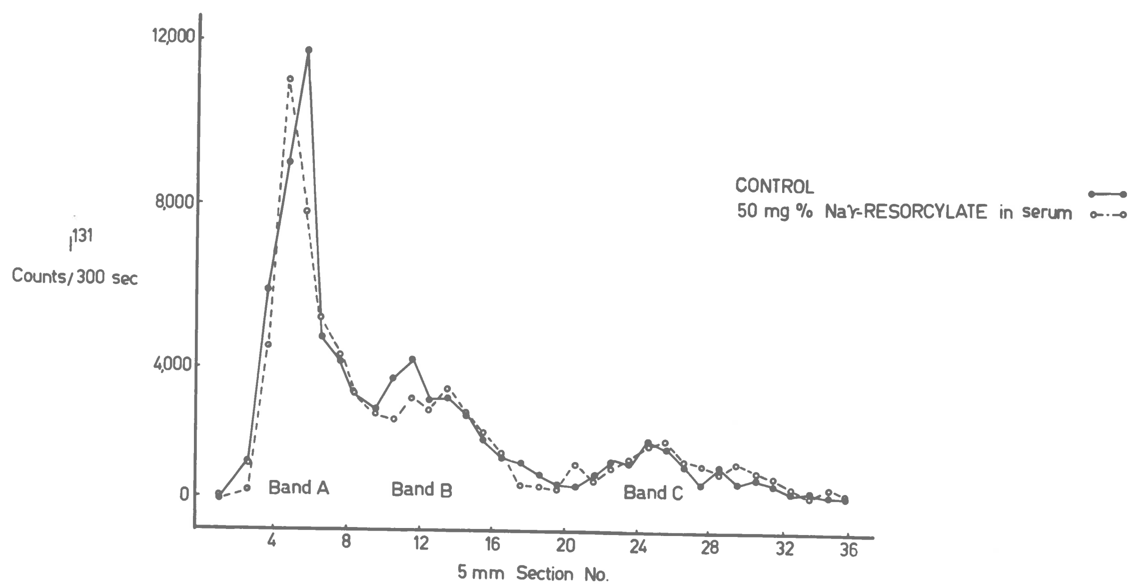
The action of 2,4-dinitrophenol in displacing radiothyroxine from band A following its administration to rats resembles that of salicylate and confirms the in vitro effect of the drug in human serum.

The failure to demonstrate a displacement of thyroxine following the administration of γ-resorcylate to rats was considered possibly to be the result of the rather low level of γ-resorcylate reached in the serum (a mean of 22.9 mg. per 100 ml. for the three rats) compared to the mean drug concentration of 40.5 mg. per 100 ml. in the three salicylate treated rats. However, the in vitro addition to the control serum samples of 50 mg. of γ-resorcylate per 100 ml. still failed to produce the expected displacement of thyroxine (Figure 22).

It was decided therefore to determine the effect of γ-resorcylate on human serum by starch gel electrophoresis.

The in vitro action of salicylate and γ-resorcylate in displacing thyroxine from bands A and B to band C was qualitatively different from the in vivo effect with salicylate when thyroxine was displaced from band A to band B. It is considered that the in vitro effect resulted from the large reservoir of the drugs which were added to the buffer used to prepare the starch gel, and the electrode buffer.

IN VITRO EFFECT ON THYROXINE BINDING OF γ-RESORCYLATE ADDED TO RAT SERUM



RADIOACTIVITY QUANTITATED IN SERIAL SECTIONS OF STARCH GEL ELECTROPHORETIC STRIP

FIGURE 22.

3. THE EFFECT OF γ-RESORCYLATE ON THYROXINE BINDING IN HUMAN SERUM DETERMINED BY STARCH GEL ELECTROPHORESIS

IN VITRO STUDIES

The distribution of thyroxine between the binding sites of normal human serum (subject L) was determined by starch gel electrophoresis in borate buffer. The same serum was then analysed after the addition of p-hydroxybenzoic acid and γ-resorcylic acid to the buffer at a concentration of 50 mg./100 ml.

The normal distribution of thyroxine on four binding sites was confirmed (Blumberg et al, 1961). These were band 1, corresponding to TBPA, band 2 on albumin, band 3 in the post albumin region and band 4 in the α-globulin region. As the radioactivity carried on bands 3 and 4 was the smallest fraction and could not be readily separated into two distinct peaks by counting the serially sectioned gel, it was treated as a single fraction.

The findings were the same as those reported following the in vitro addition of the drugs to rat serum (Table 46). The addition of p-hydroxybenzoic acid caused a small displacement from band 1 (TBPA) of 9.7 per cent of the total radioactivity which was mainly recovered on the post albumin region (bands 3 and 4). When γ-resorcylic acid was added to the buffer, all the radioactivity on bands 1 and 2 was displaced to bands 3 and 4.

<u>IN VIVO STUDIES</u>

The serum samples from two human subjects (K and M) obtained before and two hours after treatment with γ-resorcylate, which induced a displacement of thyroxine from TBPA demonstrable by paper electrophoresis, were analysed in the starch gel system.

The results (Table 46) indicate, as was the case with rats using starch gel electrophoretic separation, that there was no displacement of radiothyroxine in human serum following the administration of γ-resorcylate. The <u>in vitro</u> and <u>in vivo</u> effects of γ-resorcylate on thyroxine binding in human serum are shown in Figure 23.

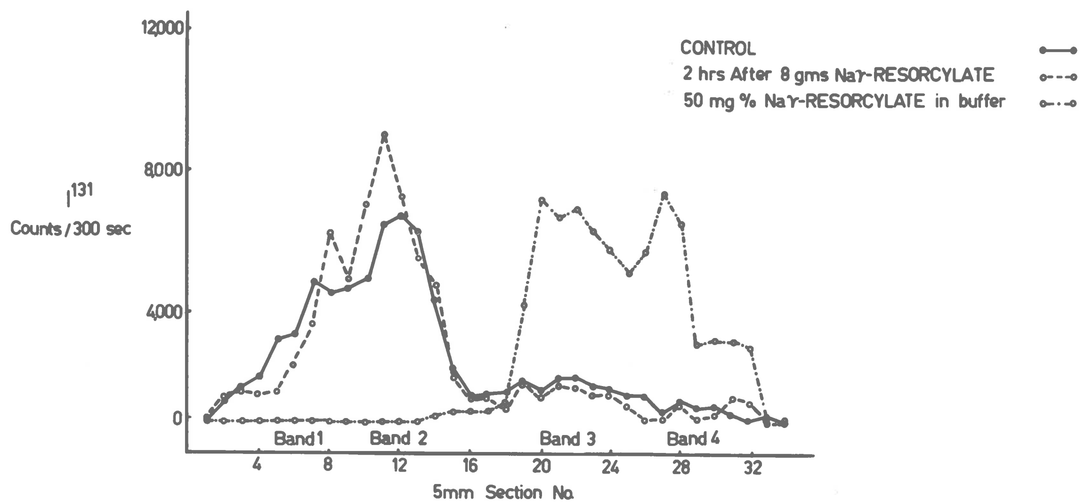
The failure to demonstrate a displacement of thyroxine from the TBPA site (band 1) by γ-resorcylate in the starch gel system in the samples of human serum, in view of the fact that these samples showed a displacement when separated in paper electrophoresis, was therefore considered to be an artefact due possibly to the migration of the drug off the gel under the influence of the electric current. It was concluded that the failure to demonstrate a displacement by γ-resorcylate in rats occurred for the same reason.

