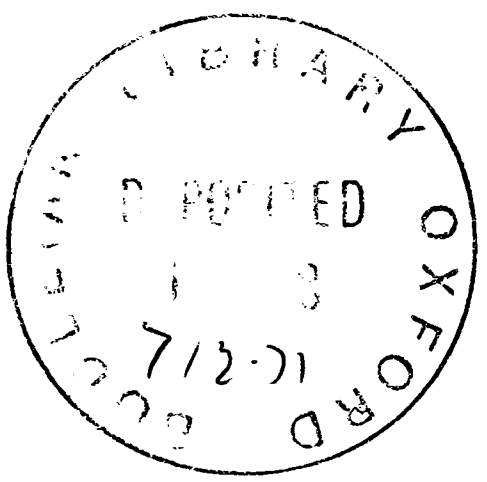

SOME PERIPHERAL EFFECTS OF THYROID STIMULATING SUBSTANCES

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AMMENDEMENTS

The following corrections are submitted:-

p.4. Line 10 should read: mice (Adams et al, 1961, Munro, 1959)....

p.7 Lines 10-15 should read: On the other hand, Volpe et al (1974) suggested that the expression of cell mediated immunity(1) in Graves' disease is attributable to a clone of T-lymphocytes(2) which induce B-lymphocytes(3) to produce autoantibody. They postulated an inherited disorder of immunological surveillance as the underlying defect permitting the emergency of this 'forbidden' clone by somatic mutation.

The following footnotes are to be added:-

(1) e.g. T-lymphocytosis, macrophage inhibition factor production by lymphocytes in the presence of thyroid extract.

(2) Thymus-dependent lymphocytes

(3) Bursa-equivalent lymphocytes

p.13 Line 25 should read: of organification (e.g. methimazole, propyl thiouracil (3)).

The following footnote is to be added: 3. P.T.U.

p.14 Line 17: for (Wollman and Read, 1959) read (Wollman and Reed, 1959).

p.16 Line 8: for pentasephoshate read pentose phosphate

Line 10: for Tondeut-Montenez read Tondeur-Montenez

p.18 Line 7: for porcine read purine

Line 14: for pirimidine read pyrimidine

p.21 Line 3: for Tong (1976) read Tong (1974)

p.23 Line 22: for endotheline read endothelium

p.27 Line 3: for phosphorylase C read phospholipase C

Line 7: for iodotyronines read iodotyrosines

Line 19: for parallel read parallels

Line 20: for was read is

p.29 Line 16: for hydroxytryphamine read hydroxytryptamine

p.30 Line 14: for suggests read suggest

Line 15: for was read is

p.31 Line 18: for terbutilene read terbutiline

p.32 Line 7: for was read is

p.40 Footnote 1: for Sumloch Compucorp 3421 read Sumlock Compucorp 342

p.43 Line 6: for successfull- read successfully

p.48 Line 6: for mg read µg

Fig. 2.10 legend: add "Each point represents a single fraction"

p.50 Line 9: for 17.5 kev read 75 kev

p.51 Line 6: for lyanogum read Cyanogum

p.58 Line 6: for Bourke et al (1974) read Manley et al (1974)

p.61 Line 11: for 6.87 g sodium barbitone read 6.87 g/l sodium barbitone

p.62 Line 1: for Pasteru read Pasteur

Line 6: for 10 c/cm read 10 v/cm

p.69 Line 12: for triiodothyroxine read triiodothyronine

Fig. 3.5. legend: The lettering of the groups in the table should read ABCD from above.

p.87 Line 26: for had read has

p.87 Line 11: for control read controls.

Fig. 3.20 Footnote should read: Intercepts not significantly different $0.05 > p > 0.1$.

p.106 Line 22: for decays read decayed

Line 25: for peaks read peaked

p.111 Lines 6-7: should read: Results were analysed using Student's, Dunnett's or Duncan's test as described in appendix 1.

p.120 Line 17: for specific read non-specific

p.139 Line 3: delete rate

p.154 Footnote 1 should read: Generously supplied by Dr L. Reichert, N.I.A.M.D., Bethesda, Maryland, U.S.A.

p.160 Line 20: for clotting read gelling

p.164 Footnote 1 should read: Serum was taken on two occasions each from C.P.G., M.J., and R.N. The IgG produced from these are referred to as C.P.G.1 IgG, C.P.G.2 IgG etc.

Footnote 2 should read: Kindly supplied by Professor D. Doniach, The Middlesex Hospital, London.

p.171 Line 22: for µg/ml read mg/l

p(ii) Line 13: should read $t = \dfrac{\bar{y}_i - \bar{y}_1}{S_m\sqrt{2/n}}$

p(iii) Line 19: should read for R'p = SZp read R'p = sZp

(iv) Line 12: for part 2 read part 3

(v) Line 21: should read $S_m^2 = \dfrac{y_s^2 + y_u^2 - T_d^2/n}{N_u - k_u + N_s - k_s}$

(xiv) Footnote (3) should read 'Nembutal' (0.067 mg/100 g.i.p.);
Abbott Laboratories Limited, Queenborough, Kent

(xvii) Line 19: should read: at between 12.5-75 kev (^{125}I) and 314-414 kev (^{131}I)

To my wife Ann, for all her help and support.

INDEX

PREFACE

ABSTRACT

PART 1. THYROID STIMULATORS AND THYROID STIMULATION

1.1. The nature and significance of thyroid stimulators 1
1.1.1. Physiological control of thyroid function 1
1.1.2. Pathological thyroid stimulators 3
1.1.3. Pharmacological thyroid stimulation 8

1.2. The effects of thyroid stimulators 10
1.2.1. T.S.H. 10
1.2.2. L.A.T.S. and T.S.I. 23
1.2.3. Biogenic amines 29
1.2.4. Receptor relationships 32

1.3. The scope of the experimental work 33

PART 2. GENERAL METHODS

2.1. The McKenzie bioassay 34
2.1.1. Introduction 34
2.1.2. Materials 35
2.1.3. Method 37
2.1.4. Treatment of results 38

2.2. The preparation of iodinated T.S.H. 40
2.2.1. Introduction: methods available for protein labelling 40
2.2.2. Materials 43
2.2.3. Methods 45
2.2.4. The estimation of specific activity 47
2.2.5. Rechromatography of T.S.H.-^{125}I 48

2.3. The properties of labelled T.S.H. 49
2.3.1. Gel filtration on Sephadex G-100 50
2.3.2. Polyacrylamide gel electrophoresis (P.A.G.E.) 51
2.3.3. Immunoreactivity 52
2.3.4. Biolgical activity 56
2.3.5. Conclusions on the purity of T.S.H. labelled with ^{125}I by the chloramine T method 57

2.4. The preparation of IgG from human plasma or serum 58
2.4.1. Introduction 58
2.4.2. Method 59
2.4.3. Protein estimation 60
2.4.2. Immunoelectrophoresis 61

2.5. The iodination of human IgG 62

2.6. The physicochemical properties of IgG-^{125}I 64

PART 3. AN INVESTIGATION INTO THE TIME COURSE OF ACTION OF T.S.H. AND L.A.T.S. IN VIVO

3.1. Introduction 66

3.2. Studies on the decay rate of injected b-T.S.H. in mice 71
3.2.1. Introduction 71
3.2.2. Materials and methods 71
3.2.3. The decay rate of T.S.H.-^{125}I in the mouse 73
3.2.4. A comparison of methods used for the removal of contaminating iodide from blood or plasma 74
3.2.5. The fate of injected label 75
3.2.6. The fate of purified T.S.H.-^{125}I following intravenous injection 78
3.2.7. The decay rate of purified T.S.H.-^{125}I from the blood and plasma of mice following intravenous injection 79
3.2.8. The effects of thyroxine and large quantities of exogenous T.S.H. on the decay curve for T.S.H.-^{125}I 80
3.2.9. Discussion 82

3.3. The time course of action of T.S.H. on the thyroid experiments using injections of anti-T.S.H. antiserum at varying times after T.S.H. in the mouse 84
3.3.1. Introduction 84
3.3.2. Method 85
3.3.3. Results 86
3.3.4. Discussion 86

3.4. The decay rate of intravenously injected L.A.T.S. and normal IgG in mice 88
3.4.1. Introduction 88
3.4.2. Materials and methods 89
3.4.3. The decay rate of injected h-IgG-^{125}I in the mouse - the effect of thyroxine and injected serum 91
3.4.4. The decay of total h-IgG as measured by single radial immunodiffusion 93
3.4.5. The decay of L.A.T.S. activity after intravenous injection 95
3.4.6. Discussion 96

3.5. The influence of T.S.H. and L.A.T.S. on the decay rate of thyroxine-^{125}I in the mouse 97
3.5.1. Introduction 97
3.5.2. Materials and methods 98
3.5.3. The pattern of decay of intravenously injected T_4-^{125}I and iodide-^{13}I^{1} 99
3.5.4. A comparison of the effects of intraperitoneal and intravenous routes of administration of labelled thyroxine on the disposal rate in normal and thyroxine-treated mice 101
3.5.5. The effect of T.S.H. on the decay of T_4-^{125}I in the mouse 102
3.5.6. The effect of L.A.T.S. or control IgG on T_4-^{125}I disposal rate 103
3.5.7. Discussion 103

3.6. Discussion and conclusions 106

PART 4. RECEPTORS IN THE MOUSE THYROID

4.1. Introduction 110

4.2. Materials and methods 111

4.3. The effects of adrenaline, noradrenaline, isoprenaline (I.P.N.A.) and 5-hydroxytryptamine (5-H.T.) on mouse thyroid secretion in vivo 112

4.4. The effect of 5-H.T. and I.P.N.A. on protein bound ^{131}I (P.B.^{131}I) and T_4-^{125}I decay rate 113

4.5. Did 5-H.T. and I.P.N.A. stimulate the thyroid directly or via the release of T.S.H. by the pituitary gland? 115

4.6. The effect of P.B.A. on the responses to T.S.H., L.A.T.S., 5-H.T. and I.P.N.A. 116

4.7. The effect of P.B.A. on blood radioiodine level in the "McKenzie" mouse 121

4.8. The effect of dl-propranolol on the responses to T.S.H., L.A.T.S., 5-H.T. and I.P.N.A. 122

4.9. Discussion 123

PART 5. STUDIES ON THE BINDING OF T.S.H. BY THYROID GLAND HOMOGENATES

5.1. Introduction 130

5.2. The aims of the study 132

5.3. The choice of system 133
5.3.1. Labelling procedure 133
5.3.2. Tissue preparation 133

5.4. Homogenisation procedure 134

5.5. First attempts to demonstrate binding of T.S.H. by thyroid homogenates 135
5.5.1. First experiment 136
5.5.2. Second experiment 137
5.5.3. Comments 138

5.6. The time course of T.S.H.-^{125}I uptake and the effect of temperature 141

5.7. The effect of iodination method 143

5.8. The effect of incubation damage on T.S.H.-^{125}I uptake 144

5.9. Was the bound label iodide or T.S.H.? 145
5.9.1. Was T.S.H.^{125}I deiodinated during the incubation? 145
5.9.2. The effects of goitrogens and carrier T.S.H. on T.S.H.-^{125}I and iodide-^{131}I ($^{131}I^-$) uptake by bovine thyroid homogenates 146
5.9.3. Comments 147

5.10. Was the binding of T.S.H.-^{125}I reversible? 147

5.11. Does T.S.H.-^{125}I bind to other tissues? 149

5.12. The interaction of T.S.H.-^{125}I with other thyroid cell fractions 151
5.12.1. Microsomal fraction 151
5.12.2. Soluble fraction 153

5.13. The effects of graded doses of T.S.H. and other pituitary hormones on the binding of T.S.H.-^{125}I to bovine thyroid homogenates 154

5.14. Estimates of the association constant (K_A) 156

5.15. The effect of washing and prolongation of centrifugation on T.S.H.-^{125}I binding by bovine thyroid homogenates 158
5.15.1. The effect of washing the homogenate before use 159
5.15.2. The effect of prolongation of centrifugation 159
5.15.3. Estimation of the loss of label on washing the pellet 160

5.16. The uptake of T.S.H.^{125}I by human thyroid homogenates 161

5.17. The effects of L.A.T.S. on T.S.H.^{125}I binding to thyroid homogenates 162
5.17.1. Preliminary experiments 163
5.17.2. Further experiments on the effect of L.A.T.S. IgG on T.S.H.^{125}I binding by human and bovine thyroid homogenates 164

5.18. The effect of graded doses of L.A.T.S. 166

5.19. The effect of L.A.T.S. on the slope of the Scatchard plot 167

5.20. Does L.A.T.S. bind T.S.H.? 168
5.20.1. Direct approach 168
5.20.2. Indirect approach: The effect of pre-incubation with L.A.T.S. IgG on the subsequent uptake of T.S.H.-^{125}I in the absence of L.A.T.S. homogenate by a bovine thyroid 169

5.21. The effects of 5-H.T., I.P.N.A. and dl-propranolol on T.S.H.-^{125}I binding by bovine thyroid homogenates 171

5.22. Discussion 172

APPENDICES

Appendix 1. Statistical methods i

Appendix 2. Stock solutions vii

Appendix 3. Preparation of Sephadex columns x

Appendix 4. Human sera xi

Appendix 5. The preparation of anti-T.S.H. antibodies xiv

Appendix 6. A method for removal of iodide from small samples of blood or plasma by chromatography on microcolumns of amberlite I.R.A. 400 ion exchange resin. xvi

REFERENCES

PREFACE

This thesis describes original experiments carried out between September 1970 and September 1973. The work was not completed, however, because of the sudden loss of suitable laboratory facilities during the summer of 1973. Some of the work in part 5 was presented to the Royal Society of Medicine on 17th May 1973, but otherwise it has not be reported. Preparation of this thesis began in September 1973 but was protracted because of lack of time during clinical studies and house officer appointments at the Radcliffe Infirmary, Oxford, but completion was eventually achieved during an appointment as a departmental demonstrator in the Department of Human Anatomy from February to August 1977.

I am greatly indebted to Mrs. P. Jeffs for her expert technical assistance. Dr. D. Smyth and Dr. Rosalind Pitt-Rivers of the National Institute for Medical Research, Mill Hill, Professor Deborah Doniach of the Middlesex Hospital and Dr. R. W. Hiorns of the Department of Biomathematics, Oxford, generously allowed me some of their precious time for discussion. My gratitude goes to Professor I. Doniach of the London Hospital and the pathology departments of Northampton General and Stoke Mandeville Hospitals for human thyroid tissue, to Professor D. Doniach of the Middlesex Hospital, Dr. T. D. R. Hockaday of the Radcliffe Infirmary, Oxford, Dr. Jacques Mahaux of the Clinique Medicale Universitaire de l'hôpital Brugmann, Brussels, and Dr. W. M. Priest of the Warnford General Hospital, Leamington Spa, for human sera,

to Professor J. G. Pierce of the University of California, Los Angeles, for his gift of highly purified b-T.S.H., and Professor L. Reichert of the National Institute of Health, Bethesda, Maryland, for a selection of pituitary hormone preparations.

I am indebted to the late Professor G. W. Harris and Professor G. Weddell in whose department the work was performed, and to the other members of staff of the Department of Human Anatomy, especially Dr. K. Brown-Grant, Dr. H. M. Charlton, Dr. G. Fink, Dr. F. Greig and Dr. P. C. B. McKinnon, for advice and encouragement. I should like to thank Dr. C. W. Burke of the Radcliffe Infirmary, Oxford, for useful criticism and for offering a ray of hope when all was thought to be lost. I am grateful to Dr. D. J. El Kabir for acting as supervisor and allowing me the freedom of his laboratory. Financial support was generously supplied by the Medical Research Council and a Theodore Williams Scholarship in Anatomy.

May 1977 C. P. G.

ABSTRACT

T.S.H. is the major physiological thyroid stimulator. Pathological thyroid stimulators may occur in certain disease states. In human Graves' disease thyroid stimulating immunoglobulins are produced and may be the cause of thyrotoxicosis. Some of these (L.A.T.S.-P) are human specific in their actions whereas others (L.A.T.S.) can affect the thyroids of other mammals. Pharmacological thyroid stimulation may be produced by a variety of substances including catecholamines and indolealkylamines. The physiological significance of this is considered. Following a brief review of the effects of these substances it is concluded that T.S.H. and L.A.T.S. probably act on the same thyroid receptor whereas separate receptors exist for catecholamines and indolealkylamines.

Experimental work is described in 3 parts:

1. A comparison of the time course of action of T.S.H. and L.A.T.S. on the mouse thyroid in vivo

The effects of T.S.H. and L.A.T.S. on thyroid secretion in vivo differ in their time course. L.A.T.S. is probably long acting in vivo because of its slow plasma decay rate but previous estimates had been only semiquantitative.

In experiments in mice the disappearance from the circulation of intravenously injected b-T.S.H.-^{125}I labelled by a modification of the chloramine T method was followed. Label rapidly disappeared from the circulation, decaying to 50% at 5-6 minutes and to less than 3% at 40 minutes. T.S.H.-^{125}I leaving the circulation was probably degraded since label

rapidly reappeared as iodide. No labelled T.S.H. fragments were detected by gel filtration of plasma after injection of T.S.H.-^{125}I. The observed rate of decay was not affected by thyroxine pretreatment or the concomitant injection of large quantities of unlabelled T.S.H. but was profoundly affected by the purity of the label.

The prolongation of the thyroid response beyond the disappearance of T.S.H. from the circulation was unlikely to be due to persistently receptor-bound hormone since injections of anti-T.S.H. antiserum more than 10 minutes after T.S.H. had no effect.

The plasma decay of L.A.T.S. IgG in mice was followed using radioiodinated IgG, single radial immunodiffusion and bioassay. IgG disappeared with a half life in excess of 90 hours following a distribution phase of approximately 24 hours. Iodination by the chloramine T method was found to increase the rate of IgG decay. The distribution volume was increased by the concomitant injection of human serum. Thyroxine speeded IgG decay. L.A.T.S. decay paralleled IgG decay over 24 hours although a small discrepancy was noted at 72 hours.

Since the response in the McKenzie assay may be affected by thyroxine metabolism,the effects of T.S.H. and L.A.T.S.-IgG were tested on thyroxine decay rate in mice. No significant changes were noted.

The short-acting nature of T.S.H. is attributable therefore to its rapid plasma decay rate, the pattern of response being determined by intrathyroid mechanisms, whereas L.A.T.S. is

long-acting because of its slow plasma decay rate.

2. Experiments on the actions of various biogenic or synthetic amines on the mouse thyroid in vivo

In confirmation of previous reports 5-hydroxytryptamine (5-H.T.) and isopropylnoradrenaline (I.P.N.A.) were able to elicit a response in the McKenzie assay. Both agents affected both total and protein bound circulating radioiodide in parallel but failed to affect the decay rate of injected T_4-^{125}I. In contrast to earlier reports, attempts to demonstrate thyroid stimulation following adrenaline and noradrenaline were unsuccessful.

The response to I.P.N.A. could be specifically inhibited by the β-receptor blocking agent, dl-propranolol. In suitable doses the α-receptor blocking agent, phenoxybenzamine (P.B.A.), selectively abolished the response to 5-H.T. without affecting the response to other stimulators. Evidence was also put forward suggesting that P.B.A. may have a mild thyroid stimulatory effect of its own.

These results suggested that the mouse thyroid contained receptors for 5-H.T. and β-adrenergic agents in addition to receptors for T.S.H. They failed to distinguish between T.S.H. and L.A.T.S. receptors.

3. The binding of T.S.H. by thyroid homogenates

Attempts were made to iodinate bovine T.S.H. with minimal loss of biological activity by modifications of the chloramine T method. The resultant T.S.H.-^{125}I ran essentially

as a single peak on Sephadex G-100 column chromatography and on polyacrylamide gel electrophoresis and was more than 90% immunoprecipitable, but retained only 30-50% of the biological activity in the unlabelled material.

T.S.H.-^{125}I, thus prepared, bound specifically to 2000g sediments and microsomal (105,000g) fractions of bovine thyroid homogenates. The binding of 2000g fractions was up to 90% inhibitable by excess carrier T.S.H. and 70% of specifically bound label could subsequently be displaced by excess carrier T.S.H. Highly purified T.S.H. and International Standard T.S.H. were equally effective for the inhibition of binding and the effects of other pituitary hormone preparations were related to their bioassayable thyrotropic activity. Scatchard analysis yielded a straight line with some reduction in slope at high carrier T.S.H. concentration ("tail-off"). K_A was calculated to be between 4.7×10^8 and 9.0×10^8 M^{-1}. Binding was also demonstrated using human thyroid homogenates. Results were similar but "tail-off" of the Scatchard plot was more marked. K_A was between 4.5×10^8 and 1.2×10^9 M^{-1}.

Binding to either bovine or human thyroid homogenates was inhibited by 3 L.A.T.S. containing IgGs from patients with thyrotoxicosis, but not by 2 L.A.T.S.-negative IgGs from patients undergoing treatment for thyrotoxicosis, 4 control IgGs from 2 euthyroid subjects and IgG prepared from a pool of serum with high antithyroid (antimicrosomal) antibody titres. The effect of L.A.T.S. was dose related and was not attributable to inactivation of T.S.H.-^{125}I. Bovine serum

albumin inhibited binding to human thyroid homogenates but increased binding to bovine thyroid homogenates. 5-H.T., I.P.N.A. and dl-propranolol were without effect on saturable binding to bovine thyroid homogenates.

The results are discussed in relation to subsequent literature. It is proposed that T.S.H. binding sites within thyroid homogenates are thyroid receptors. These observations support the theory that L.A.T.S. acts via combination with T.S.H. receptors, whereas 5-H.T. and I.P.N.A. act independently.

PART 1

THYROID STIMULATORS AND THYROID STIMULATION

- a review of current concepts

1.1. THE NATURE AND SIGNIFICANCE OF THYROID STIMULATORS

A great variety of substances can increase secretion by the mammalian thyroid gland. The most important of these is the pituitary hormone, thyrotropin (T.S.H.), which is the physiological thyroid stimulator[1], but further mechanisms for thyroid activation are gaining recognition. Certain others, for instance thyroid stimulating antibodies and T.S.H.-like substances produced by tumours, may be important in the pathogenesis of human thyrotoxicosis. Yet others may be of interest from a pharmacological point of view. This part of the thesis deals with the nature and significance of these substances, with particular reference to their mode of action. A general scheme of the various influences on the thyroid is presented in figure 1.

1.1.1. Physiological control of thyroid function

The thyroid-pituitary axis. In mammals thyroid secretion appears to be maintained by the double chain glycoprotein, T.S.H. (M.W. 28,500)(Pierce, 1971). The plasma levels of free thyroid hormones control its secretion by the pituitary to complete a servo loop, which tends to maintain free thyroid hormone levels constant (Brown-Grant, 1956; Reichlin, 1966; Reichlin et al, 1972). The setting of the thyroid-pituitary axis is regulated by the anterior hypothalamus via the tripeptide, thyrotropin releasing factor (T.R.F.), which is secreted into hypothalamico-hypophysial portal system (Martin, 1974). This provides the neuroendocrine route for increased thyroid secretion response to stimuli such as reduced environmental

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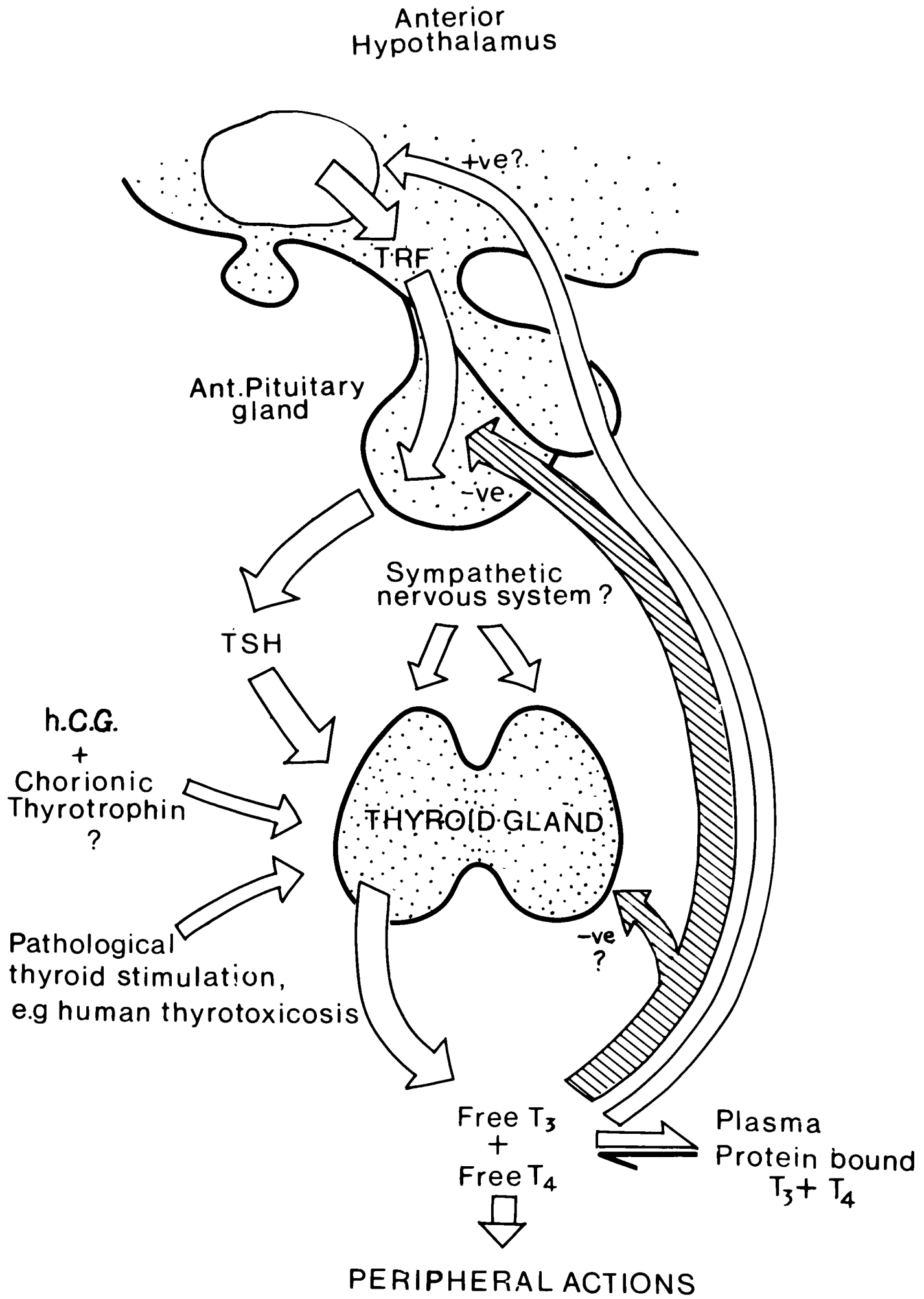
(1) For the purpose of the present work a thyroid stimulator is taken to be a substance capable of enhancing thyroid hormone secretion rate by a direct action on the gland

FIGURE 1

Factors involved in thyroid control


Anterior
Hypothalamus
+ve?
TRF
Ant.Pituitary
gland
-ve
Sympathetic
nervous system ?
TSH
h.C.G.
+
Chorionic
Thyrotrophin
?
THYROID GLAND
Pathological
thyroid stimulation,
e.g human thyrotoxicosis
-ve
?
Free T_3
+
Free T_4
Plasma
Protein bound
T_3+ T_4
PERIPHERAL ACTIONS

temperature, at least in the short term (Martin, 1974; Reichlin et al, 1972). Thyroid hormones have their major effect at pituitary level, by inhibiting the stimulatory action of T.R.F. (Bowers et al, 1967. 1968a, b), but, surprisingly, the effect on hypothalamic T.R.F. production seems to be, if anything, stimulatory (Reichlin et al, 1972).

Recently, a direct inhibitory effect of thyroid hormones on the thyroid has been reported (Gafni et al, 1976; Shishiba et al, 1976; Takasu et al, 1974), but its significance remains to be demonstrated. There is also some evidence for a thyroid antoregulatory mechanism relating thyroid activity to iodide availability (Ingbar, 1972; Sherwin and Tong, 1974, 1975; Van Sande et al, 1975).

The autonomic nervous system. There is now some evidence for a direct regulation of the thyroid by the sympathetic nervous system, at least in some species. Sympathetic nerve fibres have been demonstrated not only along thyroid blood vessels, but also closely apposed to thyroid follicles in a variety of species, including mice and man (Melander et al, 1973b, 1974, 1975b, 1976; Tice and Craveling, 1975), and electrical stimulation of the cervical sympathetic trunk has been shown to enhance thyroid secretion in thyroxine suppressed mice (Melander et al, 1972, 1974) and in man (Melander et al, 1976). This was a direct effect on the thyroid since colloid droplets appeared unilaterally following unilateral sympathetic stimulation (Melander et al, 1972). The ability of noradrenaline to induce thyroid endocytosis and protein bound iodine release in mice adds further support (Ericson et al, 1970; Melander, 1969, 1971a). Melander et al (1974) attributed the differences

from results obtained by previous workers (Harrison, 1964; Söderberg, 1958) to great interspecies and age variations in thyroid sympathetic innervation. The physiological significance of these observations is not known, but it has been suggested that a tonic sympathetic discharge contributes to the maintenance of the resting thyroid secretion of mice, since chemical or surgical sympathectomy resulted in a temperary diminution of radioiodine release (Melander et al, 1974).

Vagal stimulation may also give rise to a modest increase in P.B.^{131}I in thyroid venous blood from dogs or rabbits with prelabelled thyroids (Ishii et al, 1968; Söderberg, 1958). A mild stimulatory effect of the vagal transmitter, acetyl choline, is evident from the data of Brown and Munro (1967) on mouse thyroid radioiodine release in vitro, but this has not been confirmed in vivo (Melander, 1971a). The significance of these findings remains uncertain.

1.1.2. Pathological thyroid stimulators

L.A.T.S. and T.S.I. In recent years the cause of the hyperthyroidism in human Graves' disease has been hotly debated (Hoffenberg, 1974a, b; McKenzie, 1968, 1974; Munro, 1971). However, it is now almost universally agreed that thyroid stimulating immunoglobulins (T.S.I.) are produced by lymphoid tissue as part of an autoimmune disease process resulting in thyroid hyperplasia, hypertrophy and hypersecretion.

This stems from the original discovery by Adams and Purves (1956) that the serum from certain patients with Graves' disease contained a substance capable of stimulating thyroid iodine release in guinea-pigs but which differed from T.S.H.

in the time course of its action in vivo. This became known as the long-acting thyroid stimulator (L.A.T.S.). Its existence was confirmed by McKenzie (1958a, 1960, 1961) using a similar bioassay in mice (McKenzie, 1958b), which was subsequently adopted by a variety of laboratories (with minor modifications) as the standard method for assay (Adams, 1961; El Kabir et al, 1966; Kriss et al, 1964; Major and Munro, 1962; Pinchera et al, 1965; Snyder et al, 1964). The effect on the thyroid was direct since it was active both in hypophysectomised mice (Adams et al, 1-61; Munro, 1959) and in mouse thyroids in vitro (Brown and Munro, 1967). It was also shown to work in the rat (Purves and Adams, 1960) and rabbit (Adams, 1965). Apart from the longer time course of action in vivo, which was attributed to its longer plasma half life (Adams, 1960; McKenzie, 1961), its effect on a variety of parameters of thyroid function resembled those of T.S.H. (see section 1.2.2).

L.A.T.S. was later shown to be an immunoglobulin of the IgG class (Dorrington and Munro, 1965, 1966a; Kriss et al, 1964; McKenzie, 1967), and could be detected in the medium of phyto-haemagglutinin-cultured lymphocytes from patients with Graves' disease (McKenzie, 1967; Miyai et al, 1967). Furthermore, human thyroid subcellular fractions were capable of adsorbing L.A.T.S. (Beall and Solomon, 1966; Beall et al, 1969; Benhamou-Glynn et al, 1969; Berumen et al, 1967; Chopra et al, 1971; Dirmikis and Munro, 1973; Dorrington et al, 1966b; McKenzie, 1967; Ochi and De Groot, 1970; Schleusener et al, 1971; Smith, 1970, 1972), and it was proposed that the active component was not only the antigen for L.A.T.S. but also the receptor through which it exerted its action (Chopra et al, 1971).

The major objection to the theory that L.A.T.S. causes thyrotoxicosis was the failure to detect it in a large proportion of patients with thyrotoxic Graves' disease (McKenzie, 1968). Even when the IgG fraction was concentrated ten or fifteen fold, the yield of positive L.A.T.S. assays was only 62% or 80%, respectively (Carneiro et al, 1966a; Chopra et al, 1970b). Furthermore, detectable L.A.T.S. levels correlated poorly with indices of thyroid function (Carneiro et al, 1966a, b; Chopra et al, 1970a; Hoffmann and Hetzel, 1966; Silverstein and Burke, 1970) and L.A.T.S. had even been detected in euthyroid relatives of thyrotoxic patients (Wall et al, 1969) and in patients with asymptomatic atrophic thyroiditis (Bastenie et al, 1967). Therefore a causative role for L.A.T.S. in human thyrotoxicosis would seem doubtful (Chopra et al, 1970b; McKenzie, 1972).

More recently, the matter has been clarified by Adams and Kennedy (1967, 1971) who found that the inactivation of L.A.T.S. by human thyroid homogenates could be prevented by the addition of serum of IgG from L.A.T.S.-negative thyrotoxic patients. This was interpreted(1) as evidence for another thyroid stimulator, L.A.T.S.-protector (L.A.T.S.-P), which could compete for the L.A.T.S. receptor in human thyroid but which was unable to affect the thyroid of bioassay animals (Adams and Kennedy, 1971). The existence of a species-specific human thyroid stimulator in L.A.T.S.-negative thyrotoxic serum or IgG has been confirmed directly in vitro (Holmes et al, 1976; McKenzie and Zakarija, 1976; Onaya et al, 1973; Shishiba et al, 1973) and in vivo (Adams et al, 1974a). Thyroid stimulating immunoglobulins (T.S.I.)

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(1) Following a suggestion by Prof. D. Doniach

which are probably identical with L.A.T.S.-P, have also been detected using a sensitive radio-receptor assay. The results correlate well with assays of direct stimulatory effect (measured by cA.M.P. production) in human tissue in vitro (Hall et al, 1975).

L.A.T.S.-P or T.S.I. have been detected in the unconcentrated IgG fraction from 80-100% of patients with untreated Graves' disease (Adams et al, 1974b; Dimikis et al, 1976; Hall et al, 1975) and a significant correlation existed between L.A.T.S.-P levels and the severity of thyrotoxicosis as measured by thyroid ^{131}I uptake (Adams et al, 1974b). Clague et al (1976) have also reported a good correlation between thyroid autonomy and the presence of T.S.I. The lymphoid origin of T.S.I. has been confirmed by its detection in the medium of phytohaemogglutinin stimulated Graves' disease lymphocytes (Knox et al, 1975) and in the medium of lymphocytes from 16 out of 20 patients with Graves' disease when cultured with normal thyroid tissue (Knox et al, 1976). However, surprisingly, 4 out of 17 control cultures were also active in the latter experiments.

Therefore, the present view of the pathogenesis of Graves' disease is that a breakdown of immunological tolerance results in the formation of a variety of thyroid-stimulating antibodies, some of which are human specific (T.S.I. or L.A.T.S.-P) but some of which are more active in other species, such as the mouse (L.A.T.S.)(Hall et al, 1975). Simultaneously other immunoglobulins may be produced, such as antimicrosomal antibodies, which may result in some degree of thyroid

destruction (Doniach and Roitt, 1965), and exophthalmogenic IgG, which has been implicated in the pathogenesis of ophthalmic Graves' disease (Dandona and El Kabir, 1970; Singh and McKenzie, 1971a, b; St. Etienne et al, 1976; Winand and Kohn, 1973). It seems possible that other manifestations, such as pretibial myxoedema, may be mediated by yet other antibodies.

The role of cell mediated immunity in Graves' disease is not clear, although it has been postulated to be the major factor in the pathogenesis of ophthalmopathy (Mullin et al, 1977). On the other hand, the defects of cell mediated immunity which have been reported in Graves' disease may be evidence of a thymus dependent lymphocyte (T-cell) abnormality underlying a defect of immunological surveillance, which permits the emergence of autoantibody producing cells by somatic mutation (Volpé et al, 1974). However, since serum T_3 and T_4 levels following steroid therapy in Graves' disease fall too rapidly to be accounted for by changes in T.S.I. levels (Williams et al, 1975), other immunological factors, perhaps direct thyroid stimulation by activated T-lymphocytes or by a locally produced lymphocyte factor, may still be involved.

Ectopic sources of T.S.H.-like substances. There have been occasional reports of thyrotoxicosis associated with high circulating T.S.H. levels attributable to pituitary neoplasms (Faglia et al, 1972; Hamilton et al, 1970; Jailer and Holub, 1960; Lamberg et al, 1969; O'Donell et al, 1973). Further cases of hyperthyroidism have been associated with trophoblastic disease, such as hydatidiform mole (Dowling et al, 1960; Kock et al, 1966; Hershman and Higgins, 1971; Miyai et al, 1976),

choriocarcinoma (Miyai et al, 1976; Cohen and Utiger, 1970; Odell et al, 1963) and embryonal carcinoma of the testis (Steigbigel et al, 1964). Trophoblastic tissue seems to produce two thyroid stimulators: one is probably identical with human chorionic gonadotropin (h-C.G.)(Kenimer et al, 1975; Nisula and Kettslegers, 1974) and predominates in hydatidiform mole (Hershman et al, 1970), whereas the other, human chorionic thyrotropin (h-C.T.), is similar to pituitary h-T.S.H. in its molecular weight but differs in its reactions with antisera (Hennen et al, 1969; Hershman and Stones, 1969). During normal pregnancy, the levels of circulating h-C.T. and h-C.G. increase, especially during the first trimester, and would appear to "take over" thyroid control to some degree, allowing a fall in circulating pituitary T.S.H. (Braunstein and Hershman, 1976; Hennen et al, 1969) without causing hyperthyroidism(1). In trophoblastic disease, however, h-C.G. may reach such levels that hyperthyroidism results despite a potency of only 1/5000 of that of human pituitary T.S.H. (Kenimer et al, 1975).

1.1.3. Pharmacological thyroid stimulation

Catecholamines. Pharmacological activation of thyroid glands has been demonstrated in response to a variety of substances. The catecholamines, adrenaline, noradrenaline, isoprenaline and dopamine have been shown to enhance thyroid secretion in mice (Ericson et al, 1970; Melander, 1969, 1970, 1971a). Noradrenaline is probably the transmitter involved in

(1) Free thyroid hormone levels are within the normal range in pregnancy (Osathanondh et al, 1976)

sympathetic stimulation of the thyroid gland (Melander et al, 1972) but no physiological role has been demonstrated for other catecholamines.

Indolealkylamines and histamine. 5-H.T. enhances thyroid endocytosis and protein-bound iodide release in mice (Ericson et al, 1970; Melander, 1969, 1970, 1971a). Histamine has also been shown to induce thyroid activation in mouse thyroids in vitro (Onaya et al, 1977), although Melander (1970) could not achieve this in vivo. Both amines are present in the thyroid mast cells of rodents[1] (Melander et al, 1971) and their release may mediate the increase in blood flow in response to T.S.H. (Clayton and Masuoka, 1968; Clayton and Szego, 1967; Melander, 1975c). It has also been suggested that they contribute to the secretory response to T.S.H., at least in rats, since this is reduced following depletion of mast cell amines by compound 48/80 (Melander and Sundler, 1972).

Other substances. Increased thyroid secretion has been reported following antidiuretic hormone in rabbits (Garcia et al, 1964), glucagon and thyroglobulin in mice (Burke, 1970; Burke and Szabo, 1972). Cholera enterotoxin (Mashiter et al, 1973) and a factor identified in clostridium perfringens (Macchia et al, 1967) are active in vitro but the physiological or pathological significance of these findings is doubtful.

Cyclic A.M.P. or its derivative, dibutyryl cyclic A.M.P., and prostaglandins are also potent thyroid stimulators. However, these will be considered separately since there is evidence that they may be involved in the response of the thyroid to T.S.H.

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(1) Calf thyroid mast cells contain histamine and dopamine but not 5-H.T. (Melander et al, 1973)

1.2. THE EFFECTS OF THYROID STIMULATORS

In the above discussion thyroid stimulators were taken to be substances capable of enhancing thyroid secretion rate. However, they affect many other processes within the thyroid and some in other tissues. A knowledge of the effects of these substances is essential to the understanding of their modes of action. Therefore, these are briefly reviewed and compared below.

1.2.1. T.S.H.[1]

Thyroid structure and ultrastructure[2]. The first histological sign of stimulation following T.S.H. appears to be the rapid discharge of exocytotic vesicles (0.1-0.3μ diameter) at the apical cell surface (Ekholm et al, 1975). Immature (uniodinated) thyroglobulin, synthesised within the rough-surfaced endoplasmic reticulum and Golgi region, is thereby transferred to the colloid space (Bjorkman and Ekholm, 1973; Bjorkman et al, 1974, 1976; Ekholm et al, 1975, 1976; Haddad et al, 1971; Nadler et al, 1964; Whur et al, 1969).

Following this, and perhaps dependent on it (Ericson et al, 1976), pseudopodia appear at the apical cell border, reaching out to engulf colloid to form colloid droplets (endocytotic vesicles, 1-2μ diameter)(Ketelbant-Balasse et al, 1976; Wetzel et al, 1965; Wollman et al, 1964). This occurs within 5 min. in rats (Wollman et al, 1964) and mice (Shishiba et al, 1967) but between 10 and 30 min. in the dog (Nève and Dumont, 1970a). Colloid droplets contain iodinated thyroglobulin (Bauer and Meyer, 1964, 1965; Nadler et al, 1962; Sheldon et al, 1964; Stein and Gross, 1964; Wollman and Spicer, 1963) which is subsequently digested following fusion with primary

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(1) Comprehensive reviews - Dumont 1971, Tong 1974
(2) Review - Nadler 1974

lysosomes (Wetzel et al, 1965; Wollman et al, 1964; Wollman, 1969)[1] to release thyroid hormones. The resultant secondary lysosomes move towards the base of the cell, becoming smaller and denser, and have been postulated to revert to the state of primary lysosomes (Wetzel et al, 1965; Wollman, 1969). The life span of a colloid droplet has been calculated to be in the region of 3-26 min. in rats (Wollman and Lowenstein, 1973).

In mice the increase in intracellular colloid droplets after an injection of 0.5mU T.S.H. reaches a peak at 30 min. and continues for over 2 hr. (Shishiba et al, 1967), but later with doses greater than 10mU (Gafni and Gross, 1975). A similarly delayed peak with large doses has also been noted in rats (Wollman et al, 1964). The size of the peak is also dose related and a T.S.H. bioassay has been developed on this basis using guinea-pigs (De Robertis, 1948). T.S.H. also stimulates colloid droplet formation in vitro (Junquiera, 1947; Nève and Dumont, 1970b; Shishiba et al, 1970) and, interestingly, may enhance phagocytosis of latex beads by isolated thyroid cells (Sato et al, 1972).

After several hours of stimulation colloid volume decreases, thyroid cell height increases, and nuclear volume increases. The rough surfaced endoplasmic reticulum becomes dilated, the Golgi apparatus prominent, and free ribosomes and small (exocytotic) vesicles become numerous (Gedda, 1960; Nève and Dumont, 1970a; Wetzel et al, 1965; Wissig, 1964). Chronic thyrotropin administration in vivo induces cell division both in the follicular cells (Gedda, 1960) and in the

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(1) The concept of colloid droplets with lysosomes has recently been challenged since few combining particles can be seen in the E.M. (Nalder, 1974)

stromal elements of the thyroid (Wollman et al, 1968).

T.S.H. may also influence the development and structural integrity of its target gland: Isolated thyroid cells lose their polarity and grow in a monolayer, but if T.S.H. is included in the medium within 2-3 days of preparation they reaggregate into follicles (Fayet et al, 1971; Lissitzky et al, 1971; Winand and Kohn, 1975b). A glycoprotein aggregation factor, synthesised and released into the medium under the influence of T.S.H., may be involved (Giraud et al, 1974).

Thyroid blood flow increases rapidly following thyrotropin administration in cats, rats and rabbits (Clayton and Szego, 1967; Melander et al, 1975c; Söderberg, 1958) and this may be mediated by mast cell amine release (see section 1.1.3).

Thyroid secretion(1). The secretory products appearing in thyroid venous blood after T.S.H. are mainly T_4 and iodide with smaller quantities of T_3 in the rat (Inoue et al, 1967; Taurog et al, 1964) although traces of iodotyrosines can be detected after prolonged stimulation in the dog (Masuoka et al, 1964). The iodide is derived from deiodinated iodotyrosines (Green, 1968; Greer and Grimm, 1968). Some intact thyroglobulin is secreted into thyroid lymph and this is increased by T.S.H. (Daniel et al, 1967a, b, c), but the mechanism of this is not understood (Wollman, 1969).

Thyroid secretion in vivo is conveniently studied by observing the change in circulating radioiodine levels in mice whose thyroid iodine stores have been prelabelled with radioiodide (McKenzie, 1958b). An intravenous injection of T.S.H.

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(1) Review - Greer and Haibach (1974) and Wollman (1969)

produces an increase in blood radioactivity which starts within 5 min. and is maximal at 2-3 hr. with small doses (Shishiba et al, 1967) but somewhat later with doses in excess of 10mU (Gafni and Gross, 1975; Kriss et al, 1964; McKenzie and Williamson, 1966). The size of response is also dose-dependent, allowing the system to be used as a bioassay, which has been popular with investigators (section 2.1). The radioactivity in the peripheral blood is mainly thyroxine and iodide, with smaller quantities of triiodothyronine. T.S.H. increases all fractions approximately in proportion (Pinchera et al, 1965).

In vitro radioiodine release has been shown to be increased by T.S.H. in a variety of systems, including hyperplastic guinea-pig thyroid (Bottari et al, 1963; Kirkham, 1962), intact mouse thyroid (Brown and Munro, 1967; Williams and Wolff, 1971), intact rat thyroid lobes (Tonoue et al, 1970) and dog thyroid slices (Williams et al, 1970). In all systems the resting thyroid secretion is predominantly iodoprotein (e.g. 60-70%, Williams and Wolff, 1971) and iodide (30-40%) but iodothyronine can be detected after T.S.H. (up to 12%, Williams and Wolff, 1971)[1].

Iodide transport[2]. The ability of the thyroid to concentrate iodide independently of organification has usually been studied by measuring the partition of radioiodide between the thyroid and serum or medium in the presence of an inhibitor of organification (e.g. methimazole, popylthiouracil-P.T.U.) in vivo or in vitro (Franklin et al, 1944; Schaschner et al, 1944; Tong, 1964a; Vanderlaan and Vanderlaan 1947). The

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(1) In contrast to the in vivo situation T.S.H. fails to increase iodoprotein release in vitro (Williams and Wolff, 1971)

(2) Review - Bastomsky 1974

thyroid/serum (T/S) iodide ratios in vivo range from 25 in normal rats to 127 in goitrous rats (treated with P.T.U. for 20 days) or 5 in the atrophic glands of hypophysectomised animals (Halmi, 1954).

The effect of T.S.H. on the T/S ratio in rats (in vivo) is biphasic, with an initial decrease the T/S ratio lasting several hours, followed by a delayed increase (Halmi et al, 1960). A similar pattern has also been observed in isolated bovine thyroid cells (Knopp et al, 1970, Scranton and Tong, 1967; Wilson et al, 1968), but only a decrease in T/M (thyroid/medium) ratio is seen in canine and mouse thyroids in vitro (Ahn and Rosenberg, 1970; Williams and Malayan, 1975).

The decrease in T/S ratio is due to an increased permeability of the cell membrane to iodide (Halmi et al, 1960; Scranton and Tong, 1967; Williams and Malayan, 1975) whereas the delayed increase probably reflects enhanced activity of the iodide pump (Scranton and Tong, 1967; Wollman and Read, 1959). T.S.H. appears to increase the V_{max} (i.e. the number of pumping sites) of the iodide pump via new R.N.A. and protein synthesis (Knopp et al, 1970).

T.S.H. also enhances the activity of a thyroid Na^+/K^+-dependent, ouabain-sensitive A.T.P.-ase, which may be indirectly linked to the iodide transport mechanism (Brunberg and Halmi, 1966; Wolff, 1960; Wolff and Halmi, 1963).

Thyroglobulin synthesis[1]. T.S.H. has been shown to increase incorporation of labelled amino acids into thyroglobulin in the rat at intervals of 4-24 hr. after administration (Cavalieri and Searle, 1967; Pavlovic-Hournac et al, 1967; Pavlovic-Hournac and Delbauffe, 1973) and in isolated bovine thyroid cells within 2 hr. (Stolc and Tong, 1966). The

(1) Review - Ui 1974

mechanism is discussed below under "protein synthesis".

Iodide organification[1] is enhanced by T.S.H. within minutes of administration to rats (Ekholm et al, 1976) or dogs (Nagataki et al, 1961; Rosenberg et al, 1965). This has also been seen in thyroid slices in vitro (Ahn and Rosenberg, 1970; Kerkof and Tata, 1969; Kondo, 1961a, b; Rodesch et al, 1969). Isolated cells also respond (Tong, 1964c) but lose this ability over the days following preparation unless cultured with T.S.H. (Lissitzky et al, 1971; Winand and Kohn, 1975b). Ahn and Rosenberg (1970) suggested that the rapid effect on iodination was due to increased hydrogen peroxide availability since hydrogen peroxide also enhanced organification except when maximally stimulated by T.S.H. However, Ekholm et al (1976) have postulated an increased availability of peroxidase at the apical cell membrane since (a) peroxidase was found in exocytotic vesicles by E.M. histochemistry, (b) apical vesicles discharged rapidly after T.S.H. administration in the rat, thus transferring peroxidase to the apical cell membrane, (c) vinblastine prevents both exocytosis and increased iodine organification in response to thyrotropin.

Iodothyronine synthesis is increased within 30 min. of T.S.H. injection in rats (Shimoda et al, 1966). Increased coupling may occur in previously labelled thyroglobulin (Greer et al, 1974).

Respiration. T.S.H. rapidly increases thyroid slice oxygen consumption in vitro, even in substrate-free media (Freinkel, 1957). This is probably a result of increased mitochondrial respiration (Dumont, 1971; Lamy et al, 1967).

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(1) Review - Taurog 1974

Carbohydrate metabolism. Glucose uptake is increased by thyrotropin (Field et al, 1960; Gilman and Rall, 1968b; Scott et al, 1966b) and this may be due partly to an increased transport into thyroid cells (Tarui et al, 1963). Glucose oxidation in thyroid slices and isolated cells occurs mainly via glycolysis (as measured by glucose-6-^{14}C oxidation) although the rapid increase (within 10 min.) after high concentrations of T.S.H. occurs mainly via the pentasephosphate pathway(1) (as measured by glucose-1-^{14}C oxidation) (Dumont and Tondeut-Montenez, 1965; Field et al, 1966; Gilman and Rall, 1968b; Merlevede et al, 1963; Tong, 1964b). This may be secondary to an increased $NADP^+$ availability (Dumont, 1971; Tong, 1974). T.S.H. also enhances pyruvate oxidation via the Krebs cycle (Gilman and Rall, 1968b) and depresses incorporation of G-^{14}C into glycogen, fatty acids and protein (Dumont and Tondeur-Montenez, 1965).

Phospholipid synthesis. Thyrotropin rapidly enhances the incorporation of phosphate-^{32}P into thyroid phospholipids in vivo (Freinkel, 1957; Kerkof and Tata, 1967; Ochi and De Groot, 1968), and in vitro (Altman et al, 1968; Burke, 1968b; Freinkel, 1957; Florsheim et al, 1957; Jacquemin and Haye, 1970; Kerkof and Tata, 1969; Pastan and Macchia, 1967; Scott et al, 1966a, b). This is probably due to an increased phospholipid synthesis per se rather than an increased ^{32}P uptake since T.C.A. soluble ^{32}P is unchanged (Florsheim et al, 1957; Kerkof and Tata, 1969) and since the incorporation of ^{14}C labelled precursors into phospholipids is also enhanced (Scott et al, 1968).

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(1) In bovine and human thyroid slices low concentrations of T.S.H. (<1mU/ml) may decrease G-1-^{14}C oxidation (Gilman and Rall, 1968b; Merlevede et al, 1963; Dumont, 1971)

Protein synthesis. Uptake of the nonmetabolisable amino acid, α amino-isobutyrate (A.I.B.), is increased by T.S.H. by thyroid slices in vitro (Debons and Pittman, 1962, 1966; Segal et al, 1966) and isolated thyroid cells (Tong, 1964b), and this has been taken to suggest that amino acid transport in general is increased. However, Segal et al (1966) could not demonstrate this for a variety of other amino acids.

T.S.H. enhances the incorporation of labelled amino acids into protein in rats and guinea-pigs in vivo (Pavlovic-Hournac et al, 1967; Pavlovic-Hournac and Delbauffe, 1973; Raghupathy et al, 1963; Wagar et al, 1963), and in thyroid slices (Lecocq and Dumont, 1972; Wagar et al, 1973). The effect in isolated thyroid cells is rapid (within 1 hr.) and is not modified by actinomycin D (Tong, 1967).

In rats, T.S.H. increases labelled amino acid incorporation into thyroglobulin and total protein equally but hypophysectomy results in a selective decrease in incorporation into thyroglobulin (Pavlovic-Hournac and Delbauffe, 1973). Tong (1974) proposed a dual control of protein synthesis by thyrotropin: a rapid generalised stimulatory effect on protein synthesis at translational level and a delayed thyroglobulin-selective effect involving new R.N.A. synthesis. Some of this proposal has recently been confirmed by Sherwin and Tong (1976). They found a rapid (<15 min.) increase in ^{3}H-leucine incorporation into protein in isolated bovine thyroid cells, which was dependent on the continual presence of T.S.H. and was unaffected by actinomycin D (translational regulation), and a more delayed increase which persisted after T.S.H. removal and could be prevented by actinomycin D (transcriptional regulation).

T.S.H. stimulation of protein synthesis at translational level is also demonstrated by the increased proportion of polysomes in dog thyroids after T.S.H. (Lecocq and Dumont, 1973) and the increased ability of ribosomes from T.S.H. stimulated beef thyroids to incorporate phenylalanine-^{14}C into protein in the presence of polyuridine m-R.N.A. (de Nayer, 1976).

Porcine, pyrimidine and nucleic acid synthesis. Increased incorporation of labelled precursors into R.N.A. has been observed after T.S.H. in vivo (Kerkof and Tata, 1967; Ochi and De Groot, 1968) and in vitro (Adiga et al, 1971; Hall and Tubmen, 1965; Kerkof and Tata, 1969; Lindsay et al, 1969; Wilson and Wright, 1970).

Hall and Tubmen (1965) and Lindsay et al (1969) presented evidence that the rates of purine and pirimidine nucleotide biosynthesis, respectively, were limited by the availability of ribose 5-phosphate provided by the pentose phosphate pathway. Both groups concluded that the increased incorporation of labelled nucleotide precursors into the R.N.A. of bovine thyroid slices within 1-2 hr. of T.S.H. administration was secondary to activation of the pentose phosphate pathway.

R.N.A. synthesis may also be stimulated at a later step since ^{32}P incorporation in porcine thyroid slices (Kerkof and Tata, 1969) and adenine-^{14}C or iodine-^{14}C incorporation in isolated bovine thyroid cells (Wilson and Wright, 1970) are enhanced by T.S.H. in the absence of an increase in perchloric acid-soluble radioactivity. Moreover, Adiga et al (1971) reported an enhanced R.N.A. polymerase activity in isolated nuclei from porcine thyroid slices incubated with T.S.H.

After chronic stimulation by T.S.H. (14 days) in guinea-pigs, the increase in absolute levels of R.N.A. is out of proportion to the hyperplasia (Matavinovic and Vickery, 1959).

Thyroid membrane potential. T.S.H. has been shown to cause a rapid hyperpolarisation of mouse thyroid cells in small doses (Batt and McKenzie, 1976) and a rapid depolarisation of mouse or rat thyroid cells in larger doses (Konno and McKenzie, 1970; Williams, 1970). The latter was accompanied by an increased membrane conductance and probably reflected a non-specific increase in thyroid membrane permeability. Indeed, increased influx of $^{24}Na^{+}$ (Solomon, 1961) or $^{45}Ca^{2+}$ (Hachiya et al, 1976) and efflux of $^{131}I^{-}$ ions (Halmi et al, 1960; Scranton and Tong, 1967; Williams and Malayan, 1975) have been demonstrated after T.S.H.

In contrast to some other glands, such as the adrenal medulla or posterior pituitary (Douglas, 1968), the depolarisation is not necessary for activation since high $\{K^{+}\}$ media do not evoke secretion (Williams, 1972a) or phospholipogenesis (Oka and Field, 1966). Moreover, Ca^{2+} influx is not obligatory since T.S.H. induced secretion, endocytosis or phospholipogenesis, is not reduced in Ca^{2+}-free media[(1)] (Dekker and Field, 1970; Oka and Field, 1966; Willems et al, 1971; Williams, 1972b).

The mode of action of T.S.H. There is now convincing evidence that most of the effects of T.S.H. are mediated by cyclic 3', 5'-adenosine monophosphate (cA.M.P.), which probably

(1) However, high $\{K^{+}\}$ media may evoke G-1-^{14}C oxidation and Ca^{2+}-free media inhibit T.S.H. stimulated G-1-^{14}C oxidation (Dekker and Field, 1970) and iodide organification (Willems et al, 1971)

acts as the intracellular "second messenger" (Dumont, 1971; Schell-Frederick and Dumont, 1970; Tong, 1974). A T.S.H.-sensitive adenyl cyclase is located in thyroid plasma membranes (Wolff and Jones, 1971; Yamashita and Field, 1970) and T.S.H. increases cA.M.P. levels in a variety of thyroid preparations (Gilman and Rall, 1968a; Kaneko et al, 1970; Knopp et al, 1970; Onaya et al, 1973; Tonoue et al, 1970). The rise in cA.M.P. must be secondary to the increase in adenyl cyclase activity since phosphodiesterase activity is unchanged by T.S.H. (Pastan and Katzen, 1967).

cA.M.P. or its less polar dibutyryl derivative (D.B.cA.M.P.) can reproduce most of the actions of T.S.H. on the thyroid, including endocytosis (Nève and Dumont, 1970b; Pastan and Wollman, 1967; Rodesch et al, 1969; Willems et al, 1970), cellular aggregation (Fayet et al, 1970; Giraud et al, 1974; Lissitzky et al, 1971; Winand and Kohn, 1975b), secretion (Bastomsky and McKenzie, 1967; Ensor and Munro, 1969; Kendall-Taylor and Munro, 1970a; Tonoue et al, 1970; Willems et al, 1970), iodide trapping (Wilson et al, 1968), iodide organification (Kerkof and Tata, 1969; Rodesch et al, 1969: Wilson et al, 1968), sugar uptake (Tarui et al, 1963), glucose-1-^{14}C oxidation[1] (Gilman and Rall, 1968b; Pastan and Macchia, 1967; Rodesch et al, 1969), α amino-isobutyrate uptake (Wilson et al, 1968), protein synthesis (De Nayer, 1976; Lecocq and Dumont, 1972; Wilson et al, 1968), ribosomal pattern (Lecocq and Dumont, 1973), R.N.A. synthesis (Adiya et al, 1971; Kerkof and Tata, 1969; Wilson and Wright, 1970) and membrane hyperpolarisation (but not depolarisation)(Batt and McKenzie, 1976). However, the effects

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(1) There are some discrepancies between some of the reports for G-1-^{14}C oxidation (see Dumont, 1971; Tong, 1974). Moreover in bovine tissue D.B.cA.M.P. mimics only the inhibition of G-1-^{14}C oxidation (Gilman and Rall, 1968c)

on phospholipid synthesis are small and inconsistent (Jacquemin and Haye, 1970; Kerkof and Tata, 1969; Pastan and Macchia, 1967). Thus, Dumont (1971), Tong (1976) and Field (1975) concluded that cA.M.P. mediated most intracellular actions of T.S.H. with the probable exception of the enhancement of phospholipid synthesis and possibly the stimulation of G-1- ^{14}C oxidation at high levels of T.S.H. Stimulation by the methylxanthines(1) phosphodiesterase inhibitors (Ensor and Munro, 1969; Kendall-Taylor, 1970; Willems et al, 1970) and the potentiation of the response to submaximal T.S.H. concentrations by methylxanthines (Bastomsky and McKenzie, 1967; Ensor and Munro, 1969; Rodesch et al, 1969) support this concept.

cA.M.P. dependent protein kinases have been identified within the thyroid and their activity was increased by T.S.H. (Field, 1975; Kobayashi and Harada, 1976; Spaulding and Burrow, 1972, 1974; Yamashita and Field, 1972b). Whereas T.S.H. fails to enhance total protein phophorylation, increased ^{32}P incorporation into certain fractions has been reported (Lamy et al, 1976; Tirard and Rocques, 1976). By analogy with other more thoroughly investigated systems, this probably represents the next step in the stimulatory chain (Field, 1975).

Cyclic 3', 5'-guanosine monophosphate (cG.M.P.) is also present in the thyroid (Van Sande et al, 1976; Yamashita and Field, 1972b). cG.M.P. or D.B.cG.M.P. can enhance thyroid protein synthesis but not other aspects of thyroid metabolism (Macchia and Varrone, 1971; Pisarev et al, 1971). However, since guanyl cyclase activity and cG.M.P. levels are not increased by T.S.H. (Field, 1975; Van Sande et al, 1976;

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(1) e.g. caffeine, theophylline

Yamashita and Field, 1972c) cG.M.P. is unlikely to be involved in the thyrotropic response.

The role of prostaglandins. T.S.H. and D.B.cA.M.P. have been reported to increase prostaglandin (P.G.) synthesis within the thyroid (Burke et al, 1973; Haye et al, 1973). P.Gs. can imitate a variety of the actions of thyrotropin, including endocytosis and secretion (Burke, 1970; Dekker and Field, 1970; Rodesch et al, 1969) and act on thyroid adenyl cyclase via a receptor which is different from that for T.S.H. (Takasu et al, 1976; Wolff and Jones, 1971; Wolff and Cooke, 1973). T.S.H. activation of adenyl cyclase or G-1-^{14}C oxidation is not dependent on P.G. synthesis since it occurs when P.G. synthesis is blocked by indomethacin (Boynaems et al, 1976; Haye et al, 1973; Mashiter et al, 1974; Wolff and Moore, 1973). Burke (1974), Burke et al (1973) and Haye et al (1973) have suggested that P.Gs. may be involved in a feedback mechanism regulating adenyl cyclase responsiveness to T.S.H. on the basis of experiments with P.G. inhibitors, but this is highly speculative. Their true role is still elusive.

Receptors. T.S.H. has recently been shown to bind to a variety of thyroid preparations, including cell membranes. This activity is closely related to adenyl cyclase and probably represents the initial reaction with the thyroid (Field, 1975). This aspect forms part of the experimental work of this thesis and is presented in greater depth in part 5.

Extrathyroid effects of T.S.H. In common with a variety of other hormones T.S.H. increases lipolysis in adipocytes (Freinkel, 1961; Hart and McKenzie, 1971; Rodbell, 1964;

Rudman et al, 1963; White and Engel, 1958). It is also capable of inducing weight and histological changes, increased ^{35}S uptake and incorporation into sulphated glycosaminoglycans, enhanced hyaluronic acid synthesis and adenyl cyclase activity in mouse and guinea-pig harderian glands (Lamberg et al, 1956; Pochin, 1952; Singh and McKenzie, 1969, 1970; Sisson and Miles, 1967; Winand and Kohn, 1975a). Interestingly, Singh and McKenzie (1969, 1970) also noted increased uptake of ^{35}S by pancreas and salivary gland. Recent evidence suggests that the T.S.H. receptors for thyroid and harderian gland have different molecular weights (St. Etienne et al, 1976).

1.2.2. L.A.T.S. and T.S.I.

Thyroid structure and ultrastructure. L.A.T.S. containing sera or IgG enhance thyroid endocytosis in mice in vivo (Shishiba et al, 1967), in vitro (Shishiba et al, 1972) and in canine thyroid slices (Shishiba et al, 1970). The latency of the colloid droplet response to L.A.T.S. in mice is longer than for T.S.H. in vivo (7 min. cf. 4 min.) (Shishiba et al, 1967) but since there was no difference with "equivalent" doses in canine thyroid slices, Shishiba et al (1970) concluded that this was due to slower penetration of the thyroid capillary endotheline by L.A.T.S. in vivo. The in vivo response to L.A.T.S. is, as expected, prolonged (Shishiba et al, 1967). At E.M. level the histological changes after L.A.T.S. resemble those after T.S.H. with no evidence of cell damage (McKenzie, 1967). After 72 hours L.A.T.S. exposure in vitro mitosis is increased in rat

thyroids (Garry and Hall, 1970) and after repeated injections in vivo, mouse thyroids show hypertrophy and hyperplasia with increased cell height and decreased colloid space (McKenzie, 1960; Ochi and De Groot, 1969b; Sharard, 1968). As after T.S.H., thyroid mast cells are diminished in number (Melander and Sundler, 1973). L.A.T.S. can also mimic T.S.H.-induced aggregation in isolated porcine thyroid cells (Fayet et al, 1973).

Thyroid secretion. L.A.T.S. increases thyroid radioiodine release in guinea-pigs (Adams and Purves, 1956), rats (Purves and Adams, 1960), rabbits (Adams, 1965) and mice (Adams et al, 1961, Munro, 1959; McKenzie, 1958a) in vivo and by mouse thyroids in vitro (Brown and Munro, 1967; Ensor et al, 1971). The in vivo response has a longer latency than for T.S.H. (Shishiba et al, 1967) and has a longer time course, peaking at between 7 and 24 hr. (McKenzie, 1974) but the in vitro responses are similar (Brown and Munro, 1967; Ensor et al, 1971). The chromatographic pattern of radioiodinated compounds in the serum following administration of L.A.T.S. to mice with prelabelled thyroids resembles that after T.S.H., with labelled T_4, T_3 and iodide increasing in proportion (Pinchera et al, 1965).

Iodine metabolism. L.A.T.S., like T.S.H., increases the C/M iodide ratio in isolated bovine thyroid cells after a 4½ hr. incubation (Edmunds et al, 1970) but the temporal characteristics of this were not studied. Total thyroid radioiodine accumulation and organification are increased by L.A.T.S. (Bastomsky and McKenzie, 1968; McKenzie, 1960).

Intermediary metabolism. Like T.S.H., L.A.T.S. enhances

a variety of other thyroid metabolic functions but these effects have not been so comprehensively studied. Oxidation of G-1-^{14}C via the pentose phosphate pathway and ^{32}P incorporation into phospholipids are enhanced in vitro (Burke, 1968a, b, c; Field et al, 1968; Ochi and De Groot, 1968; Scott et al, 1966b; Shishiba et al, 1970). However, Scott et al (1966b) noted that ^{32}P incorporation into phosphatidyl inositol was specifically enhanced by T.S.H. whereas a more generalised stimulation of phospholipogenesis was seen with L.A.T.S. However, this does not indicate separate sites of action since only a single (not necessarily equivalent) dose of each stimulator was used and Jaquemin and Haye (1970) have shown that T.S.H. also produces a generalised enhancement of phospholipogenesis at high doses. Protein and R.N.A. synthesis are also increased by L.A.T.S. in vivo or in vitro (Adiga et al, 1971; El-Khatib et al, 1973; Ochi and De Groot, 1968). Interestingly, El-Khatib et al (1973) found that L.A.T.S. was active in isolated thyroid cells (bovine or porcine) which were unresponsive to high concentrations of T.S.H. Since trypsin can split thyroid receptors from the cell membrane (Tate et al, 1976; Winand and Kohn, 1975b) there may have been a loss or modification of T.S.H. receptors during preparation, suggesting different receptors for T.S.H. and L.A.T.S. However, alternative explanations would be that L.A.T.S. can exert its effects at lower occupancy or can combine with trypsin inactivated receptors.

Cell membrane effects. Like T.S.H., L.A.T.S. causes a cellular depolarisation in mouse thyroids in vitro (Konno and

McKenzie, 1970) but the hyperpolarisation with low concentrations of T.S.H. or cA.M.P. (Batt and McKenzie, 1976) has not been reported. $^{45}Ca^{2+}$ uptake by mouse thyroids is increased by L.A.T.S. and T.S.H. (Hachiya et al, 1976). Since neither depolarisation (Batt and McKenzie, 1976) nor $^{45}Ca^{2+}$ uptake (Hachiya et al,1976) were reproduced by cA.M.P. or D.B.cA.M.P., L.A.T.S. and T.S.H. may have a common site of action on the membrane independent of cA.M.P.

Adenyl cyclase activity. L.A.T.S. enhances adenyl cyclase activity in isolated bovine thyroid plasma membranes (Yamashita and Field, 1972a), human (Kendall-Taylor, 1973) and canine thyroid slices (Kaneko et al, 1970), mouse thyroids in vitro (Kendall-Taylor, 1972) and bovine, canine (Levey and Pastan, 1970) and ovine thyroid homogenates (Burke, 1968). This results in an increase in absolute cA.M.P. levels, as seen in dog (Kaneko et al, 1970) and human thyroid slices (Onaya et al, 1973). Phosphodiesterase activity is unchanged (Levey and Pastan, 1970).

Thus L.A.T.S., like T.S.H., may act via cA.M.P. The enhancement of L.A.T.S.-induced secretion and iodide accumulation in mouse thyroids in vivo by theophylline supports this (Bastomsky and McKenzie, 1968).

The initial reaction of L.A.T.S. with the thyroid. The great similarity of T.S.H. and L.A.T.S. action has led to the hypothesis that both act on the same receptor (Burke, 1971). Attempts have been made to separate their actions using various inhibitory substances such as actinomycin D (McKenzie, 1967), iodide (Ochi and De Groot, 1969a),chlorpromazine, propranolol,

phentolamine (Burke, 1969b; Kendall-Taylor and Munro, 1970b; Onaya and Solomon, 1969), prostaglandin inhibitors (Burke, 1974; Melander et al, 1973a), and phosphorylase C (Burke, 1969a) have failed to show any differences. However, some differences in susceptibility of L.A.T.S.- and T.S.H.-induced G-1-^{14}C oxidation and phospholipogenesis to the inhibitory effects of iodide or the enhancing effect of iodotyronines have been noted (Burke, 1968a, b), but their significance is uncertain since very few stimulator dose levels were used. On the other hand, El Kabir et al (1964) briefly reported that a preparation of carbamylated T.S.H., which was itself inactive, but which could prevent the effect of up to 4 times the quantity of untreated T.S.H. in vivo or in vitro, prevented the response to a L.A.T.S. serum in the McKenzie assay. This certainly suggests a common or, at least, closely related receptor site.

Human, bovine and mouse thyroid homogenates are capable of taking up L.A.T.S. activity (Adams and Kennedy, 1971; Ochi and De Groot, 1970). A large variety of human thyroid functions take up L.A.T.S. but the absorbing activity paralleled 5' nucleotidase in the various fractions suggesting that it was originally present in the cell membrane (Beall et al, 1969). It has been suggested that this represents the L.A.T.S. receptor (Chopra et al, 1971). If this is so, then the inhibition of L.A.T.S. uptake by human thyroid microsomes after pretreatment with T.S.H. (Burke, 1967; Ochi and De Groot, 1970) is evidence that it may also be the T.S.H. receptor. More precise experiments showing the reverse, inhibition of T.S.H. binding to cell fractions, are described in part 5 of this thesis and

have also been reported by others (Lissitzky et al, 1973; Manley et al, 1974b; Mehdi et al, 1973; Mehdi and Nussey, 1975). These provide further evidence that T.S.H. and L.A.T.S. receptors may be identical.

Extrathyroid effects of L.A.T.S. Like T.S.H., L.A.T.S. can stimulate lipolysis in guinea-pig epidydymal fat pad (Hart and McKenzie, 1971) but, surprisingly, it fails to affect the guinea-pig or mouse harderian gland (Dandona and El Kabir, 1970; Singh and McKenzie, 1969, 1971b; Winand and Kohn, 1973). Despite the ineffectiveness in the latter system L.A.T.S.-containing IgGs have displayed a remarkable series of stimulatory actions in other systems. The mouse adrenal cortex may be activated (Dandona et al, 1971; El Kabir and Hockaday, 1969; El Kabir et al, 1971), pituitary L.H. may be released in the rat (Dandona et al, 1975) and even R.N.A. metabolism and membrane permeability in Escherichia coli may be affected (Mehdi et al, 1971). This may be taken as evidence that L.A.T.S. has more widespread effects, perhaps at cell membrane, but since the activities on the adrenal, pituitary and thyroid did not correlate (Dandona et al, 1975) these actions may be due to one or more coexisting species of immunoglobulin.

Species specificity and the effects of T.S.I. L.A.T.S.-containing IgGs seem to be active in a variety of mammals (McKenzie, 1974) but not in chicks (Lepp and Oliner, 1967; Ochi and De Groot, 1970; Shishiba et al, 1972). However, the specificity of different L.A.T.S. samples may vary since occasional L.A.T.S.-containing IgGs have been reported to be inactive in bovine thyroid tissue (Amir et al, 1973; Wolff and Jones, 1971) and since the slopes of the dose response

lines for different samples in the McKenzie assay may differ (McKenzie, 1974). Thus L.A.T.S. is probably not a single molecular species but a group of heterogenous immunoglobulins with a common action on the mouse thyroid.

Human-specific, non-L.A.T.S. IgG moieties (L.A.T.S.-P) probably represent a further part of the thyroid stimulatory antibody spectrum. These can induce endocytosis or cA.M.P. accumulation by human thyroid slices in vitro (Holmes et al, 1976; McKenzie and Zakarija, 1976; Onaya et al, 1973; Shishiba et al, 1973) and human thyroid radioiodine release in vivo (Adams et al, 1974a), but actions on other aspects of human thyroid metabolism have not been studied.

1.2.3. Biogenic amines

Thyroid structure. Adrenaline, noradrenaline (N.A.), isopropylnoradrenaline (I.P.N.A.), dopamine (D.A.) and 5-hydroxytryphamine (5-H.T.), are all able to induce mouse thyroid endocytosis in vivo (Ericson et al, 1970; Melander, 1971a). N.A. also increases endocytosis in human thyroid slices in vitro (Melander et al, 1976). However, Pastan and Wollman (1967) reported that adrenaline was inactive in dog thyroid slices. Recently, histamine has also been shown to induce endocytosis in mouse thyroids in vitro (Onaya et al, 1977).

Thyroid secretion is enhanced by catecholamines in the dog in vivo (Ackerman and Arons, 1958, 1962), by catecholamines or 5-H.T. in the mouse in vivo (Ericson et al, 1970; Melander, 1969, 1970, 1971a) and human thyroid slices in vitro (Melander et al, 1976). In one report, adrenaline and 5-H.T. increased

radioiodine release from mouse thyroids in vitro although the authors failed to comment on this (Brown and Munro, 1967; table 3). Histamine is also active in this system (Onaya et al, 1977).

Iodide transport and organification. Adrenaline, N.A., I.P.N.A. and 5-H.T. have been shown to increase iodide accumulation and organification by isolated bovine thyroid cells (Maayan and Ingbar, 1968, 1970; Maayan et al, 1971, 1973; Melander et al, 1973b) but the time course of these effects have not been described.

Intermediary metabolism, protein and R.N.A. synthesis. Adrenaline and 5-H.T. increase glucose-1-^{14}C oxidation by thyroid slices and isolated cells (Altman et al, 1966; Pastan et al, 1962; Hupka and Dumont, 1963; Maayan and Ingbar, 1970; Maayan et al, 1973) but the results in thyroid slices suggested that this was not a specific receptor-mediated effect. The incorporation of labelled precursors into protein and, in the case of catecholamines, into R.N.A., is also increased by catecholamines and 5-H.T. in isolated cells (Maayan and Ingbar, 1970; Maayan et al, 1971, 1973). In contrast, ^{32}P incorporation into phospholipids is unchanged or depressed by amines (Altman et al, 1966; Maayan and Ingbar, 1970; Maayan et al, 1973).

Adenyl cyclase activity or cA.M.P. is enhanced by catecholamines in bovine thyroid clices (Gilman and Rall, 1968a), human thyroid slices (Sato et al, 1976), isolated bovine thyroid cells (Maayan and Ingbar, 1970) and bovine thyroid cell membranes (Marshall et al, 1975b). 5-H.T. is also effective in isolated thyroid cells (Maayan et al, 1971). Protein

kinase activation has been reported to be enhanced by I.P.N.A. (Spaulding, 1976).

Amine receptors. The effect of catecholamines on adenyl cyclase or cA.M.P. levels is inhibited by β-receptor blockers (Gilman and Rall, 1968a; Marshall et al, 1975b; Sato et al, 1976). Therefore the mediation of β-agonist stimulation by cA.M.P. seems likely. In contrast, other effects of catecholamines in vitro are inhibited by α-receptor blockers(1) (Maayan and Ingbar, 1968; Maayan et al, 1970, 1971, 1973; Melander et al, 1973b). Since theophylline does not potentiate α-receptor mediated effects these may be independent of cA.M.P. (Maayan and Ingbar, 1968).

The stimulation of endocytosis and secretion in mice by 5-H.T. adrenaline or N.A. is prevented by α-receptor blocking agents, whereas the actions of I.P.N.A. are inhibited by β-receptor blockers (Ericson et al, 1970; Melander, 1971a, b). The β-receptors on the mouse thyroid are of the β_2 class since they are selectively stimulated by β_2 agonists (e.g. terbutilene) but not by β_1 agonists (e.g. I.T.P.(2))(Melander et al, 1975b).

α-receptor blocking agents have also been reported to reduce the secretory response to T.S.H. and L.A.T.S. in vivo and this may suggest a link between α-receptors and T.S.H. receptors (Ericson et al, 1970; Melander, 1971a, b). However, at high concentrations, in vitro, both α- and β-receptor blockers

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(1) Maayan et al (1973) reported some inconsistencies in the effects of adrenergic blocking agents on adrenaline stimulated functions. Stimulation of iodide uptake and organification was inhibited by phentolamine, whereas that of G-1-^{14}C oxidation could be inhibited by phentolamine or propranolol. In contrast, enhanced R.N.A. synthesis was unaffected by either agent. They suggest the existence of several adenyl cyclase systems to explain this.

(2) 1-isopropylamine-3-(2-thiazoloxy)-2-propranolol.

inhibit the response to T.S.H., L.A.T.S. or cA.M.P. (Burke, 1969; Kendall-Taylor and Munro, 1970b; Levey et al, 1969; Onaya and Solomon, 1969).

The effects of histamine on the mouse thyroid in vitro are inhibited by the H_2-receptor antagonist, metiamide, but not by the H_1-receptor blocker, chlorpheniramine. Moreover, the H_2-receptor agonist, 4-methylhistamine, was active in contrast to the H_1-receptor agonist, 2-methylhistamine, suggesting that only H_2-receptors exist on thyroid follicular cells (Onaya et al, 1977).

1.2.4. Receptor relationships

The evidence above shows that L.A.T.S. may be viewed as a true thyroid stimulator since it has been shown to mimic most of the actions of T.S.H. Like thyrotropin, it probably exerts most of its effects via cA.M.P. and the available evidence indicates that the initial reaction with the thyroid cell membrane is at, or close to, the T.S.H. receptor.

The data for biogenic amines is less comprehensive and more complex. Many of the effects of T.S.H. may be reproduced but in many systems the maximal response is smaller. There are probably at least four amine receptor types on the thyroid (α, β, 5-H.T. and histamine-H_2). The linkage of these with adenyl cyclase is not clear but it seems likely that β and possibly also 5-H.T. may act in this way. Whether any linkage or structural inter-relationship exists between receptors remains to be discovered.

1.3. THE SCOPE OF THE EXPERIMENTAL WORK

The major interest in thyroid receptors stems from their probable role in the pathogenesis of Graves' disease. At the time of the experimental work described in this thesis[1] there was evidence (reviewed above) to suggest the existence of a variety of receptor species on thyroid cells but it appeared probable that L.A.T.S. acted via the T.S.H. receptor. However, this remained unproven and, moreover, the relationship between L.A.T.S. or T.S.H. receptors and amine receptors was not entirely clear. For these reasons, attempts were made to explore further some of the similarities and differences between the thyroidal effects of these stimulators, within the limits of available resources. These are presented in three sections:

1. Part 3 describes an investigation into the difference in time course between the actions of L.A.T.S. and T.S.H. on thyroid secretion in the mouse, extending previous studies by others (Adams, 1960; McKenzie, 1961).
2. Part 4 describes experiments investigating the actions of biogenic amines on the mouse thyroid secretion and the effects of receptor-blocking drugs on the response to these and other thyroid stimulators.
3. Finally, a direct approach was made to investigate receptor specificities by studying the binding of radiolabelled T.S.H. to thyroid homogenates and testing the effects of other thyroid stimulators on its magnitude. These experiments are presented in part 5.

Methods relevant to more than one section are presented in part 2.

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(1) September 1970 - September 1973

PART 2

GENERAL METHODS

Materials and methods are described for each experiment in the relevant sections. However, a few techniques were used repeatedly throughout the work. These are described separately here.

2.1. THE McKENZIE BIOASSAY

2.1.1. Introduction

In 1955 Adams and Purves described a new method for the assay of thyrotropin. Guinea pigs were given radioiodine to label thyroid iodine and were maintained on thyroxine to suppress endogenous T.S.H. production. The assay was performed three days later by injecting T.S.H. intravenously and measuring the increase in blood radioactivity after three hours. This method was slow and costly and has been superceded by that introduced by McKenzie (1958b) which uses iodine deficient mice instead of guinea pigs. This method has been widely accepted and many workers have adopted it with a variety of modifications in technique, design or treatment of results (Table 2.1).

The main advantages of this bioassay are its speed and convenience, whilst its precision and sensitivity match those of other in vivo assays (Condliffe and Robbins, 1967). Greater sensitivity and precision may be obtained with in vitro assays but interference by normal plasma constituents makes them unsuitable for plasma T.S.H. measurements (Bakke et al, 1957; Bourke et al, 1973; Bottari et al, 1963; Brown and Munro,

Table 2.1 Variants of the McKenzie assay - a comparison

Reference	Animals	Diet	Isotope	T.S.H. Suppression	No. mice per group	Bled at	λ	Sensitivity to b.T.S.H.	Data Treatment
McKenzie (1958)	♀ albino mice	Dog biscuits (low iodine)	8μCi. ^{131}I s.c.	Thyroid powder in diet for 3 days (after ^{131}I)	5	2hr.(T.S.H.)	0.24	25μU	R.I.[1] Analysis of variance
Adams (1961)	♀ mice	Low iodine for 3 weeks M.T.U. for 1 wk; Dist. H_2O 1 wk.	1.5μCi. ^{131}I i.p.	10μg. T_4 i.p. 8-16hr. after ^{131}I. Pancreatic digest of thyroid tablets in drinking water for 3 days.	16 - 28	2hr.(T.S.H.) or L.A.T.S.) 16hr. (L.A.T.S.)	Not Stated	Not Stated	R.I.
Major and Munro (1962)	White Swiss mice 15-20g.	Low iodine diet (50μg/kg.)	6μCi. ^{131}I s.c.	10μg. T_4 s.c., 3 days and 1 day before assay	5	3hr.) (T.S.H.) 8hr.) 12hr.) (L.A.T.S.)	Not Stated	Not Stated	R.I.
Kriss et al. (1964)	♀ white mice 20-25g.	2wk. low iodine diet	5μCi. ^{131}I i.p.	15μg. T_4 i.p. on days 1, 4 and 5 (after ^{131}I) (Assay day 5)	4	3hr. 24hr. 48hr. (L.A.T.S. injected i.p.)	Not Stated	Not Stated	R.I.
Snyder et al. (1964)	Mice	10 days on low iodine diet	4μCi. ^{131}I ? route	0.12% dessicated thyroid in diet for 3 days (after ^{131}I) 10ug. T_4 s.c. on 2nd day	6	2hr.) (L.A.T.S.) 8hr.)	Not Stated	Not Stated	Modified R.I.[2]
Sakiz and Guillemin (1964)	♀ S.A. mice	Low iodine diet	15μCi. ^{131}I i.p.	10μg. T_4 i.p. 5hr. after ^{131}I. 5μg. T_4 24hr. later. Assay done 48hr. after last T_4 injection	Not Stated	2hr. (T.S.H.)	Not Stated	Not Stated	Log c.p.m. Analysis of covariance
Pinchera et al. (1965)	Swiss Webster mice 15g.	"Remington" diet	13-15μCi. ^{125}I ? route	Assay 3-5days after ^{125}I. 10μg T_4 i.p., 24hr. before assay	6	2hr.) (L.A.T.S.) 8½hr.)	Not Stated	50μU	3 or 4 point bioassay Analysis of variance
Rerup and Melander (1965)	White Swiss mice 20g.	Normal pellet diet	8μCi ^{131}I s.c.	20μg. T_4 s.c. with ^{131}I. Repeated 72hr. later. Assay next day.	5	2hr. (T.S.H.)	a) 0.23 (randomised groups) b) 0.15 (crossover)	17mU	Log R.I. Randomised groups or crossover design
El Kabir et al. (1966)	White Swiss 15-20g.	Dog biscuit (low iodine) for 10 days	6μCi. ^{131}I s.c.	10μg. T_4 i.p. 6hr. after ^{131}I. 10μg. T_4/day in drinking water. 10μg T_4 i.p. on evening of day 3 or 4. Assay day 4 or 5	8	2hr. 7hr. 24hr. (L.A.T.S.) 2hr.(T.S.H.)	Not Stated	Not Stated	R.I.
Good and Stenhouse (1966)	white mice	Dog biscuit (100μgI/kg)	5-6 μCi ^{131}I s.c.	10μg T_4 s.c. with ^{131}I. 0.1% dessicated thyroid extract in drinking water for 3 days	5 - 6	3hr. (T.S.H.)	0.188	6μU	Log. R.I. Latin square
McKenzie and Williamson (1966)	♀ white mice 15-18g.	10 days on low iodine diet	15μCi ^{125}I i.p.	10μg T_4 with ^{125}I folowed by 0.066% thyroid U.S.P. in diet for 3 days	6	2hr. 9hr. (L.A.T.S.)	Not Stated	Not Stated	Log c.p.m. Analysis of covariance
Florsheim et al. (1970)	♀ white Swiss	2wk. iodine deficient diet	12μCi ^{125}I i.p.	6μg./ml. T_4 in drinking water for 72 or 90hr.	8 - 12	3hr.(T.S.H.) 21hr. (L.A.T.S.)	Not Stated	Not Stated	R.I.

[1] R.I. = Response Index = $\frac{\text{c.p.m.}_{xhr}}{\text{c.p.m.}_{0hr}}$

[2] Modified R.I. = $100\left[\frac{(\text{c.p.m.}_{8hr.})}{(\text{c.p.m.}_{0hr.})_U} - \frac{(\text{c.p.m.}_{8hr.})}{(\text{c.p.m.}_{0hr.})_C} + 1\right]$

where U = unknown and C = mean of results of control mice

1967; Kirkham, 1962; Manley et al, 1969). The McKenzie assay has the added advantage of distinguishing between L.A.T.S. and T.S.H. by the pattern of response (McKenzie, 1960, 1968; Major and Munro, 1962).

For these reasons the McKenzie assay has been used widely for the measurement of T.S.H. activity, detection of L.A.T.S. activity and also for investigation of thyroid stimulation by other substances (e.g. Burke, 1968d, 1970; Burke and Szabo, 1972; Melander, 1971a). At the time of embarking on the work described in this thesis, one particular variant of the McKenzie bioassay (El Kabir et al, 1966) had been used successfully for more than five years in the Department of Human Anatomy, Oxford. This method was therefore employed throughout this work with minor modifications.

Applications:

1. The bioassay of labelled and unlabelled T.S.H. (Section 2.3.4)
2. The bioassay of L.A.T.S. (Section 3.4.5)
3. The quantitation of anti-T.S.H. antiserum potency (appendix 5)
4. An investigation into the time course of the response to T.S.H. and L.A.T.S. in the assay is presented in Part 3.

2.1.2. Materials

Animals: A colony of albino mice was bred in the Department of Human Anatomy from an original stock of the C.F.L.P. strain(1). This had been found to give the best assay precision in a previous trial of four different strains of mice. The breeding stock was kept on a normal pellet diet(2) and deionised water

(1) Carworth Europe Ltd., Huntingdon
(2) 41B, Dixon Ltd., Ware, Herts.

prepared in an 'Elgastat' deioniser. The latter was necessary since Oxford tap water had previously been found to prevent the uptake of radioiodine by the thyroids of mice, probably because of its high chlorine content (Salaman, 1968). Mice were weaned at three weeks onto a diet of commercial dog biscuits(1), known to have a low iodine content(2), and deionised water. Male or female mice were used between 10 and 24 days after weaning. The weights of these animals were usually in the range 20-30g. Whenever possible either males alone or females alone were used for a single assay. Often, however, this was not possible and the sexes had to be mixed, with the precaution that equal numbers of each sex were allocated to each treatment.

Solutions: (1) 20µg/ml thyroxine injection was prepared at the start of each assay, dissolving 2µg thyroxine(3) in 20ml normal saline by adding N sodium hydroxide solution drop-wise and making up to 100ml with normal saline. (2) 3.3µg/ml thyroxine drinking water was prepared by dissolving 10mg thyroxine in 20ml deionised water, adding alkali, and making up to 3 litres with deionised water.

Standards: International standard bovine T.S.H. (Int.Std. T.S.H.)(4) tablets were stored in a dessicator at -10 to -20°C. Standard solutions of 100mU/ml in 1% B.S.A.(5) were prepared at intervals of less than three months and stored in 0.2-0.5ml aliquots in stoppered vials at -10 to -20°C. Appropriate dilutions (usually in the range 17-150mU/100ml with a log dose interval of log 3) were

(1) Vim's dog biscuits
(2) 1.7 p.p.m. iodine as assayed on 19.12.69. by Huntingdon Research Laboratories Ltd., Huntingdon
(3) Sodium thyroxine tetrahydrate, Sigma Chemical Co., Kingston-upon-Thames
(4) M.R.C. Standards Division, Mill Hill
(5) Bovine Cohn fraction V, Sigma Chemical Co., Kingston-upon-Thames

made up from these vials the night before the assay, stored overnight at 4°C and warmed to room temperature before injection.

No L.A.T.S. standards were used since these are of doubtful meaning (McKenzie, 1974), although in some experiments sera were ascribed an arbitrary standard potency for descriptive purposes. All human sera were assayed on arrival and stored at -10 to -20°C until use. The characteristics of sera are described in appendix 4.

2.1.3. Method (Figure 2.1)

On the morning of day 1 mice were taken from the colony and injected with 6μCi $Na^{131}I$ in 0.5ml saline (i.p.). The same evening 10μg thyroxine in saline solution (0.5ml) were injected (i.p.) and the mice were started on thyroxinised drinking water (3.3μg.ml). On the evening of day 3 a further injection of 10μg thyroxine (i.p.) was given and the actual assay was performed on the morning of day 4. In some cases the second dose of thyroxine was given on day 4, the assay being performed on the morning of day 5.

The mice were divided into treatment groups of 8, numbered with coloured pens. 0.1ml blood was taken from the orbital sinus of each mouse using a 'Gold line' pipette(1) and mixed with 0.5ml aliquots of tap water in stoppered plastic counting vials(2). A dilute heparin(3) solution was used to rinse the pipette between bleeds. Following bleeding the mice were left

---

(1) Gold line pipettes P2220, Scientific Supplies, Vine Hill, London, E.C.1
(2) 3ml flat bottomed tubes PT10944, Luckham Ltd., Burgess Hill, Sussex
(3) 'Pularin' heparin injection B.P. (25000IU/ml), Evans Medical Ltd., Speke, Liverpool

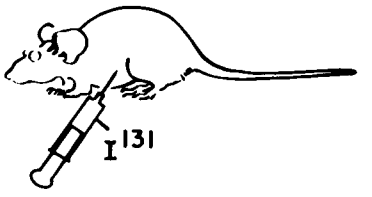
FIGURE 2.1

The McKenzie bioassay: method

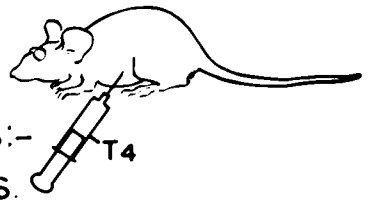
THE Mc KENZIE ASSAY

DAY 1

Morning – Inject 6 μC of tracer I^{131} I.P. in 0·5 mls. saline.



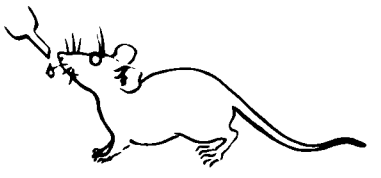
Afternoon – Suppression of Endogenous TSH production begins:-



1) Inject 10 μg T_4 in 0·5 mls. saline I.P.
2) Give mice 10 μg T_4/day in drinking water.

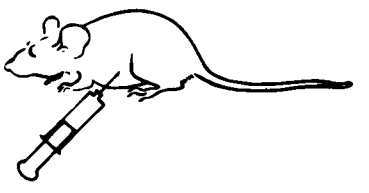
DAY 1 —— DAY 4

10 μg T_4 /day given to each mouse in drinking water



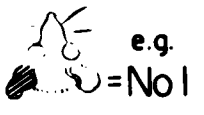
DAY 3

Evening – Inject 10 μg T_4 in 0·5 mls. saline. I.P.



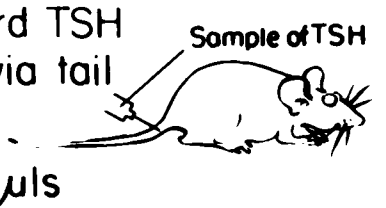
DAY 4 — ASSAY

1) Mice numbered in groups of 8



2) 100 μls blood taken by orbital puncture — <u>0 hr sample</u>

3) 0·5 mls sample or standard TSH injected into each mouse via tail vein



4) 2 hrs after injection - 100 μls blood again removed by orbital puncture. — <u>2 hr sample</u>

in a heated cage[1] at 35-40°C for 5 minutes to promote dilation of the tail veins. They were then injected with stimulator or control solutions via a suitable tail vein. The injection volume was usually 0.5ml although smaller quantities were administered in some experiments. The mice were left on a dog biscuit diet with thyroxinised drinking water before bleeding again 2hr. following injection. For L.A.T.S. detection the mice were also bled at 7hr. and 24hr. In this way, a single person could deal with 120 mice per assay and two people could deal with up to 200 mice.

The blood samples were counted for 5 or 10 minutes in a well type scintillation spectrometer (Packard autogamma at 290-410kev or Nuclear Chicago autogamma at 314-414kev) giving initial (0hr.) counts of about 1000/5 min. Counts of less than twice background level (which was usually about 350/5 min) were discarded. Results were expressed in terms of the response index ($R.I._{x\ hr.}$) or its logarithm, where

$$R.I._{x\ hr.} = \frac{x\ hr.\ counts}{0\ hr.\ counts}$$

(all counts corrected for background). Figure 2.2 shows the results of a typical experiment and demonstrates the time course of the responses to L.A.T.S. and T.S.H. The very obvious heteroscadasticity of the results when plotted as R.I. (fig. 2.2a) was eliminated when replotted as $\log_{10}$R.I. (fig. 2.2b).