TABLE 46

THE IN VITRO AND IN VIVO EFFECT OF γ-RESORCYLATE ON THYROXINE BINDING IN HUMAN SERUM DETERMINED BY STARCH GEL ELECTROPHORESIS

Rat No.	Sample	% Total Radioactivity on Thyroxine Binding Sites			% Total Radio-Thyroxine displaced from Thyroxine Binding Sites	
		1	2	3 + 4	1	2
IN VITRO ADDITION TO BUFFER (50 mg.%)						
L	Control	36.6	50.0	13.4		
	p-hydroxy benzoate	26.9	51.5	21.6	9.7	
	γ-resorcylate	-	-	100.0	36.6	50.0
IN VIVO ADMINISTRATION OF γ-RESORCYLATE (8 g.)						
K	Before treatment	33.2	45.7	21.1		
	After 2 hours	32.2	48.0	19.8		
M	Before treatment	33.2	49.1	17.7		
	After 2 hours	33.3	49.9	16.7		
Mean	Before treatment	33.2	47.4	19.4	N.S.	
	After tretment	32.8	48.9	18.3		

IN VIVO AND IN VITRO EFFECT OF γ-RESORCYLATE ON THYROXINE BINDING IN NORMAL MAN



RADIOACTIVITY QUANTITATED IN SERIAL SECTIONS OF STARCH GEL ELECTROPHORETIC STRIP

FIGURE 23.

PART IV

CONCLUSIONS

Evidence has been presented in this chapter that the *in vitro* addition of salicylate and 2,4-dinitrophenol to the buffer during the electrophoresis of normal human serum interfered with the binding of thyroxine to TBPA; the thyroxine displaced was bound mainly to TBG. These results confirm the findings of Wolff, Standaert and Rall (1961).

The addition of γ-resorcylate to the electrophoretic buffer produced a displacement of thyroxine from TBPA similar in extent to that produced by salicylate. The addition of p-hydroxybenzoate to the buffer, on the other hand, produced only a small displacement.

An examination of the effect *in vivo* of these drugs in man on the binding of thyroxine to the serum proteins, carried out on aliquots of the serum samples which were used for the estimation of free thyroxine, showed that both salicylate and γ-resorcylate displaced thyroxine from TBPA to TBG. There was no change induced in the distribution of thyroxine on the binding sites when these subjects were given p-hydroxybenzoate.

It is concluded, therefore, that the increase in free thyroxine which results from the administration of salicylate and γ-resorcylate to human subjects is brought about by the displacement of thyroxine from the TBPA fraction.

Although only one thyroxine binding site could be detected in rat serum by paper electrophoresis, three such sites were consistently demonstrated when the electrophoresis was performed in starch gel.

An investigation of the effect of salicylate, γ-resorcylate and p-hydroxybenzoate, added *in vitro* to the buffer during electrophoresis of rat serum, led to the conclusion that the first two of these drugs displaced thyroxine from the binding sites detected in a fast moving albumin fraction (band A) and also in a slower moving albumin fraction (band B), while p-hydroxybenzoate produced a much smaller displacement from band A alone.

Examination of the effect of these drugs and also 2,4-dinitrophenol *in vivo* revealed that, in rats, both salicylate and 2,4-dinitrophenol produced a displacement of thyroxine from band A, while p-hydroxybenzoate had no such effect; γ-resorcylate, which produced a displacement *in vitro* greater than that produced by salicylate, had no effect on thyroxine binding *in vivo*. An *in vivo* effect of γ-resorcylate in man could not be detected by starch gel electrophoresis although a displacement of thyroxine from TBPA had been demonstrated when the samples were analysed by paper electrophoresis.

These studies with rats have served to confirm the finding that, in man, salicylate and γ-resorcylate *in vivo* displace thyroxine from one of the specific thyroxine binding proteins in serum.

It is concluded, therefore, that the increase in circulating free thyroxine produced by these drugs results from the displacement of thyroxine from a specific thyroxine binding protein in the serum.

CHAPTER VI

DISCUSSION

CHAPTER VI

DISCUSSION

An investigation of the effect of 2,4-dinitrophenol and of salicylate and related compounds on the plasma PBI in rats was reported in Chapter II of this thesis. The administration of these drugs to normal rats produced a significant depression in plasma PBI, confirming the previously published reports of such an effect of 2,4-dinitrophenol in rats (Goldberg et al, 1951, 1955) and in man (Castor and Beierwaltes, 1956), and of salicylate in man (Austen et al, 1958; Wetzel et al, 1962).

Sodium γ-resorcylate also produced a significant depression in plasma PBI when administered to normal rats in a dosage which produced a plasma level of the drug comparable with that obtained following salicylate treatment. This finding has not been previously documented.

There was no significant depression in plasma PBI when rats were treated with sodium p-hydroxybenzoate.

Similar findings were obtained from studies of the effects of the drugs on the plasma PBI of thyroidectomized rats maintained on thyroxine. The percentage fall in plasma PBI was of the same order in both normal and thyroxine maintained rats, indicating that a peripheral action of the drugs would alone account for the depression. This was in contrast to evidence, obtained in similar studies in man, that the depression in PBI produced by salicylate comprised both a central and a peripheral component (Wetzel et al, 1962).

Indirect evidence of a central action, that is a depression in the release of TSH from the pituitary, operating to produce the fall in plasma PBI had been reported in rats for 2,4-dinitrophenol by Goldberg and co-workers (1955, 1957) and in man for salicylate by Wolff and Austen (1958) and Wetzel and co-workers (1962).

This finding of simultaneously lowered circulating thyroid hormone and depression of TSH release constitutes a disruption of the negative feed-back system postulated to control thyroid-pituitary interrelations. According to this concept, a depression in circulating thyroid hormone would be expected to stimulate the release of TSH from the pituitary.

Both 2,4-dinitrophenol and salicylate increase metabolic rate and oxygen consumption in the whole animal and uncouple oxidative phosphorylation in isolated mitochondrial preparations. It was concluded by Goldberg and co-workers (1957) that the inhibition of TSH release from the pituitary by 2,4-dinitrophenol was related to the metabolic stimulating property of the drug, acting on the hypothalamus either directly or indirectly via the peripheral metabolism. This was consistent with the concept of the control of pituitary function by the hypothalamus proposed by Harris (1955). Further support for such a mechanism of action of 2,4-dinitrophenol was obtained by Reichlin (1960) who demonstrated that the depression in thyroid function produced by 2,4-dinitrophenol in normal rats was significantly reduced in rats with lesions in the hypothalamus.

Wolff and Austen (1958), in a further examination of the mechanism of action of salicylate on thyroid function, demonstrated that not only salicylate and 2,4-dinitrophenol but also the dihydroxy benzoic acids, γ-resorcylic and gentisic acids, reduced the release of labelled hormone from the rat thyroid, indicating a depression in the release of TSH from the pituitary. These dihydroxy benzoic acids unlike salicylate and 2,4-dinitrophenol do not increase metabolic rate or oxygen consumption in intact rats nor uncouple oxidative phosphorylation in isolated mitochondrial preparations. Wolff and Austen concluded that the action of salicylate and 2,4-dinitrophenol in depressing the release of TSH was not related to their metabolic stimulating or uncoupling properties.