2.1.4. Treatment of results

For simple experiments comparing the effects of two or more treatments, the mean and S.E.M. of the $\log_{10}$R.I. (or, in

---

(1) Constructed by Mr. J. Glozier, Dept. Human Anatomy, Oxford

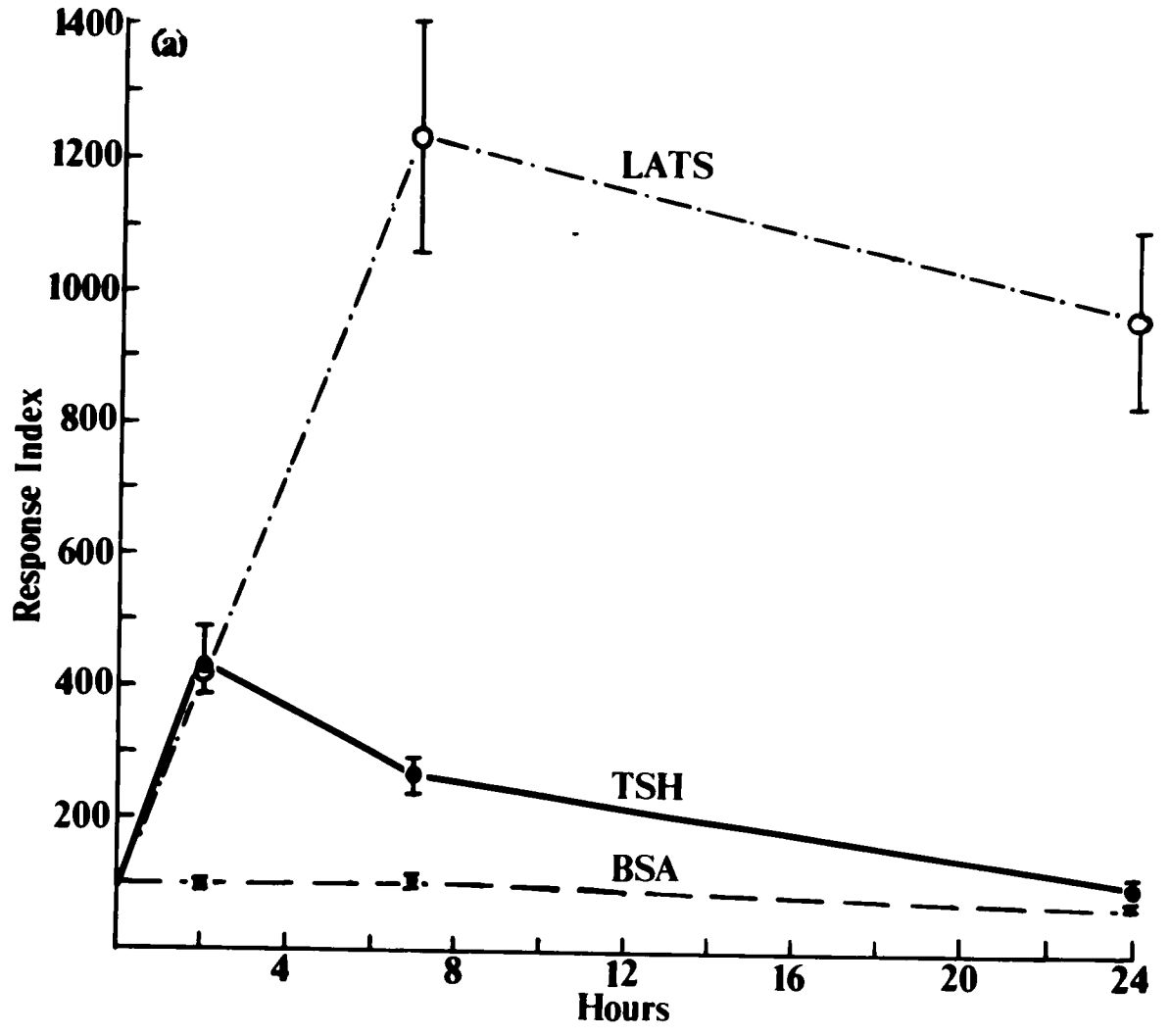
FIGURE 2.2

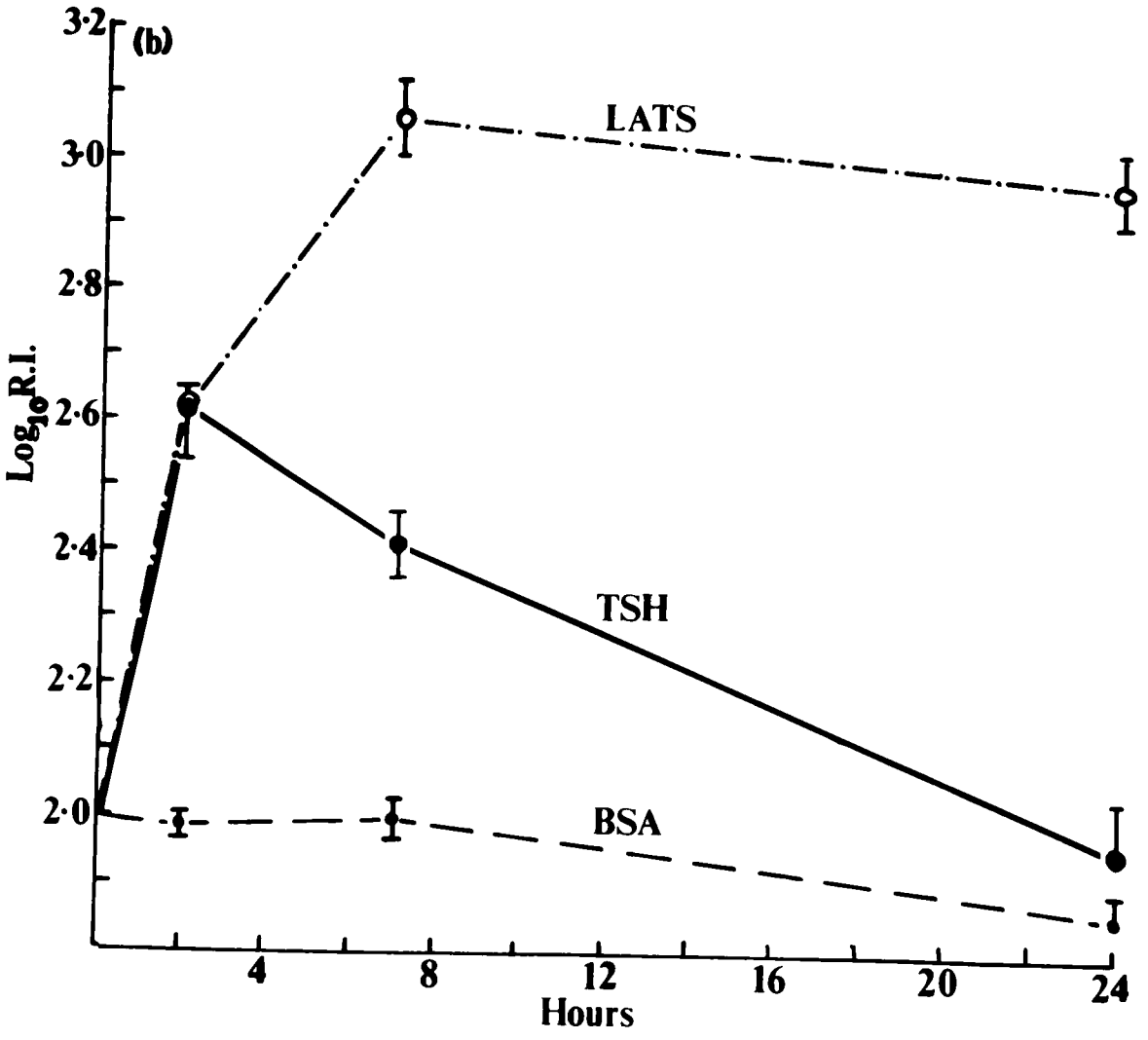
The McKenzie bioassay: the time course of response to T.S.H. (0.5mU), L.A.T.S. serum (R.C. diluted 1 in 3 with 1% B.S.A.) and 1% B.S.A.

(a) Time vs. R.I.

(b) Time vs. $\log_{10}$R.I.

Each point represents mean ± S.E.M.


(a)
1400
1200
1000
800
600
400
200
Response Index
LATS
TSH
BSA
4
8
12
16
20
24
Hours


(b)
3·2
3·0
2·8
2·6
2·4
2·2
2·0
Log10R.I.
LATS
TSH
BSA
4
8
12
16
20
24
Hours

some early experiments, the R.I.) were calculated for each group which were compared using Student's 't' test (for two groups) or Dunnett's 't' test or Duncan's test (for comparison of several groups). Except where insufficient animals were available, a standard dose of T.S.H. (0.5mU) was included and the response to it plotted on a quality control chart (fig. 2.3).

When graded doses were used, $\log_{10}$ dose vs. $\log_{10}$R.I. lines were calculated and subjected to analysis of variance by the method of Bliss (1952). Heteroscadasticity was usually absent (Bartlett's test) having been eliminated by log transformation. (Statistical methods are described in more detail in appendix 1.)

For T.S.H., many of the $\log_{10}$ dose vs. $\log_{10}$R.I. curves were not strictly linear over three doses, although nonlinearity rarely reached significance (fig. 2.4 and table 2.2). This resulted in lower slopes (b) and indices of precision (λ) for all 3 doses than for the lower 2 doses within the same assay. The reduction in slope with higher doses is also shown by the 5 dose curve in figure 2.5.

In contrast, the $\log_{10}$ dose vs. response lines for L.A.T.S. were linear over a wide range (figs. 2.6, 2.7 and table 2.3). The 7 hour R.I. ($R.I._{7\ hr.}$) was used for these since it was the most sensitive index of L.A.T.S. response (fig. 2.8).

When solutions of unknown potency were assayed, a variety of experimental design was used. Where possible a 3 + 2 or 2 + 2 design was employed. In the case of 3 + 2 T.S.H. assays, when the curve was markedly nonlinear and the unknowns fell on the steeper, lower part of the curve, the highest standard T.S.H.

FIGURE 2.3

Quality control chart for response to standard 0.5mU T.S.H. dose between January 1972 and August 1973.

Each point represents mean ± S.E.M. of $\log_{10}$R.I.$_{2\ hr.}$ for a given experiment. (Open circles, female mice; closed circles, male mice; half open circles, mixed sexes.) Horizontal lines represent unweighted mean ± S.D. of mean $\log_{10}$R.I.$_{2\ hr.}$ values.

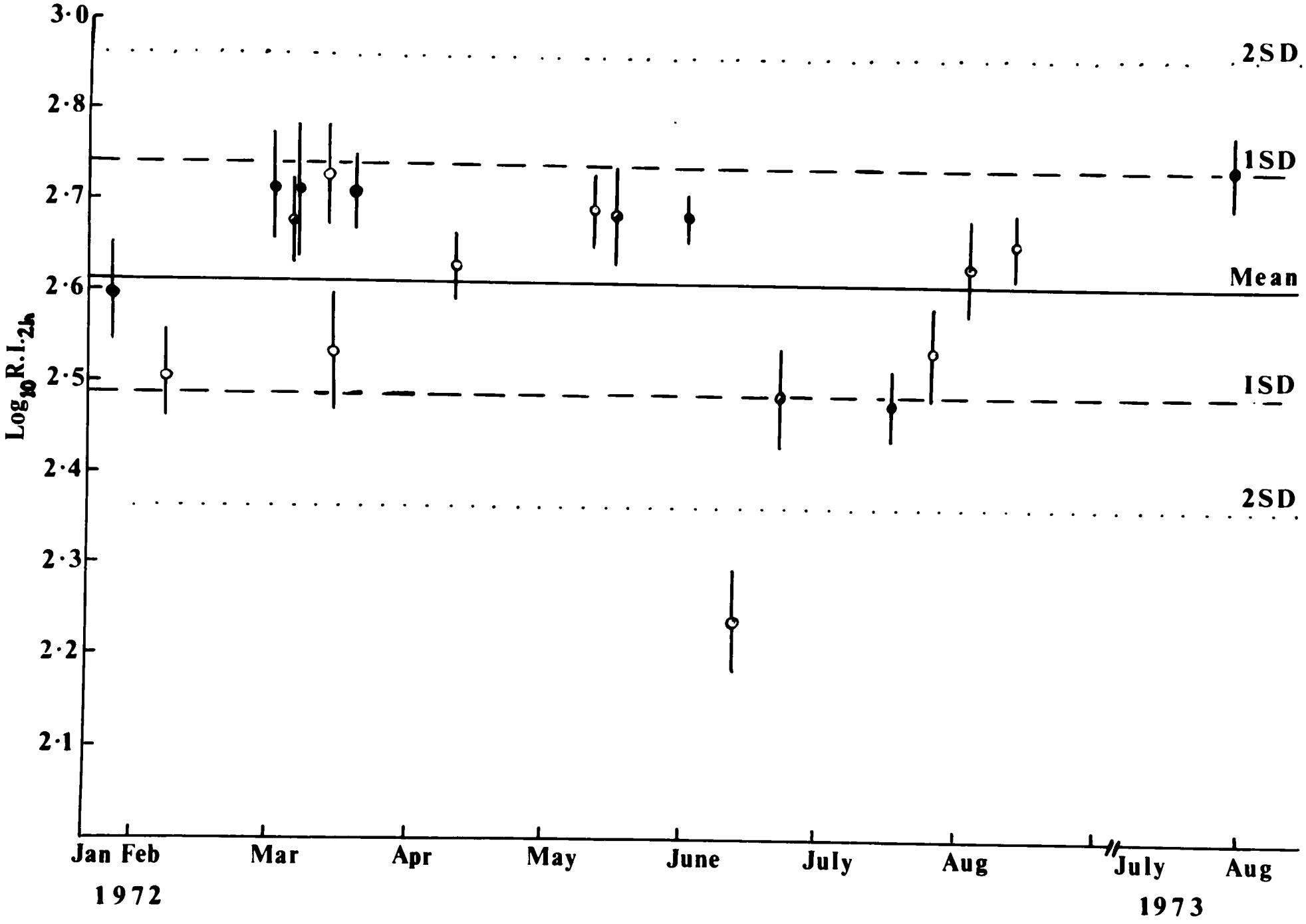

3·0
2·8
2·7
2·6
2·5
2·4
2·3
2·2
2·1
Log_{10}R.I.$_{2h}$
2SD
1SD
Mean
1SD
2SD
Jan Feb
1972
Mar
Apr
May
June
July
Aug
July
1973
Aug

FIGURE 2.4

$\underline{\text{Log}_{10}\text{ dose vs. log}_{10}\text{R.I. curves for T.S.H.}}$
$\underline{\text{in the eight experiments of Table 2.2A}}$.

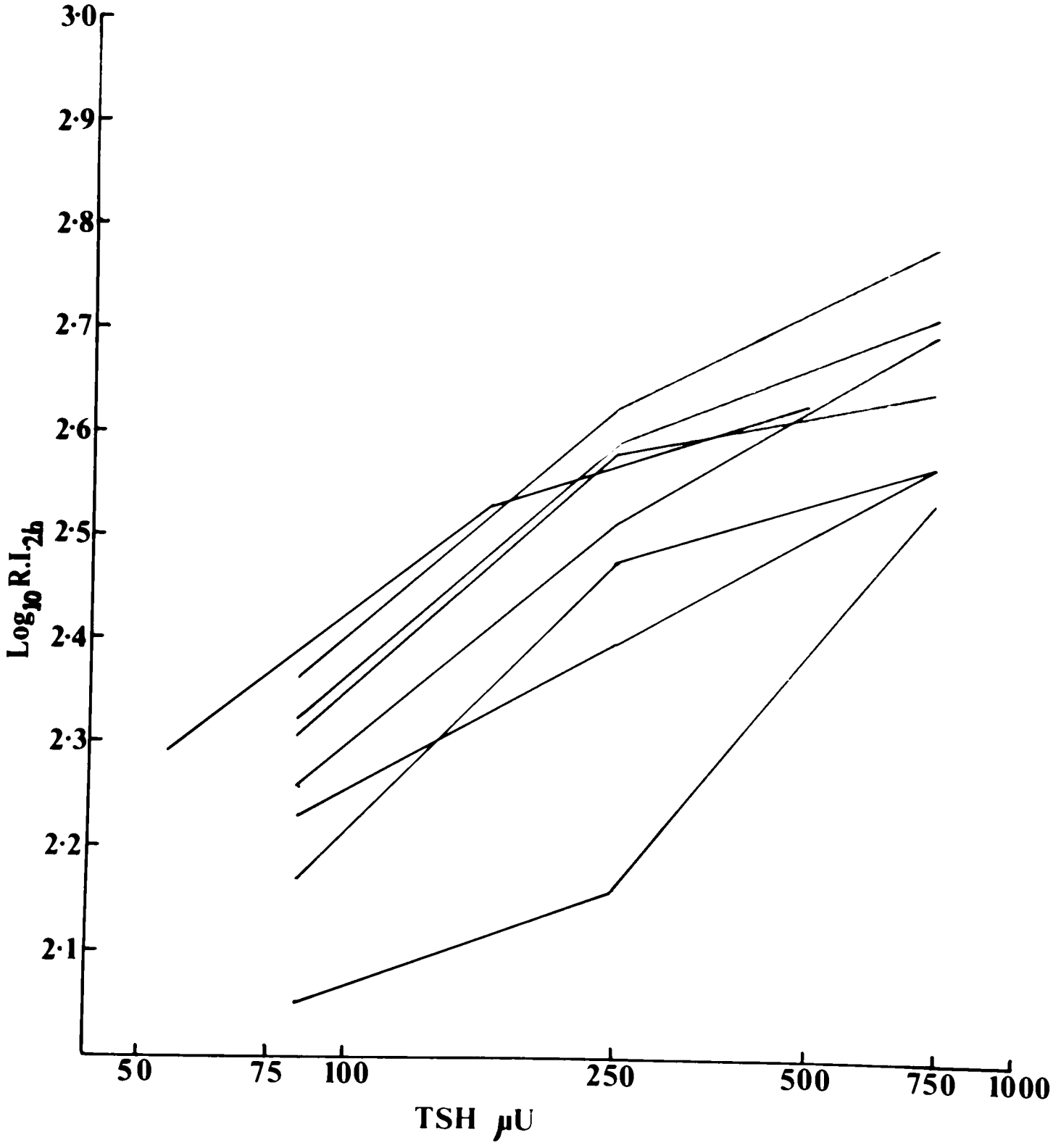

3·0
2·9
2·8
2·7
2·6
2·5
2·4
2·3
2·2
2·1
Log_{10} R.I.$_{2h}$
50
75
100
250
500
750
1000
TSH μU

TABLE 2.2. Log_{10} dose vs. log_{10} R.I.$_{2\ hr.}$ line characteristics for Int. Std. T.S.H.

A. Three dose T.S.H. assays

Assay date	Doses μU	Sex of mice	Three doses					Lower two doses only		
			Non-linearity	Hetero-scadasticity	Slope	Sig. of slope	λ	Slope	Sig. of slope	λ
2.3.72.	85,250,750	F	N.S.	N.S.	0.434	p<0.001	0.296	0.560	p<0.01	0.217
10.4.72.	56,167,500	F	N.S.	N.S.	0.354	p<0.001	0.318	0.510	p<0.01	0.259
25.4.72.	85,250,750	F	p<0.05	N.S.	0.523	p<0.001	0.224	0.241	p<0.05	0.369
4.5.72.	85,250,750	M	N.S.	N.S.	0.464	p<0.001	0.326	0.551	p<0.01	0.286
9.5.72.	85,250,750	M	N.S.	N.S.	0.441	p<0.001	0.277	0.552	p<0.001	0.175
19.5.72.	85,250,750	M	p<0.05	N.S.	0.341	p<0.001	0.429	0.587	p<0.001	0.193
11.7.72.	85,250,750	M	N.S.	p<0.05	0.353	p<0.001	0.329	0.351	p<0.01	0.267
14.9.72.	85,250,750	F	N.S.	N.S.	0.425	p<0.001	0.311	0.642	p<0.001	0.203
	Mean ± S.E.M.				0.417 ±0.063		0.314 ±0.058	0.499 ±0.134		0.246 ±0.063

B. Two dose assays

Assay date	Doses	Sex of mice	Heteroscadasticity	Slope	Sig. of slope	λ
11.6.71.	85,250	M	N.S.	0.503	p<0.01	0.274
14.10.71.	85,250	F	N.S.	0.753	p<0.001	0.163
21.9.72.	85,250	M	N.S.	0.714	p<0.001	0.167
28.11.72	85,250	M	N.S.	0.584	p<0.001	0.194
	Mean ± S.E.M.			0.639 ±0.116		0.200 ±0.052

FIGURE 2.5

$\underline{\text{Log}_{10}\text{ dose vs. log}_{10}\text{R.I.}_{2\text{ hr.}}\text{ curve for a five dose T.S.H.}}$ $\underline{\text{McKenzie assay}}$.

Each point represents mean ± S.E.M. of eight mice except where specified.

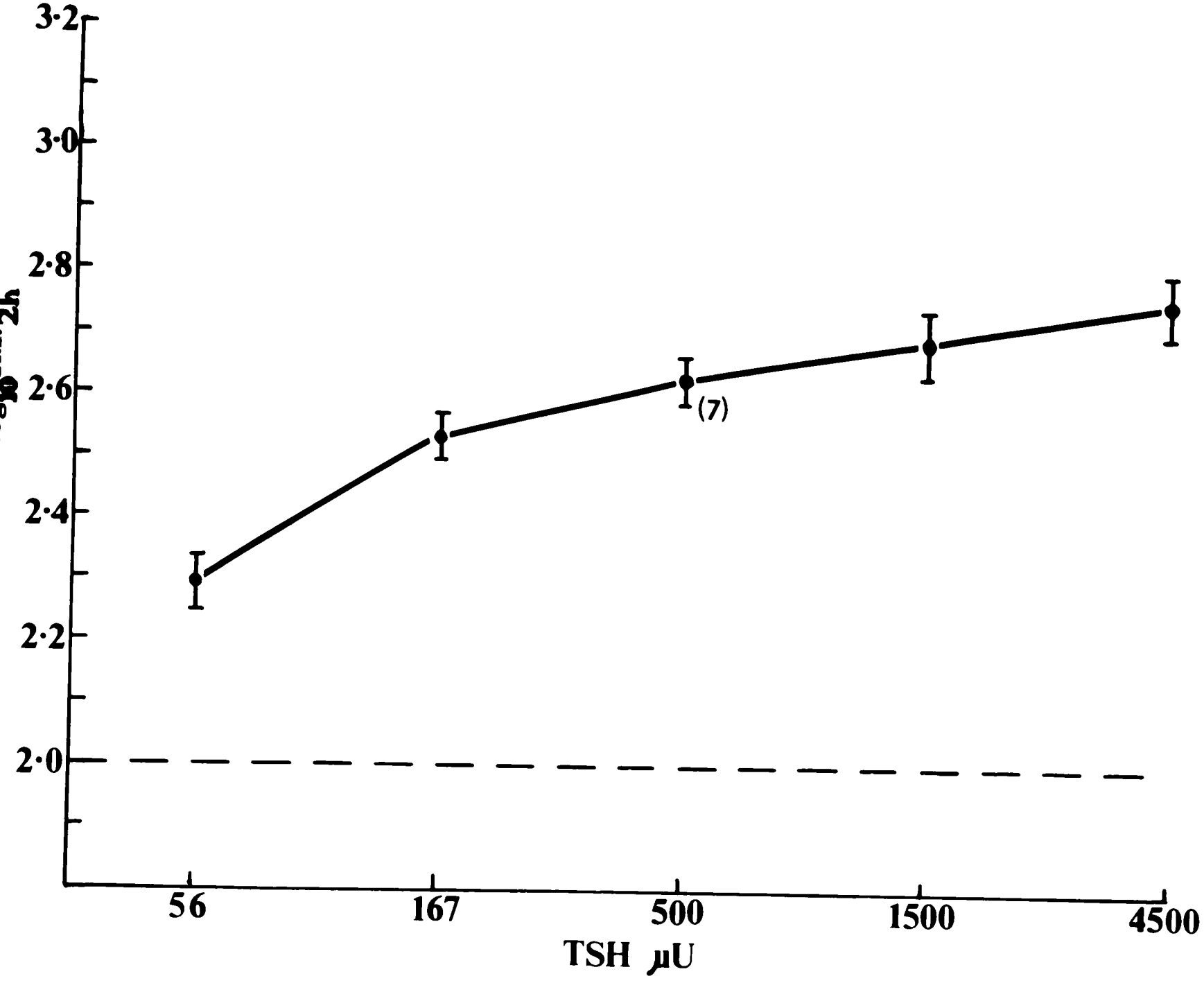

3·2
3·0
2·8
2·6
2·4
2·2
2·0
Log_{10}R.I.$_{2h}$
(7)
56
167
500
1500
4500
TSH μU

FIGURE 2.6

$\underline{\text{Log}_{10}}$ $\underline{\text{dose vs. log}_{10}}$ $\underline{\text{R.I.}}_{7}$ $\underline{\text{hr. curve for a L.A.T.S. serum (R.N.2) in the McKenzie assay.}}$

All dilutions performed in 5% B.S.A. Injection volume 0.5ml.

Each point represents mean ± S.E.M. of seven mice.

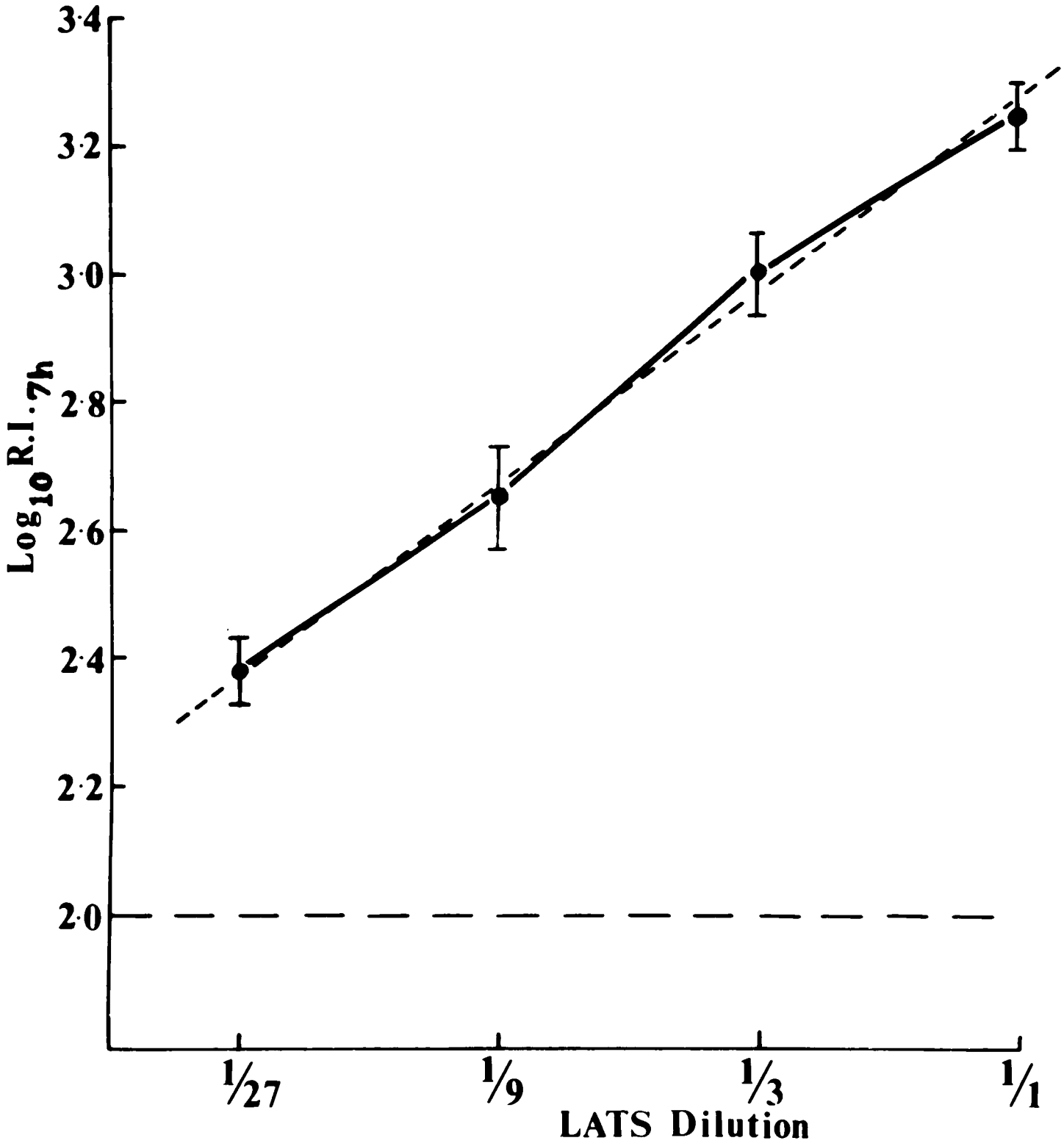

3·4
3·2
3·0
2·8
2·6
2·4
2·2
2·0
Log10R.I.7h
1/27
1/9
1/3
1/1
LATS Dilution

FIGURE 2.7

$\underline{\text{Log}_{10}}$ $\underline{\text{dose vs. log}_{10}}$ $\underline{\text{R.I.}}_{7\text{ hr.}}$ $\underline{\text{curves for L.A.T.S. sera}}$ in the four experiments in Table 2.3. All dilutions performed in 5% B.S.A. Injection volume 0.5ml.

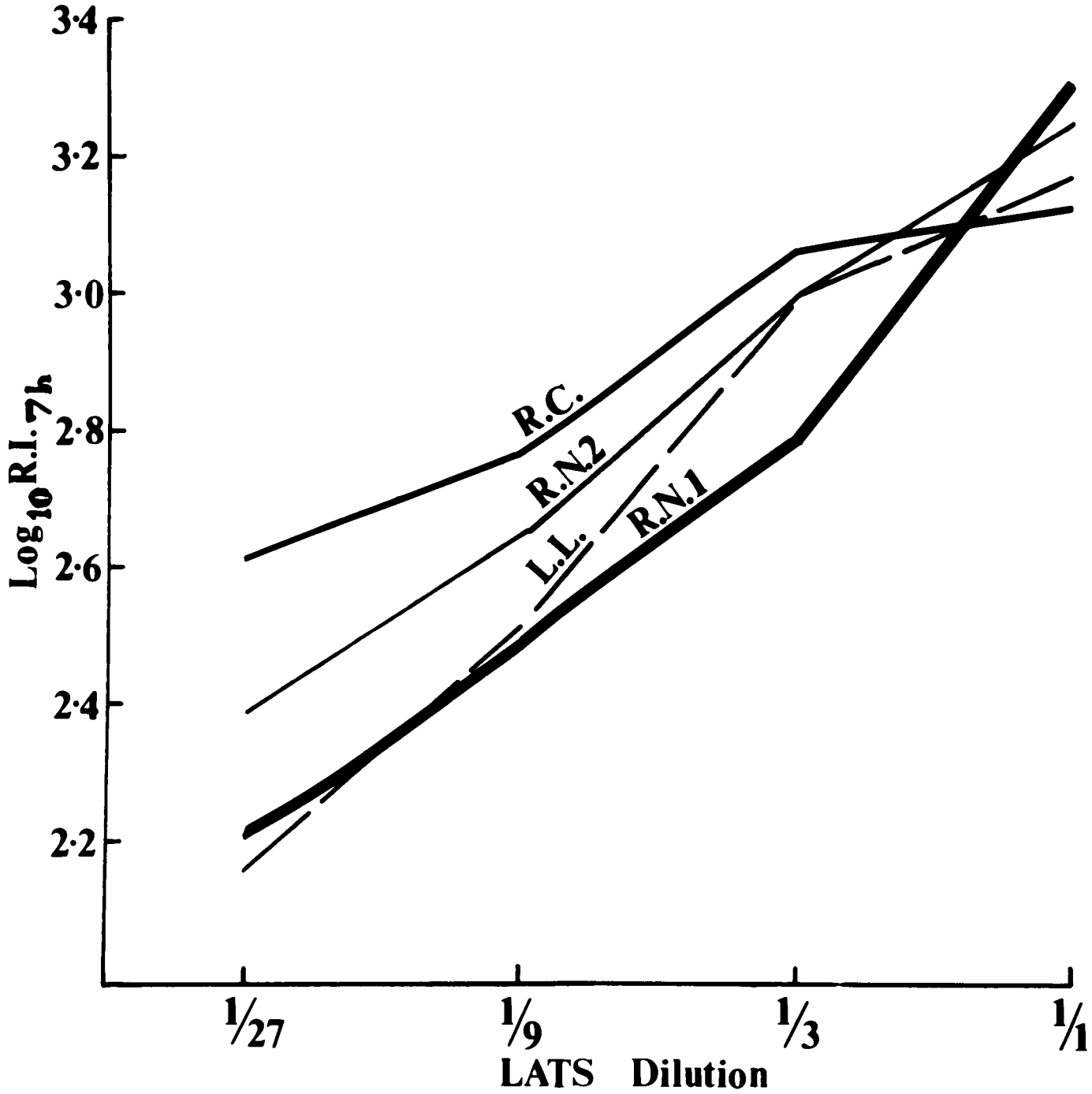

3·4
3·2
3·0
2·8
2·6
2·4
2·2
Log_{10}R.I. 7h
R.C.
R.N.2
L.L.
R.N.1
1/27
1/9
1/3
1/1
LATS Dilution

TABLE 2.3. Log_{10} dose vs. log_{10} R.I.$_{7\ hr.}$ line characteristics for four different L.A.T.S. sera in the McKenzie assay

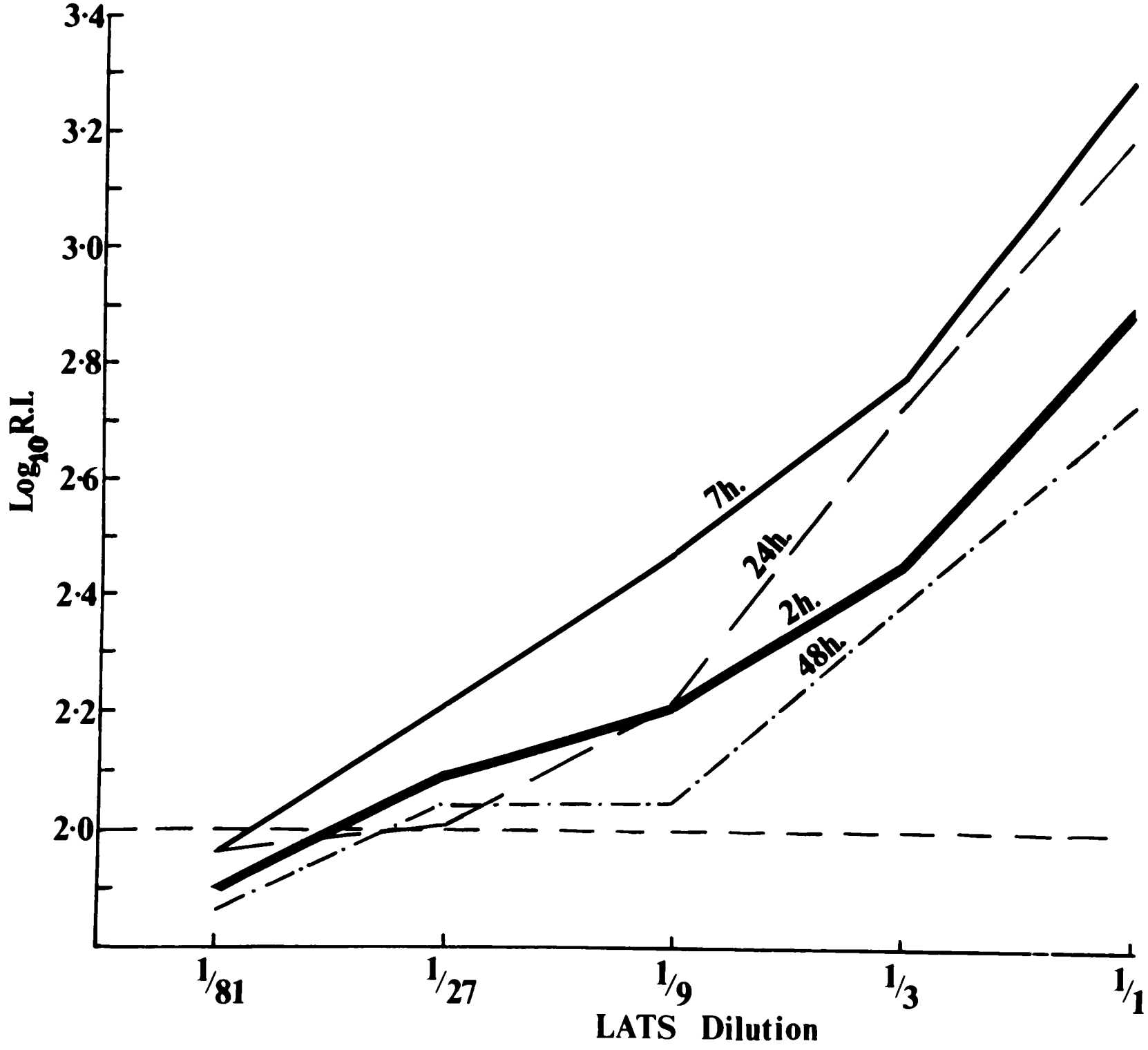
All sera injected in dilutions of 1 in 1, 1 in 3, 1 in 9 and 1 in 27. 0.5ml injected per mouse.

Assay date	L.A.T.S. serum	Sex of mice	Non-linearity	Heteroscadascity	Slope	Significance of slope	λ
10.12.71.	R.N.1	F	N.S.	N.S.	0.748	$p<0.001$	0.269
20.1.72.	L.L.	F	N.S.	N.S.	0.741	$p<0.001$	0.249
25.5.72.	R.N.2	M + F	N.S.	N.S.	0.615	$p<0.001$	0.261
25.7.72.	R.C.	M	N.S.	N.S.	0.479	$p<0.001$	0.379

FIGURE 2.8

Comparison of the $\log_{10}$ dose vs. $\log_{10}$R.I. curves for different response times following injection of L.A.T.S. (R.N.1) in the McKenzie assay.

All dilutions performed in 5% B.S.A. Injection volume 0.5ml.


3·4
3·2
3·0
2·8
2·6
2·4
2·2
2·0
Log10R.I.
7h.
24h.
2h.
48h.
1/81
1/27
1/9
1/3
1/1
LATS Dilution

dose was discarded and the assay treated as a 2 + 2 design. In some experiments single dose unknowns were employed, either because insufficient was available or because the number of available mice limited the assay capacity. Potencies and their confidence limits were subsequently calculated by the method of Bliss (1952) with the aid of a desk calculator(1). The steps in calculation are laid out in appendix 1.

2.2. THE PREPARATION OF IODINATED T.S.H.

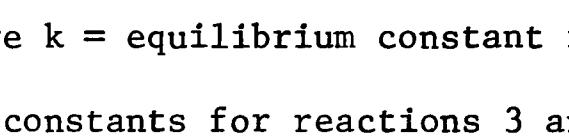
2.2.1. Introduction: methods available for protein labelling

A great variety of methods have been made available for the introduction of radioactive elements into proteins. Biological systems have been employed for the incorporation of tritium (^{3}H)(Fontaine, 1970), carbon-14 (^{14}C) or sulphur-35 (^{35}S)(Goldsworthy and Volwiler, 1957) into proteins, but these methods were uneconomical, capable of producing only low specific activity labelling and suitable only for proteins which are easily isolated. Of the earlier chemical methods used, mustard gas -^{35}S-sulphone or ^{32}P-phosphoryl chloride (Francis et al, 1951) proved unsatisfactory due to denaturation or alteration in properties of the proteins. Tritium has been incorporated into proteins by an exchange reaction (von Holt et al, 1960) and ^{14}C-acetyl groups have been added by a reaction with ^{14}C acetic anhydride (Collipp et al, 1965). ^{3}H-alanyl h-C.G. prepared by the addition of N carboxy alanine anhydride-^{3}H to h-C.G. has been shown to retain full biological and immunological potency (Eshkol, 1969) and, more recently, a

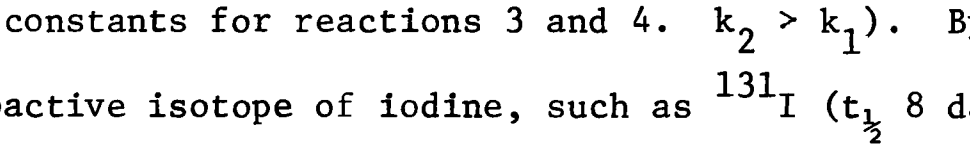
(1) Olivetti Programmer 101 or Sumlock Compucorp 3421 'Statistician'

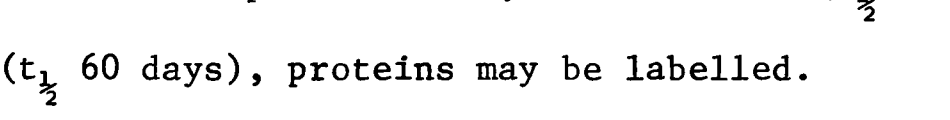
highly successful method has been reported for the introduction of ^{3}H into the sialic acid residues of h-C.G. by oxidation with potassium periodate followed by reduction with boron hydride-^{3}H, giving specific activities of 1-2μCi/mg with 80% retention of biological activity (Vaitukaitis et al, 1971).

The ability of iodine to react with proteins has been recognised for 100 years (Boehm and Berg, 1876). The reaction occurs by substitution of iodine for hydrogen in the phenolic rings of tyrosine residues and is mediated by hydroiodous ions (H_2OI^+) (Hughes, 1957):

1. 

2. $I_2 + H_2O \rightleftharpoons H_2OI^+ + I^-$

3. 

4. 

(where k = equilibrium constant for reaction 1. k_1 and k_2 are rate constants for reactions 3 and 4. $k_2 > k_1$). By using a radioactive isotope of iodine, such as ^{131}I ($t_{\frac{1}{2}}$ 8 days) or ^{125}I ($t_{\frac{1}{2}}$ 60 days), proteins may be labelled.

There are many reasons for the popularity of iodination. According to Freeman (1967) its advantages are:

"1. Labelling can be performed in vitro, thus enabling very high specific activities to be reached.

2. The label remains firmly bound to the selected protein and does not exchange with other protein molecules .....

..... 3. If iodination is performed under carefully controlled conditions, the labelled proteins can be indistinguishable from native proteins by either in vitro or in vivo tests."

In addition:

4. Many of the techniques for iodination are swift and simple to perform.

5. Radioactive iodine is readily available at relatively low cost.

Various methods have been devised for accomplishing the iodination reaction (table 2.4). In most cases they rely on the oxidation of iodide to iodine in the presence of the protein. The chloramine T method of Greenwood et al (1963) is currently the most widely used (although others are gaining in popularity) and has been applied to a variety of proteins, especially the polypeptide hormones (Hunter, 1967, 1971). However, many oxidising agents, including chloramine T, may "damage" protein molecules independently of the iodination process (Hunter, 1971). Hughes (1957) suggests three possible sources of structural change in protein molecules during iodination:

1. -SH group oxidation,
2. Iodination of histidyl or tryptophan groups,
3. Thyroxine formation.

Some of the newer techniques, such as the lactoperoxidase method (Marchalonis, 1969; Miyachi et al, 1971) electrolytic iodination (Rosa et al, 1964; Rosa, 1966; Donabedian et al, 1972) or the method of Butt (1972) which uses iodine vapour generated by the action of chloramine T on iodide, appear to achieve high specific activity labelling without causing significant inactivation on physicochemical, immunochemical or biological grounds.

TABLE 2.4. Methods employed for iodination of proteins

Authors	Method	Isotope	Protein	Specific activity
Hughes and Straessle (1950)	KI_3	I^{127}	h-serum albumin	-
Francis et al (1951)	$NH_3/KI/KIO_3$	I^{131}	Gamma globulin	Not stated (low)
Borson et al (1953)	Nitrous acid	I^{131}	h-serum albumin	150 Ci/mg
Gilmore et al (1954)	Ammonium persulphate	I^{131}	b- and h-serum albumin	Not stated (low)
Veall et al (1955)	$NH_3/I/KIO_3$	I^{131} and I^{132}	h-serum albumin	Not stated (low)
McFarlane (1956)	H_2O_2	I^{131}	e-, h- and b-serum albumin	Not stated (low)
McFarlane (1958)	I Cl	I^{131}	plasma protein	Not stated
Samols and Williams (1961)	I Cl (micro)	I^{131}	h-insulin	300-600mCi/mg
Greenwood et al (1963)	Chloramine T	I^{131}	h-GH	290-590mCi/mg
Rosa et al (1964)	Electrolytic	I^{131}	h-fibrinogen	50 Ci/mg
Rosa (1966)	Electrolytic	I^{125}	h-serum albumin	40-60 Ci/mg
Heideman et al (1965)	Distillation of iodine	I^{131}	h-TSH	0.4-1.6mCi/mg
Marchalonis (1969)	Lactoperoxidose	I^{125}	h-IgG	5mCi/mg
Miyachi et al (1972)	Lactoperoxidose	I^{125}	h-GH, h-FSH, h-LH	50mCi/mg
Donabedian et al (1972)	Electrolytic	I^{125}	h-GH, h-TSH, p-insulin	Not stated (1 atom/mol)
Butt (1972)	Iodine vapour from reaction of chloramine T and iodide^{125}	I^{125}	h-FSH and other pituitary hormones	50-100mCi/mg

Despite the attraction of these more recent techniques, the chloramine T method was used here. The reasons for this were:

1. It is a quick, simple and effective method capable of producing high specific activities.
2. The method had been used successfull- for 2-3 years in the Department of Human Anatomy, Oxford, for the iodination of L.H. and more recently F.S.H., Prolactin and A.C.T.H. for use in radioimmunoassay.
3. By reducing the reaction temperature, the reaction time and the concentration of chloramine T, Tsuruhara et al (1971) had successfully used the method to label h-L.H. and h-C.G. to high specific activities with the preservation of biological activity for use in receptor binding studies. Similarly, chloramine T iodinated h-L.H. and h-C.G. had been used by Leidenberger and Reichert (1972a, b) for their highly successful radio-ligand receptor assay.

The original experimental plan was to investigate the use of the chloramine T method to label b-T.S.H. for use in plasma decay (part 3) and receptor binding studies (part 5), but to change to one of the newer techniques, such as the lacto-peroxidase method, if biologically active T.S.H.-^{125}I could not be produced. Unfortunately, despite the imperfections of the label yielded by the chloramine T method, the investigation of other techniques could not be undertaken due to lack of time.

2.2.2. Materials

T.S.H.: Highly purified b-T.S.H. (40IU/mg) was a gift

from Prof. J. G. Pierce, University of California, Los Angeles. This was dissolved in 0.05M phosphate buffer (pH7.5) to a concentration of 500µg/ml. Aliquots of 12-15µl were frozen in small stoppered plastic vials and stored for up to 12 months at -10°C to -20°C. The biological activity of the preparation was confirmed by McKenzie assay (table 2.5).

Iodide-^{125}I[1] was chosen for most iodinations since the shelf life of ^{125}I iodinated proteins is longer than that of ^{131}I labelled material, thus allowing fewer iodinations and less hurried experimentation (Hunter, 1971).

Buffers: 0.5M sodium phosphate[2] pH7.5; 0.05M sodium phosphate pH7.5; 0.05M sodium phosphate pH7.5-0.1M saline[2] (P.B.S.); 5% B.S.A.[3] in P.B.S. All buffers, except those used in the reaction mixture, contained merthiolate[4] (0.1g/l) to prevent bacterial growth.

Chloramine T[4] was taken from below the surface of the reagent in the jar and made up to 1mg/ml in 0.05M phosphate pH7.5 immediately before the reaction was started.

Sodium metabisulphite[5]: 3mg/ml solution in 0.05M phosphate pH7.5 made up immediately before the reaction was started.

Transfer solution containing 10mg bromphenol blue[5], 1g potassium iodide[6], 10g sucrose[6] and 100ml distilled water.

Sephadex G-50 (fine) was left overnight to swell in distilled water and the medium was changed to P.B.S. by

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(1) ^{125}I for protein iodination, 1.M.S.30, The Radiochemical Centre, Amersham
(2) Analytical reagent, B.D.H. Chemicals Ltd., Poole
(3) Cohn fraction V, Sigma Chemicals Ltd., Kingston-upon-Thames
(4) Laboratory reagent, B.D.H. Chemicals Ltd., Poole
(5) Laboratory reagent, B.D.H. Chemicals Ltd., Poole
(6) Analytical reagent, B.D.H. Chemicals Ltd., Poole

TABLE 2.5. The biological activity of pure b.T.S.H. (Pierce)

McKenzie assays of pure b.T.S.H. against int. std. T.S.H.

Date	Assay Design	λ_c	Potency(IU/mg)	95% Conf.limits
14.9.72.	3 + 1	0.30	38.1	19.1 - 77.3
28.11.72.	2 + 2	0.25	44.5	28.9 - 71.7

repeated decantation and addition of P.B.S. A 20 x 1cm column was then poured and approximately 50ml P.B.S. were run through it followed by 2-5ml 5% B.S.A.-P.B.S. and 20-30ml P.B.S.

2.2.3. Methods

In an attempt to reduce damage to a minimum, lower concentrations of chloramine T were employed than originally described by Greenwood et al (1963). Methods A and B below, based on the work of Tsuruhara et al (1972) and Leidenberger and Reichert (1972a), were used for the production of T.S.H.-^{125}I for plasma decay and receptor binding studies. Method C was similar to that of Greenwood et al (1963) and was used to prepare T.S.H.-^{125}I of very high specific activity for use in the radioimmunoassay described in section 2.3.

Method A (50µg chloramine T, 60 sec. reaction time, room temperature)

A plastic autoanalyser vial(1), covered with parafilm, was used to house the reaction and the procedure was performed in a lead-shielded fume cupboard(2). 100µl 0.5M phosphate pH7.5, 2.5µg T.S.H. (in 5µl) and 1mCi. ^{125}I (approximately 10µl) were pipetted(3) into the reaction vial, which was then counted as described below. The reaction was started by adding 5µl of the chloramine T solution. Mixing was facilitated by vigorous tapping until termination of the reaction at 60 sec. by the addition of 5µl sodium metabisulphite solution. Two drops of transfer solution were added and the contents of the

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(1) Technion Instrument Co., Basingstoke, Hants.
(2) Constructed by Mr. J. Glozier, Dept. Human Anatomy, Oxford
(3) All reagents except ^{125}I added using capillary pipettes (Volupettes, A. R. Horwell Ltd., London N.W.6). ^{125}I added with a 10 or 25µl Hamilton syringe (Hamilton Manufacturing A.G., Switzerland).

reaction vessel layered on the top of the Sephadex G-50 (fine) column, rinsing the reaction vessel and transfer pipette with buffer(1). The column was eluted with P.B.S., collecting 1ml fractions into plastic tubes(2) except for the iodinated protein peak which was collected in 0.5ml fractions into 0.5ml P.B.S.-1% B.S.A. (to prevent surface adsorption of labelled T.S.H.).

The empty reaction vessel plus the transfer pipette combined, and the eluted fractions were counted using an Ecko spectrometer(3) used in conjunction with an Ecko scaler(3). Samples were changed manually and placed in a well 10cm from the crystal. Because of the appreciable "dead time" of the counter and the high levels of radiation used, the counts registered were not linearly related to the dose. Therefore a correction curve, constructed by Dr. Fenella Greig and D. Nabarro by counting the fixed volume of doubling dilutions of an ^{125}I source, was employed (Nabarro, 1973). Furthermore, the difference in shape between the autoanalyser vials and LP3 tubes necessitated a further correction factor, which was also obtained from their data, and the counts were expressed in terms of LP3 tube counts (Nabarro, 1973).

The elution pattern from one such iodination is shown in figure 2.9. The tubes containing the main part of the protein peak were combined and stored in a refrigerator at 0-4^{o}C until use.

Method B (5µg chloramine T, 45 sec. reaction on ice)

This method was employed for all experiments using T.S.H. ^{125}I in part 3 and a few early experiments in part 5.

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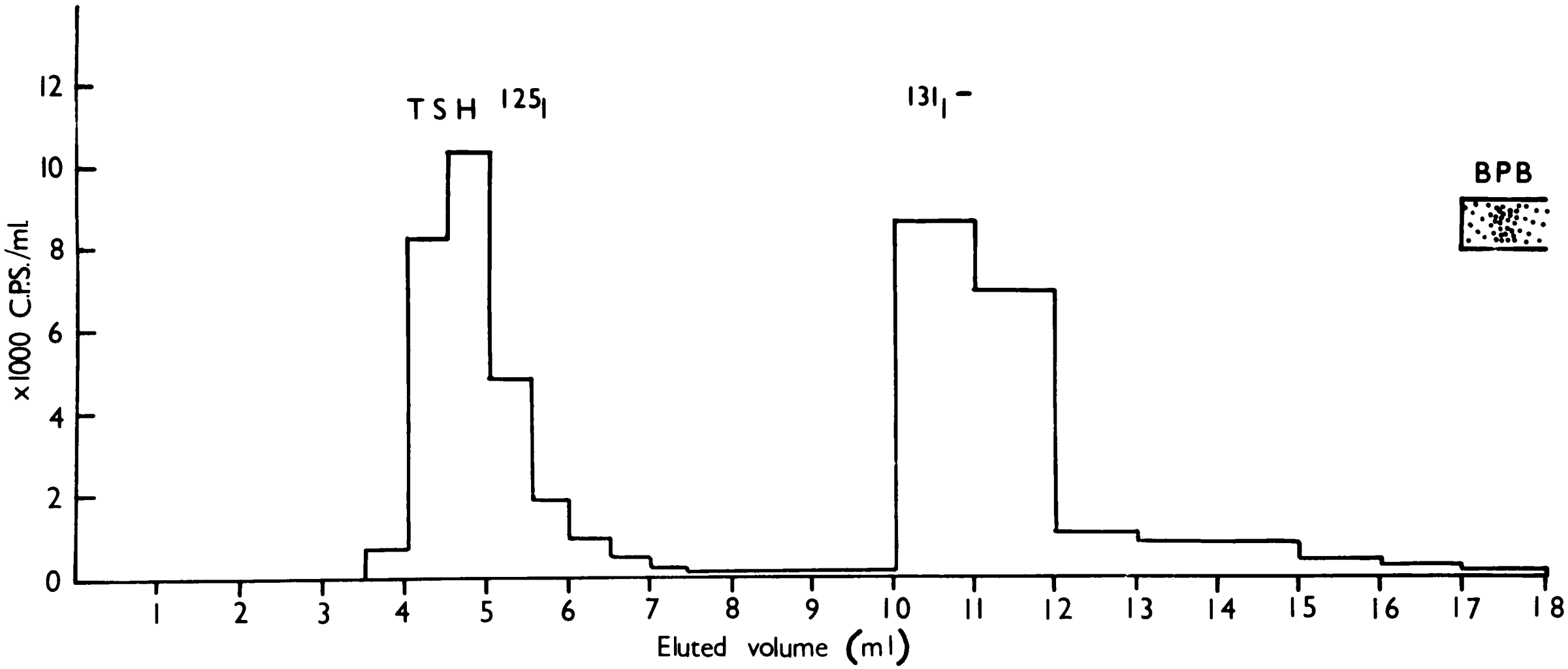
(1) In several experiments these steps were omitted for convenience and the specific activity left uncalculated
(2) LP3 tubes, Luckham Ltd., Burgess Hill, Sussex
(3) Ecko Ltd., Southend, Essex

FIGURE 2.9

The pattern of elution of the reaction mixture on Sephadex G-50 (fine) following iodination (by method A)


TSH 125I
131I –
BPB
12
10
8
6
4
2
0
x1000 C.P.S./ml
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
Eluted volume (ml)

The protocol was the same as for method A except for the following:

1. The iodination reaction was performed on ice,
2. The reaction time was reduced to 45 seconds.

It was hoped that these even gentler conditions would further reduce iodination damage and preserve biological activity at the expense of high specific activity.

Method C (50µg chloramine T, 60 sec. reaction time, room temperature)

In one experiment (section 2.3.3) ^{131}I labelled T.S.H. was prepared for use in radioimmunoassay by the method of Greenwood et al (1963). This procedure was the same as method A except for the following:

1. The reaction mixture consisted of 25µl 0.5M phosphate, 1.3µCi ^{131}I in 5µl chloramine T in 50µl.
2. The reaction was terminated by the addition of 125µg sodium metabisulphite in 25µl.

2.2.4. The estimation of specific activity

The calculation of specific activity presented a problem because the losses of protein were difficult to assess. Possible sources of this were:

1. Protein adsorbed to the reaction vessel. This may be estimated by measuring the radioactivity remaining in the vessel after repeated washes with buffer.
2. Protein adsorbed onto the column during gel filtration. This was minimised by presaturating the column with B.S.A. However, losses may still have occurred. Furthermore, whilst the total loss of radioactivity on the column was easily

obtained, the relative proportions of labelled protein and free radioiodide could not be calculated. Total losses on the column were small in comparison with the total radioactivity but significant in comparison with the yield of labelled protein. However, for convenience this figure was ignored and the total yield (mg) of protein (Y_p) was calculated as:

$$Y_p = \frac{C_p \times W_p}{(C_p + C_v)}$$

(where C_p = counts in protein peak;
C_v = counts adsorbed to reaction vessel;
W_p = added protein = 2.5µg)

and the specific activity (S.A.) was:

$$S.A. = \frac{C_p \times R_I}{C_I \times Y_p}$$

(where C_I = initial counts in reaction vessel;
R_I = radioactivity used for iodination)

The estimates thus obtained were:

Method A: 70 - 250µCi/mg (11 iodinations)

Method B: 25 - 110µCi/mg (12 iodinations)

Method C: 600µCi/mg (^{131}I) (1 iodination)

Assuming that the specific activity of ^{125}I was 17µCi/µg[1] and that the molecular weight of b-T.S.H. is 28,500 (Pierce, 1971) the substitution levels achieved with methods A and B were 1.0-3.5 and 0.5-1.5 atoms/molecule, respectively.

2.2.5. Rechromatography of T.S.H.-^{125}I

The appearance of "incubation damage" (Hunter, 1967, 1971) following iodination restricted the useful life of T.S.H.-^{125}I labelled by method A for receptor binding studies to 10-14 days. Therefore, T.S.H.-^{125}I was repurified on Sephadex G-100 10-14 days after preparation, prolonging use to 3-4 weeks (see section 5.8).

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(1) Stated to be 14µCi/µg by The Radiochemical Centre, Amersham, but was usually approximately 17µCi/µg. (Glover, J.S. personal communication)

Method. Approximately 0.25ml of the original T.S.H.-^{125}I (undiluted since first chromatography on Sephadex G-50), after adding a few crystals of sucrose to increase its density, was layered under a head of buffer on a 22 x 1cm column of Sephadex G-100 (prepared as described in appendix 3) and eluted at 20ml/hr with P.B.S. - 0.1% B.S.A.(1). 0.5ml fractions were collected manually in plastic tubes(2). Samples of 10µl were taken into a second set of tubes each containing 1ml water for counting in a Nuclear Chicago autogamma analyser at 12.5-75kev(3) for 4 min or 10,000 counts.

Figure 2.10 shows the elution pattern obtained. In order to eliminate aggregated T.S.H.^{125}I the ascending phase of the protein peak was discarded. The tubes containing maximum counts and those corresponding to the first part of the descending phase (in the case shown, fractions 11-15 inclusive) were combined and stored at 0-4^{o}C before further use (within 7 days).

2.3. THE PROPERTIES OF LABELLED T.S.H.

It is well known that iodination of polypeptide hormones may alter their properties (Hunter, 1967, 1971). Therefore, the homogeneity of T.S.H. labelled by method A or B was tested by both gel filtration on Sephadex G-100 and by polyacrylamide gel electrophoresis. Immunoreactivity and biological activity were also measured and compared in order to assess the suitability of the label as a tracer for receptor binding or plasma decay studies.

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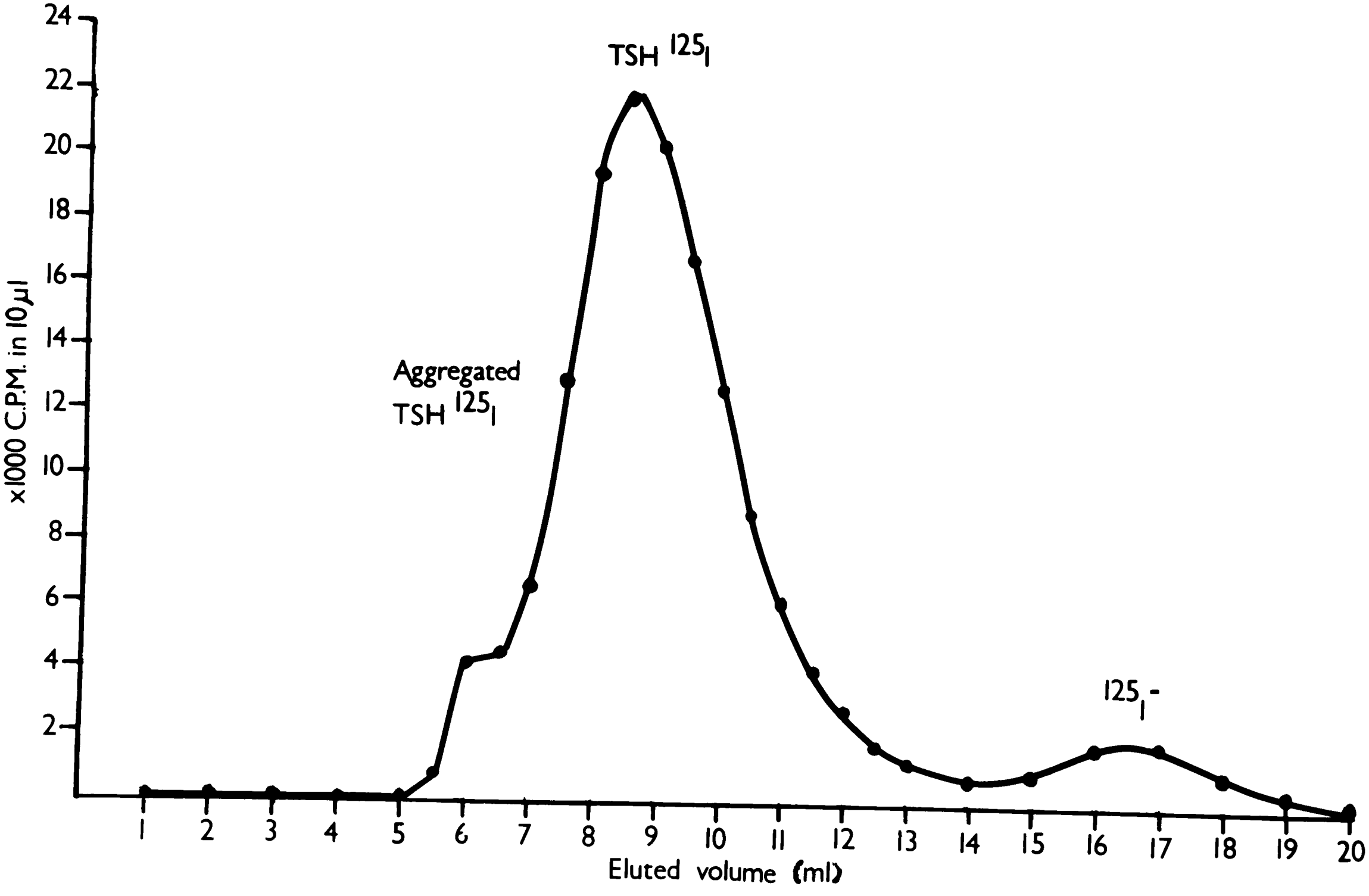
(1) Containing 0.1g/1 sodium merthiolate (B.D.H. laboratory reagent)
(2) LP3 tubes, Luckham Ltd., Burgess Hill, Sussex
(3) Calibrated with a ^{137}Cs standard source as directed by the manufacturers (Nuclear Chicago Ltd., analytical information no.32, August 17, 1970)

FIGURE 2.10

Rechromatography of T.S.H.-^{125}I

14 days after iodination by method A 0.25ml T.S.H.-^{125}I layered on 22 x 1cm column of Sephadex G-100. Eluted with P.B.S. -0.1% B.S.A. at 20ml/hr. Fractions 11-15 combined and retained for further use.


TSH 125I
Aggregated TSH 125I
125I-
x1000 C.P.M. in 10µl
24
22
20
18
16
14
12
10
8
6
4
2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Eluted volume (ml)

2.3.1. Gel filtration on Sephadex G-100

Method. Shortly after iodination, usually within 24 hours, about 50,000 c.p.m. of T.S.H.-^{125}I in 50-100µl P.B.S., containing sucrose[1] to increase its density, were layered under a head of buffer on a 100 x 1cm solumn of Sephadex G-100[2], prepared as described in appendix 3, and eluted at 12-20ml/hr with P.B.S. -0.1% B.S.A. Fractions of 1ml were collected in flat bottomed plastic counting vials, with the aid of a fraction collector[3] and counted for 4 mins. or 10,000 counts between 12.5 and 17.5kev in a Nuclear Chicago autogamma analyser.

Results. Figure 2.11 shows a typical elution pattern from an early experiment. T.S.H. labelled by method A or B ran essentially as a single peak, although a variable small proportion (usually <5%) eluted with the void volume. The latter probably represented molecular aggregates of T.S.H. In later iodinations, by either method, the proportion of aggregates was higher than initially, reaching over 6% in one sample of fresh T.S.H.-^{125}I. Furthermore, two extra peaks could be distinguished from the main T.S.H. peak in these experiments (figs. 3.3-3.6). By comparison with the elution patterns obtained by Nabarro (1973) for L.H.-^{125}I on calibrated 100 x 1cm columns of Sephadex G-100 S.F., it is suggested that these two peaks represented polymers and dimers of T.S.H.-^{125}I, respectively. The label also contained a small proportion of iodide ^{125}I (<5%). The proportion of both aggregates and iodide were noted to increase with time following iodination.

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(1) B.D.H. analytical reagent. A few grains per ml
(2) Pharmacia (G.B.) Ltd., Ealing, London W.5
(3) Central automatic fraction collector with 84 tube capacity, Scientific Supplies Ltd., Vine Hill, London E.C.1

FIGURE 2.11

The chromatography of T.S.H.-^{125}I on Sephadex G-100

T.S.H.-^{125}I iodinated by method B to a specific activity of 80mCi/mg 105 x 1cm column of Sephadex G-100 eluted at 18ml/hr with P.B.S. -0.1% B.S.A. The void volume is shown by the point of maximum concentration of dextran blue 2000[1] in a previous column run.

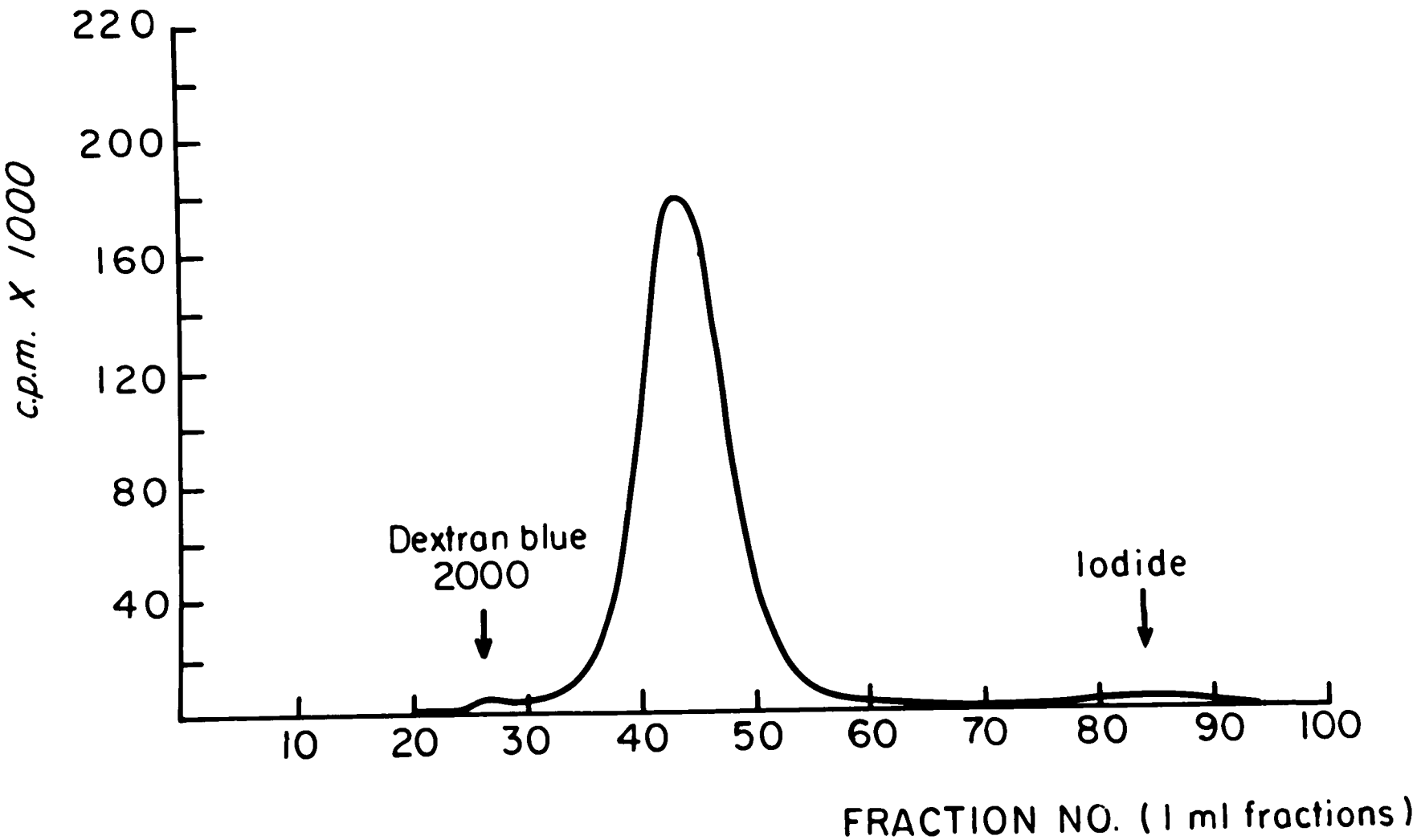
(1) Pharmacia (G.B.) Ltd., Ealing, London W.5

SEPHADEX G-100 CHROMATOGRAPHY OF TSH^{125} (SPECIFIC ACTIVITY 80 mCi/mg)

Column_105 X 1 cm

Buffer_0·05 M Phosphate pH 7·5 with 0·1 M NaCl, 0·1 gm % BSA and 10^{-4} Merthiolate

Flow rate_18 ml / hr



2.3.2. Polyacrylamide gel electrophoresis (P.A.G.E.)

In one experiment samples of T.S.H.-^{125}I, iodinated by methods A and B, were subjected to electrophoresis on a 7% polyacrylamide gel at pH9.5 as described by Liao et al (1969).

Method. The following solutions were freshly prepared:

A: 2.8g lyanogum 41[(1)] in 10ml distilled water.

B: 6.05g tris[(2)] + 0.2ml N,N,N´,N´, tetramethyl diamine[(1)] in 100ml distilled water adjusted to pH8.9-9.0 with ortho-phosphoric acid[(2)].

C: 14mg ammonium persulphate[(1)] in 10ml.

Buffer: 0.012M sodium glycinate pH9.5 was prepared as described in appendix 2.

The gel was prepared by mixing solutions A, B and C in the proportions 1:1:2, by volume, and pipetting 1ml aliquots into 0.5 x 6.0cm glass tubes, sealed with a rubber cap at one end, to give a 4.8cm column of gel. After setting, the caps were removed, the surface of the gel washed with buffer and the tubes inserted into a "Quickfit" P.A.G.E. apparatus[(3)] with buffer in both upper and lower compartments. 0.3ml of a solution of freshly labelled T.S.H. was then mixed with a few crystals of sucrose[(4)] and bromphenol blue[(5)] and 5-10µl of the resultant solution were layered on top of the gel with a finely drawn out Pasteur pipette. A current of 2 mα per tube was passed for 1 hr. at room temperature, by which time the band of bromphenol blue had reached the end of the tube. Following the run, gels were removed from the tubes, carefully cut into 2mm thick slices and counted for 1 min. at 12.5-75kev in a Nuclear Chicago autogamma analyser.

---

(1) B.D.H. laboratory reagent
(2) B.D.H. analytical reagent
(3) Quickfit and Quartz Ltd.
(4) B.D.H. analytical reagent
(5) B.D.H. laboratory reagent

Results. Figure 2.12 compares the patterns produced by T.S.H.-^{125}I labelled using method A and T.S.H.^{125}I labelled by method B. Both ran as single broad bands, the higher specific activity label travelling in advance. This was attributable to the increase in negative charge imparted to the molecule by increased levels of iodine substitution. There was no evidence of the heterogeneity reported for pure T.S.H. by Liao et al (1969) but this was probably attributable to the superimposition of heterogeneity due to varying iodine substitution levels and to the imprecision of the slicing technique. The patterns of triplicates were, however, similar. There was a small quantity of origin material present in the pattern produced by T.S.H.-^{125}I labelled by method B but the nature of this was uncertain.

2.3.3. Immunoreactivity

Anti-T.S.H. antibodies were raised in guinea pigs as described in appendix 5 and were used to assess the immunological activity of T.S.H.-^{125}I in two ways:

1. By finding the maximum percentage precipitable by excess anti-T.S.H. antibody.

2. By comparing the slopes of inhibition of binding curves produced by pure T.S.H. and T.S.H.-^{125}I in a radioimmunoassay using T.S.H. iodinated with ^{131}I by method C as the labelled antigen.

1. Precipitation of T.S.H.-^{125}I by excess antibody

Method. In one experiment, tubes(1) were set up in duplicate containing 0.5ml P.B.S.-0.1% B.S.A., 50µl of

(1) LP3 tubes, Luckham Ltd., Burgess Hill, Sussex

FIGURE 2.12

Polyacrylamide gel electrophoresis of T.S.H.-^{125}I under conditions of Liao et al (1969).

T.S.H.-^{125}I labelled by method A. - S.A. 150mCi/mg

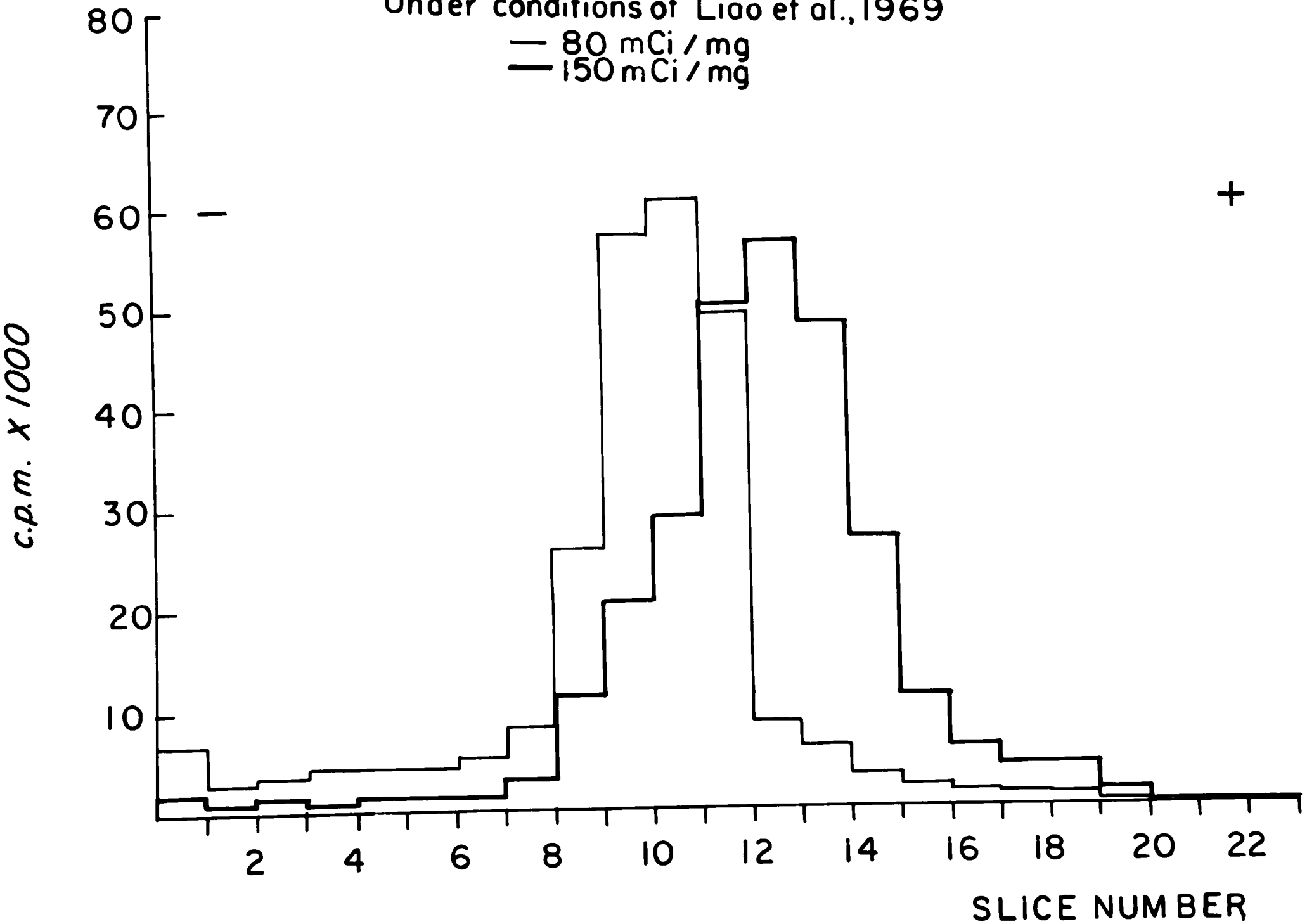
T.S.H.-^{125}I labelled by method B. - S.A. 80mCi/mg

POLYACRYLAMIDE GEL ELECTROPHORESIS OF TSH I^{125}

Under conditions of Liao et al., 1969

— 80 mCi / mg

— 150 mCi / mg



T.S.H.-^{125}I iodinated by method A (A17) or B (B18), containing approximately 20,000 c.p.m. (0.15-3mU T.S.H.),and 100µl 1 in 10 anti-T.S.H. antiserum (guinea pig 2, appendix 5) and incubated for three days at 4°C. 200µl of goat anti-guinea pig gamma globulin (precipitating antibody)(1) were added and the incubation continued for a further 24 hours. The tubes were then counted (12.5-75kev, 10^4 counts) and centrifuged for 45 mins. at 2,300g at 4°C(2). The supernatants were aspirated with a modified Pasteur pipette attached to a suction pump and the precipitate redissolved in 0.8ml 2N NaOH before recounting.

Results. As can be seen in table 2.6, T.S.H. iodinated by either method was more than 90% precipitable when the precipitating serum was added within three days of iodination. However, 7 days later, A17 was attenuated, giving only 81% precipitability, whereas B18 was relatively unchanged. This was probably attributable to incubation damage which naturally would have been greater in the higher specific activity preparation.

2. T.S.H. radioimmunoassay

Method. An antiserum dilution curve was first constructed as follows: Tubes(3) were set up on ice in triplicate each containing 0.5ml P.B.S.-0.1% B.S.A., 100µl of T.S.H.-^{131}I (iodinated by method C 150,000 c.p.m.). 100µl of an anti-T.S.H. antiserum at dilutions of 1 in 100 to 1 in 2,560,000 and 100µl normal guinea pig serum(4) were added with the aid of a

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(1) M.R.67, Wellcome Reagents Ltd., Beckenham, Kent
(2) M.S.E. Mistral, general purpose head 62303, multiple tube carriers, 3,100 r.p.m.
(3) LP3 tubes, Luckham Ltd., Burgess Hill, Sussex
(4) 100µl P.B.S.-0.1% B.S.A. were added instead of normal guinea pig serum to tubes containing 1 in 100 anti-T.S.H. antiserum to keep the total added serum to 1µl

TABLE 2.6. The precipitation of T.S.H.-^{125}I by excess antibody

Method	Iodination no.	Days after iodination[1]	% precipitable[2]
A	17	3	90 (88,91)
		10	81 (83,79)
B	18	3	94 (94,95)
		10	93 (94,92)

1. Time of addition of precipitating antibody

2. Mean (of duplicates)

repeater pipette(1). The tubes were incubated for 72 hours at 4°C before the precipitation of bound hormone by adding 200µl of goat anti-guinea pig gamma globulin(2) at a dilution of 1 in 12 (chosen empirically, using the manufacturer's recommendations as a guide). After a further 24 hours at 4°C the tubes were centrifuged for 45 mins. at 2,300g at 4°C. The tubes were aspirated and counted at 314-414kev in a Nuclear Chicago autogamma analyser. Figure 2.13 shows the antiserum dilution curve obtained. From this, the working antiserum dilution to be used for subsequent radioimmunoassay was selected at 1 in 30,000 (giving 50% bound).

Inhibition of binding by T.S.H. and T.S.H.-^{125}I labelled by method A or B was investigated. In order to circumvent the need for an investigation into the specificity of the antiserum (especially with regard to other pituitary hormones) highly purified ('pure') b-T.S.H. was used as the standard. The protein peak from each iodination was diluted to 33ml with P.B.S.-0.1% B.S.A., giving solutions A17 and B18. Tubes were set up in triplicate on ice, adding the following reagents in turn: 0.5ml P.B.S.-0.1% B.S.A., 0.5ml 'pure' b-T.S.H. solution at concentrations from 36.8pg/ml to 37.5ng/ml, or 0.5ml T.S.H.-^{125}I solution A17 or B18 at dilutions of up to 1 in 256; 15,000 c.p.m. T.S.H.-^{131}I in 100µl; 100µl, 1 in 30,000 anti-T.S.H. antiserum; 100µl, 1 in 100 normal guinea pig serum (all dilutions made in P.B.S.-0.1% B.S.A.). In three control tubes anti-T.S.H. antiserum was replaced by 100µl buffer.

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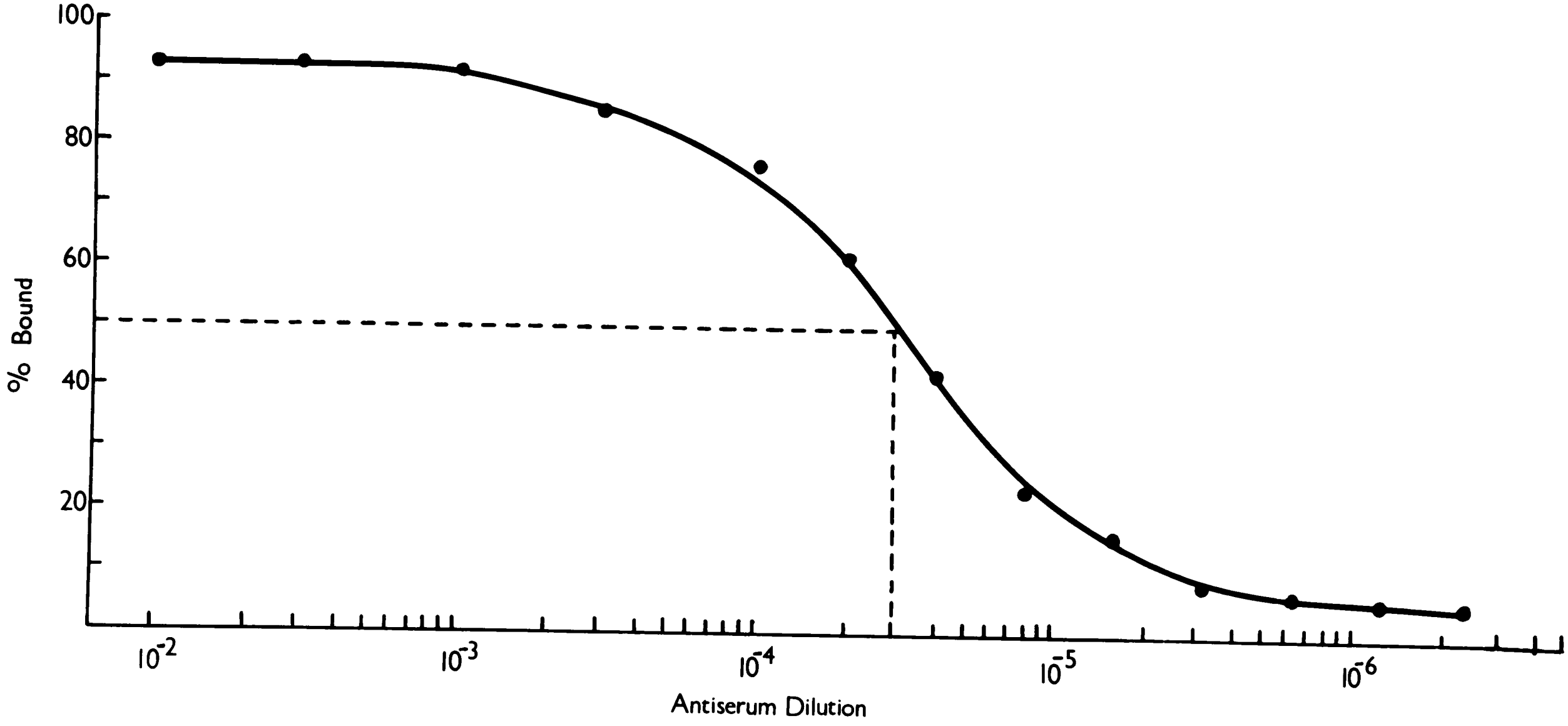
(1) Schwartz-Mann Biopette, Becton-Dickinson Ltd., Runcorn, Cheshire

(2) Precipitating serum for radioimmunoassay, M.R.67, Wellcome Reagents Ltd., Beckenham, Kent

FIGURE 2.13

Antiserum dilution curve for T.S.H. radioimmunoassay

(see Section 2.3.3.(2))


100
80
60
40
20
% Bound
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
Antiserum Dilution

The tubes were incubated for 72 hours at $4^{o}C$ before the addition of 200µl of diluted precipitating antibody(1). After a further 24 hours at $4^{o}C$ they were centrifuged, the supernatants aspirated and the tubes counted for ^{131}I as described above. At the energy range monitored (314-414kev), ^{125}I did not register significantly. Two such experiments were performed, the second antibody being added 3 (expt. 1) and 10 (expt. 2) days, respectively, after the iodination of the T.S.H.-^{125}I. In the first of these an unforeseen shortage of precipitating serum reduced the tubes to duplicates.

Results. The inhibition of binding obtained was plotted in terms of % bound vs. $\log_{10}$ /added T.S.H./ (figs. 2.14, 2.15) and in terms of logit (% bound) vs. $\log_{10}$ /added T.S.H./ (figs. 2.16, 2.17), where logit (% bound) was calculated as $\ln (B_x-B_o)/(B_{max}-B_x)$ (B_x = bound counts, B_o = bound counts in absence of antiserum, and B_{max} = bound counts in absence of carrier T.S.H.). Good inhibition of binding curves were obtained for T.S.H. and T.S.H.-^{125}I in both experiments but the logit transformation failed to linearise them. Mathematical verification of parallelism, analysis of variance or determination of potency and its confidence limits were precluded by the presence of both curvature and obvious heteroscadasticity. Nevertheless, by inspection, the three curves in each experiment (figs. 2.16, 2.17) ran parallel. Comparison of the 50% bound values (logit(% bound) = 0) gave estimates for immunological potency of T.S.H.-^{125}I A17 and B18 as 18.8ng/ml and 26.0ng/ml (table 2.7) corresponding to iodination yields of 25 and 35%

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(1) Precipitating serum for radioimmunoassay, M.R.67, Wellcome Reagents Ltd., Beckenham, Kent

FIGURE 2.14

Radioimmunoassay of T.S.H. (expt. 1)

Inhibition of binding curves for T.S.H. and T.S.H.-^{125}I (A17 and B18).

Second antibody added three days after iodination of T.S.H.-^{125}I.

Each point represents the mean of duplicates.

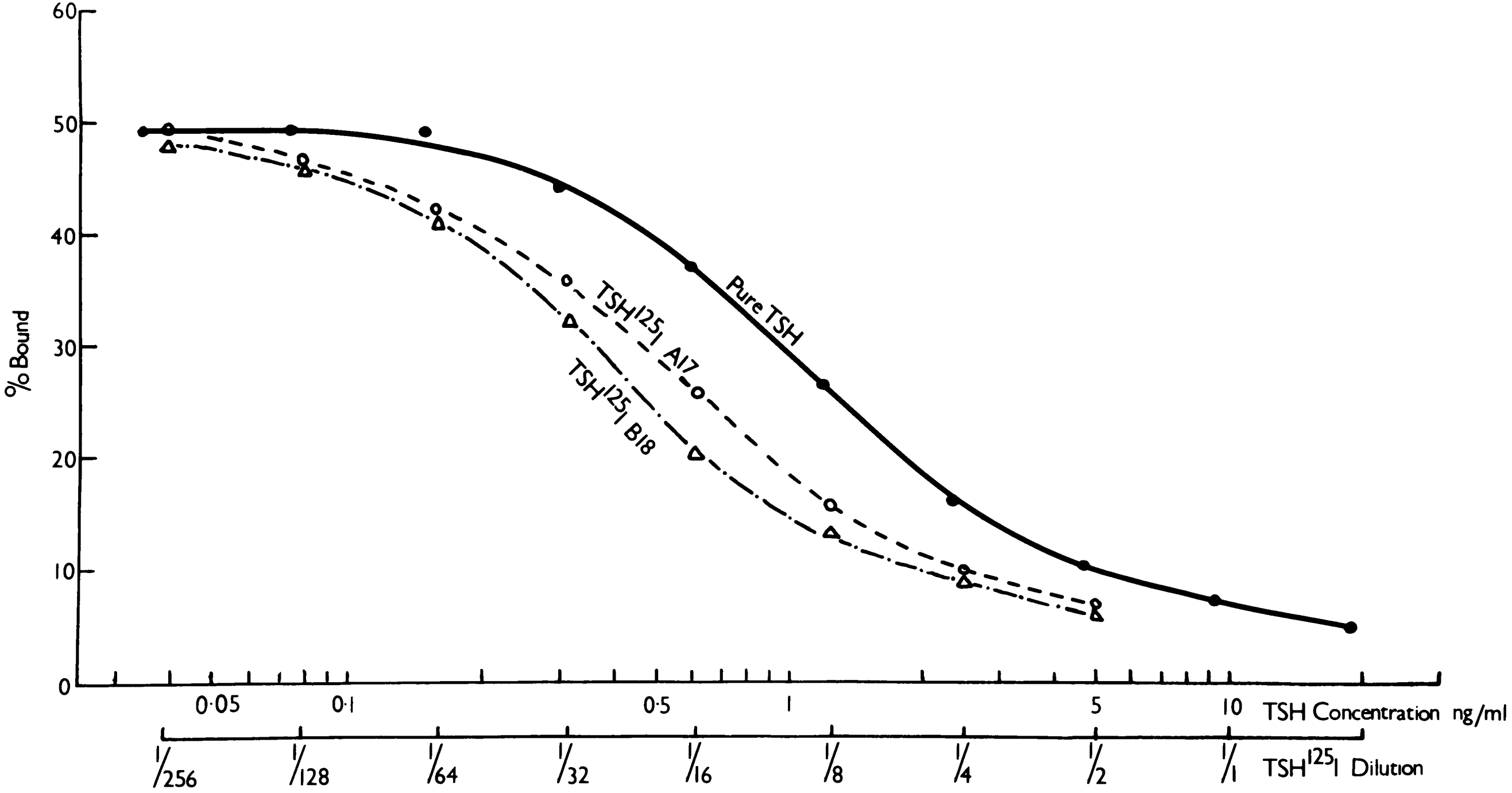
60
50
40
30
20
10
0
% Bound
Pure TSH
TSH125I A17
TSH125I B18
0·05
0·1
0·5
1
5
10
TSH Concentration ng/ml
1/256
1/128
1/64
1/32
1/16
1/8
1/4
1/2
1/1
TSH125I Dilution

FIGURE 2.15

Radioimmunoassay of T.S.H. (expt. 2)

Inhibition of binding curves for T.S.H. and T.S.H.-^{125}I (A17 and B18).

Second antibody added ten days after iodination of T.S.H.-^{125}I.

Each point represents the mean of triplicates.

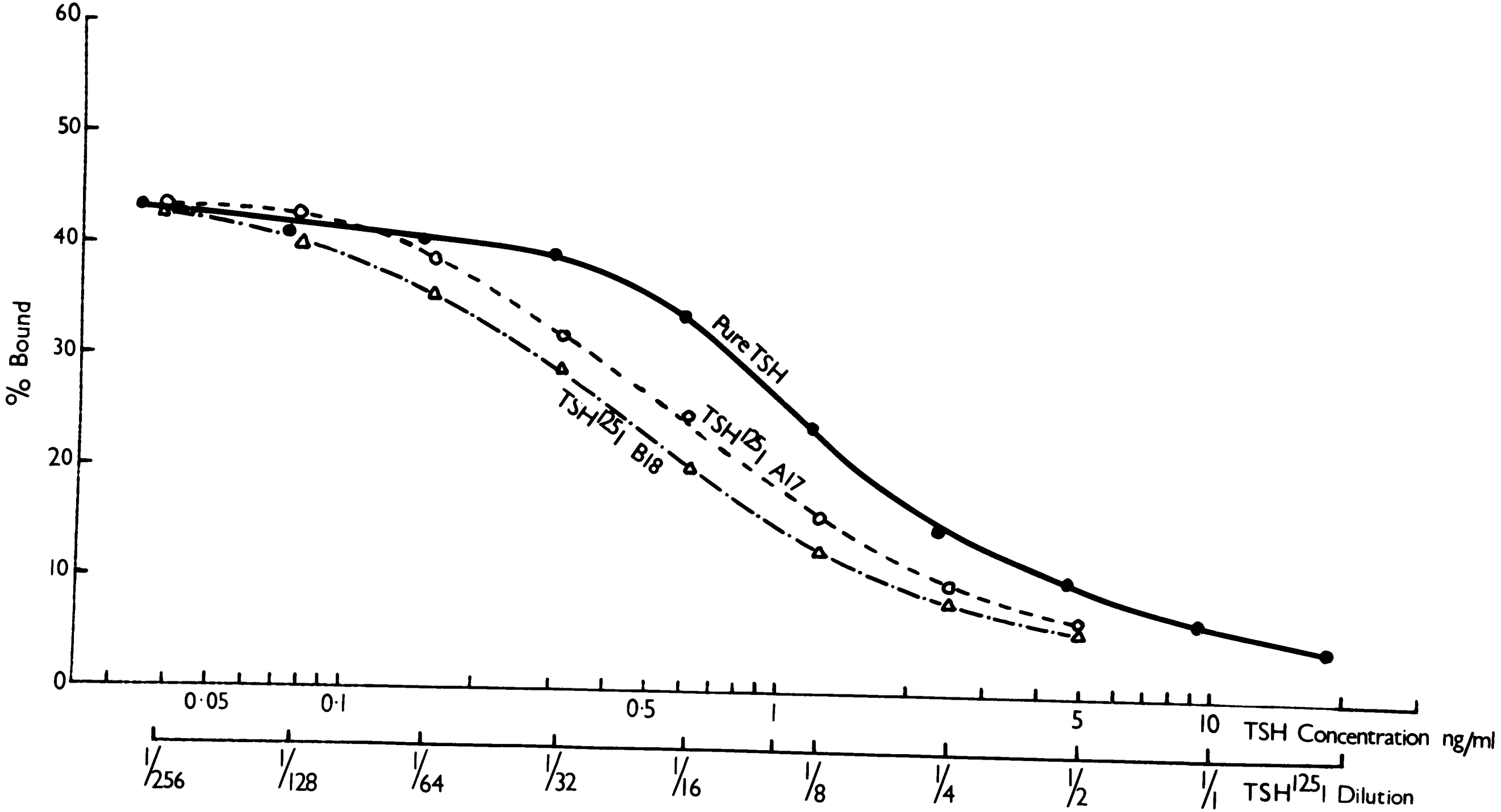
% Bound
60
50
40
30
20
10
0
Pure TSH
TSH125I A17
TSH125I B18
0·05
0·1
0·5
1
5
10
TSH Concentration ng/ml
1/256
1/128
1/64
1/32
1/16
1/8
1/4
1/2
1/1
TSH125I Dilution

FIGURE 2.16

Radioimmunoassay of T.S.H. (Experiment 1)

Logit binding curves for T.S.H. and T.S.H.-^{125}I (A17 and B18).

Second antibody added three days after iodination of T.S.H.-I^{125}.

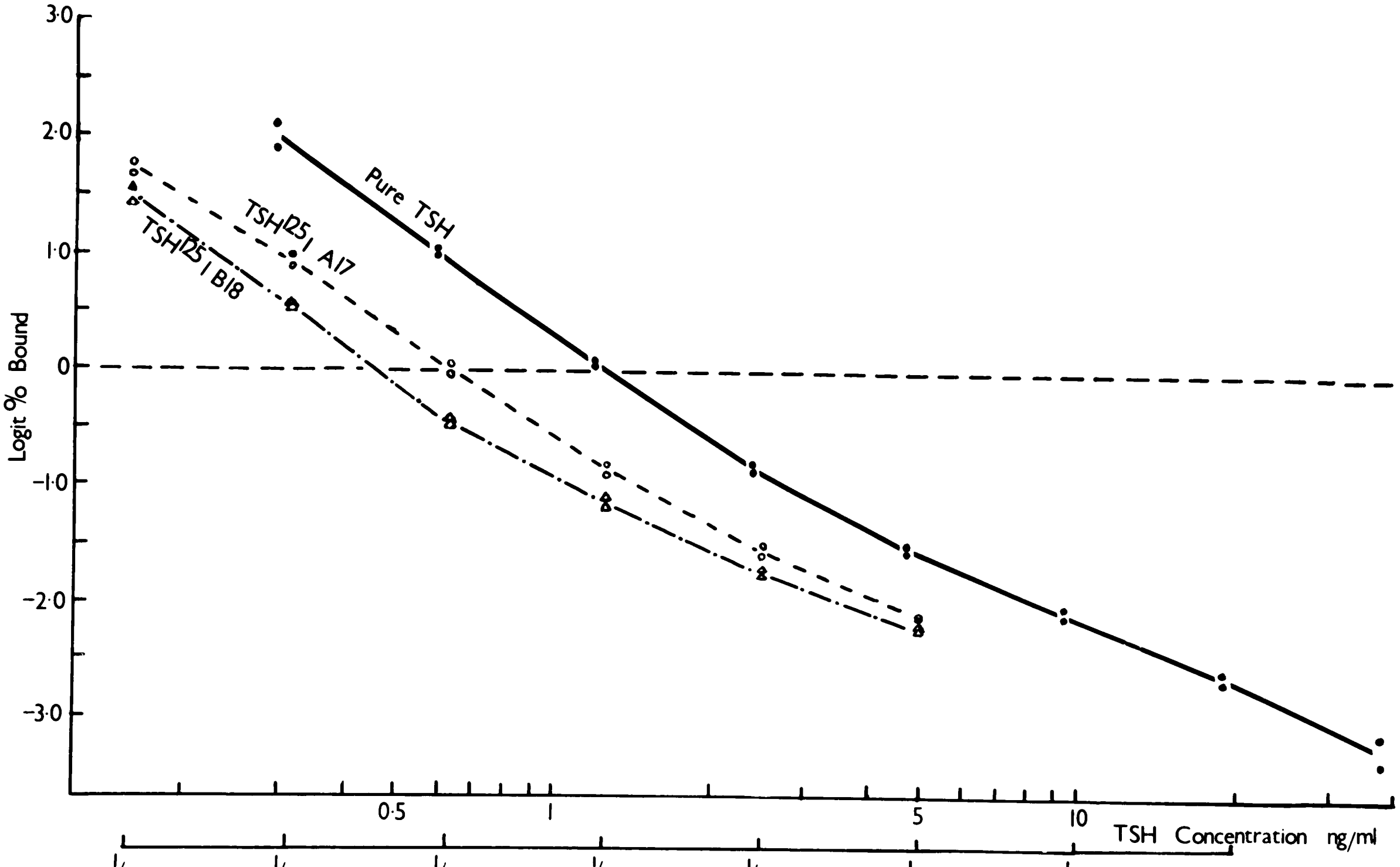
3·0
2·0
1·0
0
-1·0
-2·0
-3·0
Logit % Bound
Pure TSH
TSH I125 I A17
TSH I125 I B18
0·5
1
5
10
TSH Concentration ng/ml

FIGURE 2.17

Radioimmunoassay of T.S.H. (Experiment 2)

Logit binding curves for T.S.H. and T.S.H.-^{125}I (A17 and B18).

Second antibody added three days after iodination of T.S.H.-^{125}I.