Because of the importance of this finding in relation to the physiological control of thyroid function, the action of these drugs on pituitary function was therefore re-examined. TSH in the plasma of rats both before and after treatment with these drugs was estimated directly by bioassay, in order to confirm the indirect evidence of a depression in TSH release (Chapter III).

Modifications were made in the method for the bioassay of TSH (McKenzie, 1958) which resulted in increased sensitivity and precision, enabling the level of TSH in plasma from normal rats to be estimated. It was demonstrated that 2,4-dinitrophenol, salicylate and γ-resorcylate significantly depressed the level of circulating TSH in normal rats, in association

with a depression of plasma PBI. Sodium p-hydroxybenzoate was without effect on either circulating TSH or plasma PBI.

Thus, a simultaneous depression in both circulating thyroid hormone and TSH was produced not only by 2,4-dinitrophenol and salicylate but also by γ-resorcylate. The three drugs therefore exerted a similar action in disrupting the negative feedback regulation of the thyroid-pituitary axis.

It is concluded, therefore, that the metabolic stimulating properties of salicylate and 2,4-dinitrophenol are not responsible for this disruption, since γ-resorcylate does not possess such metabolic stimulating properties.

The affinity of specific serum proteins for thyroxine is of such intensity that the greater proportion of the total thyroxine circulating is carried bound to these proteins, the remainder being in the free or unbound state. Robbins and Rall (1960) postulated that the circulating free thyroxine was the physiologically active moiety of the thyroid hormone, and that the bound thyroxine served as an inactive storage form. These investigators further proposed that free thyroxine might act as the regulator of the negative feedback system controlling thyroid-pituitary interrelations.

Christensen (1959) had reported that the _in vitro_ addition of salicylate and 2,4-dinitrophenol to serum equilibrated with radiothyroxine, during dialysis in a special apparatus, resulted in an increase in the rate of dialysis of the radiothyroxine. This finding was consistent with an action of the drugs by displacement, into the free state, of radio-

thyroxine bound to a specific thyroxine binding protein. An increase in free thyroxine would account for the increased fractional rate of disappearance of injected radiothyroxine and the fall in plasma PBI resulting from the administration of salicylate and 2,4-dinitrophenol to man and rats. The depression in TSH produced by these drugs might also be explained by this finding if the level of free thyroxine controls the negative feedback regulation of the thyroid-pituitary axis.

An investigation of the effect of salicylate and related drugs on free thyroxine both in vitro and in vivo was therefore carried out using the dialysis method of Christensen (1959a). The results of these studies are reported in Chapter IV. It was demonstrated that the in vitro addition of salicylate and γ-resorcylate to either normal human serum or normal rat serum resulted in an increased rate of dialysis of radiothyroxine indicating an increase in free thyroxine. The increase in the rate of dialysis of radiothyroxine following the in vitro addition of p-hydroxybenzoate was smaller than that obtained with the other drugs.

Because of the change in equilibrium of the total circulating thyroid hormone, consequent upon the depression in plasma PBI produced by the administration of salicylate and γ-resorcylate, it was not possible to predict the in vivo effect of the drugs on free thyroxine from the in vitro findings. An examination of the in vivo effect of the drugs on free thyroxine was therefore made. The level of circulating

free thyroxine in normal human subjects was shown to be elevated within two hours of the administration of either salicylate or γ-resorcylate. The administration of p-hydroxybenzoate was without effect.

The effect of more prolonged administration of the drugs was determined in experiments using normal rats. Following treatment for 52 hours with 2,4-dinitrophenol, salicylate and γ-resorcylate, the free thyroxine was increased above the control value in each case, in spite of the intense depression in plasma PBI. The mechanism producing the acute increase in free thyroxine in man within two hours of a single dose of salicylate and γ-resorcylate was therefore still operative following repeated dosage of the drugs to rats over a much longer period. The level of circulating free thyroxine in rats was unaffected by treatment with sodium p-hydroxybenzoate or sodium benzoate.

Only those drugs which depressed the plasma PBI were shown to cause a simultaneous elevation in free thyroxine. This was the case with 2,4-dinitrophenol, salicylate and γ-resorcylate. Moreover, those drugs which did not depress the plasma PBI, namely, sodium p-hydroxybenzoate and sodium benzoate did not affect the free thyroxine level.

As was mentioned previously, Christensen (1959) postulated that the increase in the rate of dialysis of radiothyroxine, produced by the _in vitro_ addition of salicylate and 2,4-dinitrophenol to the serum, depended upon the ability of the drugs to displace, into the free state, thyroxine bound

to one of the specific binding sites in the serum proteins.

Following the demonstration that TBPA was a normal component of human serum proteins, Ingbar (1960) reported that the *in vitro* addition of salicylate to the trismaleate buffer at pH 8.4 during paper electrophoresis of human serum, resulted in a displacement of thyroxine from TBPA to TBG. Subsequently Wolff, Standaert and Rall (1961) confirmed this finding and further demonstrated that 2,4-dinitrophenol produced a similar displacement of thyroxine.

An examination of the effect of 2,4-dinitrophenol and of salicylate and related drugs on the binding of thyroxine to the serum proteins in both man and rats was therefore undertaken (Chapter V). The various drugs were added *in vitro* to the ammonium carbonate buffer at pH 8.4 during paper electrophoresis of normal human serum. A large displacement of thyroxine from TBPA to TBG was observed with 2,4-dinitrophenol, salicylate and γ-resorcylate. The displacement observed when p-hydroxybenzoate was added to the electrophoretic buffer, while appreciable, was considerably less than that observed with the other drugs, and was consistent with the slight *in vitro* effect of the drug in the dialysis system.

An investigation of the *in vivo* effect of these drugs on the binding of thyroxine to the serum proteins in man was also carried out. The analyses were performed on aliquots of the serum samples which had been used for the estimation of free thyroxine. It was shown that the administration of

salicylate and γ-resorcylate to human subjects caused a displacement of thyroxine from TBPA to TBG. There was no change induced in the distribution of thyroxine on the binding sites when these subjects were given p-hydroxybenzoate.

However, the physiological significance of TBPA has been questioned. Several workers (Christensen and Litonjua, 1961; Myant and Osorio, 1962) demonstrated by conventional paper electrophoresis of normal human serum at pH 7.4 that virtually no binding of thyroxine to TBPA occurred. Furthermore, Osorio (1962) employing paper electrophoresis at pH 7.4 demonstrated that salicylate inhibited the binding of thyroxine by albumin and TBG. Nevertheless, Ingbar (1963) in a study of the physiological role of TBPA showed that the failure to detect binding of thyroxine to TBPA in paper electrophoresis at pH 7.4 was the result of the greater affinity of paper than of TBPA for thyroxine at this pH. When electrophoresis of the serum proteins was carried out in agar gel at pH 7.4, thyroxine binding by TBPA was consistently demonstrated. Ingbar concluded that TBPA transports a significant proportion of the thyroxine in plasma at physiological pH. He further postulated that the weaker binding affinity of TBPA than of TBG might give TBPA a metabolic significance beyond that expected from the proportion of thyroxine in the blood which it actually binds. Thus, the lability of thyroxine binding by TBPA to decreases in pH might constitute a mechanism for the transcapillary passage of thyroxine or for its delivery to

regions of increased metabolic expenditure such as exercising muscle. Ingbar stated, "this concept which suggests that TBG is the 'savings account' and TBPA the 'checking account' of thyroxine economy, although speculative at present, would serve both to reconcile the majority of available data and to provide a teleological rationale for the existence in plasma of two major thyroxine binding proteins".