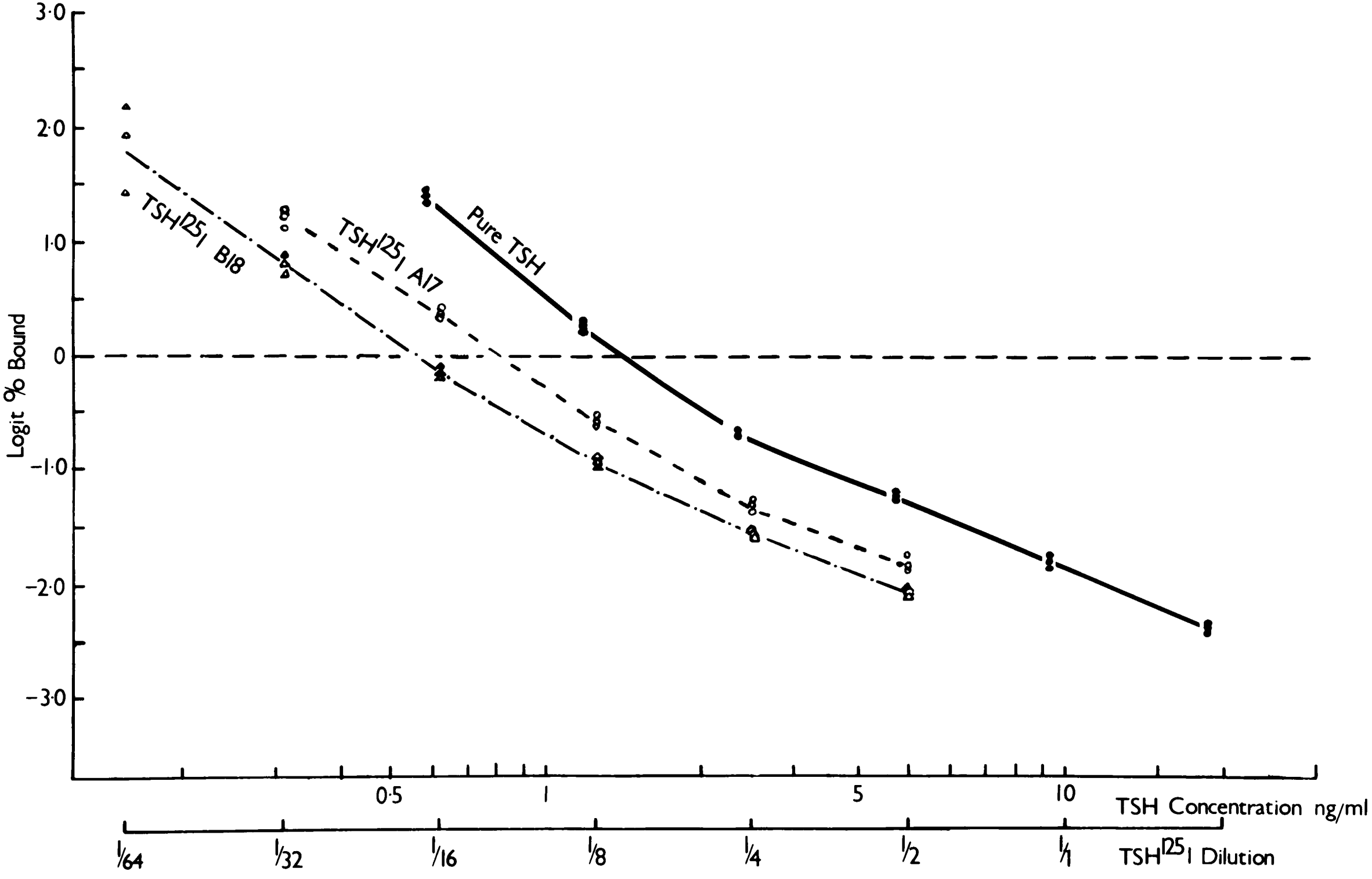
3·0
2·0
1·0
0
-1·0
-2·0
-3·0
Logit % Bound
TSH125I B18
TSH125I A17
Pure TSH
0·5
1
5
10
TSH Concentration ng/ml
1/64
1/32
1/16
1/8
1/4
1/2
1/1
TSH125I Dilution

TABLE 2.7. The immunological potency of T.S.H.-^{125}I by radioimmunoassay

Iodination	Time of addition of second antibody (days) (1)	Potency (ng/ml)	Mean (ng/ml)	% yield
A17	3	20.0	)	
			)18.8	25
A17	10	17.6	)	
B18	3	27.5	)	
			)26.0	35
B18	10	24.5	)	

1. Time following iodination of T.S.H.-^{125}I

respectively, with little obvious diminution of potency over the 7 days between experiments. The yields of T.S.H. from these iodinations calculated from the iodination data as described in section 2.2.4. were 62 and 41% for A17 and B18, respectively. However, these latter figures were probably unreliable for the reasons stated in section 2.2.4.

2.3.4. Biological activity

In several experiments portions of T.S.H.-^{125}I peak were diluted x8.33 and x25 for bioassay within 24 hours by the McKenzie method. 'Pure' b-T.S.H. was used as standard at concentrations of 42,125 and 375ng/100ml, except in one early experiment where International Standard T.S.H. was used. The yields of biologically active T.S.H. from the iodinations were subsequently calculated and compared with the yields from two "dummy" iodinations in which the addition of radioiodide, chloramine T and sodium metabisulphite was omitted.

The results are shown in table 2.8. Unfortunately, the λ values were higher than in previous assays, resulting in wide confidence limits and the rejection of data from two further assays. However, the results suggested that the yield of biologically active T.S.H. was substantially reduced by iodination, being 11.6-18.2% in comparison with 36-55% for the "dummy" iodinations. Thus it would seem that approximately 70% of the biological activity of the T.S.H. was lost during iodination.

Two of the T.S.H.-^{125}I preparations (A17 and B18) were also assayed immunologically (section 2.3.3). Table 2.9 compares the results. These confirmed the substantial

TABLE 2.8. The biological activity of T.S.H.-^{125}I

Iodination	S.A.(mCi/mg)	Sex of mice	Assay[1]	λ_c	Yield%	95% Conf.
Dummy 1	-	F	3 + 2	0.27	55	34-84
Dummy 2	-	F	2 + 2	0.30	36	23-52
A17	110	M	2 + 2	0.38	11.6	6.0-34.2
A3	250	M	2 + 2[2]	0.31	13.4	8.5-19.9
B8	65	F	3 + 2	0.38	14.2	6.7-25.6
B13	30	F	2 + 2	0.19	10.2	11.2-23.2
B18	40	F	2 + 1[3]	0.28	18.2	5.1-34.6

1. All assays except last in series 3 + 2 in design but top dose omitted from calculation in four cases where appreciable departure from linearity found in 3 point curves.

2. Int. std. T.S.H. used as standard.

3. 3 + 1 assay top dose of standard left out of calculation as in note 1 above.

TABLE 2.9. Comparison of biologically and immunologically measured T.S.H. activity following iodination

Iodination	Yield (%) Bioassay	Immunoassay	Bioassay/immunoassay
A17	11.6	25	0.46
B18	18.2	35	0.52

diminution in biological activity of T.S.H. following iodination. However, assuming that immunological potency was unchanged by iodination, as was suggested by the high (>90%) precipitability of the label by excess anti-T.S.H. antibody and the parallelism of the inhibition of binding curves (section 2.3.3), the biological activity of T.S.H.-^{125}I labelled by chloramine T methods A or B was reduced by approximately 50% compared with that of untreated 'pure' T.S.H.

2.3.5. Conclusions on the purity of T.S.H. labelled with ^{125}I by the chloramine T method

b-T.S.H. iodinated with ^{125}I by method A or method B was found by physicochemical or immunological means to be at least 90% undamaged. Despite this, biological activity was substantially reduced (to between 30 and 50%)[1]. Very low specific activity b-T.S.H.-^{125}I prepared by the reaction of T.S.H. with iodine vapour, has been shown to retain full biological activity (Heideman et al, 1965). Since the levels of substitution in this report were similar to those achieved here ($\sim$ 1 atom/mol), the iodination method, rather than the introduction of iodine into the molecule, per se, was probably responsible for the reduction of biological activity in the present experiments. It was therefore concluded that oxidation of b-T.S.H. by exposure to chloramine T under the present conditions caused its partial inactivation and that gentler methods of oxidation were required for the preparation of fully active b-T.S.H. However, Goldfine et al (1974) have since succeeded in labelling T.S.H. with ^{125}I without loss of

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(1) According to Field (1975), Amir et al have also found only 30-40% retention of biological activity with iodination of T.S.H. by a low dose chloramine T method

biological activity by reducing the chloramine T concentration even further (to 10μg/ml) and employing stoichiometric quantities of reactants over a 30 minute reaction time. T.S.H. iodinated with lactoperoxidase has also been found to retain full biological activity (Field, 1975; Verrier et al, 1974). An alternative approach has been used by Bourke et al (1974a) who purified T.S.H.-^{125}I labelled by the chloramine T method by adsorption onto a concentrated thyroid receptor preparation, following by dissociation with thiocyanate.

During the assessment of its purity, iodinated T.S.H. was being used in two types of experiment:

1. Studies on the decay rate of T.S.H.-^{125}I in the blood following injection into mice (part 3).
2. Studies on the binding of T.S.H.-^{125}I to thyroid cell fractions (part 5).

Since useful results were obtained in both categories and these agreed with the data of others (where comparisons were possible) this imperfect tracer was used to complete existing data. It is recommended that future studies in these fields should employ T.S.H. iodinated by gentler oxidation. Indeed, this was envisaged but prevented by a shortage of time.

2.4. THE PREPARATION OF IgG FROM HUMAN PLASMA OR SERUM

2.4.1. Introduction

In several experiments into the effects of L.A.T.S., where the presence of other plasma proteins might interfere (parts 3 and 5), the IgG fractions isolated from L.A.T.S. and control plasma were used. In addition, purified IgG was

iodinated as described in section 2.5 for use in experiments to compare the plasma half-life of IgG and L.A.T.S. (section 3.4).

2.4.2. Method

IgG was prepared from human plasma or serum by the method of Baumstark et al (1964) with slight modifications. 10g D.E.A.E.-Sephadex (A-50)(1) were allowed to swell in 1-2 litres of distilled water for at least 30 mins. The 'fines' were decanted 2-3 times with resuspension in further 1 litre aliquots of distilled water. The gel was filtered through a Buchner funnel and washed with 1 litre 0.5N NaOH(2) followed by 1-2 litres distilled water, 1 litre 0.5N HCL(2) and a further 1-2 litres distilled water, leaving the gel in the chloride form. It was then resuspended in 1-2 litres distilled water and the pH adjusted to 6.5 by adding 1N NaOH(2) dropwise, monitoring pH with a meter (previously calibrated using standard solutions). The fines were again decanted and the gel refiltered on a Buchner funnel, washing again with 0.01M phosphate buffer, pH6.5.

The damp gel was divided into two roughly equal portions. One half was stirred with 25ml of the serum or plasma for 1 hr. on ice. If the mixture was too stiff, buffer was added until the stirrer moved freely. The mixture was filtered on a Buchner funnel and washed five times with 10ml aliquots of buffer. The filtrate was stirred for 1 hr. on ice with the other half of the D.E.A.E.-Sephadex, filtering again and washing the gel with 5 x 20ml aliquots of buffer.

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(1) Pharmacia (G.B.) Ltd., Ealing, London W.5
(2) B.D.H. analytical reagent

The volume of the filtrate was measured in a measuring cylinder and anhydrous sodium sulphate[1] was added at a concentration of 18g/100ml, stirring until it completely dissolved. The IgG was spun down[2] and resuspended in 5-10ml saline (for use in experiments in part 3) or phosphate buffered saline pH7.5 (P.B.S.) (for use in experiments in part 5). Excess sodium sulphate was removed by dialysis against at least 2 litres saline or P.B.S. for 24 hr. Denatured protein was removed by centrifugation and the solution frozen and stored at -10 to -20°C after putting aside a small quantity (0.2ml) for protein estimation and electrophoresis.

2.4.3. Protein estimation

The concentration IgG was measured by the method of Lowry et al (1951) as follows:

Method. Immediately before the protein estimation two reagents were made up from stock solutions:

Reagent A: 10ml 0.8% sodium hydroxide[3] + 10ml 4% sodium carbonate[3] + 0.4ml 1% sodium potassium tartrate[4] + 0.4ml 0.5% copper sulphate (pentahydrate) solution.

Reagent B: 1ml Folin Cioculteau reagent[4] + 3ml distilled water.

12.5, 25, 50 and 100mg/l bovine serum albumin[5] solutions were used as standards. 1ml reagent A was mixed with 1ml distilled water (blank), standard protein solution (standards) or test solution diluted to give a concentration in the order of 50mg/l (usually 5µl IgG in 1ml distilled water). Ten minutes later, 0.2ml of reagent B was added and the tubes were

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(1) B.D.H. analytical reagent
(2) M.S.E. Mistral 20°C general purpose head 62303 with 8 x 50ml buckets, 2800 r.p.m. (2000g) for 30 mins.
(3) B.D.H. analytical reagent
(4) B.D.H. laboratory reagent
(5) Cohn fraction V, Sigma Chemical Co., Kingston-upon-Thames

read against the water blank in a spectrophotometer[1] at 750nμ after a further 30 minutes. Duplicate estimations were made and the protein concentration of the test solutions read off the standard curve.

2.4.2. Immunoelectrophoresis

An estimate of the purity of prepared IgG was obtained by immunoelectrophoresis performed by a modification of the microtechnique of Scheidegger (1955) as described in the L.K.B. 6800 A operating manual.

Method. A 0.07M veronal/acetate buffer pH8.6 was used (containing 6.87g sodium barbitone[2], 1.23g/l barbitone[2] and 2.73g/l sodium acetate[2] with 0.1g/l sodium mertholate[2] as antibacterial). A 1% agarose gel containing 40g/l dextran, to improve the definition of precipitin lines (Ceska and Grossmuller, 1968; Hellsing, 1969), was prepared by heating 1g agarose[3], 4g dextran 10[4], 25ml buffer and 75ml distilled water to about 90°C on a hot plate with constant stirring. 12ml of the solution were pipetted onto three clean microscope slides in one compartment of a special carrier[5] holding six slides to give a layer approximately 2mm thick. The gel was left for 10 minutes to set and a further 30 minutes were allowed in a damp compartment for hardening. The circular wells (1mm diameter, separated by 7mm) with a 1mm trough midway between were cut with the aid of an L.K.B. immunoelectrophoresis gel cutter[6]. The wells were filled with IgG solution using a drawn

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(1) Unicam 600 with 1cm x 3ml glass curvettes, Unicam Instruments, Cambridge
(2) B.D.H. laboratory reagents
(3) B.D.H. laboratory reagent
(4) Pharmacia (G.B.) Ltd., Ealing, London W.5
(5) Constructed by the workshop, Department of Human Anatomy
(6) L.K.B. Instruments Ltd., Sweden

out Pasteru pipette until the liquid was level with the gel and the carrier containing the slides put across an electrophoresis tank(1) filled with 0.07M veronal/acetate buffer pH8.6 using strips of filter paper soaked in buffer to connect the ends of the carriers to the buffer compartments. A potential difference of 250v (10c/cm) was applied for 1 hour, when the carriers were removed and the troughs filled with 0.1ml horse anti-human serum(2). In early experiments controls were also run using sheep anti-human IgG(3), anti-human IgA(4) and anti-human IgM(5) at appropriate concentrations (50%, 50% and 10%, respectively), selected empirically. Precipitin lines were viewed by darkfield illumination after 18-24 hours in a damp compartment.

Results. No contamination by any other protein fraction could be detected in any IgG sample prepared as above. Examples are shown in figure 2.18.

Comments. As is evident from the lower photograph (B) the precipitin lines for the prepared IgG were shorter than those for the native serum, the anodal (acidic) portion having been removed during preparation. However, this should not have affected the L.A.T.S. potency of the prepared IgG since L.A.T.S. activity can be detected throughout a wide range of isoelectric pH (Adlkofer et al, 1973; Miyai and Werner, 1966; Smith et al, 1969).

2.5. THE IODINATION OF HUMAN IgG

In experiments to investigate the blood decay rate of

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(1) Shandon Scientific Co. Ltd., Willesdon, London N.W.10
(2) M.R.40, Wellcome Reagents Ltd., Beckenham, Kent
(3) M.R.54, Wellcome Reagents Ltd., Beckenham, Kent
(4) M.R.80, Wellcome Reagents Ltd., Beckenham, Kent
(5) M.R.55, Wellcome Reagents Ltd., Beckenham, Kent

FIGURE 2.18

Immunoelectrophoresis of IgG

A. C.P.G. Plasma
← Anti h-serum
C.P.G. IgG

B. C.P.G. Plasma
← Anti IgG
C.P.G. IgG

A

B

L.A.T.S. in the mouse, h-IgG was labelled with ^{125}I by the chloramine T method (Greenwood et al, 1963) for use as a tracer (section 3.4).

Method. The materials and methods were similar to those for T.S.H. iodination by method A (section 2.2.3), with slight differences.

1. The reaction mixture consisted of 100µl 0.5M phosphate pH7.5, 25µg h-IgG in 5µl P.B.S. and 500µCi ^{125}I in 5µl.
2. The reaction was started by the addition of 5µg chloramine T solution in 5µl and terminated after 60 seconds by the addition of 15µg sodium metabisulphite (in 5µl). In one experiment iodinations were also performed using 2.5 and 10µg chloramine T, the reaction being stopped by adding 7.5 and 30µg sodium metabisulphite, respectively.
3. A 20 x 1cm column of Sephadex G-50 (fine), presaturated with B.S.A., was used to remove radioiodide and other reactants, as described earlier (section 2.2.3).

The estimation of specific activity

Since there was little adsorption of protein onto the walls of the reaction vessel (<15%)(1), as judged by counts remaining in the vial following the reaction, the calculation was simpler. Losses of protein or radioactive iodine were ignored and the specific activity (mCi/mg) was calculated as:

$$S.A. = \frac{C_p \times R_I}{C_I \times W_p}$$

(where C_p = counts in protein peak
R_I = initial radioactivity = 0.5mCi
C_I = initial corrected counts(2)
W_p = added protein = 0.025mg)

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(1) Probably because of the larger quantity of protein used here
(2) Corrected for nonlinearity of scaler response and geometry as described in section 2.2.3

Table 2.10 compares the specific activities of IgG-^{125}I prepared using 2.5, 5 and 10µg chloramine T on the same day with the same batch of radioiodine. 2.5µg gave very poor iodination but there was little to choose between the results using 5 and 10µg chloramine T. Thus 5µg chloramine T was chosen for use in further iodinations to give adequate labelling with minimal exposure to oxidising agent. The specific activities achieved in three subsequent iodinations ranged from 5.8 to 12.0mCi/mg.

2.6. THE PHYSICOCHEMICAL PROPERTIES OF IgG-^{125}I

In an attempt to assess the purity of IgG labelled as above samples were subjected to gel filtration on Sephadex G-200 columns.

Method. A 130 x 1cm column of Sephadex G-200(1) in 0.1M tris(2) HCL(3)/0.2M NaCl(3) buffer pH8.0 was prepared as described in appendix 3 and calibrated with dextran blue 2000(1). 10ml 5% B.S.A. in buffer were eluted through the column to saturate it with protein thus preventing adsorption of labelled IgG. Approximately 10^6c.p.m. IgG-^{125}I were mixed with 0.3ml human serum plus a few crystals of sucrose and layered on the column under a head of buffer. The column was eluted at 10-12ml/hr and 1ml fractions were collected in flat bottomed plastic counting vials with the aid of a fraction collector. Tubes were counted for 4 minutes or 10,000 counts at 12.5-75kev in a Nuclear Chicago autogamma.

Results. A typical elution pattern is shown in figure 2.19. The small quantity of label (<1% in fig. 2.19) eluting

(1) Pharmacia (G.B.) Ltd., Ealing, London W.5
(2) B.D.H. laboratory reagent
(3) B.D.H. analytical reagent

TABLE 2.10. Comparison of specific activity of IgG iodinated with ^{125}I by the chloramine T method at different chloramine T concentrations

Chloramine T added (µg)	% iodination	S.A. (mCi/mg)
2.5	7.5	1.3
5	25	5.0
10	31.5	6.3

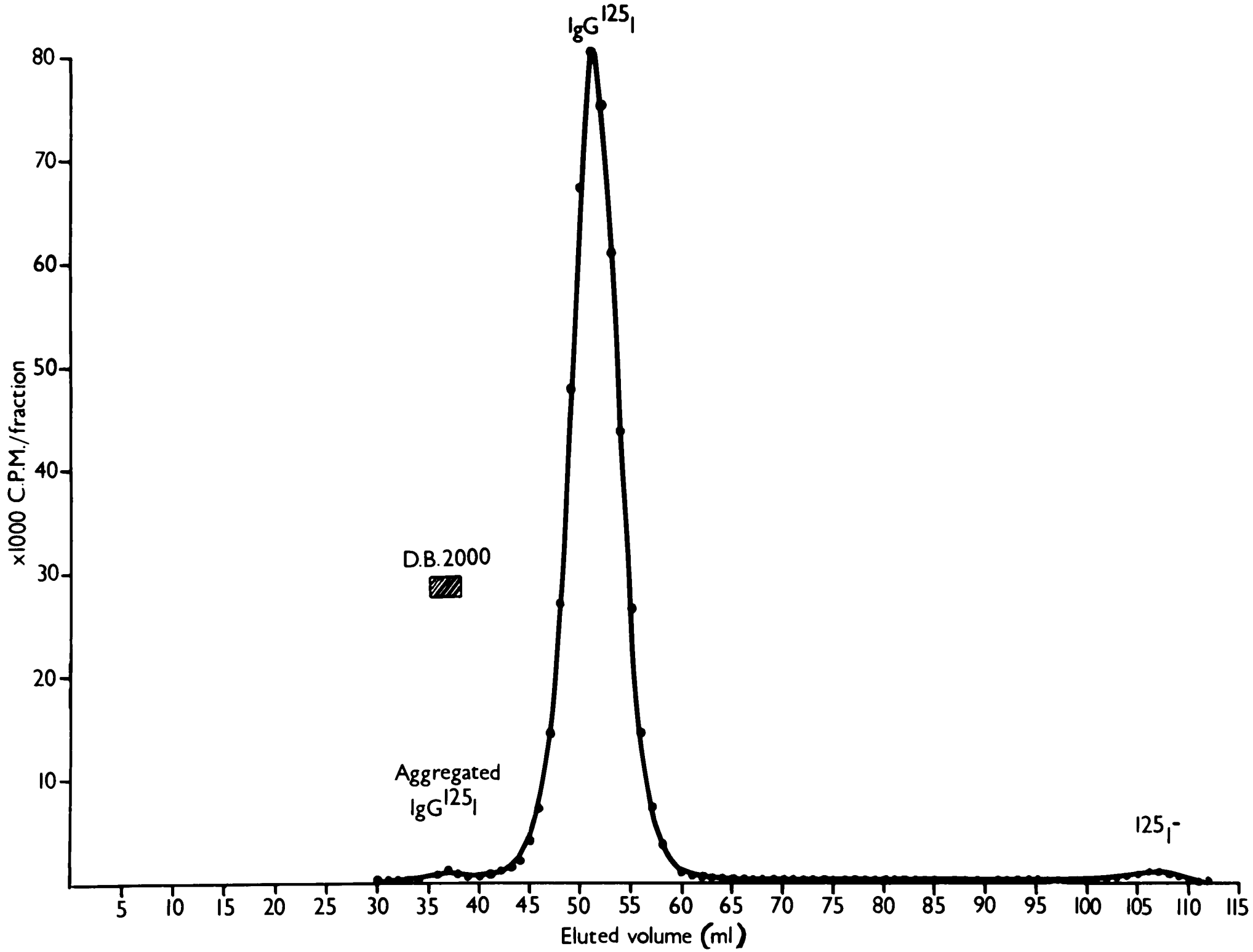
Reaction volume = 115µl

25µg protein + 500µCi ^{125}I

FIGURE 2.19

Gel filtration of IgG-^{125}I on Sephadex G-200

IgG (N.) iodinated to a specific activity of 10mCi/mg 130 x 1cm column of Sephadex G-200 eluted at 12ml/hr with Tris HCL/NaCl buffer pH8.0. (Presaturated with 10ml 5% B.S.A.) The void volume is shown by the point of maximum concentration of dextran blue 2000 in a previous column run.


IgG 125I
80
70
60
50
40
30
20
10
x1000 C.P.M./fraction
D.B.2000
Aggregated IgG 125I
125I-
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115
Eluted volume (ml)

with the void volume varied between different iodinations of the same IgG sample suggesting that this represented denatured or aggregated IgG-^{125}I rather than contaminating IgM. This percentage was always low and never exceeded 3% of the total counts.

Conclusions. Despite the purity on chromatography such material was unsuitable for IgG or L.A.T.S. studies because

1. Evidence of an altered plasma decay rate of IgG-^{125}I compared with IgG was accumulated in the study described in part 3.

2. During the work presented in this thesis Mori and Kriss (1971) showed that iodination by the chloramine T method inactivated L.A.T.S. IgG even at low chloramine T concentrations.

The way in which these difficulties were overcome is described in section 3.4.

PART 3

AN INVESTIGATION INTO THE TIME COURSE OF ACTION OF T.S.H. AND L.A.T.S. IN VIVO

3.1. INTRODUCTION

In early experiments the major feature distinguishing L.A.T.S. from T.S.H. was its longer time course of action, as reflected by the prolonged increase in blood radioactivity in the guinea pig or mouse assays (Adams, 1958; Adams and Purves, 1955, 1956; McKenzie, 1958a, b, 1960). The possible causes for such a difference are:

1. Prethyroid. A difference between the decay rates of L.A.T.S. and T.S.H.

2. Intrathyroid. (a) A slower onset of action of L.A.T.S. than of T.S.H., (b) A slower decline in activity following removal of L.A.T.S. than of T.S.H.

3. Post-thyroid. A reduction in the decay rates of the circulating radioactive products released by the thyroid in the presence of L.A.T.S. or T.S.H.

1. A difference between the decay rates of L.A.T.S. and T.S.H.

Adams (1960) showed that L.A.T.S. activity remained far longer than T.S.H. in the blood of rats following intravenous injection, decaying with a $t_{\frac{1}{2}}$ of approximately 7.5 hours, whereas both bovine and human T.S.H. fell to less than 5% of basal levels within 1 hour. McKenzie (1961) confirmed this in mice although the L.A.T.S. serum used was not potent and the bioassay responses were very variable. Similarly, the observed decay of plasma L.A.T.S. activity in human neonates with thyrotoxicosis (following removal from the maternal source of L.A.T.S.) is very slow with a $t_{\frac{1}{2}}$ of 4-14 days (Maisey and Stimmler, 1972; McKenzie, 1964; Sunshine et al, 1965) in

comparison with a $t_{\frac{1}{2}}$ of 54 minutes for h-T.S.H. in adult humans (Odell et al, 1967a, b). These results strongly suggest that the difference between the times courses of response following T.S.H. or L.A.T.S. injection can be accounted for by the slower removal of L.A.T.S. from the circulation. This is supported by the shorter time course of response in the McKenzie bioassay following injection of the active F_{ab} fraction of papain digests in comparison with that of the original L.A.T.S. IgG (Dorrington et al, 1966b; McKenzie, 1967), since removal of the F_c fragment is known to reduce substantially the half life of IgG (Speigelberg and Weigle, 1965). However, the only direct comparisons of L.A.T.S. and T.S.H. decay to date (Adams, 1960; McKenzie, 1961) were performed using bioassay and did not permit accurate assessments of their disappearance rates, nor did they allow comparison of the decay of IgG and L.A.T.S.

2. (a) A slower onset of action of L.A.T.S. than of T.S.H.

The latency of L.A.T.S. action on in vivo radioiodine release or colloid droplet formation by the mouse thyroid exceeds that of T.S.H. action by about 5 minutes (Shishiba et al, 1967). Since there is no difference in latency of action on colloid droplet formation or glucose-1-^{14}C oxidation by canine thyroid slices (Shishiba et al, 1970), the delayed action of L.A.T.S. in vivo may be due to slower diffusion of the bulkier L.A.T.S. molecules from the bloodstream to the receptor sites(1). However, this would be insufficient to

(1) A similar latency has also been reported in the in vitro guinea pig thyroid cytochemical assay of Bitensky et al (1974) and Petersen et al (1975). Delayed diffusion or slow association with the receptors could be a factor here.

account for the delayed peak of the L.A.T.S. response in the McKenzie assay.

(b) A slower decline in secretory activity following the removal of L.A.T.S. than T.S.H.

The decline of activity following removal of the tropic hormones has received little attention in endocrinology. The probable reason for this is the difficulty in removing hormones rapidly and completely, especially in vivo.

In vitro thyroid slice G-1-^{14}C oxidation (De Rubertis et al, 1975; Pastan et al, 1966), ^{32}P incorporation into phospholipids (Burke, 1968c) and cA.M.P. accumulation (De Rubertis et al, 1975; Malan et al, 1974) continue at an enhanced rate after removal from a T.S.H. stimulus. The prevention of this by the addition of trypsin or anti-T.S.H. antiserum has been taken as evidence of persistent binding of active T.S.H. by thyroid receptors. There is also some direct evidence of this from receptor binding studies (Field, 1975; see also part 5 discussion). Persistent binding of L.A.T.S. to receptors has been demonstrated by the abolition of persistently enhanced phospholipogenesis after L.A.T.S. by anti-h-IgG antiserum (Burke, 1968c). The relative rates of loss of persistently bound T.S.H. and L.A.T.S. were not studied.

Secretion by in vitro thyroid systems continues at an enhanced rate when T.S.H. is removed from the medium (El Kabir, personal communication; Malan et al, 1974; Tong, 1971; Walls, 1967). However, trypsin fails to abolish this effect, which may be due to the slow digestion of thyroglobulin within

colloid droplets (Malan et al, 1974). There is no data on the 'switch-off' of thyroid secretion following L.A.T.S. in vitro.

To the author's knowledge there is no published work on the 'switch-off' rate of the thyroid in vivo.

3. A difference in the plasma decay rate of the circulating products released by the thyroid gland in response to T.S.H. and L.A.T.S.

In the McKenzie assay, circulating radioactivity before or after stimulation is mainly in the form of thyroxine (70-80%) with smaller amounts of iodide and traces of triodothyroxine (Pinchera et al, 1965). Therefore any external influence retarding thyroxine disposal could produce an artificial 'response' or prolong and enhance an existing response to a true stimulator. Such a mechanism has been invoked to explain the appearance of an apparent stimulator in the serum of rabbits immunised against human thyroid microsomes in an attempt to produce an L.A.T.S.-like antibody (Florsheim et al, 1970; Solomon et al, 1970). However, it could not account for the difference between the time courses of T.S.H. and L.A.T.S. responses in the McKenzie assay since L.A.T.S. also produces a more prolonged increase in thyroid colloid droplets than T.S.H. (Shishiba et al, 1967). Nevertheless, little attention has been focused on whether T.S.H. or L.A.T.S. affects thyroxine disposal rate.

Sisson (1970) showed that very large doses of b-T.S.H. enhanced thyroxine degradation in thyroidectomised guinea pigs

maintained on a replacement dose of thyroxine. In contrast, Story and Perkins (1959) found no such effect in humans with intact thyroids. Florsheim et al (1970) found a variety of substances, including thyroglobulin and plasma proteins, which could affect thyroxine disposal rate, but they also failed to find any influence of T.S.H. in thyroidectomised mice given replacement doses of thyroxine. In the same study a single L.A.T.S. serum significantly slowed thyroxine decay but this effect may have been attributable to the injected plasma protein rather than L.A.T.S. per se. Moreover, the change observed was insufficient to account for the longer time course of action of L.A.T.S.

Despite incomplete data, it is undisputed that T.S.H. decays more rapidly than L.A.T.S. and it seems almost certain that this is the major cause of the difference in time course of action of the two stimulators. For this reason, attention was focused on this aspect and an attempt was made to quantify the disappearance rates using radiolabelled T.S.H. and L.A.T.S.-IgG. The decay of L.A.T.S.-IgG was also followed by measuring IgG by single radial immunodiffusion and by bioassay.

The time course of action of T.S.H. on the thyroid gland was studied by testing the effect of removing the hormone rapidly from the circulation with injections of anti-T.S.H. antiserum at varying times after T.S.H. and the results compared with the observed rapid decay rate of radiolabelled T.S.H.

3.2. STUDIES ON THE DECAY RATE OF INJECTED b-T.S.H. IN MICE

3.2.1. Introduction

T.S.H. decay rates have not been extensively studied in the past. Bakke and his coworkers (Bakke, 1963; Bakke and Lawrence, 1962) showed that the bioassayable T.S.H. activity following injection of b-T.S.H. into rats decayed exponentially with a mean $t_{\frac{1}{2}}$ of 13.7 mins. Marceau et al (1972) reported a similar pattern of decay with a mean $t_{\frac{1}{2}}$ of 8.7 mins. Fontaine (1970) obtained similar results with biologically labelled homologous T.S.H.-^{3}H, but his decay curve was significantly nonlinear. It is of interest that the decay curves of bioassayable bovine, rat or human T.S.H. are similar in the rat (Bakke, 1963) giving some validity to estimates of T.S.H. secretion rate on the basis of plasma T.S.H. levels and heterologous T.S.H. decay rates (e.g. Marceau et al, 1972). In man, decay appears to be slower, with a $t_{\frac{1}{2}}$ of 35 minutes when measured by bioassay following b-T.S.H. injection (Bakke, 1963; Bakke et al, 1962) or 54 minutes when measured by disappearance of ^{131}I labelled h-T.S.H. (Odell et al, 1967a, b).

In the mouse, the only attempt at measuring the decay rate of injected T.S.H. was that of McKenzie (1961), who showed that the serum of mice injected with 2mU b-T.S.H. was cleared of bioassayable T.S.H. within 15 min., but the results did not allow the construction of a disappearance curve.

3.2.2. Materials and methods

T.S.H.-^{125}I. Highly purified b-T.S.H., a gift from Professor J. G. Pierce(1), was labelled with ^{125}I by

(1) University of California, Los Angeles

method B (section 2.2).

Animals. Male albino mice[1] were bred in the animal house and fed an iodine poor dog biscuit diet for 2-4 weeks following weaning at 4 weeks. At the time of use, the majority weighed between 20 and 30g.

Experimental procedure. 0.5-1μCi fresh T.S.H.-^{125}I (equivalent to approximately 0.5mU unlabelled T.S.H.) was injected in 0.25ml 1% B.S.A. intravenously via the tail vein of each mouse following a short spell (2-5 min.) in the hot box to promote vasodilatation. Blood samples of 50μl were taken by orbital bleeding at accurately timed intervals of 1, 5, 10, 20, 40, 120 and 180 minutes (chosen empirically). Blood samples were taken into 0.5ml aliquots of distilled water in plastic counting vials[2] for counting at 12.5-75kev for 4 mins. or 10,000 counts by a Nuclear Chicago autogamma scintillation spectrometer. The tubes were then subjected to removal of free radioiodide by trichloracetic acid (T.C.A.) precipitation, amberlite I.R.A. 400 chromatography or immuno-precipitation before recounting. Decay curves were constructed by taking the mean of counts from 5 mice at each time interval following injection of label. In order to establish the purity of the injected label 5-10μCi of T.S.H.-^{125}I was chromatographed on a 100 x 1cm column of Sephadex G-100 as described in section 2.3.1.

T.C.A. precipitation. 0.2ml of outdated human plasma was added followed by 1.25ml 10% T.C.A.[3]. The tubes were

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(1) C.F.L.P. strain, Carworth Europe Ltd.
(2) 3ml flat bottomed tubes, PT 10944, Luckham Ltd., Burgess Hill, Sussex
(3) B.D.H. Laboratory reagent

spun in a bench centrifuge for 10 minutes and the precipitate washed twice by resuspension in 2ml 5% T.C.A. and recentrifugation.

Amberlite I.R.A. 400 chromatography. For this, blood samples were taken into 0.5ml of water-dialysed outdated human plasma and passed through microcolumns of amberlite I.R.A. 400 as described in appendix 6.

Immunoprecipitation. Blood samples were taken into 1ml 0.05M phosphate pH7.5 containing 0.1% B.S.A. in plastic counting vials(1) with the addition of 5µl anti-T.S.H. antiserum (guinea pig 2, appendix 5) followed three hours later (at 4°C) by 0.1ml goat anti-guinea pig gamma globulin(2). Eighteen hours later the tubes were centrifuged at 2,300g(3) for 45 minutes at 4°C, the supernatant aspirated and the precipitate redissolved in 0.5ml 3N sodium hydroxide solution for recounting.

3.2.3. The decay rate of T.S.H.-^{125}I in the mouse (expt. 3.1)

Results. Figures 3.1 and 3.2 show the blood decay curves over 3 hours and 40 minutes, respectively, before and after T.C.A. precipitation. The T.S.H.-^{125}I in this experiment contained approximately 1.4% molecular aggregates of T.S.H. and 1% iodide by Sephadex G-100 chromatography. Two points are evident from the curves:

1. T.S.H.-^{125}I was rapidly removed from the circulation and degraded, releasing iodide-^{125}I, which became the predominant

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(1) L.P.3 tubes, Luckham Ltd., Burgess Hill, Sussex
(2) M.R.67, Wellcome Reagents Ltd.
(3) M.S.E. Mistral 6L, general purpose head no.62303, multi-tube carriers, 3100 r.p.m.

FIGURE 3.1

The decay rate of T.S.H.-^{125}I in the mouse

(expt. 3.1)

4.77 x 10^5 c.p.m. T.S.H.-^{125}I, prepared by method B (containing 1.4% aggregates and 1% iodide). 5% T.C.A. precipitation performed as described in the text. Each point represents mean ± S.E.M. of 5 mice.

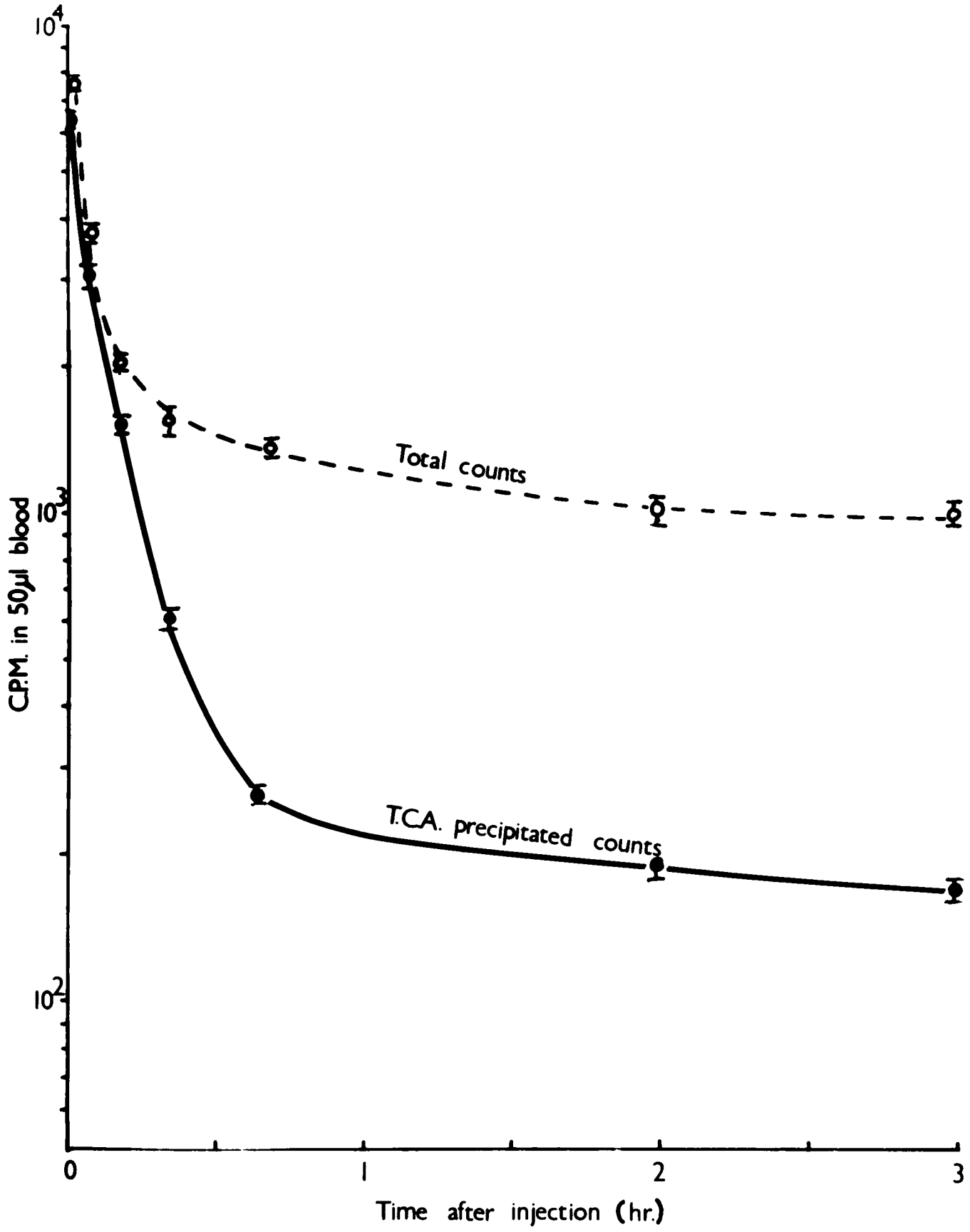
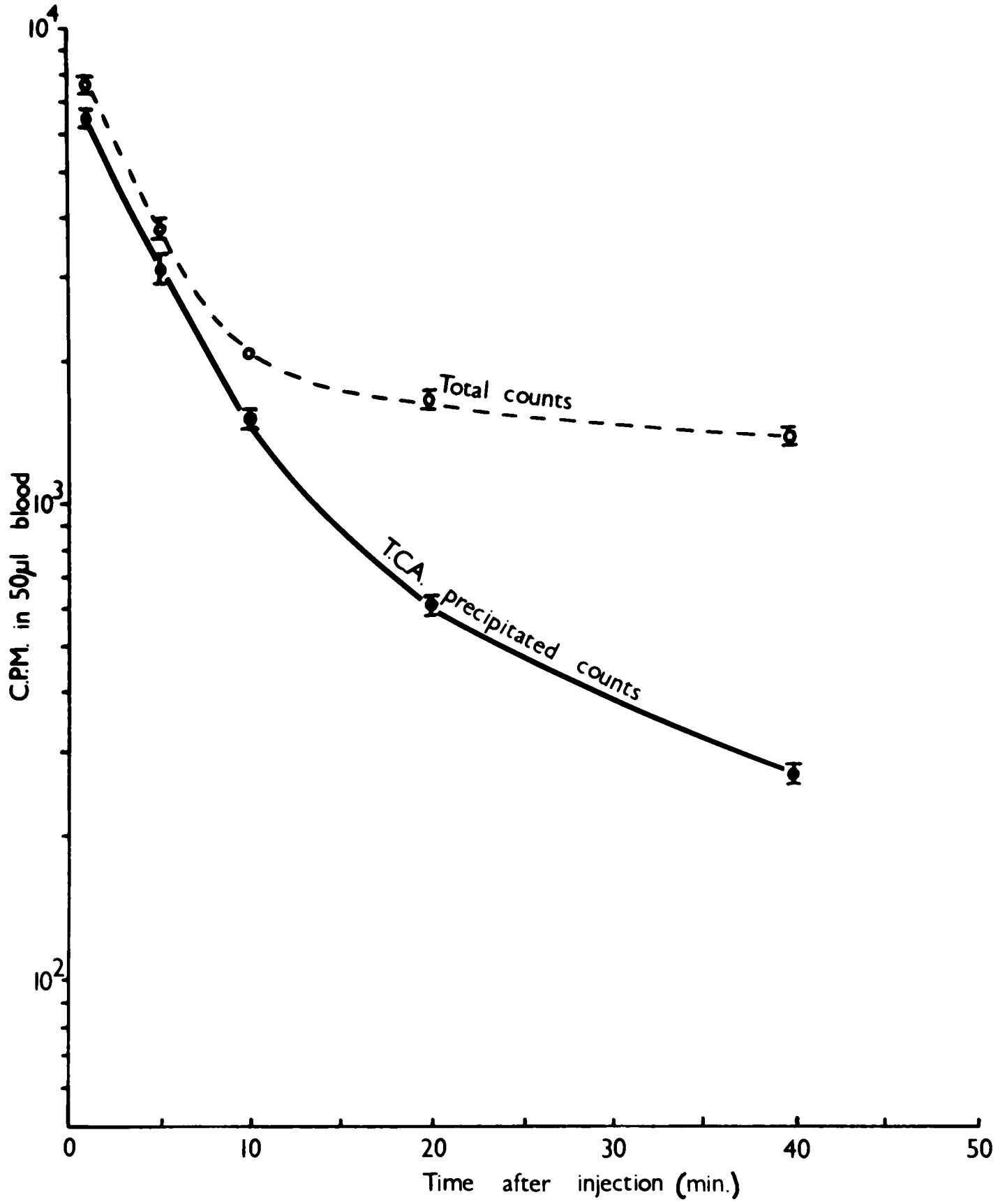

10^4
10^3
10^2
C.P.M. in 50µl blood
Total counts
T.C.A. precipitated counts
0
1
2
3
Time after injection (hr.)

FIGURE 3.2

The decay rate of T.S.H.-^{125}I in the mouse

The figure represents the same experiment as figure 3.1 (expt. 3.1) but on an expanded time scale.


10^4
10^3
10^2
C.P.M. in 50µl blood
Total counts
T.C.A. precipitated counts
0
10
20
30
40
50
Time after injection (min.)

source of circulating radioactivity at 20 minutes.

2. The decay of protein bound radioactivity was not a simple exponential. The initial rapid decay gave way to a second slower phase after 40 mintes. Since this second curve ran parallel to the total radioactivity it may have represented trapped iodide-^{125}I. Therefore the use of T.C.A. precipitation was re-examined.

3.2.4. A comparison of methods used for the removal of contaminating iodide from blood or plasma (expt. 3.2)

The possibility of contamination of precipitated counts by trapped iodide-^{125}I in the presence of a high iodide-^{125}I/ T.S.H.-^{125}I ratio made it essential to re-examine the use of T.C.A. precipitation in the present context. Therefore, the selectivity of T.C.A. precipitation was compared with that of immunoprecipitation or amberlite I.R.A. 400 chromatography by determining the remaining counts following the treatment of mouse blood or plasma containing T.S.H.-^{125}I and iodide-^{131}I(1).

Method. T.S.H.-^{125}I and iodide-^{131}I were added to blood, taken from mice by orbital puncture under ether anaesthesia, to give approximately 20,000 c.p.m. ^{125}I (12.5-75kev, Nuclear Chicago autogamma) and 10,000 c.p.m. ^{131}I (314-414kev, Nuclear Chicago autogamma) in 50µl aliquots. Half of the blood was then centrifuged and the plasma separated. Four 50µl aliquots of each blood and plasma sample were subjected to T.C.A. precipitation, immunoprecipitation or amberlite I.R.A. 400 chromatography as described above. Tubes were counted before and after treatment and corrections were

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(1) Sodium iodide-^{131}I, I.B.S.5, The Radiochemical Centre, Amersham

made for ^{131}I "crossover" onto the ^{125}I channel (by using a ^{131}I standard), radioactive decay between pre- and post-treatment counts and dilution in tubes treated by amberlite I.R.A. 400 absorption.

Results. (table 3.1) T.C.A. precipitation proved to be the least efficient procedure, especially when whole blood was used, leaving 17% of the iodide in the precipitate with a loss of 18% of T.S.H.-^{125}I counts. Immunoprecipitation appeared to have a slight advantage over amberlite chromatography for removal of iodide from plasma but the reverse was true for blood. Either method removed at least 97% of the iodide from blood or plasma. The low immunoprecipitability of the T.S.H.-^{125}I (85%) probably resulted from incubation damage, since 10 days had elapsed since its preparation.

Comments. In subsequent experiments, amberlite I.R.A. 400 chromatography was chosen as this appeared to have a slight advantage with whole blood samples. Whole blood was used for decay experiments because of the difficulty in separating plasma from the small samples of blood (50µl) necessary to allow repeated sampling without substantial blood volume depletion.

3.2.5. The fate of injected label (expts. 3.3-3.6)

Since injected T.S.H.-^{125}I was broken down rapidly, giving a nonlinear semi-logarithmic decay curve, it was of interest to see whether any labelled T.S.H. fragments could be detected in the blood stream. Therefore, in two experiments plasma samples from mice previously injected with T.S.H.-^{125}I were subjected to Sephadex G-100 chromatography.

TABLE 3.1. A comparison of the effectiveness of trichloracetic acid precipitation, immunoprecipitation and amberlite I.R.A. 400 chromatography for separation of T.S.H.-^{125}I and iodide-^{131}I

(section 3.2.3, expt. 3.2)

Medium	Method	% counts remaining(1)	
		T.S.H.-^{125}I	Iodide-^{131}I
Blood	T.C.A.	81.7 ± 2.0	16.7 ± 0.5
	Immunoprecipitation	84.9 ± 0.8	3.4 ± 1.2
	Amberlite I.R.A. 400	88.9 ± 0.5	1.2 ± 0.15
Plasma	T.C.A.	77.7 ± 3.1	4.1 ± 0.4
	Immunoprecipitation	85.1 ± 0.7	1.1 ± 0.05
	Amberlite I.R.A. 400	93.1 ± 0.5	2.9 ± 0.05

(1) Mean ± S.E.M. for 4 tubes

Method. Three pairs of mice were injected intravenously with 3-6μCi of T.S.H.-^{125}I each in 0.25ml 1% B.S.A. via the tail vein and were exsanguinated by orbital puncture under ether anaesthesia at 5, 15 or 40 minutes after injection. The blood from each pair was combined and the plasma separated. 300μl of the injection material and of each plasma were chromatographed sequentially on the same 100 x 1cm column of Sephadex G-100 as described in section 2.3.1 and the fractions counted. The proportions of aggregated material in the injected T.S.H.-^{125}I in these experiments were rather high: 6.1% (expt. 3.3) and 4.0% (expt. 3.4), respectively. The decay curve for each T.S.H.-^{125}I sample was constructed in separate experiments (performed on the same day) as described in 3.2.1 (expts. 3.5-3.6).

Results. The elution patterns obtained in expts. 3.3 and 3.4 are shown in figures 3.3 and 3.4, respectively. The decay curves appear in figure 3.5. The elution patterns show that no iodinated protein fragments appeared following injection. T.S.H.-^{125}I itself disappeared rapidly whilst labelled iodide accumulated. Some label running in advance of the T.S.H. peak was present in the original material but the proportion of this increased with time after injection. This was interpreted as a slowly-decaying contaminant rather than new material, since the proportion in plasma was dependent on the proportion in the injected label (figs. 3.3 and 3.4; see also fig. 3.8). The increase in the proportion of this material with time distorted the true decay curve and contributed to the non-linearity of the semi-logarithmic plot.

FIGURE 3.3

Sephadex G-100 chromatography of T.S.H.-^{125}I, (A), and plasma taken from mice at intervals following its injection (B) (expt. 3.3)

105 x 1cm Sephadex G-100 column. Buffer-P.B.S. containing 0.1% B.S.A. Flow rate 12ml/min. Elution pattern, A, shows that 6.1% aggregated T.S.H. was present in the injected T.S.H.-^{125}I.