Moreover, Woeber and Ingbar (1963) demonstrated by electrophoresis of human serum in agar gel at pH 7.4 that salicylate inhibited the binding of thyroxine by TBPA and not by albumin or by TBG. Thus salicylate selectively inhibits the binding of thyroxine by TBPA at physiological pH.

It is concluded, therefore, from the studies presented in this thesis, that the increase in free thyroxine produced by the administration of salicylate and γ-resorcylate to human subjects resulted from the displacement of thyroxine from the TBPA sites of the serum proteins.

Although only one thyroxine binding site could be detected in rat serum using paper electrophoresis, three such sites were demonstrated when electrophoresis was performed in starch gel. An examination of the effect of salicylate, γ-resorcylate and p-hydroxybenzoate, when added <u>in vitro</u> to the electrophoretic buffer, revealed that salicylate and γ-resorcylate displaced thyroxine from the binding sites detected in the fast moving albumin fraction (band A) and also in the slower moving albumin fraction (band B), whereas

p-hydroxybenzoate produced a much smaller displacement from band A alone.

Studies were carried out of the *in vivo* effect of these drugs and also of 2,4-dinitrophenol in rats. It was demonstrated that both salicylate and 2,4-dinitrophenol produced a displacement of thyroxine from band A, while p-hydroxybenzoate had no such effect. These studies with rats therefore confirm the finding in man that salicylate, *in vivo*, displaces thyroxine from one of the specific thyroxine binding proteins of the serum.

γ-Resorcylate which produced a displacement *in vitro* greater than that produced by salicylate had no effect on thyroxine binding *in vivo*. Failure to demonstrate an *in vivo* effect of γ-resorcylate in man using the starch gel system also occurred, despite the fact that a displacement from TBPA had been shown when the samples were analysed by paper electrophoresis.

It is concluded that the increase in circulating free thyroxine produced by 2,4-dinitrophenol, salicylate and γ-resorcylate results from the displacement of thyroxine from a specific binding site on the serum proteins.

The peripheral component in the depression in plasma PBI produced by these drugs in both man and rats would result from the displacement of thyroxine, from one of the specific binding sites on the serum proteins, into the free state, followed by its rapid excretion. It was demonstrated by Escobar del Rey and Morreale de Escobar (1958a, b) that the administration of

2,4-dinitrophenol to rats equilibrated with radioiodide, produced along with the depression in circulating radio-thyroxine an increase in the accumulation of I^{131}-labelled compounds in the intestine and faeces as the result of an increased biliary excretion of these compounds.

Osorio and Myant (1963) reported that the increased biliary excretion of I^{131}-labelled compounds which followed the administration of salicylate to rats almost equalled the loss in blood radioactivity. It was demonstrated that the biliary excretion of the metabolites of thyroxine increased in parallel with the excretion of thyroxine itself, indicating that salicylate made a greater quantity of thyroxine available to the liver but did not affect the transport and metabolism of thyroxine in the liver. Since, for the collection of the bile samples the normal enterohepatic circulation was interrupted by cannulation, experiments were carried out on intact rats in which reabsorption of biliary thyroxine could occur normally. The depression in blood radioactivity produced by salicylate in these rats was similar to that observed in cannulated rats.

Initially the increase in radioactivity in the bile could be accounted for quantitatively by the loss in radioactivity from the blood. A subsequent change in shape of the blood radioactivity concentration curve indicated that some of the radioactivity leaving the blood, as a consequence of the injection of salicylate, later returned to the blood. It was concluded that a proportion of the thyroxine, displaced from

the serum proteins by salicylate, diffused into an extravascular store such as the liver and then diffused back to the blood later when the binding sites were no longer occupied by salicylate. This phenomenon would explain the smaller peripheral effect of salicylate observed in man compared to that in rats. The more efficient enterohepatic circulation in man would possibly result in the sequestration in the liver of a larger proportion of the thyroxine displaced by salicylate. Such a mechanism would explain the finding that the repeated administration of salicylate to man did not depress the plasma PBI to hypothyroid levels as it did in rats.

After the studies reported in this thesis had been completed an account of the effect of γ-resorcylate and gentisate on the peripheral metabolism of thyroxine was published by Woeber and Ingbar (1964). It was shown that these noncalorigenic dihydroxy benzoic acids specifically inhibited the binding of thyroxine to TBPA in human serum and also lowered the plasma PBI in man, thus confirming the similar findings with γ-resorcylate reported in this thesis. Furthermore, Woeber and Ingbar demonstrated that the changes in thyroxine metabolism induced by these drugs could be ascribed only to effects on the binding of thyroxine to TBPA since they obtained no evidence of stimulation of either metabolic rate or the cellular mechanisms of thyroxine degradation.

It is concluded, therefore, that the peripheral component

in the depression in plasma PBI produced by salicylate, γ-resorcylate and 2,4-dinitrophenol in both man and rats is caused by the displacement of thyroxine, bound to one of the specific binding sites on the serum proteins, into the free state. This increased level of free thyroxine is then rapidly excreted.

The demonstration that the levels of circulating TSH and thyroid hormone were depressed simultaneously by the administration of salicylate, 2,4-dinitrophenol and γ-resorcylate indicated a disruption of the negative feedback mechanism controlling the thyroid-pituitary axis. However, the depression in circulating TSH in rats (determined by bioassay) and in man (from indirect evidence) was shown to be correlated with the increase in the level of free thyroxine produced by these drugs. Therefore, if the level of free thyroxine rather than the level of total or bound thyroxine in the blood is considered to be the physiological regulator of the negative feedback system, this apparent disruption may be explained. The increased level of free thyroxine produced by these drugs would depress the release of TSH from the pituitary.

The central component in the depression in plasma PBI in man produced by salicylate and γ-resorcylate therefore also results indirectly from the increase in free thyroxine induced by these drugs.

A depression in plasma PBI which was accompanied by a decrease in thyroidal radioiodine release has been observed in

rats following the administration of the diazo dyes trypan blue and trypan red (Yamada, 1960a, b; Shimoda et al, 1962). These workers presented evidence that the dyes competed with thyroxine for binding sites on the serum proteins indicating an increase in free thyroxine which would inhibit the secretion of TSH from the pituitary.

Penicillin has also been shown in vitro to displace thyroxine from TBPA in human serum and to increase the rate of dialysis of radiothyroxine across a semi-permeable membrane, but in acute studies with the drug in vivo, no change was observed in plasma PBI. Failure to demonstrate a depression in PBI was considered to be due to the fact that the level of the drug in the blood was too low to exert such an effect (Surks and Oppenheimer, 1963).

Although diphenylhydantoin has been shown to depress the plasma PBI slightly, no evidence of a depression in TSH was obtained (Oppenheimer et al, 1961). Further studies with this drug revealed that in vitro it displaced thyroxine from TBG. (Wolff et al, 1961) and increased the rate of dialysis of radiothyroxine across a semi-permeable membrane indicating an increase in free thyroxine (Oppenheimer and Tavernatti, 1962).