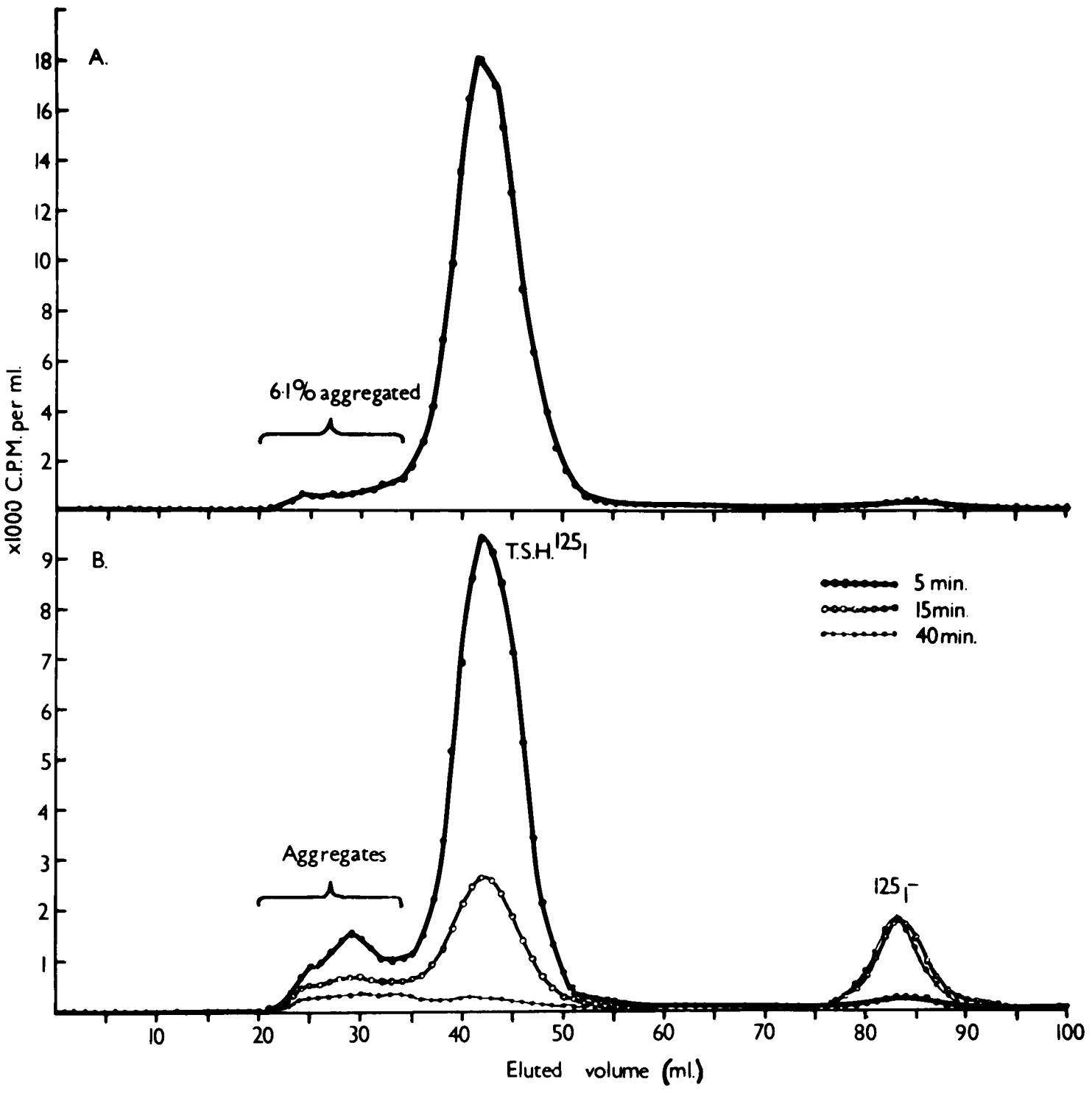

A.
B.
18
16
14
12
10
8
6
4
2
x1000 C.P.M. per ml.
6·1% aggregated
T.S.H. ^{125}I
5 min.
15min.
40min.
9
8
7
6
5
4
3
2
1
Aggregates
$^{125}I^-$
10
20
30
40
50
60
70
80
90
100
Eluted volume (ml.)

FIGURE 3.4

Sephadex G-100 chromatography of T.S.H.-^{125}I, (A), and plasma taken from mice at intervals following its injection (B) (expt. 3.4)

Conditions as for figure 3.3. 4.0% aggregates injected T.S.H.-^{125}I.

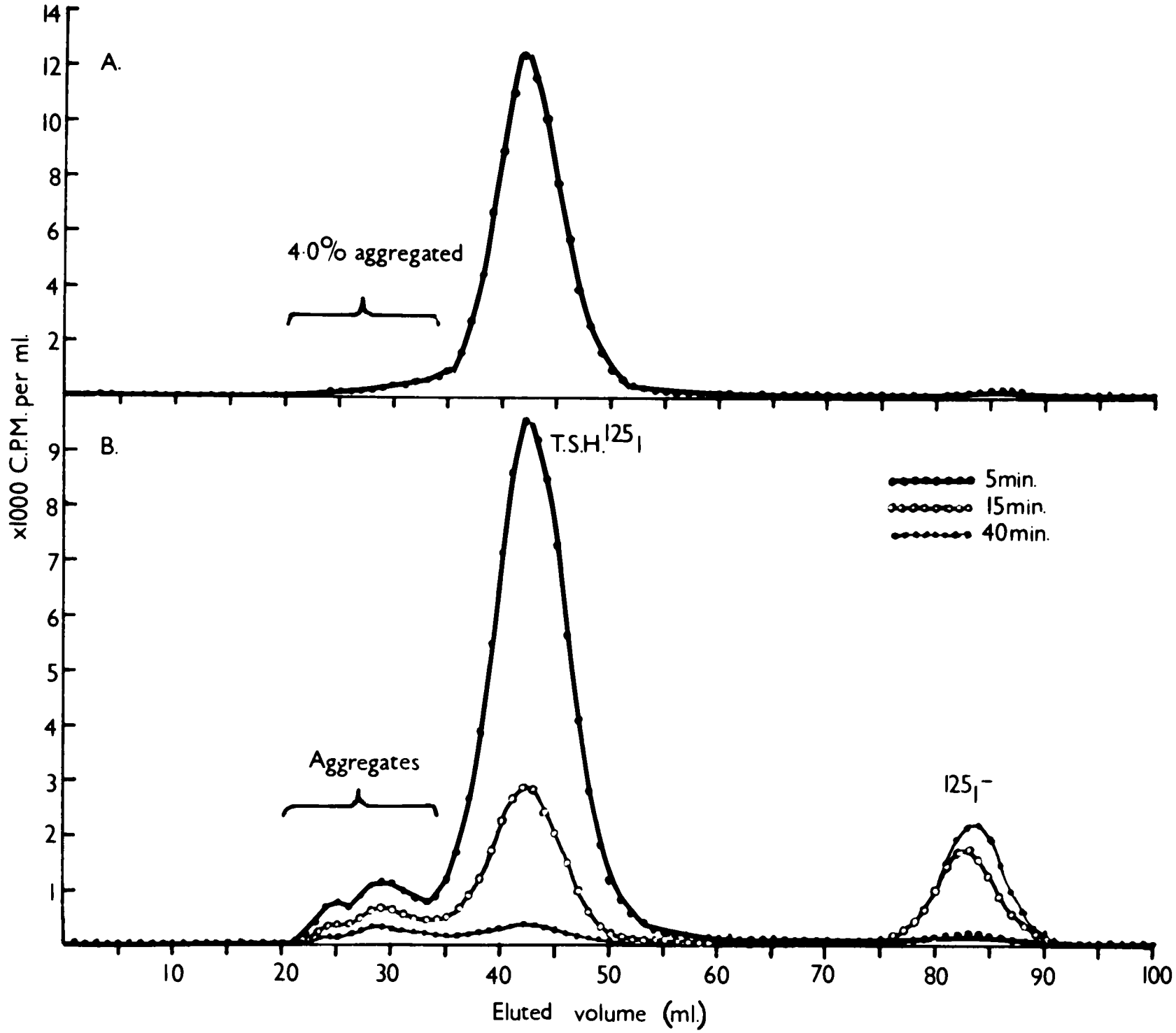

A.
4·0% aggregated
B.
T.S.H.^{125}I
5min.
15min.
40min.
Aggregates
^{125}I$^-$
x1000 C.P.M. per ml.
Eluted volume (ml.)
14
12
10
8
6
4
2
9
8
7
6
5
4
3
2
1
10
20
30
40
50
60
70
80
90
100

FIGURE 3.5

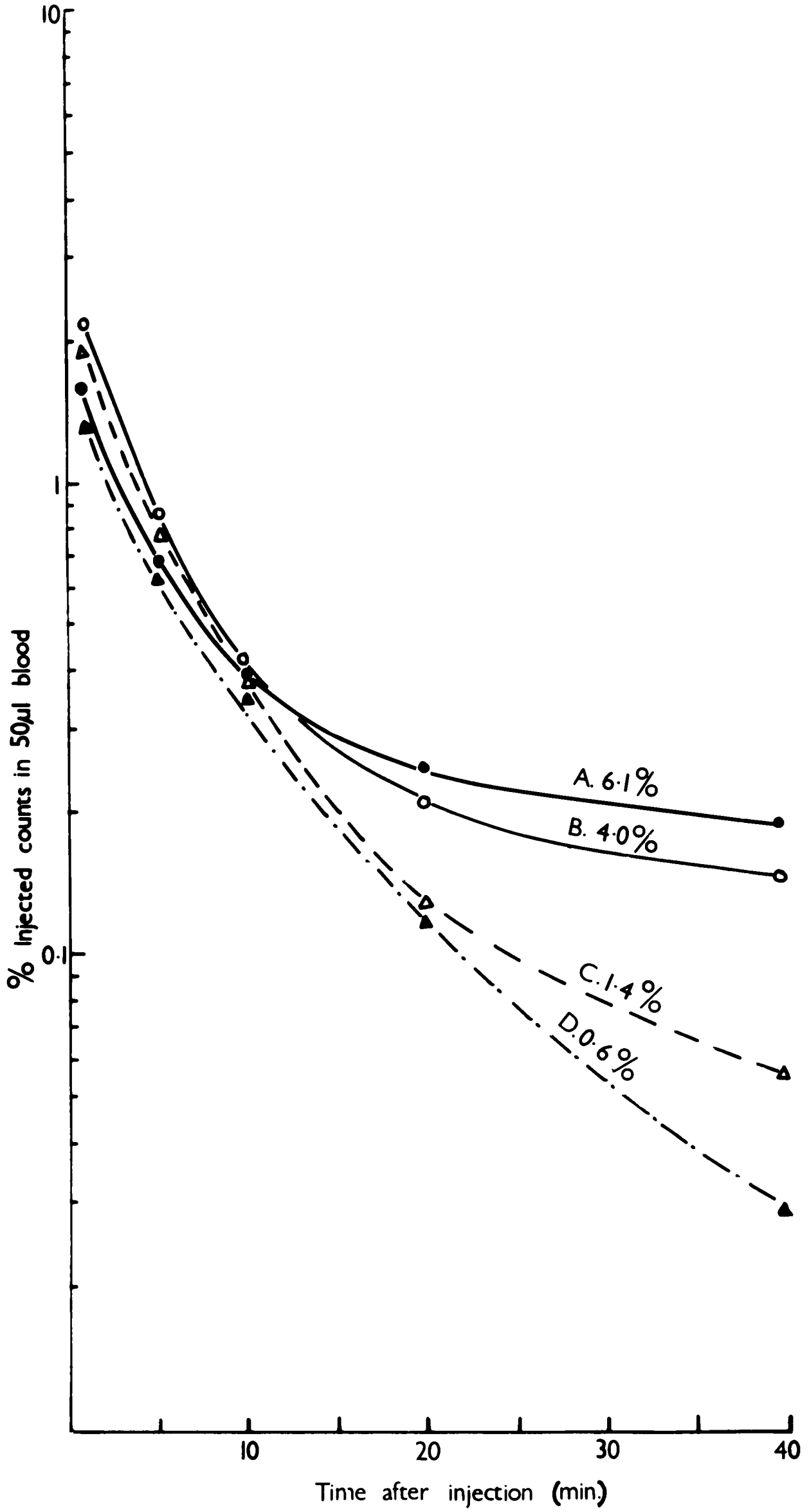
The effect of aggregated T.S.H.-^{125}I on the apparent decay curve of blood T.S.H.^{125}I in the mouse following an i.v. injection

Figures over curves represent % aggregated T.S.H. in injected T.S.H.-^{125}I.

	Expt.	Animal Wt.(1) (g)	% Aggregated	Iodide Removal
D	3.5	26.1 ± 1.2	6.1	T.C.A.
C	3.6	18.2 ± 1.0	4.0	T.C.A.
B	3.1	26.8 ± 0.4	1.4	T.C.A.
A	3.10	27.1 ± 1.2	0.6	Amberlite I.R.A. 400

Each point represents the mean of 5 mice

(1) Mean ± S.E.M. of 5 mice


10
1
0·1
% Injected counts in 50µl blood
A. 6·1%
B. 4·0%
C. 1·4%
D. 0·6%
10
20
30
40
Time after injection (min)

The slowly-decaying material ran in two peaks: one running with the void volume of the column (fraction 25) and the other running behind it (peak at fractions 29-30). As suggested in section 2.3.1, the two peaks were thought to represent aggregates (possibly polymers and dimers, respectively) of T.S.H.-^{125}I. The reasons for this were:

1. The proportion of the material varied from iodination to iodination and also increased with time after iodination (section 5.8).
2. Similar fractions had been noted after iodination of L.H. (Nabarro, 1973).
3. These fractions were precipitable by anti-T.S.H. antisera:-

In the first experiment (expt. 3.3) the immuno-precipitabilities of selected fractions were tested as described in section 3.2.1. Table 3.2 shows the results. The unchromatographed T.S.H.-^{125}I was 91% precipitable compared with 94% for the T.S.H.-^{125}I peak (fraction 42). The unchromatographed plasma fractions yielded progressively less immunoprecipitable radioactivity with the increase in the proportion of iodide. Fractions 25 and 30 (corresponding to the two peaks) were themselves immunoprecipitable, confirming their derivation from T.S.H. However, the reduced immunoprecipitability in comparison with the T.S.H. peak indicates some change in molecular structure.

TABLE 3.2. Immunoprecipitability of peak fractions on Sephadex G-100 chromatography of T.S.H.-^{125}I and mouse plasma taken following injection of T.S.H.-^{125}I (expt. 3.3)

A. Injected T.S.H.-^{125}I

Fraction	Immunoprecipitability (%)
Before chromatography	91
24	76
33	75
42	94

B. Mouse plasmas

Fraction	Immunoprecipitability (%) 5 min plasma	15 min plasma	40 min plasma
Before chromatography	87	61	26
25	70	70	62
30	85	76	62
42	95	95	88

3.2.6. The fate of purified T.S.H.-^{125}I following intravenous injection (expts. 3.8, 3.8, 3.10)

The rapid decay of T.S.H.-^{125}I and the slower decay of T.S.H. aggregates made it essential to use purified label in order to obtain a true decay curve. For this reason efforts were made to prepare aggregate-free T.S.H.-^{125}I.

Method. In one experiment T.S.H. was labelled with ^{125}I by method B except that purification of the reaction mixture was performed using a 100 x 1cm column of Sephadex G-100SF instead of the usual short Sephadex G-50 column. The T.S.H.-^{125}I peak was collected and used for injection into mice. Serum from mice injected witb label was chromatographed on Sephadex G-100 as described in section 3.2.4 and plasma decay curves were constructed as before, except for the use of amberlite I.R.A. 400 columns for iodide removal (expt. 3.10).

Results. The elution pattern from the Sephadex G-100SF column is shown in figure 3.6. Six peaks were evident. The bulk of label appeared in fraction C and was presumed to be T.S.H.-^{125}I. The heavier fractions A and B probably represented aggregates (possibly polymers and dimers, respectively) similar to those found for labelled L.H. by Nabarro (1973). Fractions D and E were of more uncertain nature and had not been noted on gel filtration of previous T.S.H.-^{125}I preparations. Perhaps they represented subunits liberated on storage of the T.S.H. (6 months had elapsed between this and the previous experiments). Peak F undoubtedly represented unchanged iodide. Peak C was

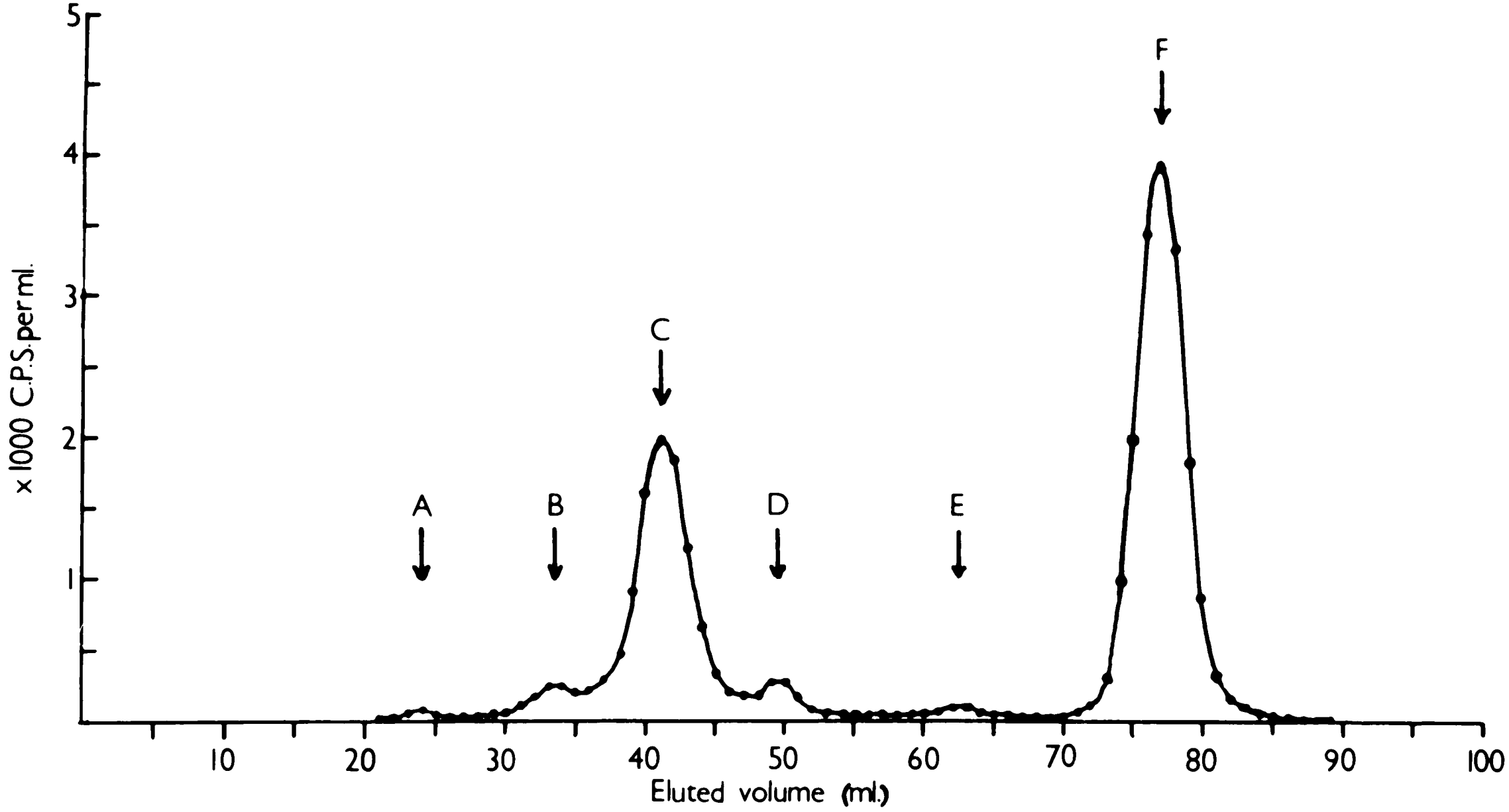
FIGURE 3.6

Chromatography of iodination reactants on Sephadex G-100 S.F. (section 3.2.5) (expt. 3.7)

T.S.H. iodinated by method B. P.B.S. -0.1% B.S.A.

Flow rate 6ml/hr.

The significance of peaks A-F is discussed in the text. Fractions 40-43 inclusive were retained for decay studies.


x 1000 C.P.S. per ml.
5
4
3
2
1
A
B
C
D
E
F
10
20
30
40
50
60
70
80
90
100
Eluted volume (ml.)

selected and used in the experiment. When rechromatographed on Sephadex G-100, it yielded a single peak, with only 0.6% of counts running between the void volume and the T.S.H.-^{125}I peak (fig. 3.7). Following intravenous injection into mice, no aggregates appeared and, at 40 minutes, less than 10% of the material ran in advance of the T.S.H.-^{125}I peak, in contrast to 60% when 6.1% aggregates were injected in experiment 3.3 (see fig. 3.8). However, with the purified label a new peak, undetected before injection, appeared in the 15 minute and 40 minute plasmas just in advance of the original T.S.H. peak. The nature of this was obscure but it probably represented another slowly decaying derivative of T.S.H., present in the original label. Unfortunately, an opportunity to allow its study did not arise. This material probably contributed to the nonlinearity of the semi-logarithmic decay curve obtained with purified T.S.H.-^{125}I (fig. 3.5D). As figure 3.5 demonstrates, removal of aggregates results in a more rapid disappearance of protein bound label. The residual nonlinearity may reflect persistent heterogeneity in the T.S.H.-^{125}I label after purification. Alternatively, single compartment, first order kinetics may not be applicable to T.S.H. decay in the mouse (see section 3.2.9).

3.2.7. The decay rate of purified T.S.H.-^{125}I from the blood and plasma of mice following intravenous injection (expt. 3.9)

In one experiment, the validity of measuring blood rather than plasma levels for decay studies was assessed.

FIGURE 3.7

Sephadex G-100 chromatography of purified T.S.H.-^{125}I (A) and plasma taken from mice at intervals following its injection (B). (expt. 3.8)

Conditions as for figure 3.3. 0.6% aggregates present in purified T.S.H.-^{125}I.

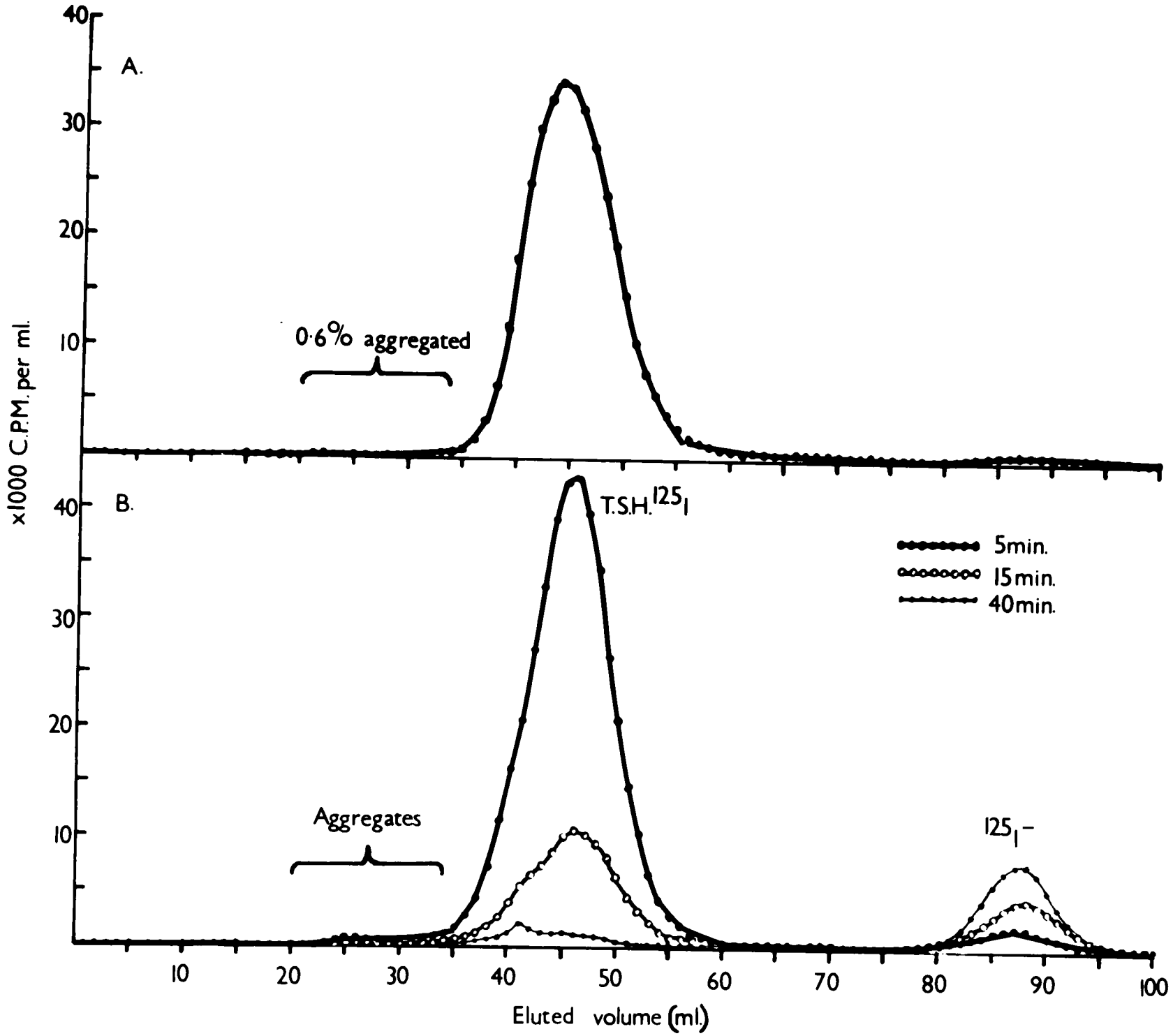
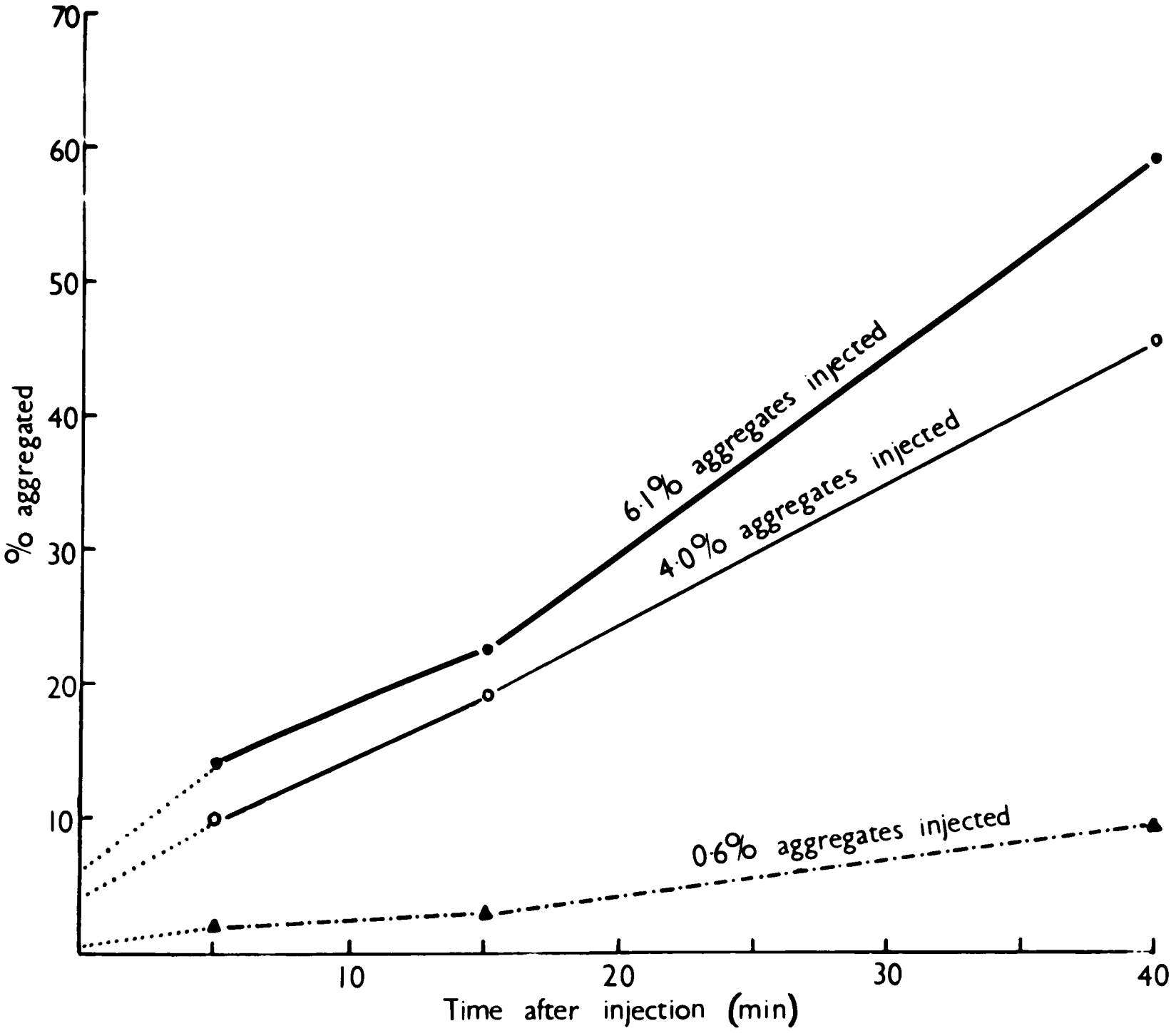

A.
B.
0·6% aggregated
T.S.H.^{125}I
5min.
15min.
40min.
Aggregates
^{125}I –
x1000 C.P.M. per ml.
Eluted volume (ml.)
10
20
30
40
50
60
70
80
90
100

FIGURE 3.8

The percentage of T.S.H.-^{125}I in aggregated form in mouse plasma at intervals following the injection of T.S.H.-^{125}I of varying degrees of purity.


70
60
50
40
30
20
10
% aggregated
6·1% aggregates injected
4·0% aggregates injected
0·6% aggregates injected
10
20
30
40
Time after injection (min)

Method. Six mice were injected intravenously with 0.5-1μCi purified T.S.H.-^{125}I. Blood samples of 50μl were removed by orbital puncture in the unanaesthetised animal at suitable intervals thereafter. Samples taken at each time interval were pooled. Duplicated 50μl samples of blood and plasma were taken from each pool for amberlite I.R.A. 400 chromatography. Plasma samples were also subjected to immunoprecipitation.

Results. Figure 3.9 shows the parallelism obtained between curves, confirming that blood levels can be used for comparative decay studies. As expected, the results for immunoprecipitation resembled those for amberlite I.R.A. 400 chromatography.

3.2.8. The effects of thyroxine and large quantities of exogenous T.S.H. on the decay curve for T.S.H.-^{125}I (expt. 3.10)

Because the time courses of T.S.H. and L.A.T.S. are usually compared in thyroxinised mice, and because thyroxine has been shown to decrease the half life of T.S.H. in rats (Bakke, 1963; Bakke and Lawrence, 1962), it was of interest to test whether thyroxine might hasten the decay of T.S.H.-^{125}I in mice. Also, since decay did not follow a simple exponential, the rate of removal of T.S.H. may be dependent on the total T.S.H. level. Therefore, the effect of large doses of T.S.H. was tested.

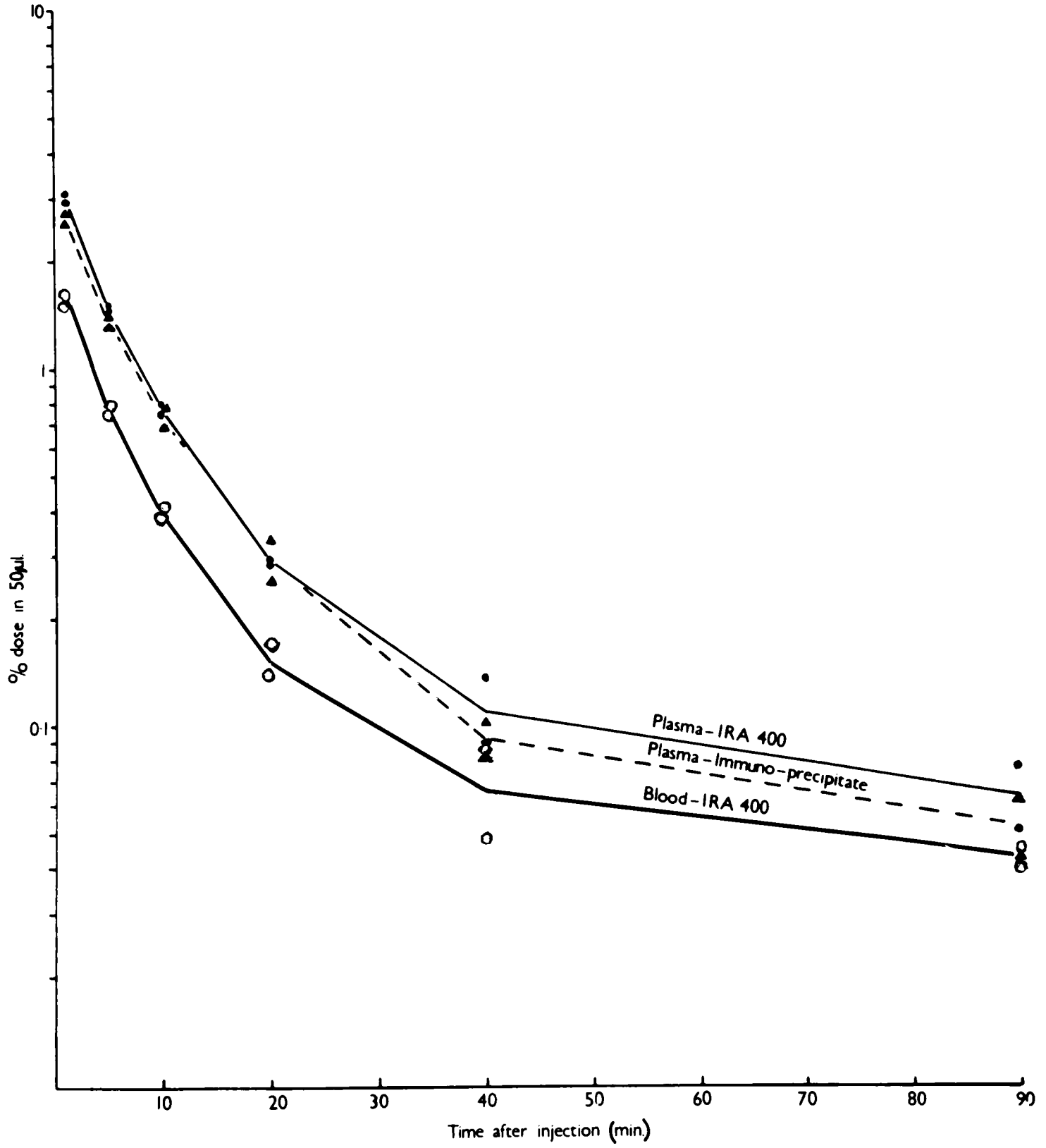
Method. Seven week old mice weighing between 20 and 30g were divided into 3 groups of five using a table of random

FIGURE 3.9

Comparison of T.S.H.-^{125}I decay in the blood and plasma of the mouse (expt. 3.9)

Purified T.S.H.-^{125}I (0.6% aggregates)

Each point represents a single determination from plasma pool. Duplicate determinations for each time interval. Plasma-I.R.A. 400: closed circles. Blood-I.R.A. 400: open circles. Plasma immuno-precipitate: triangles.


10
1
0·1
% dose in 50µl.
Plasma – IRA 400
Plasma – Immuno-precipitate
Blood – IRA 400
10
20
30
40
50
60
70
80
90
Time after injection (min.)

numbers (Fisher and Yates, 1963). Group A was given distilled water to drink for 3 days in addition to the dog biscuit diet. They received injections of saline corresponding to the thyroxine injections received by groups B and C, which were prepared as for the McKenzie assay except that the initial radioiodine injection was omitted. Each mouse in groups A and B was given an injection of approximately 0.5μCi purified T.S.H.-^{125}I (containing 0.6% aggregates corresponding to approximately 0.5mU injected hormone) in 0.25ml 1% B.S.A. The injection medium used for group C contained in addition 0.5 units of commercial T.S.H.(1). Blood samples of 50μl were taken at intervals over 40 minutes following injection and submitted to amberlite I.R.A. 400 chromatography.

Results. The decay curves are shown in figure 3.10. In table 3.3 the counts are expressed as a percentage of those at 1 minute to reduce variability due to within group variation in distribution volume. No difference could be detected between the three groups in the shape or position of the curve.

Comments. The failure of thyroxine to influence the rate of T.S.H.-^{125}I decay came as a surprise in view of the results of Bakke (1963) in rats and man. The reasons for this discrepancy may lie in the length of time for which the animals were exposed to exogenous thyroxine. This was two weeks in the experiments of Bakke (1963). The failure of T.S.H. to influence the decay rate confirms that the nonlinearity of the semilogarithmic plot was not due to a dependence on the total T.S.H. level.

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(1) 'Thytropar', Armour Pharmaceuticals Ltd., Chicago, U.S.A.

FIGURE 3.10

The effect of thyroxine and large doses of T.S.H. on the decay rate of T.S.H.-^{125}I in the mouse

(expt. 3.10)

Group	Thyroxine(1)	Exogenous T.S.H. (i.v.)	Animal wt.(g)(2)
A	-	-	27.1 ± 1.2
B	+	-	26.5 ± 0.9
C	+	0.5 IU	27.2 ± 1.4

Each point represents the mean of 5 mice.

(1) McKenzie assay preparation.

(2) Mean ± S.E.M. of 5 mice.

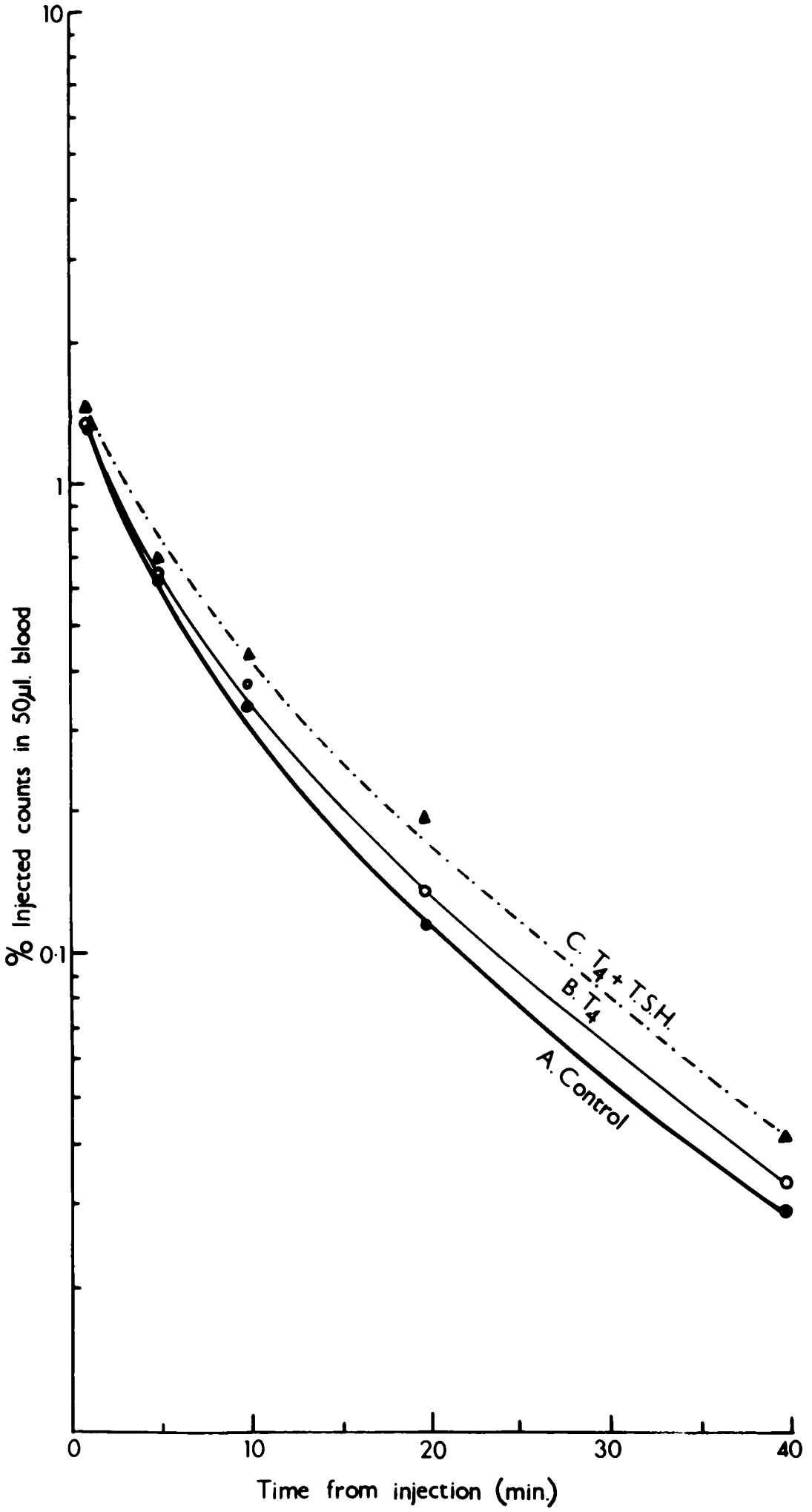
10
1
0·1
% Injected counts in 50µl. blood
C. T4 + T.S.H.
B. T4
A. Control
0
10
20
30
40
Time from injection (min.)

TABLE 3.3. The effect of thyroxine and excess T.S.H. on the decay rate of purified T.S.H.-^{125}I in mice (expt. 3.10)

1. Expressed as % injected counts in 50µl blood[1]

Group	Treatment	1 min.	5 min.	10 min.	20 min.	40 min.
A	Control	1.306 ± 0.113	0.621 ± 0.057	0.342 ± 0.040	0.114 ± 0.013	0.028 ± 0.005
B	Thyroxine	1.327 ± 0.112	0.717 ± 0.077	0.382 ± 0.053	0.136 ± 0.024	0.032 ± 0.008
C	Thyroxine + T.S.H.[2]	1.457 ± 0.021	0.793 ± 0.060	0.434 ± 0.063	0.194 ± 0.028	0.041 ± 0.006

2. Expressed as $\frac{\text{c.p.m.}_{\chi\ \text{min.}}}{\text{c.p.m.}_{\text{min.}}} \times 100$[1]

Group	Treatment	1 min.	5 min.	10 min.	20 min.	40 min.
A	Control	100	47.5 ± 1.3	26.0 ± 1.6	8.7 ± 0.5	2.1 ± 0.2
B	Thyroxine	100	53.8 ± 2.6	27.1 ± 3.8	10.2 ± 1.3	2.4 ± 0.5
C	Thyroxine + T.S.H.[2]	100	54.4 ± 3.9	29.9 ± 4.5	13.4 ± 2.0	2.8 ± 0.4

(1) Mean ± S.E.M. of 5 mice. All counts taken following passage through I.R.A. 400.
(2) 0.5 IU b-T.S.H. i.v.

3.2.9. Discussion

In the present experiments, injected T.S.H.-^{125}I disappeared rapidly in the mouse. The distribution volume, derived by extrapolation of the plasma decay curve from experiment 3.9 (fig. 3.9), was approximately 1.4ml, which probably represented the plasma volume. The subsequent disappearance of the label was nonlinear on a semilogarithmic plot. 50% of the protein bound counts present at 1 minute had disappeared 5-6 minutes later and more than 90% of the injected T.S.H.-^{125}I had been cleared from the plasma by 20 minutes. This is consistent with the results of McKenzie (1961), who failed to detect T.S.H. by bioassay at 15 minutes following the injection of a relatively small (2mU) dose.

No T.S.H.-^{125}I fragments were liberated into the plasma following injection, although the disappearing protein bound label was replaced by large amounts of radioiodide and this predominated at 40 minutes. Therefore, efficient separation of protein bound label, either by immunoprecipitation or amberlite I.R.A. 400 ion exchange resin, was essential. The true decay curve was distorted by even small quantities of aggregated label, which decayed more slowly. Sephadex G-100SF chromatography efficiently removed this contaminant, but the semilogarithmic decay plot remained nonlinear.

Single exponential decay depends on the applicability of a single compartment model (Rescigno and Segre, 1966). Some have found this to be valid for b- and h-T.S.H. decay in rats and humans (Bakke, 1963; Bakke et al, 1962; Marceau et al, 1972; Odell et al, 1967a, b) although minor deviations

from linearity are evident at long time intervals in some of the data (Bakke, 1963; Bakke et al, 1962; Odell et al, 1967a, b). However, Fontaine's (1970) decay curve for biologically labelled homologous T.S.H.-^{3}H in the rat was similar to those seen here, with a nonlinear semilogarithmic plot. In rats the distribution volume of b-T.S.H. corresponded to the expected plasma volume (Marceau et al, 1972) suggesting that either little T.S.H. was transferred to the extravascular compartment or, more likely, T.S.H. transferred to the extravascular compartment was rapidly degraded.

Lack of a simple exponential pattern in the present experiments using purified T.S.H.-^{125}I as tracer could be due to at least two possibilities:

1. The single compartment model was not appropriate, as would have occurred if T.S.H. accumulated to a significant degree in the extravascular compartment following injection. This would necessitate the use of a model, consisting of two or more compartments, whose clearance curve could be represented by the sum of two or more exponentials.
2. The purified T.S.H.-^{125}I was heterogeneous. There was some evidence of a residual, slowly-decaying contaminant in the elution patterns on Sephadex G-100 of plasma taken at intervals after the injection of purified T.S.H.-^{125}I (expt. 3.8). At 15 and 40 minutes a significant new peak appeared, running just in advance of T.S.H.-^{125}I. Whilst this peak was small in comparison with the initial T.S.H.-^{125}I peak, it constituted a major proportion of protein bound counts at 40 minutes. Its nature was not known.

A further possible source of heterogeneity was the alteration in molecular properties caused by iodination. Such changes were likely since biological potency of T.S.H.-^{125}I was reduced in comparison with highly purified b-T.S.H. (section 2.3). The same molecular modifications which reduced biological activity could have altered the susceptibility of T.S.H. to peripheral degradation(1). However, in contradiction, h-T.S.H. labelled with ^{131}I by the chloramine T method has been found to decay at the same rate as unlabelled T.S.H. (measured by radioimmunoassay) in the dog (Ridgeway et al, 1974).

Finally, heterogeneity within the original T.S.H. preparation could have contributed to nonlinearity since even the purest T.S.H. preparations show several bands on polyacrylamide gel electrophoresis (Liao et al, 1969).

3.3. THE TIME COURSE OF ACTION OF T.S.H. ON THE THYROID EXPERIMENTS USING INJECTIONS OF ANTI-T.S.H. ANTISERUM AT VARYING TIMES AFTER T.S.H. IN THE MOUSE

(expts. 3.11-3.19)

3.3.1. Introduction

The rapid decay of T.S.H.-^{125}I in the mouse is surprising in view of the longer time course of the blood radioiodine response in the McKenzie assay. The latter reaches a maximum 2-3 hours after a small dose of T.S.H.

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(1) The degradation of T.S.H. is independent of its biological activity since the thyroid does not destroy T.S.H. (Boeynaems et al, 1973; Levey and Solomon, 1957; Yamazaki et al, 1962) whereas the kidney is probably the major site of removal (Bakke, 1963; Bakke and and Lawrence, 1962; Ridgeway et al, 1974)

(<10mU) or even later with larger doses (Gafni and Gross, 1975; Kriss et al, 1964; McKenzie and Williamson, 1966). Enhanced thyroid secretion persists for at least 2 hours following T.S.H. injection since at that time the circulating radioiodine has not yet started to fall and colloid droplets are still present in increased numbers (Shishiba et al, 1967). T.S.H. could exert such a prolonged stimulatory action by virtue of persistent binding to thyroid cells after the hormone has been removed from the circulation. For this reason attempts were made to modify the response to an intravenous injection of T.S.H. by injecting excess anti-T.S.H. antiserum intravenously at intervals thereafter.

3.3.2. Method

Anti-b-T.S.H. antisera were prepared in guinea-pigs as described in appendix 5. The serum used in these experiments (guinea pig 2) was capable of totally neutralising 1mU/ml b-T.S.H. at a final dilution of 1 in 1000 as measured by bioassay (appendix 5, expt. 3.11, table 3.4). Mice were prepared for the McKenzie assay as described in section 2.1 and were used for the following experiments:

1. In seven experiments (3.12-3.18), groups of 8 male mice received injections of 0.5mU Int. Std. T.S.H. in 0.25ml i.v. (at zero time), preceded or followed by 0.25ml i.v. injections of 1% B.S.A. or antiserum at dilutions of 1 in 5 to 1 in 500. Different experiments were performed using different intervals (-5 to +40 min.) between the two injections. The $\log_{10}$R.I.$_{2\ hr.}$ values were compared for control (1% B.S.A.-injected) or antiserum-injected animals.

2. In a single experiment (3.19) the 2 hour response ($\log_{10}$R.I.$_{2\ hr.}$) to an injection of 0.5mU T.S.H. (in 0.25ml 1% B.S.A.) was compared with the response in mice which had each received an additional injection of 0.1ml anti-T.S.H. antiserum (i.v.) at a dilution of 1 in 2, 1 minute beforehand or at intervals from 1 to 40 minutes afterwards. In addition, the +40 min. group was bled at 1 hr. and the control group at 1, 3, 5 and 7 hours to gain some idea of the time course.

3.3.3. Results

Figure 3.11 taken from experiment 3.19 confirms that the T.S.H. response peaks at 2 hours. The results of experiments 3.11-3.18 are summarised in figure 3.12 and table 3.4. In these experiments three strengths of antiserum were used to be certain of administering a large excess. Since similar results were obtained with the greatest two strengths[1] it was concluded that removal of T.S.H. from the regions accessible to antibody was rapid. Anti-T.S.H. was capable of preventing the response altogether if injected 5 minutes before the stimulator, and reduced the response significantly at up to 10 minutes after T.S.H. A similar pattern emerged in the second type of experiment (3.19), as shown in figure 3.13. Moreover, when antibody was injected at 40 minutes (expt. 3.19) the pattern of response remained the same with a parallel rise between 1 hour and 2 hours.