The failure of diphenylhydantoin to depress TSH probably resulted from the fact that the blood level of the drug was not sufficiently raised. A similar finding occurred with γ-resorcylate in the studies reported in this thesis. In vitro effects on thyroxine binding and free thyroxine were readily demonstrated using the same concentration of

γ-resorcylate and salicylate. However, <u>in vivo</u>, it was necessary to use a much larger dose of γ-resorcylate than of salicylate to produce a similar effect on plasma PBI and free thyroxine.

Thus, a number of drugs have been observed to displace thyroxine into the free state from one of the binding sites of the serum proteins <u>in vitro</u>. However, in no instance were <u>in vivo</u> studies carried out to confirm the postulated rise in free thyroxine.

The studies presented in this thesis provide conclusive evidence of the validity of the <u>in vitro</u> action of salicylate, 2,4-dinitrophenol and γ-resorcylate for the situation <u>in vivo</u>; a rise in circulating free thyroxine is produced by a displacement of thyroxine from specific binding sites in the serum proteins which is correlated with a depression in circulating TSH.

Although 2,4-dichlorophenoxyacetic acid increases thyroidal uptake of radioiodine, Florsheim and Velcoff (1962) demonstrated that this effect of the drug did not involve a pituitary pathway but resulted from an intra-thyroidal action of the drug. It was also shown that administration of the drug to rats produced a significant depression in plasma PBI, but no evidence of a change in TSH output was obtained. Subsequently, Florsheim and co-workers reported that the depression in plasma PBI was related to a displacement of thyroxine from the serum proteins. Evidence was obtained that the displaced thyroxine was bound specifically in the

liver and it was postulated that 2,4-dichlorophenoxyacetic acid enhanced the binding of thyroxine to the tissue binding sites of the liver. The free thyroxine level, determined by Christensen dialysis method, was shown to be unchanged following administration of the drug. The finding that both circulating free thyroxine and pituitary secretion of TSH remained unchanged was interpreted by these workers to support the hypothesis that the pituitary thyroid feedback mechanism is controlled by the concentration of free thyroxine rather than by the total thyroxine level. (Florsheim et al, 1963).

The question of the site of action of free thyroxine in controlling the pituitary release of TSH must be considered. The injection of thyroxine into the pituitary, in quantities ineffective when injected systemically, caused almost immediate reduction in TSH secretion indicating that the pituitary is itself sensitive to small local increases in thyroxine concentration. (Van Euler and Holmgren, 1956; Yamada and Greer, 1956).

There has not been general agreement whether the hypothalamus itself is sensitive to the feedback control of thyroxine. Von Euler and Holmgren (1956) reported that the injection of thyroxine into the hypothalamus did not affect the release of TSH. However, a depression in TSH release was observed by Yamada and Greer (1956) after a latent period of about 8 hours when thyroxine was injected into the hypothalamus. Harrison (1961) could not confirm the finding of a depression in TSH release following local hypothalamic injection of

thyroxine and cautioned against the use of the relatively large volumes of material injected because of the probability of diffusion via the portal vessels to the anterior pituitary. Averill and co-workers (1963) demonstrated that injection of thyroxine into the hypothalamus of the rat produced thyroidal inhibition, but there was no latent period. By injecting radiothyroxine they demonstrated that a higher proportion of the dose was present in the pituitary after injection into the hypothalamus than after subcutaneous injection. They considered that the immediate response of a depression in TSH release following hypothalamic injection was due to an elevated concentration of thyroxine reaching the pituitary very rapidly.

Thus, the question of hypothalamic sites for the feedback control of thyroxine is still unresolved as the factors of localization and diffusion make interpretation difficult.

Nevertheless, the finding of Reichlin (1960) that lesions in the hypothalamus partially prevented the depression in thyroid function which was produced by 2,4-dinitrophenol administration to normal rats may be re-interpreted. Since a metabolic action of the drug in the depression of thyroid function may now be excluded, Reichlin's findings would be consistent with an action of free thyroxine at a hypothalamic site controlling thyroid function.

However, the possibility that the depression in TSH release produced by salicylate, γ-resorcylate and 2,4-dinitrophenol results from a direct pharmacological blockade

of the hypothalamic sites controlling TSH release from the pituitary cannot be excluded.

A depression in plasma PBI in thyrotoxic subjects induced by salicylate has been reported to be accompanied by a fall in the thyroid secretion rate (Hetzel et al, 1960) similar to that observed in normal subjects by Wolff and Austen (1958). However, other workers have not been able to confirm this finding of a depression in TSH by salicylate in thyrotoxic subjects (Myhill and Hales, 1963; Ingbar, 1964). Furthermore, Woeber and Ingbar (1964) reported a similar failure of γ-resorcylate to slow the disappearance of I^{131} from the thyroid in thyrotoxic subjects. An increase in the level of circulating free thyroxine concomitant with a displacement of thyroxine from TBPA has been demonstrated in thyrotoxic subjects following the administration of salicylate and γ-resorcylate (Good, 1964). The secretion rate from the thyroid of the thyrotoxic subject is not suppressed by the administration of thyroxine or triiodothyronine (Johnson et al, 1959), and therefore would not be suppressed by the increase in free thyroxine induced by salicylate or γ-resorcylate.

In consideration of the foregoing evidence, it is concluded that the depression in TSH release from the pituitary produced by salicylate, γ-resorcylate and 2,4-dinitrophenol is best correlated with the increase in free thyroxine induced by the displacement of thyroxine from specific thyroxine binding sites in the serum. Thus, despite the depression in

plasma PBI produced by these drugs, the circulating level of free thyroxine is elevated. The increased level of free thyroxine, acting as the regulator of the negative feedback system controlling the thyroid-pituitary axis, would be expected to depress the release of TSH by an action at pituitary and possibly hypothalamic sites controlling TSH release.

It is concluded, therefore, that the level of circulating free thyroxine, and not the total thyroxine concentration, is the physiological regulator of the feedback mechanism controlling the thyroid-pituitary axis.

B I B L I O G R A P H Y

ACLAND, J.D. (1957) Biochem. J., 66, 177.

ADAMS, D.D. (1958) J. Clin. Endocr., 18, 699.

ADAMS, D.D. and PURVES, H.D. (1953) Proc. Univ. Otago med. Sch., 31, 38.

ADAMS, D.D. and PURVES, H.D. (1955) Endocrinology, 57, 17.

ADAMS, D.D. and PURVES, H.D. (1957a) Canad.J.Biochem., 35, 993.

ADAMS, D.D. and PURVES, H.D. (1957b) Metabolism, 6, 26.

ADLER, H. (1914) Arch. Entwicklungsmech. Organ., 39, 21.

ALLEN, B.M. (1921) Anat. Rec., 20, 192.

ARON, M. (1929) Compt. rend. Soc. Biol. (Paris), 102, 682.

ARON, M. (1930) Compt. rend. Soc. Biol. (Paris), 103, 145.

ARON, M., VAN CAULERT, C. and STAHL, J. (1931) Compt. rend. Soc. Biol. (Paris), 107, 64.

AUSTEN, F.K., RUBINI, M.E., MERONEY, W.H. and WOLFF, J. (1958) J. Clin. Invest., 37, 1131.

AVERILL, R.L.W., KENNEDY, T.H. and PURVES, H.D. (1963) Proceedings Second Asia and Oceania Congress of Endocrinology (1963), 6.05.