3.3.4. Discussion

Thyroid secretion rate is probably enhanced for at least 2 hours following an injection of T.S.H., despite the rapid

(1) No significant difference by Duncan's test

FIGURE 3.11

The time course of the response to a single injection of T.S.H.(0.5mU) and the effect of an injection of anti-T.S.H. antiserum at 40 mins. afterwards (expt. 3.19).

Group	T.S.H. (0 hr)	Antiserum (40min)	Log $\frac{\text{R.I.}_{2hr.}}{\text{R.I.}_{1hr.}}$ (1)	p (2)
A	0.5mU	-	2.134 ± 0.024	N.S.
B	0.5mU	0.25ml 1/5 antiserum	2.112 ± 0.019	

(1) Mean ± S.E.M. of 8 mice.

(2) Student's 't' test.

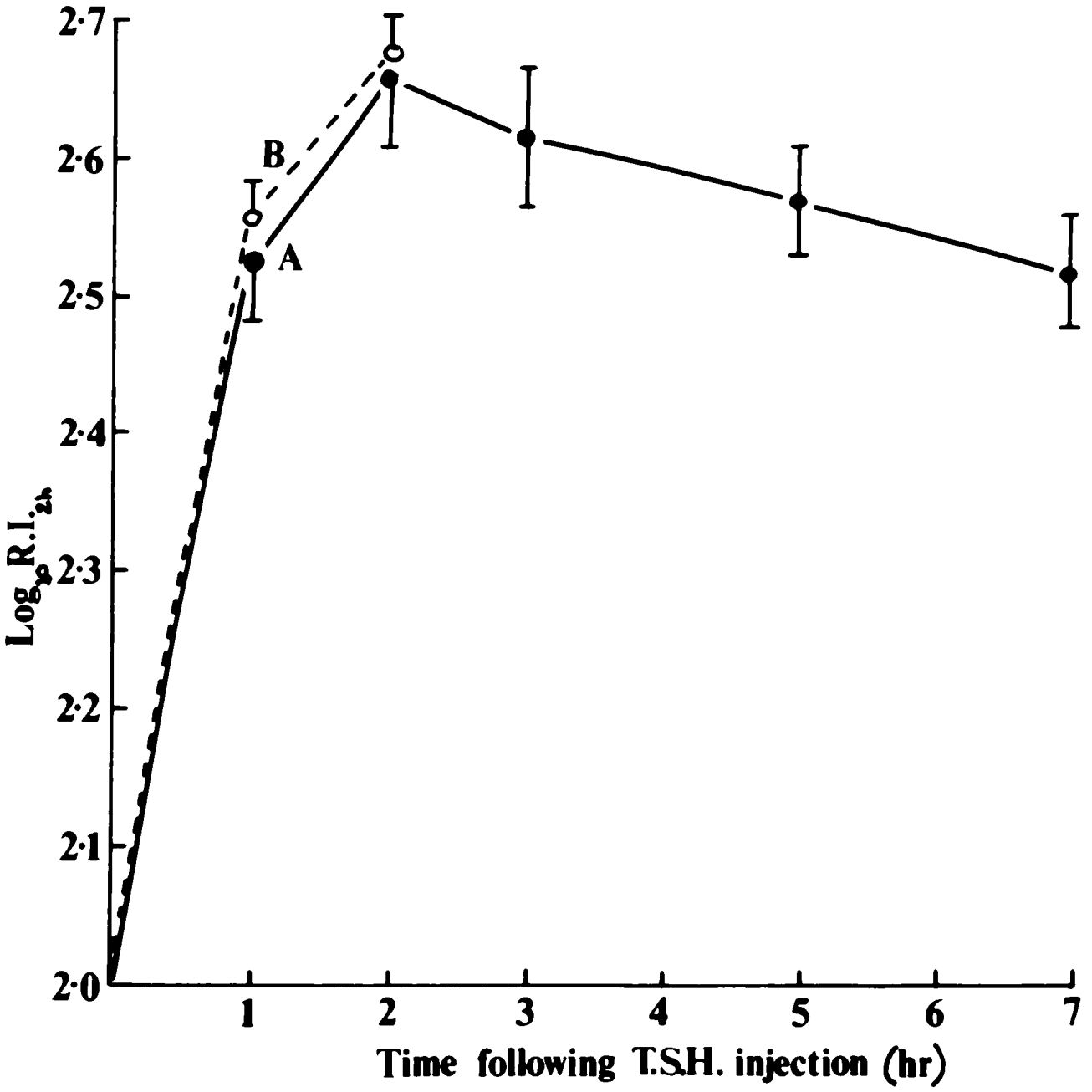

2·7
2·6
2·5
2·4
2·3
2·2
2·1
2·0
Log10 R.I.24
B
A
1
2
3
4
5
6
7
Time following T.S.H. injection (hr)

FIGURE 3.12

The effect of anti-T.S.H. antiserum on the response to T.S.H. in the McKenzie assay

(expts. 3.12-3.18)

0.5mU Int. Std. b-T.S.H. injected i.v. at time zero. 0.25ml of 1% B.S.A.(A), 1 in 500 (B), 1 in 50 (C) or 1 in 5 (D) dilutions of anti-T.S.H. antiserum injected at times before or after T.S.H. specified on abscissa.

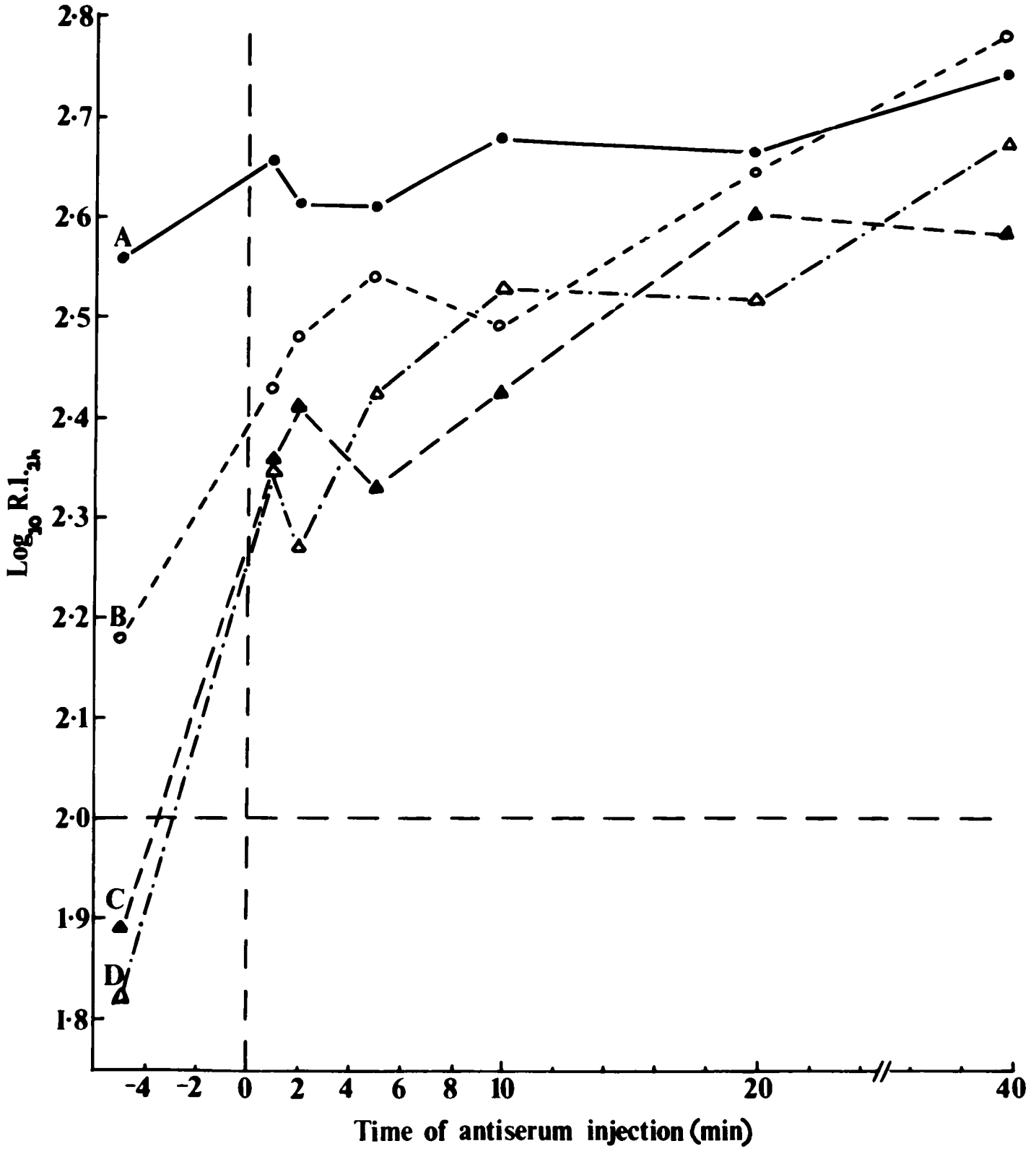

2·8
2·7
2·6
2·5
2·4
2·3
2·2
2·1
2·0
1·9
1·8
A
B
C
D
Log_{10} R.I.$_{2h}$
-4
-2
0
2
4
6
8
10
20
40
Time of antiserum injection (min)

TABLE 3.4. The effect of anti-T.S.H. antiserum on the response to T.S.H. in the McKenzie assay

(expts. 3.11-3.18)

See section 3.3. 0.5mU T.S.H. injected in at time zero in 0.25ml.

Expt.	Antiserum dil. (0.25ml injected)	Time of antiserum injection (min.)	No. of mice	$Log_{10}R.I._{2hr}$ (1)	p (2)
3.11	Control	Mixed before injection	8	2.598 ± 0.051	-
	1/5000	" " "	8	2.222 ± 0.108	<0.01
	1/500	" " "	8	1.779 ± 0.053	<0.01
	1/50	" " "	8	1.899 ± 0.050	<0.01
3.12	Control	-5 min.	6	2.559 ± 0.033	-
	1/500	" "	8	2.180 ± 0.045	<0.01
	1/50	" "	7	1.822 ± 0.043	<0.01
	1/5	" "	6	1.892 ± 0.028	<0.01
3.13	Control	+1 min.	8	2.654 ± 0.019	-
	1/500	" "	8	2.430 ± 0.057	<0.01
	1/50	" "	8	2.360 ± 0.037	<0.01
	1/5	" "	7	2.347 ± 0.055	<0.01
3.14	Control	+2 min.	8	2.612 ± 0.078	-
	1/500	" "	7	2.483 ± 0.045	N.S.
	1/50	" "	6	2.419 ± 0.052	N.S.
	1/5	" "	7	2.272 ± 0.090	<0.01

/Cont'd.

TABLE 3.4. Cont'd.

Expt.	Antiserum dil. 0.25ml injected)	Time of antiserum injection (min.)	No. of mice	$Log_{10}R.I._{2hr}$ (1)	p (2)
3.15	Control	+5 min.	8	2.608 ± 0.060	-
	1/500	" "	6	2.542 ± 0.066	N.S.
	1/50	" "	7	2.332 ± 0.073	<0.05
	1/5	" "	7	2.429 ± 0.051	N.S.
3.16	Control	+10 min.	8	2.677 ± 0.026	-
	1/500	" "	8	2.490 ± 0.046	<0.05
	1/50	" "	7	2.421 ± 0.078	<0.01
	1/5	" "	6	2.535 ± 0.036	N.S.
3.17	Control	+20 min.	8	2.664 ± 0.047	-
	1/500	" "	8	2.587 ± 0.090	N.S.
	1/50	" "	6	2.601 ± 0.053	N.S.
	1/5	" "	7	2.520 ± 0.038	N.S.
3.18	Control	+40 min.	8	2.744 ± 0.057	-
	1/500	" "	8	2.772 ± 0.047	N.S.
	1/50	" "	8	2.597 ± 0.057	N.S.
	1/5	" "	8	2.672 ± 0.033	N.S.

N.B. Heteroscadasticity insignificant in all experiments (Bartlett's test)

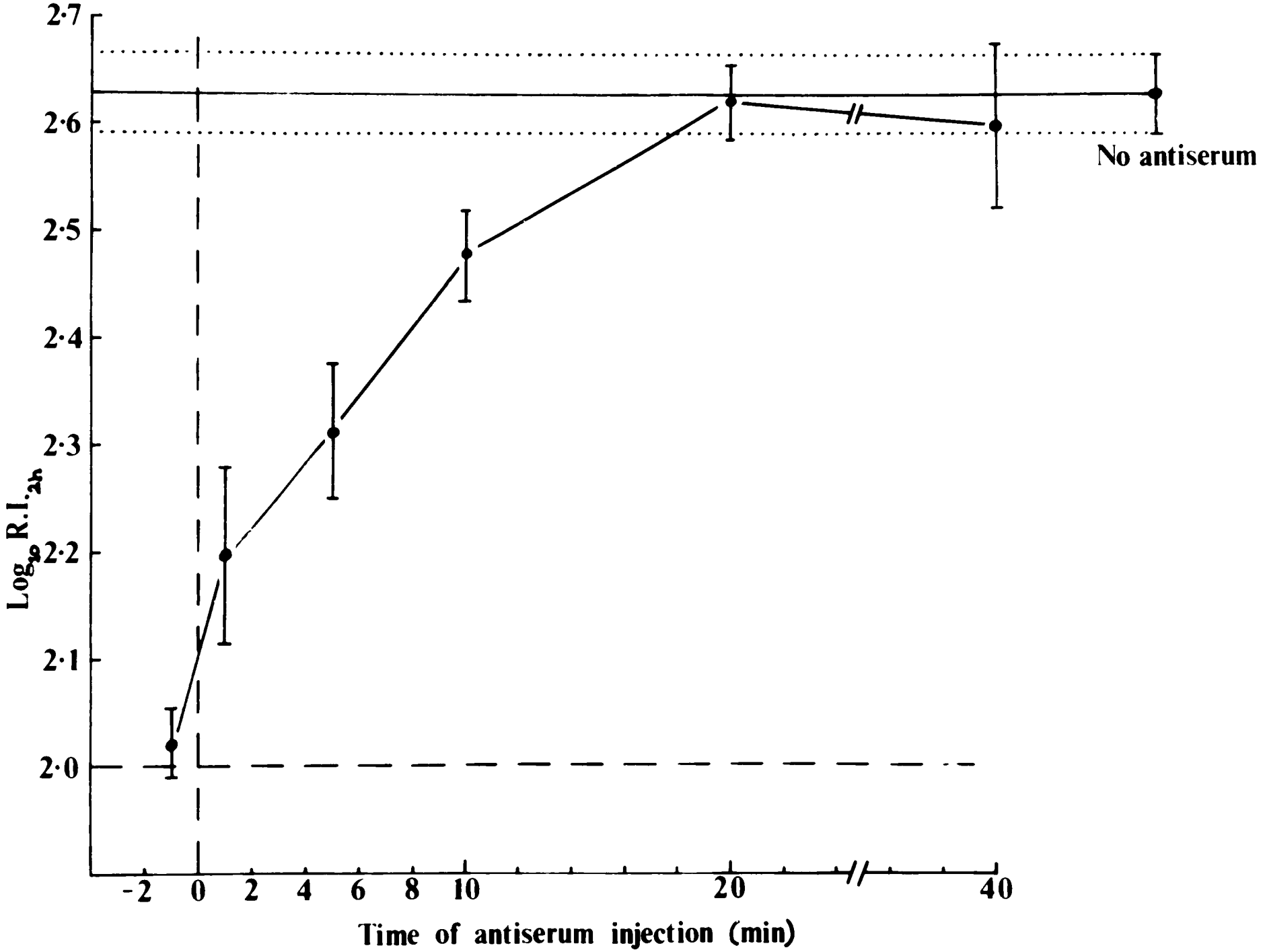
(1) Mean ± S.E.M.
(2) Difference from control (Duncan's test).

FIGURE 3.13

The effect of anti-T.S.H. antiserum on the response to T.S.H. in the McKenzie assay (2) (expt. 3.19)

0.5mU T.S.H. injected i.v. at time zero. 0.1ml of 1 in 2 dilution of anti-T.S.H. antiserum injected i.v. at intervals.

Each point represents mean ± S.E.M. of 8 mice.


2·7
2·6
2·5
2·4
2·3
2·2
2·1
2·0
Log_{10} R.I.$_{2h}$
No antiserum
-2
0
2
4
6
8
10
20
40
Time of antiserum injection (min)

disappearance of T.S.H. from the circulation over 40 minutes. The persistence of enhanced iodine release in vivo is unlikely to be due to the persistent action of receptor bound T.S.H. since anti-T.S.H. failed to modify the response after 10 minutes. The possible objection that T.S.H. receptors may be relatively inaccessible to the larger immunoglobulin molecules would seem unlikely since L.A.T.S., which is also an immunoglobulin, is able to initiate a response in mice in vivo within 10 minutes of injection (Shishiba et al, 1967).

Similar conclusions have since been reached by Malan et al (1974) on the basis of in vitro studies using prelabelled rat thyroid lobes. They found that radioiodine secretion continued and colloid droplets persisted for 2-4 hours following brief preincubation with T.S.H. even when the thyroid lobes were washed thoroughly with trypsin. In contrast, T.S.H.-enhanced cA.M.P. levels returned rapidly to baseline following the addition of trypsin. Moreover, chlorpromazine, which could inhibit new colloid droplet formation, failed to affect the continued secretion following a short preincubation with T.S.H. From this they concluded that the effect was a result, not of persistent T.S.H. action, but of the sluggishness of thyroglobulin digestion within colloid droplets formed at the time of the initial T.S.H. exposure.

Such a mechanism could account for the delayed peak of the T.S.H. response index at 2 hours whereas plasma T.S.H. had visually disappeared by 40 minutes in the mouse in vivo. The number of colloid droplets in the mouse thyroid following

an injection of 0.5mU T.S.H. also shows a persistence beyond 2 hours although the peak occurs earlier, at 30 minutes (Shishiba et al, 1967). If colloid droplets are only formed during the period of exposure to T.S.H., as suggested by Malan et al (1974), then all new colloid droplets should be formed within 40-60 minutes of injection in the mouse. Therefore at least some colloid droplets persist for 1-1½ hours, which is somewhat longer than other estimates for the lifespan of colloid droplets at 5 minutes (Wollman, 1969) or 3-26 minutes (Wollman and Loewenstein, 1973). Obviously further studies on the effects of anti-T.S.H. injection on the colloid droplet response are indicated to confirm this[1].

3.4. THE DECAY RATE OF INTRAVENOUSLY INJECTED L.A.T.S. AND NORMAL IgG IN MICE

3.4.1. Introduction

The decay rate of L.A.T.S. activity has been studied in human neonates (Maisey and Stimmler, 1972; McKenzie, 1964; Sunshine et al, 1965), rats (Adams, 1960) and mice (McKenzie, 1961). McKenzie estimated the $t_{\frac{1}{2}}$ of L.A.T.S. in the mouse by bioassay to be approximately 5½ hours, which is in contrast to estimates of the $t_{\frac{1}{2}}$ of labelled human IgG in mice, which have been in the order of 100 hours (Fahey and Robinson, 1963; Florsheim et al, 1970). For this reason it was of particular interest to follow the decay of L.A.T.S. activity and total IgG simultaneously. The most convenient method of

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(1) If this interpretation is correct then the further prolongation of the response with large doses of T.S.H. (Gafni and Gross, 1975; Wollman et al, 1964) raises the interesting question of whether more time is required to digest greater loads of thyroglobulin taken into the cells or whether T.S.H. may delay the process of secretion somehow.

following IgG decay is by using material labelled with radioiodine. However, there is evidence that iodination may alter the decay rates of plasma proteins (Margen and Tarver, 1957; Rosa et al, 1967). Thus, in some experiments the decay rate of IgG-^{125}I was compared with that of total injected IgG measured by single radial immunodiffusion.

3.4.2. Materials and methods

IgG preparation was by the method of Baumstark et al (1964), as described in section 2.4.1, from human sera taken from a strongly L.A.T.S. positive donor suffering from Graves' disease (R.N.) or normal control (C.P.G. or N.).

Iodination was performed by a modification of the chloramine T method of Greenwood et al (1963), as described in section 2.5.

Measurement of total human IgG levels in mouse plasma following IgG injection was undertaken by single radial immunodiffusion as described by Fahey and McKelvey (1965), with minor modifications. Immunodiffusion medium was prepared by dissolving 3g agarose(1) in 100ml 0.018M veronal acetate buffer pH8.6 on a hot plate, taking care to prevent boiling, and cooling to 56°C on a water bath before adding an equal volume of an appropriate dilution of sheep anti-h-IgG serum(2) in buffer at 56°C. 8ml aliquots of this solution were pipetted onto level 6.3 x 7.5cm glass slides to give layers of gel approximately 2mm thick. Fifteen minutes later the plates were transferred to a damp

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(1) B.D.H. laboratory reagent
(2) M.R.54, Wellcome Reagents Ltd.

atmosphere for 30-60 minutes to allow strengthening. 20 wells, 2.6mm in diameter, were punched out of the gel with the aid of a jig, specially constructed for the purpose in the workshop. The wells were filled with the standard or test solutions using a drawn out Pasteur pipette so that the surface of the liquid was level with the gel. After 24 hours in a damp atmosphere the plates were photographed against a dark background (fig. 3.14). The diameters of the precipitin rings in 12 x 10" enlargements were measured, taking the mean of two readings perpendicular to each other. Where large rings formed around the peripheral wells, visible distortion occurred near the edge of the plate. In these cases a single measurement was made parallel to the edge of the plate.

The appropriate antiserum concentration was determined by pilot experiment (expt. 3.20). The standard curves for one of these are shown in figure 3.15. In this experiment plates were prepared containing 0.5%, 1%, 2.5% or 5.0% anti-IgG antiserum. Dilutions of a human serum of up to 1 in 27 were used as IgG 'standards'. Ring diameters were measured directly in this experiment since great accuracy was not essential. Decreasing antiserum concentration increased sensitivity but only at the expense of clarity. Indeed, precipitin bands were too faint to be of use at antiserum concentrations of less than 2.5%. For assaying exogenous IgG in the serum of mice injected with human serum of IgG, plates were set up containing appropriate dilutions of antiserum. The 'standards' used were doubling dilutions of the same human serum diluted in mouse serum.

FIGURE 3.14

Single radial immunodiffusion method for assay of human IgG. (Photograph of precipitation rings in gel.)

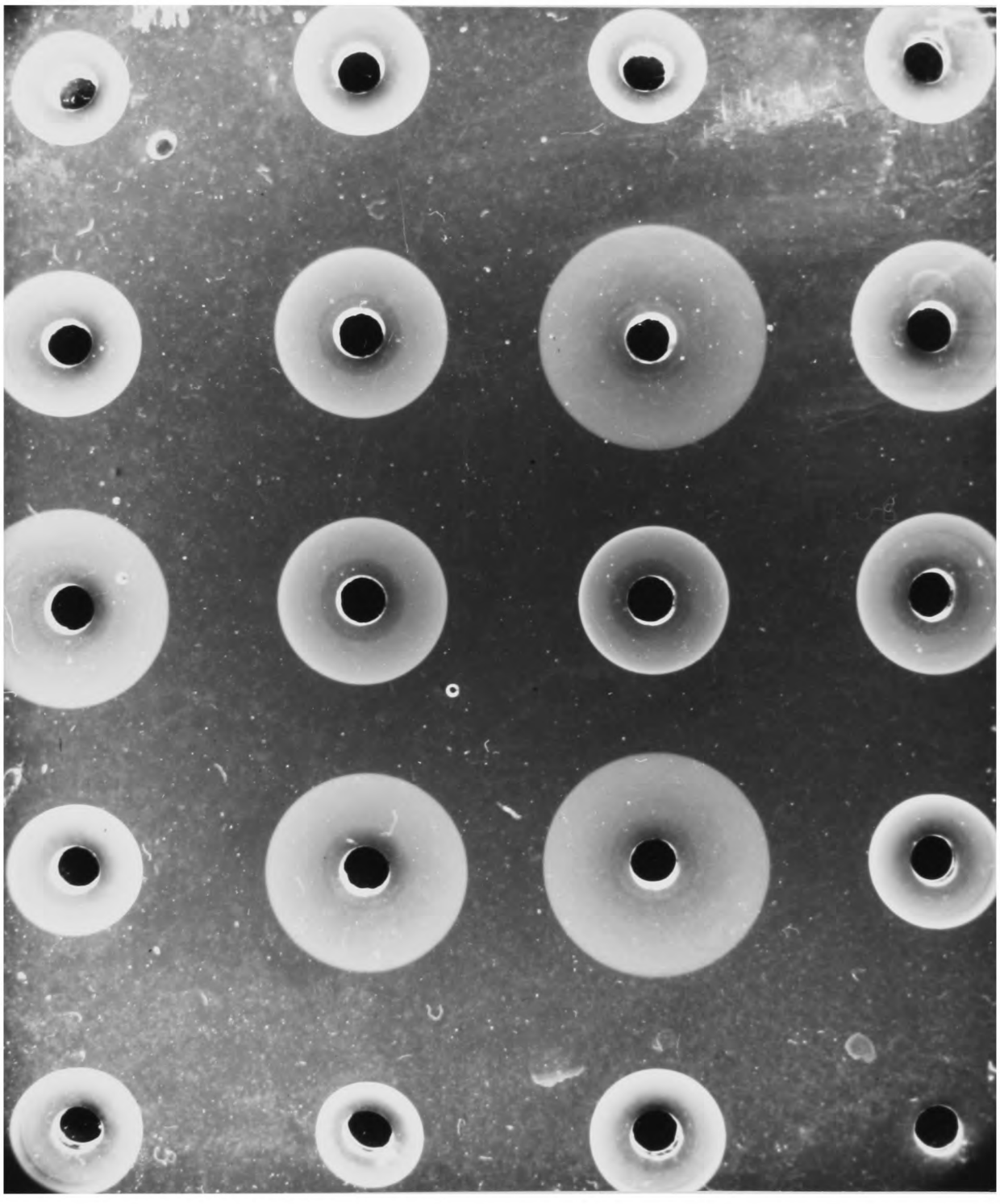


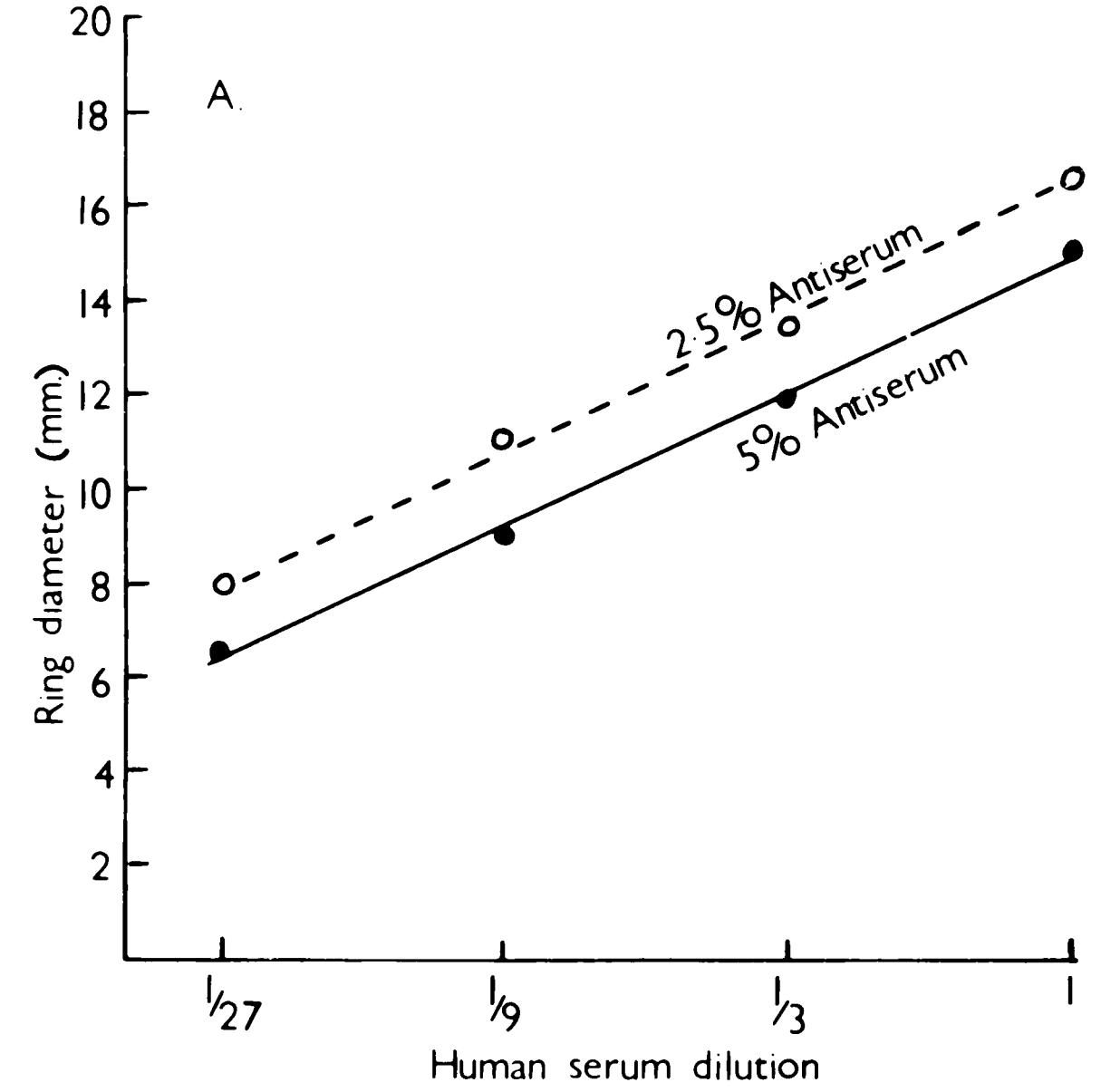
FIGURE 3.15

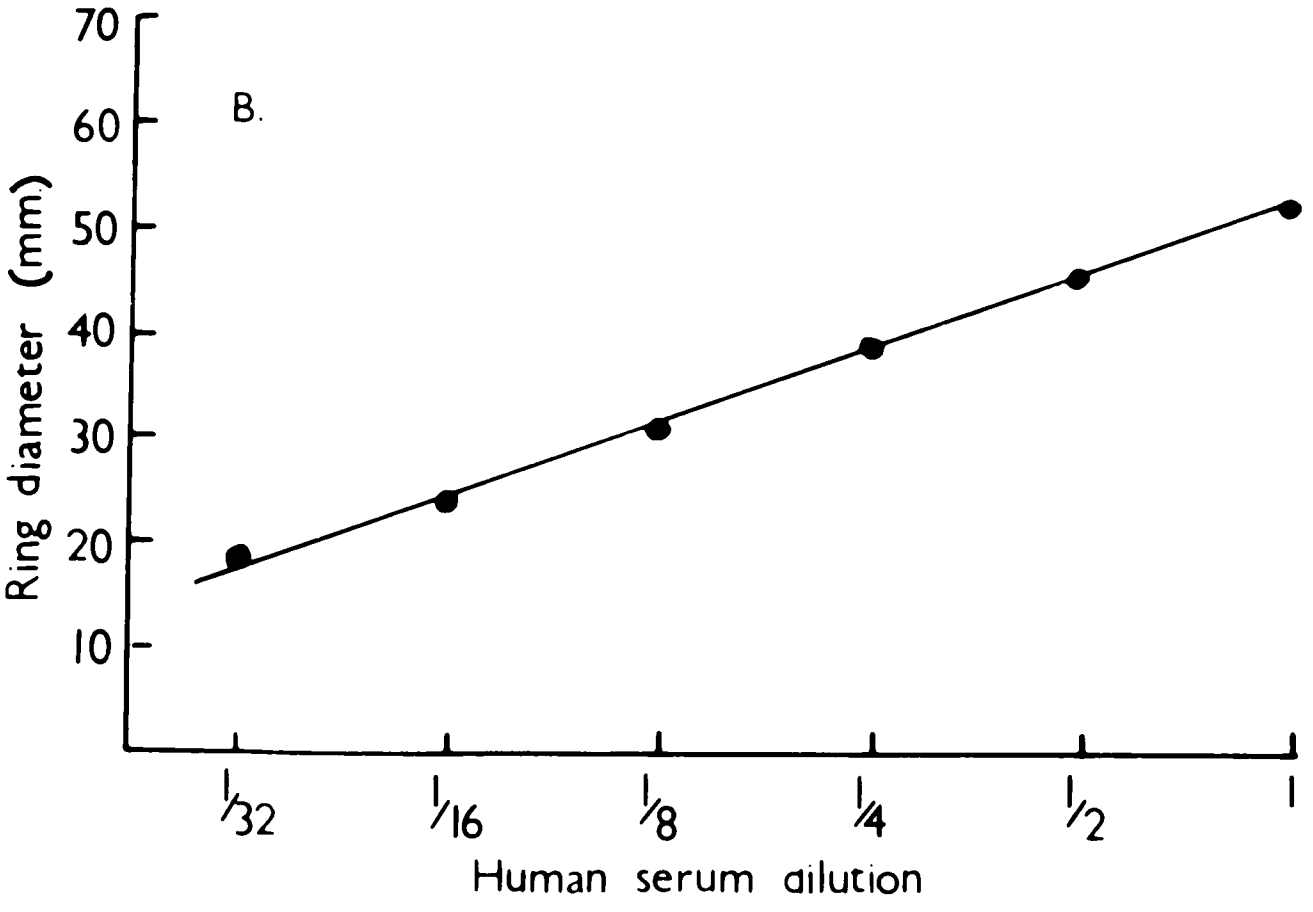
Single radial immunodiffusion method for assay of human IgG

A. Standard lines of two antiserum concentrations. Direct measurement of ring diameter.

B. Standard curve for experimental IgG determination. Measurement from 12 x 10" enlarged photographs.

Each point represents the mean of duplicates.


A.
20
18
16
14
12
10
8
6
4
2
Ring diameter (mm.)
2·5% Antiserum
5% Antiserum
1/27
1/9
1/3
1
Human serum dilution


B.
70
60
50
40
30
20
10
Ring diameter (mm)
1/32
1/16
1/8
1/4
1/2
1
Human serum dilution

Mouse serum alone formed no precipitin ring. Assays were performed in duplicate and the concentrations of unknown samples derived from the standard line and expressed as a percentage of the concentration in the parent serum. Figure 3.15 shows such a standard line.

3.4.3. The decay rate of injected h-IgG-^{125}I in the mouse - the effect of thyroxine and injected serum

A pilot experiment showed that IgG-^{125}I decayed in two phases. First, an early phase lasting 12-24 hours, then an exponential phase with a $t_{\frac{1}{2}}$ in the order of 100 hours. That the first decay phase represented equilibration with the extravascular space, rather than the more rapid decay of a labelled contaminant, was confirmed by injecting into a second mouse plasma (0.5ml), taken from one which had received an intravenous dose of IgG-^{125}I 24 hours earlier. The disappearance of radioactivity in the second mouse followed the same course as the first, with two phases of decay. Because of the particular interest in the events during the first 24 hours (i.e. during the time used for the McKenzie assay), this was studied separately.

A. Early decay (expts. 3.21-3.22)

In two experiments the decay of h-IgG-^{125}I prepared from L.A.T.S. positive (R.N.1) or L.A.T.S. negative (C.P.G.1) sera was followed over 120 or 170 hours under various conditions.

Method. Groups of 5 male mice were selected using tables of random numbers and prepared either by thyroxinisation

as for the McKenzie assay (10µg thyroxine i.p. 1 and 3 days before and 3.3µg/ml thyroxine in the drinking water from 3 days before the start of the experiment), omitting the radio-iodine injection, or by allowing them to drink distilled water throughout the experiment. Approximately 10^5 c.p.m. of freshly prepared h-IgG-^{125}I were injected intravenously in 0.5ml 1% B.S.A. or L.A.T.S.-positive (R.N.1)[1] or L.A.T.S.-negative (C.P.G.1)[2] sera. Blood samples of 50µl were taken at intervals extending to 170 hours and were counted for 4 minutes each at 12.5-75kev (Nuclear Chicago autogamma). Two such experiments were run (expts. 3.21 and 3.22) using different labelled IgG preparations (R.N.1 or C.P.G.1).

Results. The decay curves are shown in figures 3.16 and 3.17. The two phases of decay are evident in both cases. In these experiments thyroxinised and control mice yielded similar decay curves. However, there was a suggestion of an increased distribution volume when the labelled IgG was injected in either normal or L.A.T.S.-positive serum.

B. Late decay (expts. 3.23-3.24)

In two experiments the half life of IgG-^{125}I was measured under various conditions.

Method. Groups of 8 male mice were selected using tables of random numbers and treated with thyroxine as above or given distilled water to drink. Labelled IgG was injected as above in 0.5ml 1% B.S.A., 7mg/ml normal (C.P.G.1) or L.A.T.S.-positive (R.N.1) IgG or normal or L.A.T.S.-positive

---

(1) L.A.T.S. assay 851 ± 118% (2 hr.), 2150 ± 265% (7 hr.), 1650 ± 166% (24 hr.)
(2) L.A.T.S. assay 109 ± 7% (2 hr.), 118 ± 10% (7 hr.), 100 ± 14% (24 hr.)

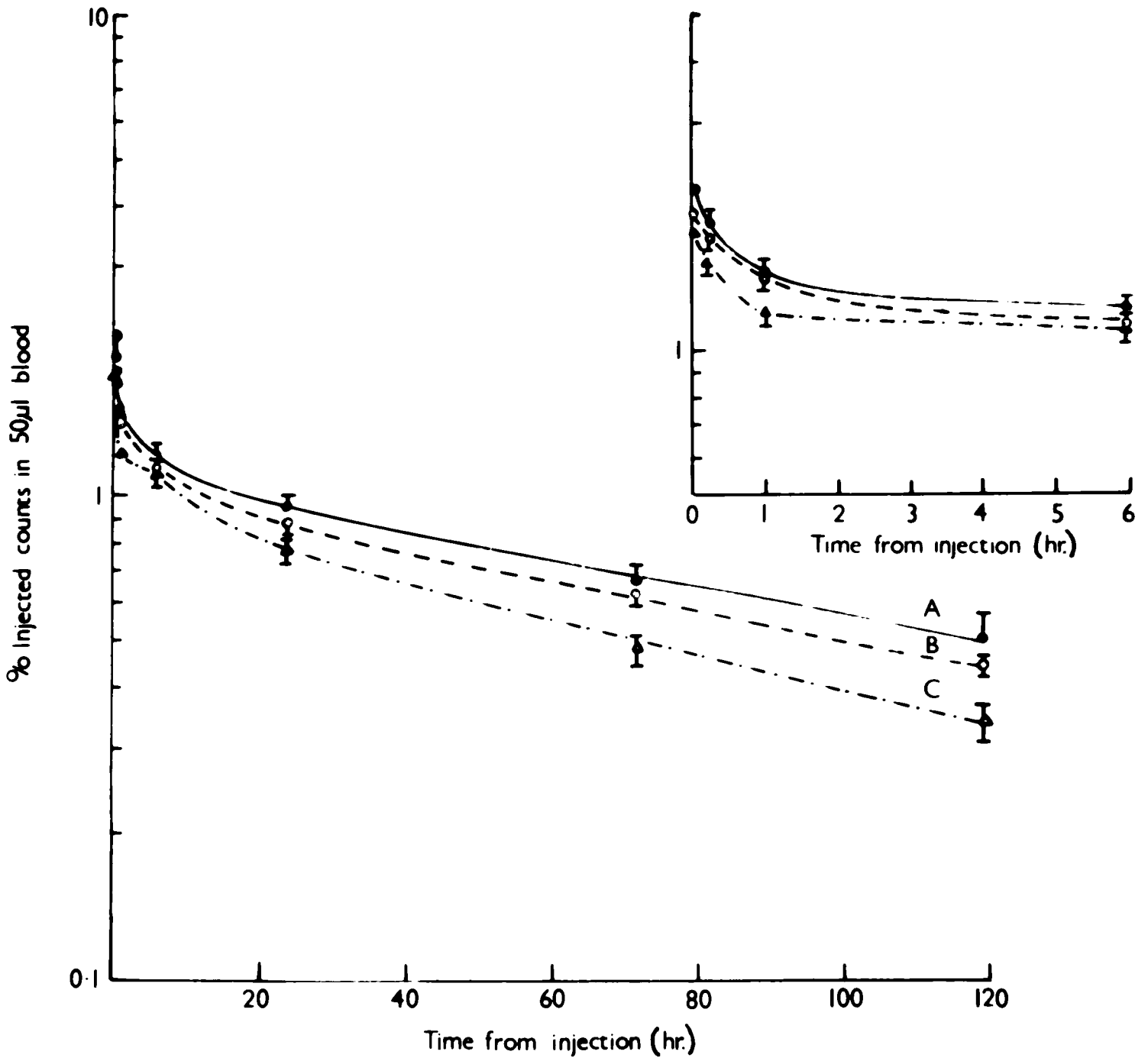
Group	Thyroxine[1]	h-IgG-^{125}I	Injected medium (0.5ml)	Animal wt.(g)[2]	$t_{\frac{1}{2}}$(hr.)[3]
A	-	L.A.T.S.(R.N.1)	1% B.S.A.	19.7 ± 0.8	104
B	+	" "	1% B.S.A.	19.9 ± 1.2	95
C	+	" "	L.A.T.S.(R.N.1) serum	17.8 ± 0.6	78

Each point represents the mean ± S.E.M. of 5 mice.
Inset shows decay over first 6 hours on expanded scale.

(1) McKenzie assay preparation.
(2) Mean ± S.E.M. of 5 mice.
(3) Determined from slope of exponential phase.

FIGURE 3.16

The early phase of IgG-^{125}I decay in mice (expt. 3.21)


% Injected counts in 50µl blood
10
1
0·1
20
40
60
80
100
120
Time from injection (hr.)
A
B
C
0
1
2
3
4
5
6
Time from injection (hr.)

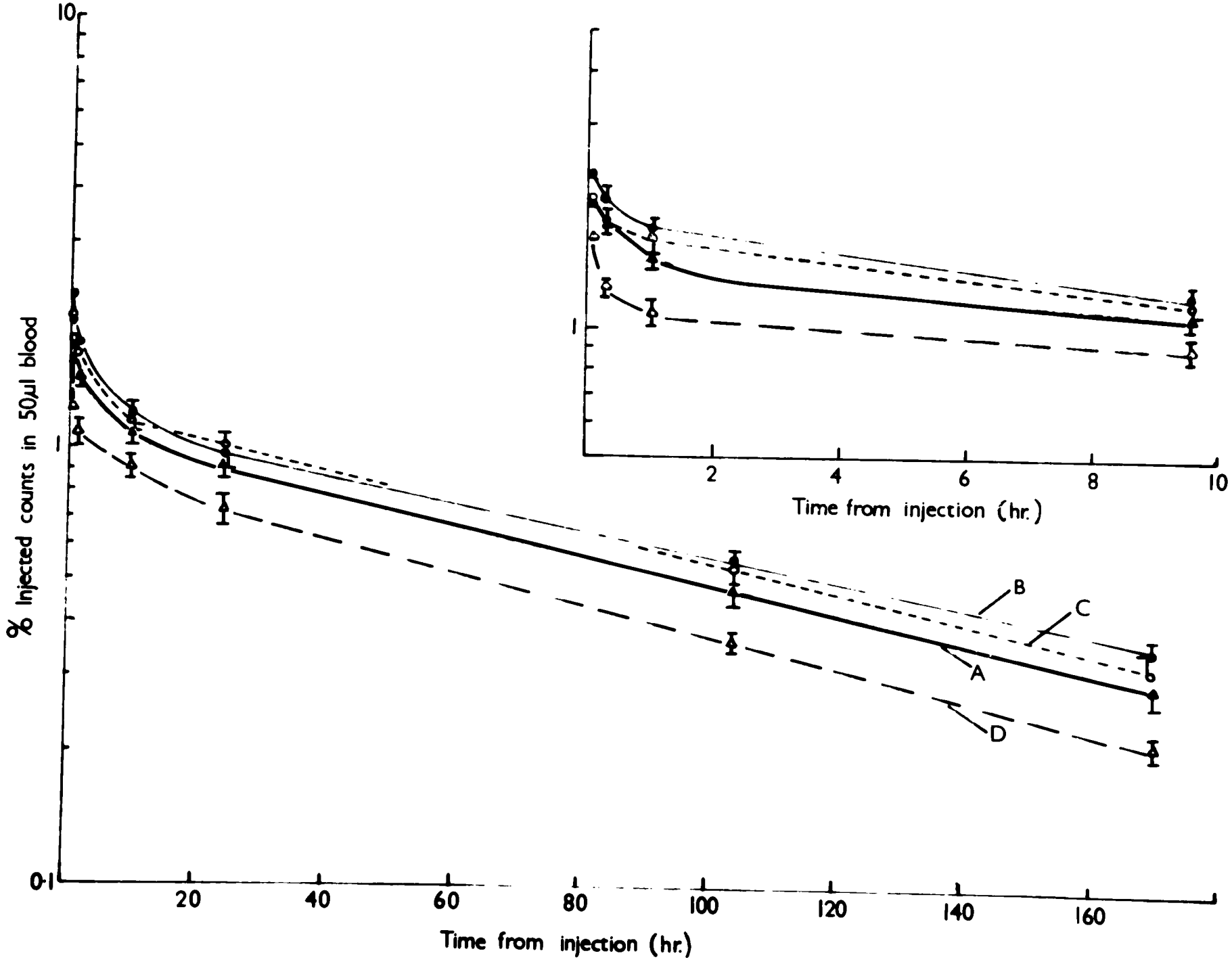
Group	Thyroxine[1]	h-IgG-^{125}I	Injected medium (0.5ml)	Animal wt.(g)[2]	$t_{\frac{1}{2}}$(hr)[3]
A	-	L.A.T.S.(R.N.1)	1% B.S.A.	19.5 ± 0.8	92
B	-	Normal(C.P.G.1)	1% B.S.A.	21.2 ± 1.6	102
C	+	Normal(C.P.G.1)	1% B.S.A.	21.1 ± 1.5	94
D	+	Normal(C.P.G.1)	Normal serum (C.P.G.1)	20.9 ± 1.2	84

Each point represents the mean ± S.E.M. of 5 mice.
Inset shows decay over first 10 hours on expanded scale.

(1) McKenzie assay preparation
(2) Mean ± S.E.M. of 5 mice
(3) Determined from slope of exponential phase

FIGURE 3.17

The early phase of IgG-^{125}I decay in mice (expt. 3.22)


10
1
0·1
% Injected counts in 50µl blood
20
40
60
80
100
120
140
160
Time from injection (hr.)
A
B
C
D
1
2
4
6
8
10
Time from injection (hr.)

serum (R.N.1). Animals were bled four times between 48 and 340 hours after injection. The mean blood radioactivity for each group at each time interval was plotted on a semi-logarithmic scale to check that decay was exponential before subjecting the data to regression analysis as described in appendix 1. In brief, this consisted of calculation of $t_{\frac{1}{2}}$ and y intercept for each mouse by the method of least squares and comparing values between groups by Duncan's test.

Results. (table 3.5) Thyroxine appeared to speed IgG-^{125}I decay in both experiments although this only reached significance in experiment 3.25 ($p<0.01$). Distribution volume was significantly increased by the injection of human serum since extrapolated zero hour counts were significantly reduced in both experiments. In experiment 3.25, thyroxine appeared to increase significantly zero hour counts but this was probably a spurious effect related to the lower mean animal weight in group 3. R.N.1 IgG-^{125}I decayed significantly faster than control IgG-^{125}I in experiment 3.25 ($p<0.05$) but this was probably unrelated to L.A.T.S., which would have been inactivated on iodination (Mori and Kriss, 1971).

3.4.4. The decay of total h-IgG as measured by single radial immunodiffusion (expt. 3.26)

In order to measure total h-IgG decay it was necessary to take plasma samples rather than blood. Because of technical difficulties (in particular, the inability to run large numbers of samples for immunodiffusion simultaneously, and the difficulty in obtaining sufficient plasma by repeated sampling from one mouse) pooled plasma had to be used.

TABLE 3.5. The effect of thyroxine (McKenzie assay preparation), human serum and human IgG on the $t_{\frac{1}{2}}$ of h-IgG-^{125}I in mice

A. Expt. 3.24

Group	No. of mice	IgG-^{125}I	Thyroxine	Injection medium	Animal Wt.(g)(1)	$t_{\frac{1}{2}}$ (hours)(1)	p(2)	0hr. % injected counts per ml. blood(1)	p(2)
1	6	R.N.	-	1% B.S.A.	22.8 ± 0.9	126.7 ± 8.9	N.S.	19.0 ± 0.8	N.S.
2	8	"	+	1% B.S.A.	24.4 ± 1.4	105.9 ± 8.1	-	19.8 ± 1.3	-
3	6	"	+	R.N.IgG(3)	24.0 ± 0.9	81.9 ± 4.0	N.S.	19.7 ± 1.4	N.S.
4	7	"	+	C.P.G.IgG(3)	21.7 ± 2.1	84.8 ± 9.3	N.S.	21.8 ± 2.4	N.S.
5	8	"	+	R.N. serum	22.6 ± 0.8	82.3 ± 6.7	N.S.	17.4 ± 1.0	N.S.
6	8	"	+	C.P.G.serum	21.8 ± 1.8	96.6 ± 4.9	N.S.	15.7 ± 1.0	N.S.