AVERILL, R.L.W., PURVES, H.D. and SIRETT, N.E. (1961) Endocrinology, 69, 735.

BAKKE, J.L., HEIDEMAN, M.L., LAWRENCE, N., and WIBERG, C. (1957) Endocrinology, 61, 352.

BAKKE, J.L., LAWRENCE, N., ARNETT, F. and MacFADDEN, W. (1961) J. Clin. Endocr., 21, 1280.

BARKER, S.B. (1946) Endocrinology, 39, 234.

BARKER, S.B., HUMPHREY, M.J. and SOLEY, M.H. (1951) J. Clin. Invest., 30, 55.

BARRAL and MARTIN (1916) Lyon Med., 125, 276.

BATES, R.W. and CORNFIELD, J. (1957) Endocrinology, 60, 225.

BATES, R.W., GARRISON, M.M. and HOWARD, T.B. (1959) Endocrinology, 65, 7.

BEIERWALTES, W.H. and ROBBINS, J. (1959) J. Clin. Invest., 38, 1683.

BLUMBERG, B.S., FARER, L., RALL, J.E. and ROBBINS, J. (1961) Endocrinology, 68, 25.

BOGDANOVE, E.M. and HALMI, N.S. (1953) Endocrinology, 53, 274.

BOGOROCH, R. and TIMIRAS, P. (1951) Endocrinology, 49, 548.

BONDY, P.K. and HAGEWOOD, M.A. (1952) Proc. Soc. exp. Biol. Med., 81, 328.

BORELL, U. (1945) Acta. Med. Scand., Suppl., 161.

BORELL, U. and HOLMGREN, H. (1949) Acta Endocr. (Kbh.), 3, 331.

BORTH, R. (1952) Ciba Foundation Colloquia on Endocrinology, 2, 45.

BOTTARI, P.M. (1957) Ciba Foundation Colloquia on Endocrinology, 11, 52.

BOTTARI, P.M. and DONOVAN, B.T. (1958) J. Physiol., 140, 36P.

BRODY, T.M. (1956) J. Pharmacol. exp. Ther., 117, 39.

BROWN, J.R. (1959) Acta. Endocr. (Kbh.), 32, 289.

BROWN GRANT, K. (1957) Ciba Foundation Colloquia on Endocrinology, 10, 97.

BROWN GRANT, K., HARRIS, G.W. and REICHLIN, S. (1957)
J. Physiol., 136, 134.

BROWN GRANT, K., VON EULER, C., HARRIS, G.W. and REICHLIN, S. (1954) J. Physiol., 126, 1.

BROWN GRANT, K., VON EULER, C., HARRIS, G.W., and REICHLIN, S. (1954a) J. Physiol., 126, 29.

CAHANE, H. and CAHANE, T. (1938) Acta. Med. Scand., 94, 320.

CAMPBELL, H.J., GEORGE, R.W. and HARRIS, G.W. (1959)
J. Physiol., 148, 5P.

CASTOR, C.W. and BEIERWALTES, W.H. (1956) J. Clin. Endocr., 15, 862.

CHAIKOFF, I.L. and TAUROG, A. (1949) Ann. N.Y. Acad. Sci., 50, 377.

CHANEY, A.L. (1940) Industr. Engng. Chem, (Anal.), 12, 179.

CHARNOCK, J.S., OPIT, L.J. and HETZEL, B.S. (1962)
Biochem. J., 83, 602.

CHRISTENSEN, L.K. (1958) Scand. J. Lab. Clin. Invest., 10, 211.

CHRISTENSEN, L.K. (1959) Nature, 183, 1189.

CHRISTENSEN, L.K. (1959a) Scand. J. Clin. Lab. Invest., 11, 326.

CHRISTENSEN, L.K. (1960) Acta. Med. Scand., 166, 133.

CHRISTENSEN, L.K. and LITONJUA, A.D. (1961) J. Clin. Endocr., 21, 104.

COCHRAN, J.B. (1952) Brit. Med. J., 2, 964.

COCHRAN, J.B. (1953) Brit. Med. J., 1, 733.

COLFER, H.F. (1949) Trans. Amer. Goiter Assoc., 376.

CORTELL, R. and RAWSON, R.W. (1944) Endocrinology, 35, 488.

CUTTING, W.C., RYTLAND, D.A. and TAINTER, M.L. (1934) J. Clin. Invest., 13, 547.

CUTTING, W.C. and TAINTER, M.L. (1933) Proc. Soc. Exper. Biol. Med., 31, 97.

D'ANGELO, S.A. (1951) Endocrinology, 48, 249.

D'ANGELO, S.A. (1955) Brookhaven Symposia in Biology, 7, 9.

D'ANGELO, S.A. (1960) Amer. J. Physiol., 199, 701.

D'ANGELO, S.A. and GORDON, A.S. (1950) Endocrinology, 46, 39.

D'ANGELO, S.A., PASCHKIS, K.E., GORDON, A.S. and CANTAROW, A. (1951) J. Clin. Endocr. 11, 1237.

D'ANGELO, S.A. and TRAUM, R.E. (1956) Endocrinology, 59, 593.

D'ANGELO, S.A. and TRAUM, R. (1958) Ann. N.Y. Acad. Sci., 72, 239.

DANOWSKI, T.S., GOW, R.C., MATEER, F.M., EVERHART, W.C., JOHNSTON, S.Y. and GREENMAN, J.H. (1950) Proc. Soc. Exp. Biol. Med., 74, 323.

DEISS, W.P., ALBRIGHT, E.C. and LARSON, F.C. (1953) Proc. Soc. Exp. Biol. Med., 84, 513.

DEL CONTE, E., RAVELLO, J.J. and STUX, M. (1955) Acta Endocr. (Kbh.), 18, 8.

DEL CONTE, E. and STUX, M. (1954) Nature, 173, 83.

DEL CONTE, E. and STUX, M. (1955) Acta Endocr. (Kbh), 20, 246.

DEMPSEY, E.W. and ASTWOOD, E.B. (1943) Endocrinology, 32, 509.

DENIS, W. and MEANS, J.H. (1916) J. Pharmacol., 8, 273.

DOWLING, J.F., FREINKEL, N. and INGBAR, S.H. (1956a) J. Clin. Endocr., 16, 280.

DUBOWITZ, L.M.S., MYANT, N.B. and OSORIO, C. (1962)
J. Physiol., 162, 358.

EINHORN, J. and LARSSON, L.G. (1959) J. Clin. Endocr., 19, 28.

EL KABIR, D.J. (1962) Nature, 194, 688.

ELZINGA, K.E., CARR, E.A. and BEIERWALTES, W.H., (1961)
Amer. J. Clin. Path., 36, 125.

ESCOBAR DEL REY, F. and MORREALE DE ESCOBAR, G. (1958a)
Acta Endocr. (Kbh.), 29, 161.

ESCOBAR DEL REY, F. and MORREALE DE ESCOBAR, G. (1958b)
Acta Endocr., (Kbh.), 29, 176.

FARER, L.S., ROBBINS, J., BLUMBERG, B.S. and RALL, J.E. (1962)
Endocrinology, 70, 686.

FLORSHEIM, W.H. and VELCOFF, S.M. (1962) Endocrinology, 71, 1.

FLORSHEIM, W.H., VELCOFF, S.M. and WILLIAMS, A.D. (1963)
Endocrinology, 72, 327.

FORTIER, C. (1951) Endocrinology, 49, 782.

FREEDBERG, I.M., HAMOLSKY, M.W. and FREEDBERG, A.S. (1957)
New England J. Med., 256, 505.

GADDUM, J.H. (1933) Spec. Rep. Ser. Med. Res. Coun. (London)
No. 183.

GADDUM, J.H. (1953) J. Pharm. Pharmacol., 6, 345.

GANONG, W.F., FREDRICKSON, D.S. and HUME, D.M. (1954)
J. Clin. Endocr. 14, 773.

GANONG, W.F., FREDRICKSON, D.S. and HUME, D.M. (1955)
Endocrinology 57, 355.

GILLILAND, I.C. and STRUDWICK, J.I. (1956) Brit. Med. J., 1, 18.

GOLDBERG, R.C. and CHAIKOFF, I.L. (1951) Endocrinology, 49, 613.

GOLDBERG, R.C., WOLFF, J. and GREEP, R.O. (1955)
Endocrinology, 56, 560.

GOLDBERG, R.C., WOLFF, J. and GREEP, R.O. (1957)
Endocrinology, 60, 38.

GOOD, B.F., HETZEL, B.S. and OPIT, L.J. (1960)
J. Endocr. 21, 231.

GOOD, B.F. (1964) Unpublished observations.

GORDON, A.H., GROSS, J., O'CONNOR, D. and PITT-RIVERS, R. (1952)
Nature, 169, 19.

GRANNER, D.K., CURTIS, B. and HALMI, N.S. (1961)
Endocrinology, 68, 1066.

GREEN, J.D. (1951) Amer. J. Anat., 88, 225.

GREEN, J.D. and HARRIS, G.W. (1947) J. Endocr. 5, 136.

GREEN, J.D. and HARRIS, G.W. (1949) J. Physiol., 108, 359.

GREENSPAN, F.S., KRISS, J.S., MOSES, L.L. and LEW, W. (1956)
Endocrinology, 58, 767

GREENSPAN, F.S. and LEW, W. (1959) Endocrinology, 64, 160.

GREER, M.A. (1951) Proc. Soc. Exp. Biol. Med., 77, 603.

GREER, M.A. (1952) J. Clin. Endocr., 12, 1259.

GREER, M. (1957) Recent Progr. Hormone Res., 13, 67.

GREER, M.A. and SHULL, H.F. (1957) J. Clin. Endocr. 17, 1030.

GUDERNATSCH, J.F. (1912) Arch. Entwicklungsmech. Organ., 35, 457.

GUILLEMIN, R. and ROSENBERG, B. (1955) Endocrinology, 57, 599.

GUILLEMIN, R., YAMASAKI, E., JUTISZ, M. and SAKIZ, E. (1962)
Compt. Rend. Acad. Sci. (Paris), 255, 1018.

HAMOLSKY, M.W., GIERLACH, Z.S. and JENSEN, H. (1951)
Amer. J. Physiol., 164, 35.

HARRIS, G.W. (1947) J. Anat., 81, 343.

HARRIS, G.W. (1955) Neural Control of the Pituitary Gland
London, Edward Arnold Ltd.

HARRIS G.W. (1959) Symposium on Comparative Endocrinology
John Wiley & Sons Inc.

HARRIS, G.W. and JACOBSOHN, D. (1952) Proc.Roy.Soc.B., 139,263.

HARRIS, G.W. and WOODS, J.W. (1958) J. Physiol., 143, 246.

HARRISON, T.S. (1961) Endocrinology, 68, 466.

HENRY, R. (1951) Ann. Pharmac. Franc., 9, 724.

HETZEL, B.S., GOOD, B.F., WELLBY, M.L. and BEGG, M.W. (1962)
Australasian Annals Med., 11, 34.

HETZEL, B.S., GOOD, B.F., WELLBY, M.L. and CHARNOCK, J.S.(1960)
Lancet, 1, 957.

HETZEL, B.S., CHARNOCK, J.S. and LANDER, H. (1959)
Metabolism, 8, 205.

HICKS, J.D. (1963) Personal communication.

HOLWEG, W. and JUNKMANN, K. (1933) Arch.ges.Physiol., 232, 148.

HOSKINS, R.G. (1949) J. Clin. Endocr., 9, 1429.

INGBAR, S.H. (1953) Endocrinology, 53, 171.

INGBAR, S.H. (1958) Endocrinology, 63, 256.

INGBAR, S.H. (1960) Ann. N.Y. Acad. Sci., 86, 440.

INGBAR, S.H. (1960a) "Clinical Endocrinology", Grune & Stratton,
N.Y., 91.

INGBAR, S.H. (1963) J. Clin. Invest., 42, 143.

INGBAR, S.H. (1964) Annual Meeting Amer. Thyroid Assoc. 22.

JAGIELLO, G.M. and McKENZIE, J.M. (1960) Endocrinology, 67, 451.

JOHNSON, D.E., SOLOMON, D.H. and GREER, M.A. (1959) J. Clin. Endocr., 19, 317.

JUNKMANN, K. and SCHOELLER, W. (1932) Klin. Wschr., 11, 1176

KHAZIN, A. and REICHLIN, S. (1961) Endocrinology, 68, 914.

KNIGGE, K.M. and BIERMAN, S.M. (1958) Amer. J. Physiol., 192, 625.

LARDY, H.A. and WELLMAN, H. (1952) J. Biol. Chem., 195, 215.

LARSON, F.C., DEISS, W.P. and ALBRIGHT, E.C. (1952) Science, 115, 626.

LEIN, S. (1952) Federation Proc., 91, 152.

LOEB, L. (1932) Endocrinology, 16, 129.

LOESER, A. and THOMPSON, W.K. (1934) Endokrinologie, 14, 144.

LORAINE, J.A. (1958) Clinical Application of Hormone Assay. Livingstone Ltd. Edin. and London.

LUTZ, C. and BAUME, S. (1917) Compt. rend. Soc. Biol. (Paris) 80, 483.

McCONNELL, E.M. (1953) Anat. Rec., 115, 175.

McKENZIE, J.M. (1958) Endocrinology, 63, 372.

McKENZIE, J.M. (1960) Physiol. Rev., 40, 398.

MEADE, B.W. (1954) Annals, Rheum. Disease, 13, 60.

MORREALE DE ESCOBAR, G. and ESCOBAR DEL REY, F. (1960) Acta Endocr. (Kbh.), 35, Supp 51, 1219.

MORREALE DE ESCOBAR, G. and EXCOBAR DEL REY, F. (1961a) J. Physiol., 159, 1.

MORREALE DE ESCOBAR, G. and ESCOBAR DEL REY, F. (1961b) J. Physiol., 159, 15.

MORTON, M.E., PERLMAN, I., ANDERSON, F. and CHAIKOFF, I.L. (1942) Endocrinology, 30, 495.

MUSSETT, M.V. and PERRY, W.L.M. (1955) Bull. Wld. Hlth. Org., 13, 917.

MYANT, N.B. (1957) J. Physiol., 135, 426.

MYANT, N.B. and OSORIO, C. (1960) J. Physiol., 152, 601.

MYHILL, J. and HALES, I.B. (1963) Lancet, 1, 802.

NIÈPCE, B. (1851) Paris : Baillière.

NIKITOVITCH-WINER, M. and EVERETT, J.W. (1958) Endocrinology, 63, 916.

ODELL, W.D. (1963) Endocrinology, 72, 39.

OPIT, L.J. (1954) Personal Communication.

OPPENHEIMER, J.H., FISHER, L.V., NELSON, K.M. and JAILLER, J.W. (1961) J. Clin. Endocr., 21, 252.

OPPENHEIMER, J.H. and TAVERNATTI, R.R. (1962) Endocrinology, 71, 496.

OSORIO, C. (1962) J. Physiol., 163, 151.

OSORIO, C. and MYANT, N.B. (1963) Endocrinology, 72, 253.

PACKER, L. (1958) Quoted in Wolff, J. and Austen F.K. (1958).

PASCHKIS, K.E., CANTAROW, A.H., EBERHARD, T. and DOYLE, D. (1950) Proc. Soc. Exp. Biol. Med., 73, 116.

PITT-RIVERS, R. and RALL, E. (1961) Endocrinology, 68, 309.

PITT-RIVERS, R. and TATA, J. (1959) The Thyroid Hormones, Pergamon Press, London.

POPA, G.T. and FIELDING, U. (1930) J. Anat., 65, 88.

POPA, G.T. and FIELDING, U. (1933) J. Anat., 67, 227.

PURVES, H. D. and ADAMS, D.D. (1960) Brit. Med. Bull., 16, 128.

PURVES, H. D. and GREISBACH, W.E. (1949) Brit. J. Exp. Path., 30, 23.

QUERIDO, A., KASSENAAR, A.A.H. and LAMEIJER, L.D.F. (1953) Acta Endocr. (Kbh.), 13, 335.

QUERIDO, A. and LAMEIJER, L.D.F. (1956) Proc. Roy. Soc. Med., 49, 209.

RAND, C.G., RIGGS, D.S. and TALBOT, N. (1952) Endocrinology, 51, 562.

RANDALL, R.V. and ALBERT, A. (1951) Endocrinology, 48, 327.

REICHLIN, S. (1957) Endocrinology, 60, 567.

REICHLIN, S. (1960) Endocrinology, 66, 327.

REICHLIN, S. (1960a) Endocrinology, 66, 340.

REICHLIN, S., BOSHANS, R.L. and BROWN, J.G. (1963). Endocrinology, 72, 334.

REICHLIN, S. and REID, A.A. (1955) Proc. Soc. Exp. Biol. Med., 89, 212.

REID, J. (1957) Scot. Med. J., 2, 91.

ROBBINS, J. (1956) Arch. Biochem., 63, 461.

ROBBINS, J. and NELSON, J.H. (1958) J. Clin. Invest., 37, 153.

ROBBINS, J. and RALL, J.E. (1955) J. Clin. Invest., 34, 1324.

ROBBINS, J. and RALL, J.E. (1957 Recent Progr. Hormone Res., 13, 161.

ROBBINS, J. and RALL, J.E. (1960) Physiol. Rev., 40, 415.

ROBBINS, J., RALL, J.E. and PETERMANN, M.L. (1954) J. Clin. Invest., 33, 959.

SALTER, W.T. (1940) The Endocrine Function of Iodine, Harvard University Press.

SALTER, W.T. and McKAY, E.A. (1944) Endocrinology, 35, 380.

SANDELL, I.M. and KOLTHOFF, E.B. (1934) J. Am. Chem. Soc., 56, 1426.

SCHULTZE, H.E., SCHÖNENBERGER, M. and SCHWICK, H. (1956) Biochem. Z., 328, 267.

SHIBUSAWA, K., NISHI, K., ABE, C. (1959) Endocr. Jap., 6, 31.

SHIMODA, S., TOMIZAWA, T., YAMADA, T. and SHICHIJO, K. (1962) Endocrinology, 71, 414

SIMKINS, S. (1937) J. Amer. Med. Assoc., 108, 2110 & 2193.

SMITH, M.J.H. and JEFFREY, S.W. (1956) Biochem. J., 63, 524.

SMITH, P.E. (1927) J. Am. Med. Assoc., 88, 158.

SMITH, P.E. and SMITH, I.P. (1922) J. Med. Research, 43, 267.

SMITH, P.E. and SMITH, I.P. (1923) Endocrinology, 7, 579.

SMITHIES, O. (1959) Biochem. J., 71, 585.

SODERBERG, U. (1958) Acta Physiol. Scand., 42, Suppl. 147.

SOMOGYI, M.A. (1930) J. Biol. Chem., 86, 655.

STERLING, K. and HEGEDUS, A. (1962) J. Clin. Invest., 41, 1031.

STERLING, K., ROSEN, P. and TABACHNICK, M. (1962) J. Clin. Invest., 41, 1021.

STIEDA, H. (1890) Beitr. pathol. Anat., 7, 535.

STURM, A. (1930) Z. ges. exptl. Med., 74, 514.

SURKS, M.I. and OPPENHEIMER, J.H. (1963) Endocrinology, 72, 567.

SWINGLE, W.W. (1921) Anat. Record, 20, 195.

TAINTER, M.L. and CUTTING, W.C. (1933) J. Pharmacol. exper. Therap., 48, 410.

TATA, J.R. (1959) Biochem. J., 72, 222.

TATA, J.R. (1959a) Clin. Chim. Acta, 4, 427.

TATA, J.R., WIDNELL, C.C. and GRATZER, W.B. (1961) Clin. Chim. Acta, 6, 597.

TREVORROW, V. (1939) J. Biol. Chem., 127, 737.

TRINDER, P. (1954) Biochem. J., 57, 301.

VAN ARSDEL, P.P. and WILLIAMS, R.H. (1956) J. Biol. Chem., 223, 431.

VAN DER LAAN, W.P. and GREER, M. (1950) Endocrinology, 47, 36.

VAN MIDDLESWORTH, L. and BERRY, M.M. (1951). Amer. J. Physiol., 167, 677.

VON EULER, C. and HOLMGREN, B. (1956) J. Physiol., 131, 125.

VON EULER, C. and HOLMGREN, B. (1956a) J. Physiol., 131, 137.

WARTHEN (1918) Int. Assoc. Med. Museums, 7, 123

WINZLER, R.J. and NOTRICA, S.R. (1952) Federation Proc., 11, 312.

WISLOCKI, G.B. (1938) Res. Publ. Ass. nerv. ment. Dis., 17, 48.

WOEBER, K.A. and INGBAR, S.H. (1963) Endocrinology, 73, 118.

WOEBER, K.A. and INGBAR, S.H. (1964) J. Clin. Invest., 43, 931.

WOLFF, J. (1951) Endocrinology, 48, 284.

WOLFF, J. and AUSTEN, F.K. (1958) J. Clin. Invest., 37, 1144.

WOLFF, J., RUBIN, L. and CHAIKOFF, I.L. (1950)
J. Pharmacol. exp. Ther., 98, 45.

WOLFF, J., STANDAERT, M.E. and RALL, J.E. (1961)
J. Clin. Invest., 40, 1373.

YAMADA, T. (1960a) Endocrinology, 67, 204.

YAMADA, T. (1960b) Endocrinology, 67, 212.

YAMADA, T. and GREER, M.A. (1959) Endocrinology, 64, 559.

YAMAZAKI, E., NOGUCHI, A., SATO, S. and SLINGERLAND, D.W.
(1961) J. Clin. Endocr., 21, 1127.