B. Expt. 3.25

Group	No. of mice	IgG-^{125}I	Thyroxine	Injection medium	Animal Wt.(g)(1)	$t_{\frac{1}{2}}$ (hours)(1)	p(2)	0hr. % injected counts per ml. blood(1)	p(2)
1	8	R.N.	-	1% B.S.A.	19.3 ± 0.8	108.0 ± 8.8	<0.05	23.3 ± 0.9	N.S.
2	6	C.P.G.	-	1% B.S.A.	20.8 ± 1.3	134.0 ± 7.8	-	21.7 ± 2.0	-
3	7	"	+	1% B.S.A.	17.8 ± 0.8	97.1 ± 6.0	<0.01	29.3 ± 1.2	<0.01
4	8	"	+	C.P.G.IgG(3)	20.8 ± 0.8	84.9 ± 4.6	<0.01	25.2 ± 0.9	N.S.
5	8	"	+	C.P.G.serum	20.8 ± 0.7	92.0 ± 6.4	<0.01	16.2 ± 1.0	<0.01

R.N.1 IgG strongly L.A.T.S. positive. C.P.G.1 IgG L.A.T.S. negative.

(1) Mean ± S.E.M.
(2) p for difference from group 2 by Duncan's test.
(3) IgG concentration 7mg/ml.

Method. Four groups of 6 male mice were treated with thyroxine and injected (i.v.) with 0.5ml of one of the following:

1. R.N.1 (L.A.T.S.-positive) h-IgG-^{125}I(1) in 5.6mg/ml R.N.1 IgG,
2. R.N.1 (L.A.T.S.-positive) h-IgG-^{125}I in R.N.1 serum,
3. C.P.G.1 (control) h-IgG-^{125}I in 5.6mg/ml C.P.H.1 IgG
4. C.P.G.1 (control) h-IgG-^{125}I in C.P.G.1 serum.

Blood samples (50µl) were taken from each mouse at 5 min., 8 hr., 27 hr., 100 hr., 196 hr. and 288 hr. after the injection and samples taken at each time were combined in heparinised tubes. After centrifugation, duplicated 25µl plasma samples were taken for counting, along with 25µl samples of each injection. Duplicated plasma samples were taken for single radial immunodiffusion as described above. Decay curves were plotted and compared.

Results. As shown in figure 3.18, similar results were obtained for L.A.T.S.-positive and L.A.T.S.-negative IgG. The $t_{\frac{1}{2}}$ and y (0 hr.) intercept values derived from these graphs are shown in table 3.6. The striking feature was the much longer $t_{\frac{1}{2}}$ of IgG when measured by immunodiffusion as compared with radiolabelled IgG. When the regression of ln IgG-^{125}I/IgG vs. time was calculated as described in appendix 1, a significant negative slope was obtained for 3 out of 4 groups. For the combined data of groups 1 and 2 (R.N.1 IgG-^{125}I) or groups 3 and 4 (C.P.G.1 IgG-^{125}I) this was highly significant ($p<0.001$ and $p<0.01$, respectively). This shows that IgG iodinated by the chloramine T method is not a suitable tracer. The greater distribution volume with concomitantly injected human serum was noted, but this was not amenable to statistical analysis.

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(1) 4×10^5 c.p.m./ml

FIGURE 3.18

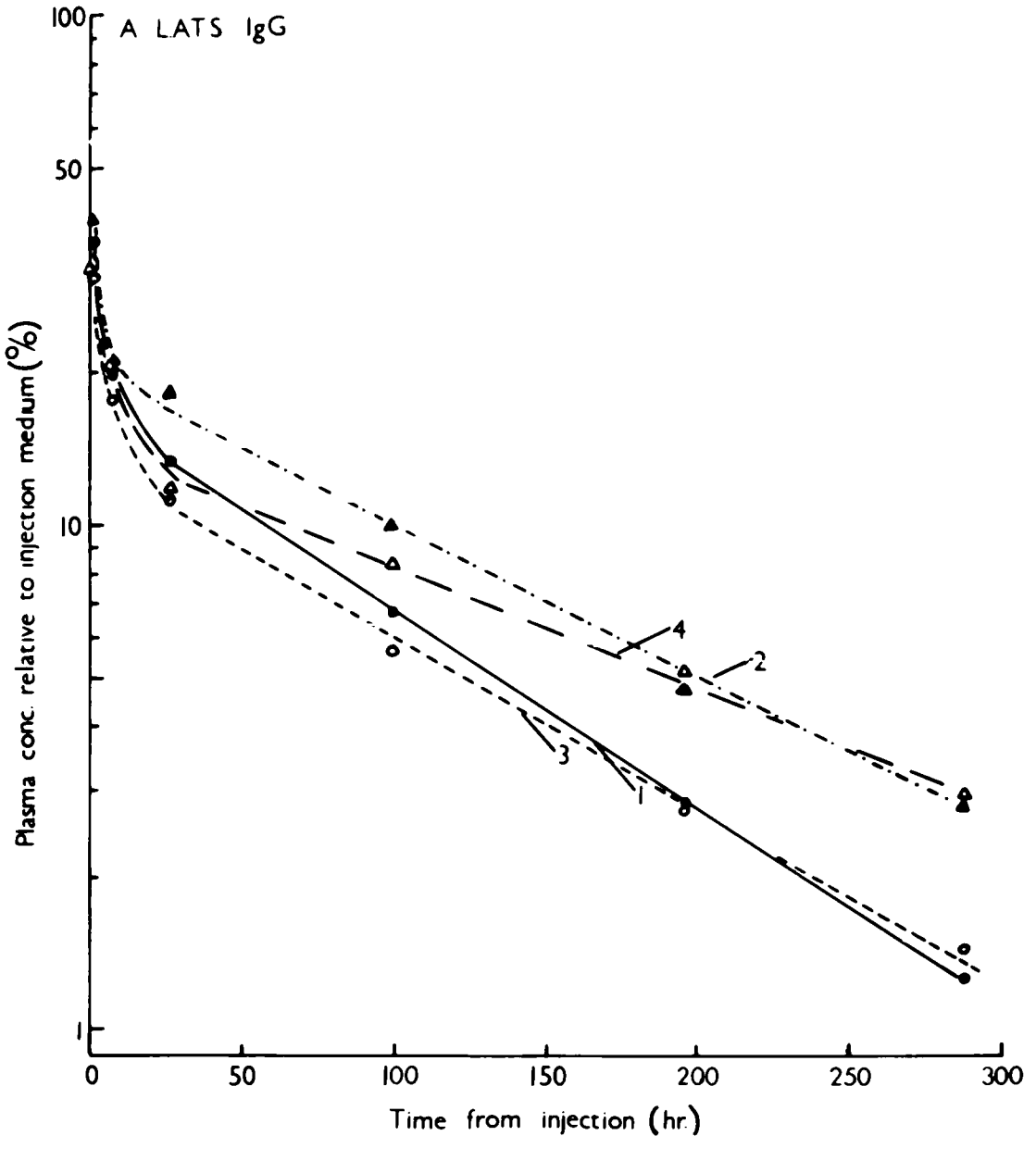
The decay of h-IgG-^{125}I and h-IgG measured by single radial immunodiffusion

A. L.A.T.S. IgG (R.N.1)

B. Control IgG (C.P.G.1)

Group	Injection	Method
1	IgG (5.6mg/ml)	IgG-^{125}I
2	IgG (5.6mg/ml)	Immunodiffusion
3	Serum	IgG-^{125}I
4	Serum	Immunodiffusion

Each point represents the mean of duplicates.


A LATS IgG
100
50
10
1
Plasma conc. relative to injection medium (%)
0
50
100
150
200
250
300
Time from injection (hr.)
1
2
3
4

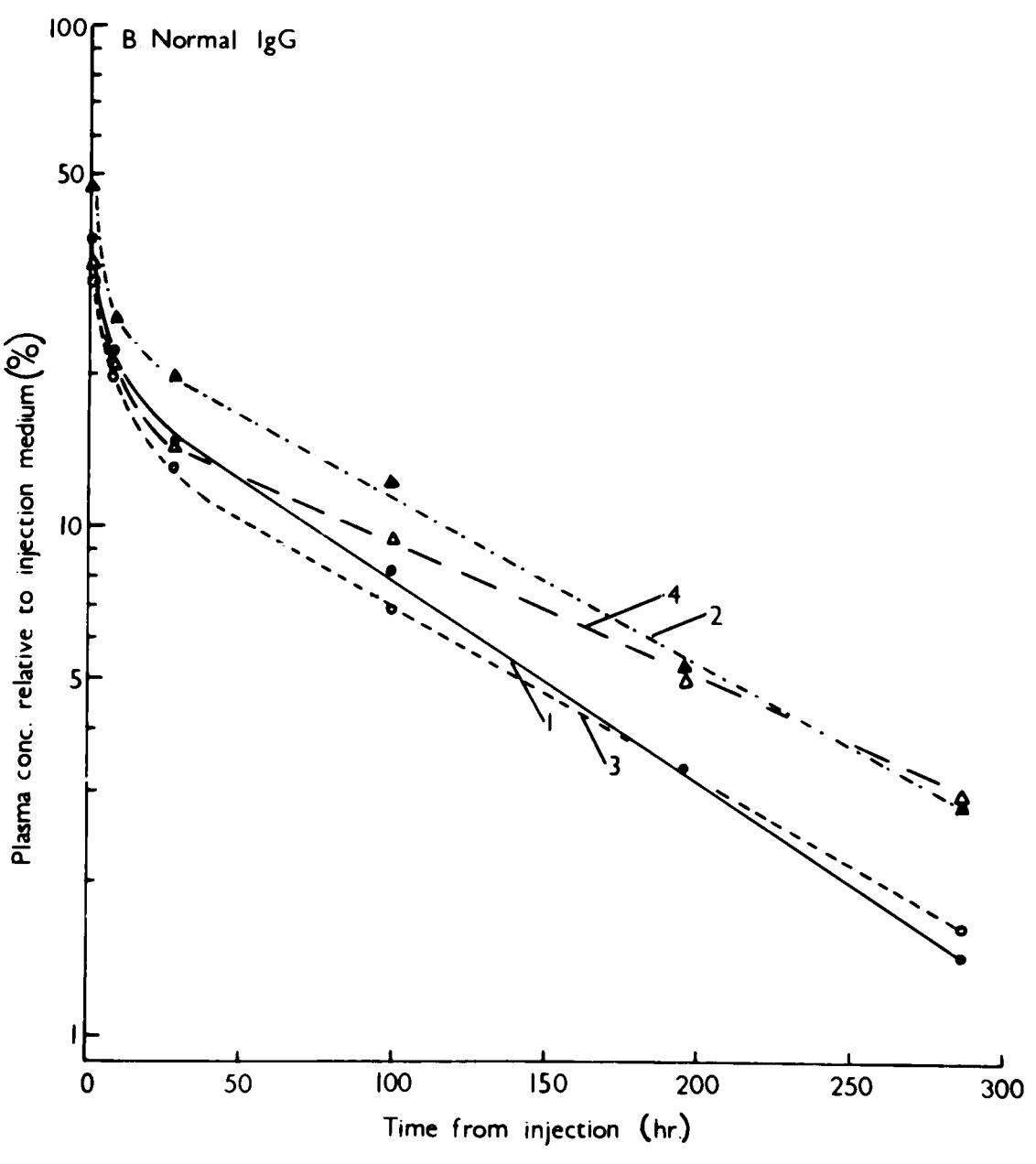

B Normal IgG
100
50
10
5
1
Plasma conc. relative to injection medium (%)
0
50
100
150
200
250
300
Time from injection (hr.)
1
2
3
4

TABLE 3.6. The comparison of h-IgG-^{125}I and total h-IgG decay

Group	IgG-^{125}I	Injection medium	0hr. intercept(%) IgG-^{125}I	0hr. intercept(%) IgG	$t_{\frac{1}{2}}$ (hr.) IgG-^{125}I	$t_{\frac{1}{2}}$ (hr.) IgG	p(1)	
1	L.A.T.S.(2)	L.A.T.S. IgG	17	20	75	104	<0.02)	) p<0.001(4)
2	L.A.T.S.(2)	L.A.T.S. serum	12	14	96	133	<0.02)	
3	Control(3)	Control IgG	20	23	75	93	<0.01)	) p<0.01 (4)
4	Control(3)	Control serum	15	17	87	110	N.S.)	

(1) p for significance of negative slope for ln IgG-^{125}I/IgG vs. time regression
(2) R.N.1 IgG
(3) C.P.G.1 IgG
(4) Combined data

3.4.5. The decay of L.A.T.S. activity after intravenous injection (expt. 3.27)

Method. In a single experiment a potent L.A.T.S. serum (R.N.1), containing 10^6 c.p.m./ml IgG-^{125}I, prepared from the same serum, was injected intravenously into 5 groups of 10 mice. These were exsanguinated under ether anaesthesia 2 min., 2 hr., 6 hr., 23 hr. or 72 hr. later.
Blood taken at each time was pooled and the plasma separated. Duplicated samples were taken for scintillation counting and determination of total h-IgG concentration by single radial immunodiffusion was performed by two separate assays. The remaining plasma was bioassayed for L.A.T.S. against the original serum at dilutions of 1 in 3, 1 in 9 and 1 in 27 in mouse plasma as described in section 2.1.

Results. (table 3.7, fig. 3.19) The decay rate of L.A.T.S. and IgG followed each other closely over the first 24 hours. However, at 72 hours the total IgG concentration just exceeded the upper 95% confidence limit for the L.A.T.S. titre. This was not amenable to statistical analysis and its significance was therefore uncertain. For this reason a second experiment using longer time intervals before exsanguination and greater numbers of mice was attempted. Unfortunately, the bioassay failed because of a poor iodine uptake, a flat dose response curve and very variable responses, with subsequent loss of a large quantity of precious L.A.T.S. serum which was not replaceable in the time available.

FIGURE 3.19

The decay of L.A.T.S., total h-IgG and h-IgG-^{125}I in mice (expt. 3.27)

L.A.T.S. assayed by McKenzie method - each point represents mean ± 95% confidence limits.

IgG-^{125}I counted at 12.5-75kev.

h-IgG measured by single radial immunodiffusion.

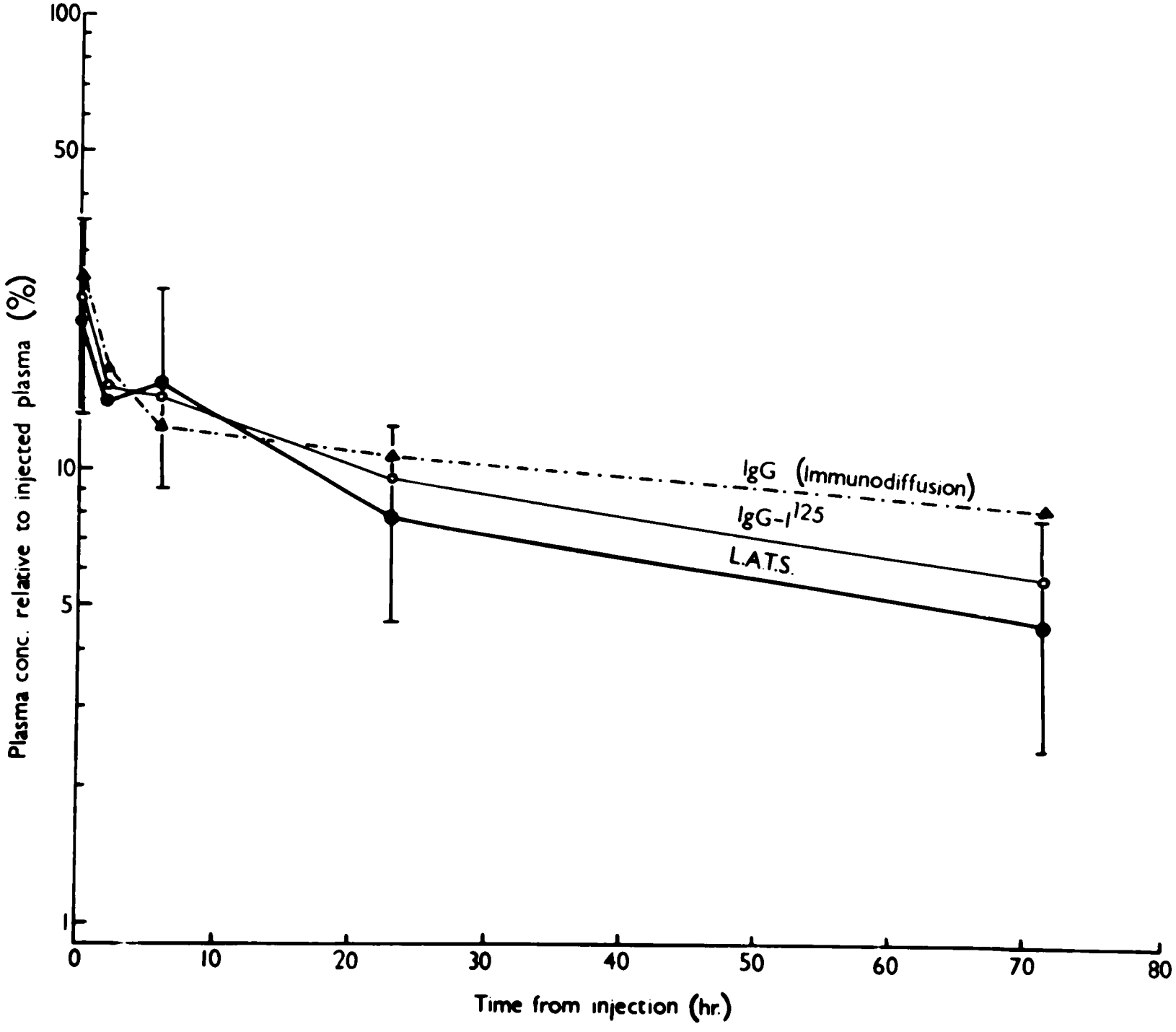

100
50
10
5
1
Plasma conc. relative to injected plasma (%)
IgG (Immunodiffusion)
IgG-I125
L.A.T.S.
0
10
20
30
40
50
60
70
80
Time from injection (hr.)

TABLE 3.7. The decay of L.A.T.S., total h-IgG and h-IgG-^{125}I in mice (expt. 3.27)

Pool	Time from injection	Concentration in plasma x 100 / Concentration in serum injected			
		IgG-^{125}I	IgG[1]	L.A.T.S.[2]	95% confidence limits
1	Control	-	-	-	-
2	2 min.	23.8	26.8 (25.0, 28.5)	21.4	13.1 - 35.0
3	2 hr.	15.2	16.5 (16.4, 16.5)	14.1	-[3]
4	6 hr.	14.5	12.5 (12.5, 12.5)	15.2	9.0 - 24.9
5	23 hr.	9.6	10.8 (10.0, 11.5)	7.8	4.6 - 12.5
6	72 hr.	5.8	8.1 (8.2, 8.0)	4.5	2.4 - 7.8

(1) Mean of two assays.

(2) λ of assay = 0.26 4 + 1 assay

(3) Confidence limits for this not calculated since S^2 for this group was significantly greater than S^2 for the standard curve (by F-ratio test). Heteroscadasticity otherwise insignificant by Bartlett's test (group 3 excluded).

3.4.6. Discussion

These experiments demonstrated the much slower decay of L.A.T.S. and IgG than of T.S.H. In experiments using radiolabelled IgG no difference in behaviour was noted between labelled L.A.T.S. and non-L.A.T.S. IgG. This is hardly surprising since active L.A.T.S. molecules comprise only a minute fraction of the total IgG (Smith, 1972) and, furthermore, iodination by the chloramine T method has been shown to inactivate L.A.T.S. (Mori and Kriss, 1971). The studies presented here add to the existing evidence that iodinated IgG is unsuitable for decay studies, since it decayed much more rapidly than total IgG measured by single radial immunodiffusion. This could be due to the more rapid disappearance of chemically modified protein or, perhaps, to deiodination in the circulation. The first possibility would appear more likely in view of the evidence that the half lives of labelled plasma proteins are extended if iodination is performed electrolytically (Rosa et al, 1967). However, there is some evidence that deiodination of iodinated albumin can occur in vivo (Margen and Tarver, 1957).

Thyroxine shortened the half life of IgG in two experiments, although this was significant only in one. Such an effect has been noted for T.S.H. in rats and humans (Bakke, 1963; Bakke et al, 1962; Odell et al, 1967a, b) and may reflect the heightened metabolism of thyrotoxic animals. The increased distribution volume of IgG when serum is injected is of some interest. This may reflect an expanded plasma

volume following the injection of a protein load. Whatever the mechanism, the plasma L.A.T.S. levels achieved in the bioassay mouse are reduced when the same quantity is injected as serum instead of purified IgG. This may account for the apparent increase in L.A.T.S. activity following the separation of the IgG fraction from some thyrotoxic sera (Bonnyns and Vanhaelst, 1969).

L.A.T.S. activity (measured by bioassay) ran parallel with h-IgG levels over 24 hours following injection. At 72 hours a minor divergence was noted although it is difficult to assess its significance on the basis of a single experiment. Nevertheless, such a divergence would not be totally unexpected in view of the ability of mouse thyroid to take up L.A.T.S., albeit in minor quantities (Adams and Kennedy, 1971). This could possibly be enhanced by extrathyroidal L.A.T.S. binding, perhaps in those tissues, such as adipose tissue, adrenal and pituitary, which have been shown to be influenced by L.A.T.S. containing IgG (Dandona et al, 1971, 1975; El Kabir and Hockaday, 1969; El Kabir et al, 1971; Hart and McKenzie, 1971).

3.5. THE INFLUENCE OF T.S.H. AND L.A.T.S. ON THE DECAY RATE OF THYROXINE-^{125}I IN THE MOUSE

3.5.1. Introduction

If the longer time course of the L.A.T.S. response than the T.S.H. response in the McKenzie assay were caused by or exacerbated by an inhibition of the disposal rate of thyroxine by L.A.T.S., this should be detectable by following the

fractional decay rate of injected labelled thyroxine in mice. This method has been used extensively for the study of thyroxine metabolism in a variety of species including man (Ingbar and Frienkel, 1955), rats (Gregerman, 1963) and mice (Florsheim et al, 1970; Wills and Schindler, 1970).

Therefore, the effect of injected T.S.H. or L.A.T.S. on the protein bound radioactivity following an injection of T_4-^{125}I was tested in thyroxinised mice (prepared as for the McKenzie assay with the omission of ^{131}I). Because of possible modification of the true fractional turnover rate by recirculation of radioiodine through the thyroid gland,or alterations of total plasma thyroxine levels in response to thyroid stimulators, some authors have advocated the use of thyroidectomised animals (Florsheim et al, 1970; Sisson, 1970). However, labelled thyroxine released from the thyroid of the 'McKenzie' mouse in response to T.S.H. or L.A.T.S. would be equally susceptible to recirculation or changes in fractional turnover with changing plasma levels. Thus, whilst the use of thyroidectomised animals may give a more precise answer to the question of whether T.S.H. or L.A.T.S. have any effect on the absolute rate of peripheral thyroxine degradation, it would be less applicable to the question of whether the stimulators affect the fractional turnover of circulating label in the McKenzie mouse. For this reason intact mice were used(1).

3.5.2. Materials and methods

Animals. Male C.F.L.P. albino mice bred in the laboratory were kept on a low iodine commercial dog biscuit diet for at

(1) Accompanying studies on thyroidectomised mice were started but not completed through lack of time

least 2 weeks after weaning.

1-Thyroxine-3'5'^{125}I (1) in aqueous propylene glycol was stored in the dark at 4°C for up to 1 month. Prior to use, iodide-^{125}I contamination was assessed by ascending chromatography on Whatmann 3MM paper with n-butanol:acetic acid:water (38:5:6) as solvent, as described in appendix 6 (section A6.2).

Disappearance of radioactivity from the blood of mice was followed by injecting 0.5-1mCi of T_4-^{125}I in 0.25 or 0.5ml saline i.v. into groups of 6-8 mice and taking 50µl blood samples at suitable intervals thereafter. Blood samples were submitted to iodide absorption on microcolumns of amberlite I.R.A. 400 as described in appendix 6. This method removed approximately 99% of the contaminating iodide with insignificant thyroxine-^{125}I losses under conditions similar to those used here. In experiments involving the calculation of the half-life, an initial equilibration phase of 6 hours was allowed before bleeding. The $t_{\frac{1}{2}}$ and y (0 hr.) intercept were calculated for each animal as described in appendix 1 and the results for each group were expressed as mean ± S.E.M. In later experiments, i.p. injection of labelled thyroxine was used for convenience since this did not alter the decay rate significantly (section 3.5.4).

3.5.3. The pattern of decay of intravenously injected T_4-^{125}I and iodide-^{13}I^{1} (expt. 3.28)

Method. A group of 5 mice was prepared as for the McKenzie assay with 10µg/ml thyroxine in water to drink and

(1) I.M.14, The Radiochemical Centre, Amersham

a control group of 5 animals was treated only with distilled water. Mice were injected with 1μCi of T_4-^{125}I and 1μCi iodide-^{131}I in saline (0.25ml) i.v., and bled at intervals up to 24 hours thereafter. Blood samples (50μl) were counted before and after amberlite I.R.A. 400 chromatography. Decay curves were constructed for protein bound ^{125}I and total ^{131}I which are shown in figures 3.20 and 3.21.

Results. The decay curves for T_4-^{125}I showed an initial equilibrium phase lasting approximately one hour and a slower exponential phase over the ensuing 24 hours. The latter showed a minor irregularity in this experiment with a faster decay at night (12-24 hours) in both groups, perhaps reflecting a greater clearance rate during the feeding period. The calculated half life ($t_{\frac{1}{2}}$) was 11.84 ± 0.50(1) for control mice as compared with 5.60 ± 0.39(1) for the thyroxinised mice. A larger distribution volume for the thyroxinised mice is evident from the lower zero hour intercept of the second decay phase. The percentage of the total ^{125}I which was protein bound remained at approximately 90% for the control group, but decreased to approximately 75% for the thyroxinised mice at 6 hours (fig. 3.22). This may be attributable to increased deiodination, releasing more iodide-^{125}I into the circulation.

The clearance of iodide was relatively slow in the present experiments with a $t_{\frac{1}{2}}$ of injected iodide-^{131}I in the order of 3.8 hours in the control mice (following an initial equilibration phase). In thyroxinised mice the decay was non-exponential but was similar to controls in overall fractional clearance rate over 12 hours (fig. 3.21).

---

(1) Mean ± S.E.M.

FIGURE 3.20

The decay rate of thyroxine-^{125}I in the mouse following intravenous injection (expt. 3.28)

Group	Exogenous thyroxine	Mouse wt.(1)	$t_{\frac{1}{2}}$(hr)(1,2)	Intercept(1) (c.p.m.)
A	-	20.4 ± 1.0	11.84 ± 0.50	53,650 ± 3,420
B	+	19.1 ± 1.1	5.60 ± 0.39(3)	43,750 ± 3,400(4)

Each point on graph represents mean ± S.E.M. Protein bound ^{125}I c.p.m. in 50µl blood (5 mice). T_4-^{125}I injected at time zero contained approximately 1% $^{125}I^-$ by paper chromatography. 5.19×10^6 c.p.m. injected per mouse.

(1) Mean ± S.E.M. of 5 mice.

(2) $t_{\frac{1}{2}}$ determined by method of least squares from last 3 points for each mouse.

(3) $t_{\frac{1}{2}}$ for two groups differ by Student's 't' test $p<0.001$.

(4) Intercepts not significantly different $0.5 > p < 0.1$.

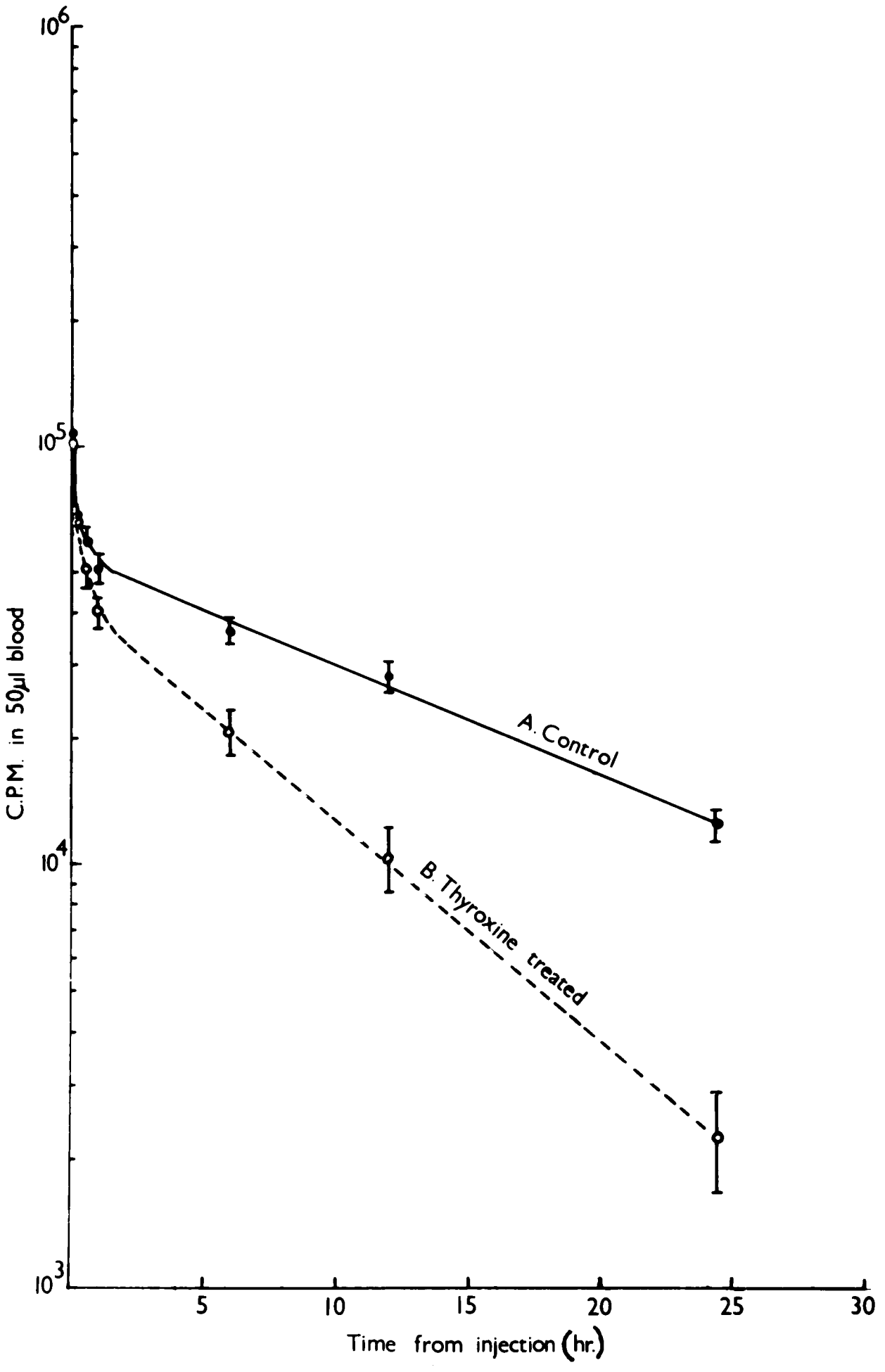

10^6
10^5
10^4
10^3
C.P.M. in 50µl blood
A. Control
B. Thyroxine treated
5
10
15
20
25
30
Time from injection (hr.)

FIGURE 3.21

The decay of iodide-^{131}I in normal and thyroxinised mice following i.v. injection (expt. 3.28)

A. Normal mice

B. McKenzie preparation (thyroxine treated)

Each point represents mean ± S.E.M. of 5 mice.

More than 90% of counts were removable by amberlite I.R.A. 400 chromatography at 12 hours. $^{131}I^-$ injected at time zero. $t_{\frac{1}{2}}$ for control group = 3.8 hours.

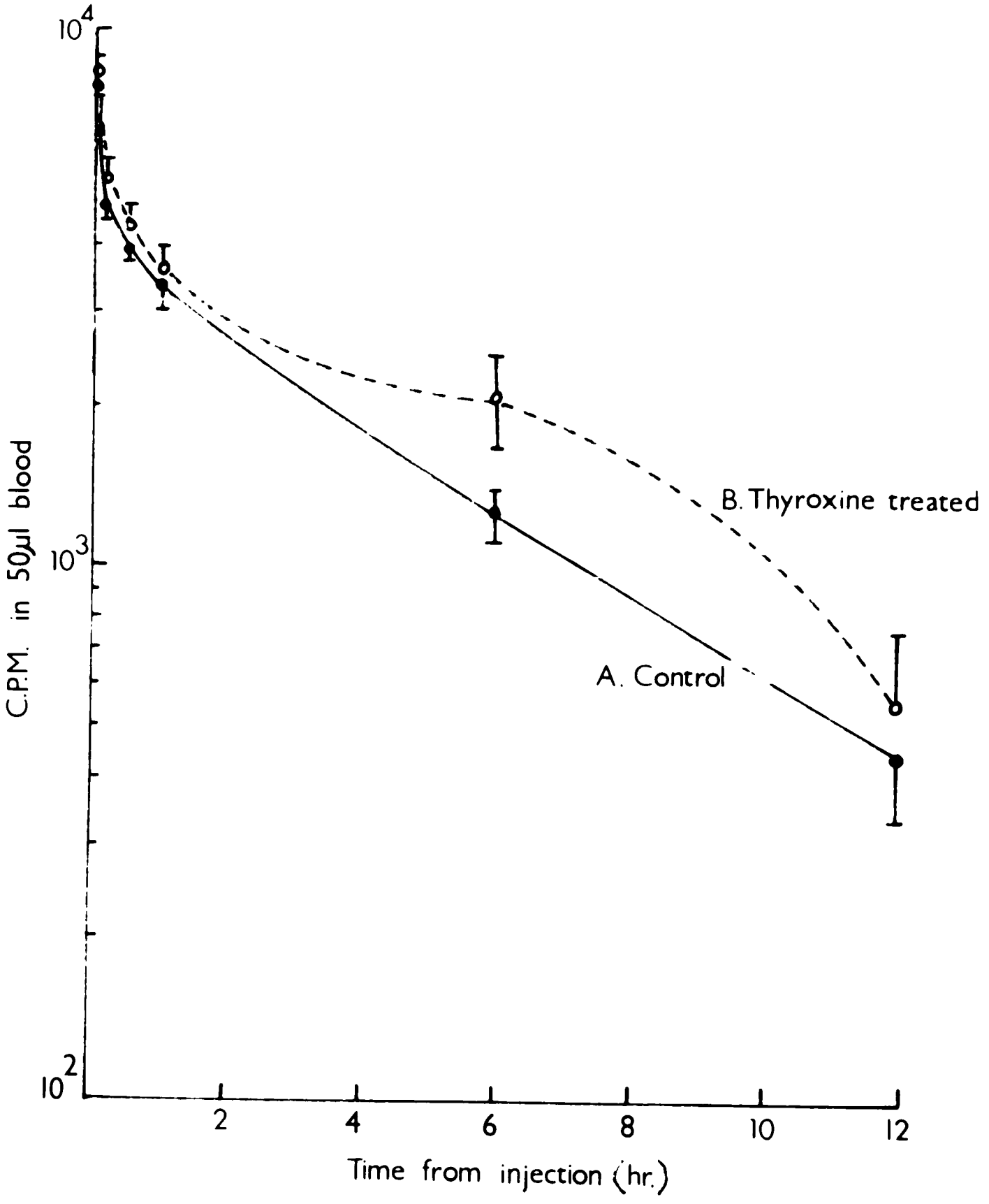

10^4
10^3
10^2
C.P.M. in 50µl blood
B. Thyroxine treated
A. Control
2
4
6
8
10
12
Time from injection (hr.)

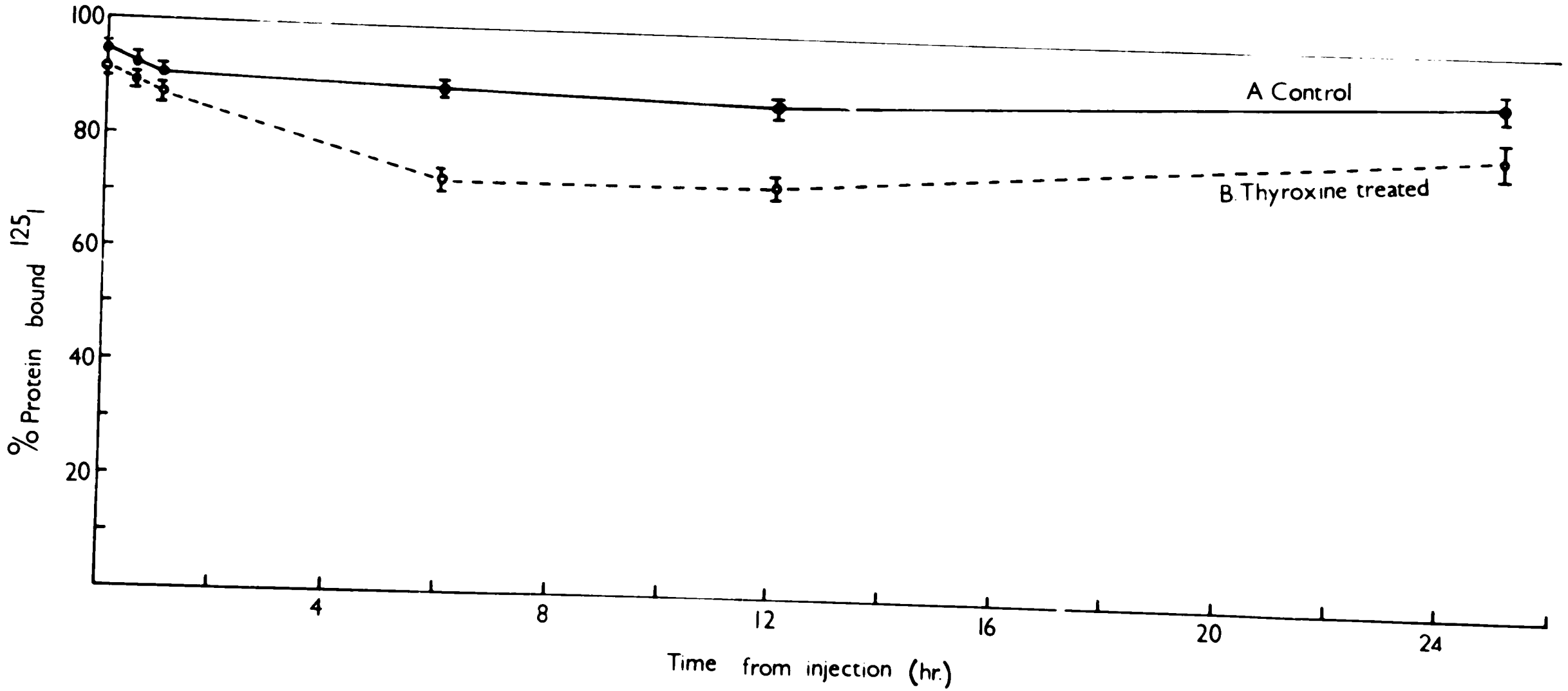
FIGURE 3.22

Thyroxine-^{125}I decay in the mouse. Percentage protein bound in whole blood at intervals following injection in the mouse as measured by amberlite I.R.A. 400 chromatography (expt. 3.28)

A. Control mice

B. McKenzie preparation (thyroxine treated)

Each point represents mean ± S.E.M. of 5 mice. Injected T_4-^{125}I contained only 1% iodide-^{125}I by paper chromatography n-butanol:acetic acid: water (38:5:6).


100
80
60
40
20
% Protein bound ^{125}I
A Control
B Thyroxine treated
4
8
12
16
20
24
Time from injection (hr.)

3.5.4. A comparison of the effects of intraperitoneal and intravenous routes of administration of labelled thyroxine on the disposal rate in normal and thyroxine-treated mice (expts. 3.29-3.30)

Intravenous administration of label is time-consuming and leads to occasional errors through extravascular injection. Intraperitoneal injection is more convenient but the label reaches the systemic circulation largely through the portal venous system, introducing the possibility of conjugation or deiodination in the liver en route. Therefore, decay of T_4-^{125}I was compared following i.p. or i.v. injections.

Method. Two experiments were performed: (1) Blood radioactivity levels were monitored in a group of thyroxinised mice following i.p. injection to find the time required for absorption and equilibration (expt. 3.29). (2) The effect of thyroxinisation was compared in groups of 6 randomly-selected mice given T_4-^{125}I i.p. or i.v. (expt. 3.30). Thyroxinisation consisted of preparation for the McKenzie assay, omitting ^{131}I. Mice whose injections of label were imperfect were omitted from the analysis.

Results. Figure 3.23 shows the pattern of radioactivity following an injection of T_4-^{131}I i.p. Rapid equilibration took place, giving way to exponential decay within 6 hours.

Figure 3.24 shows the results of experiment 3.30. Decay was indistinguishable for i.p. and i.v. injected mice except for a higher y (0 hr.) intercept for group D (i.p. thyroxinised) compared with group C (i.v. thyroxinised).

FIGURE 3.23

The pattern of decay following intraperitoneal T_4-^{131}I injection in mice (expt. 3.29)

Each point represents mean ± S.E.M. of 5 mice.

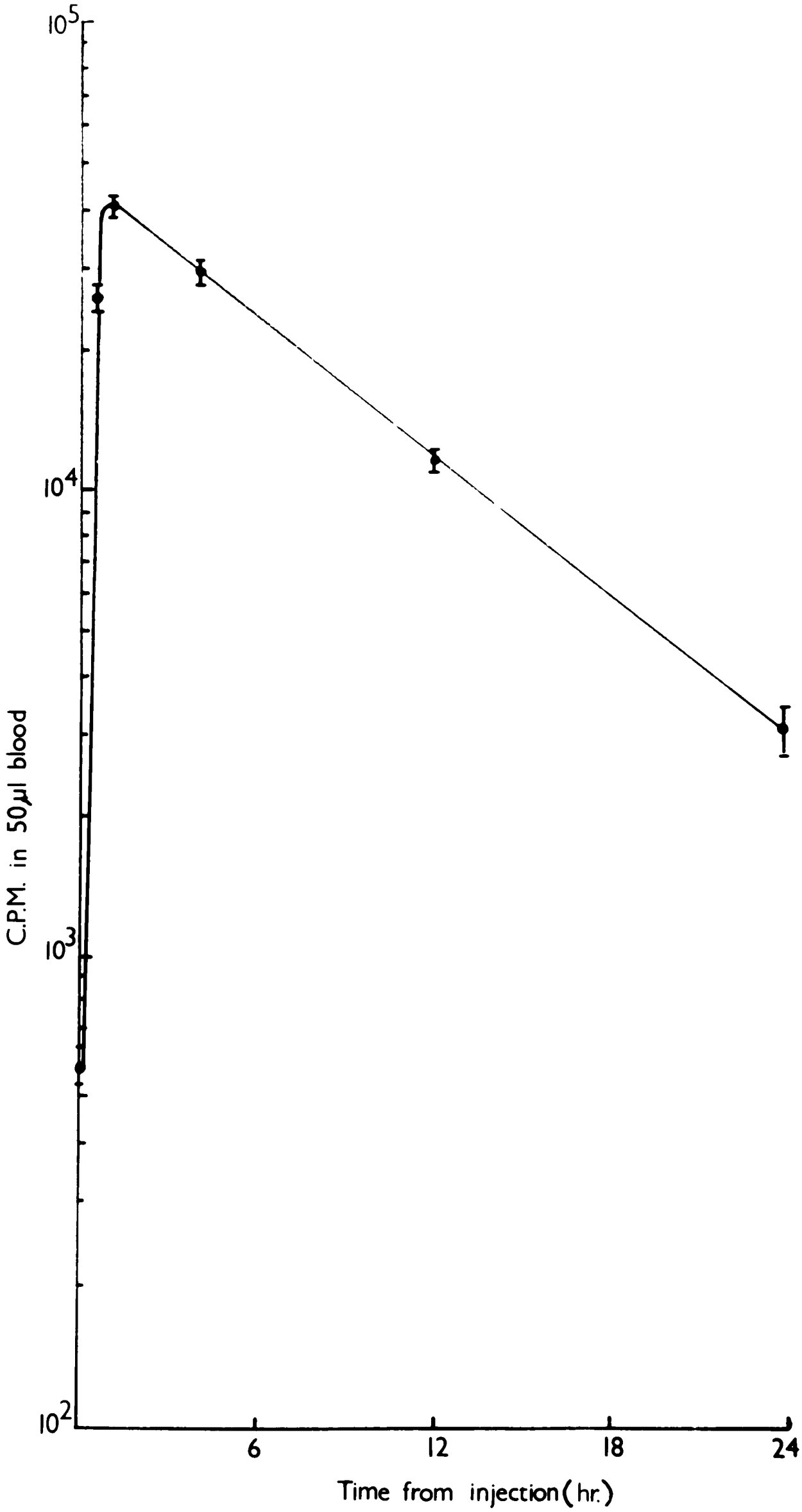

10^5
10^4
10^3
10^2
C.P.M. in 50µl blood
6
12
18
24
Time from injection (hr.)

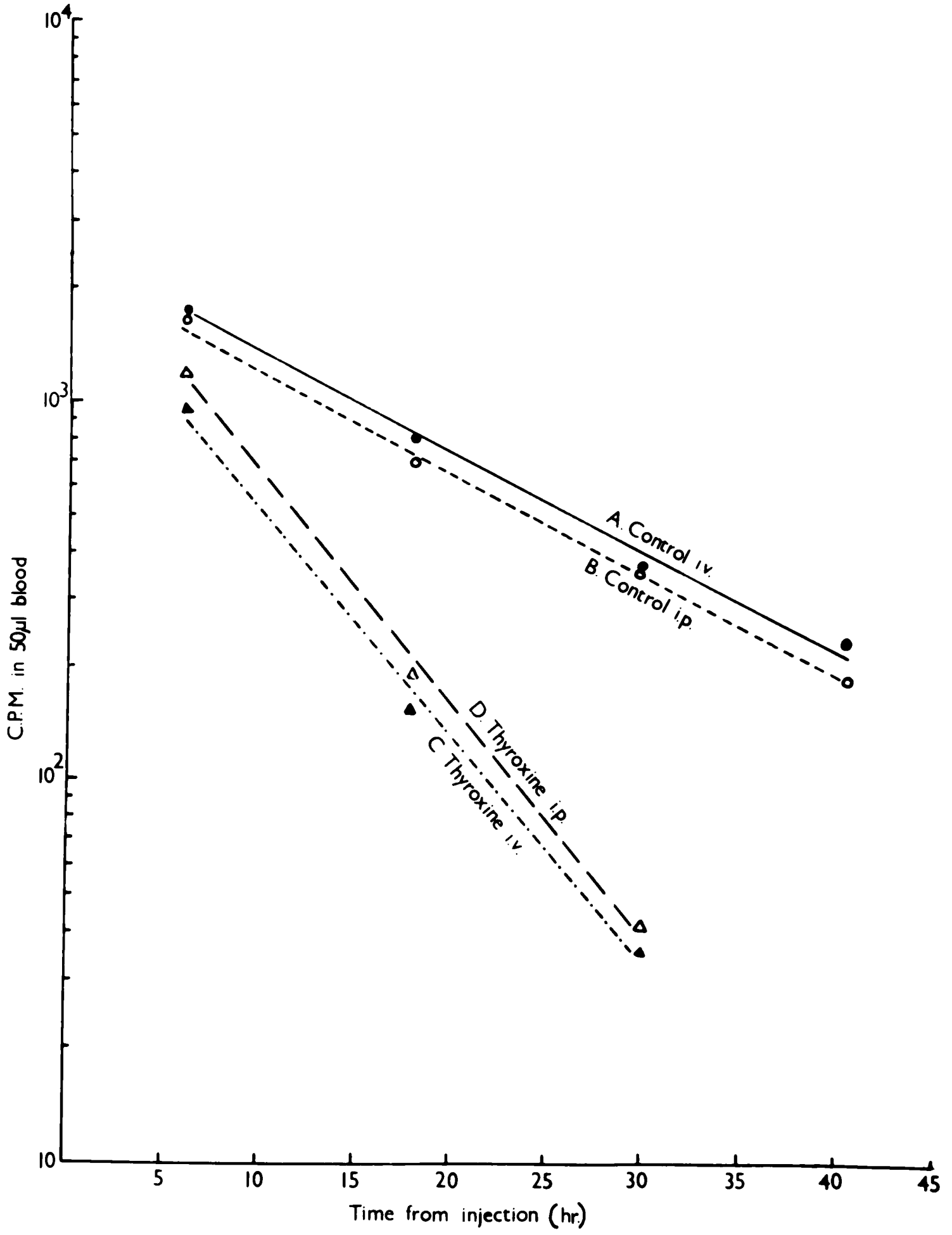
FIGURE 3.24

The decay of T_4-^{131}I in normal and thyroxinised mice following i.v. or i.p. injection (expt. 3.30)

Group	Exogenous T_4	Injection route	Animal wt.(g)(1)	$t_{\frac{1}{2}}$(hr)(1,2)	Intercept(1,3) (c.p.m.)
A	-	i.v.	24.1 ± 0.8	11.89 ± 1.01	2,351 ± 188
B	-	i.p.	24.2 ± 0.6	11.39 ± 0.65	2,272 ± 108
C	+	i.v.	26.5 ± 1.0	5.02 ± 0.19	2,106 ± 60
D	+	i.p.	25.1 ± 1.4	4.67 ± 0.38	2,868 ± 214

Each point represents mean P.B.^{125}I of 5 mice (A,D) or 6 mice (B,C).
T_4-^{131}I (injected at time zero) contained 12% $^{131}I^-$ by paper chromatography.
5.65×10^5 c.p.m. T_4-^{131}I injected in 0.25ml normal saline.

(1) Mean ± S.E.M.
(2) $t_{\frac{1}{2}}$ for A and C or B and D significantly different. No significant difference between A and B or C and D (Duncan's test).
(3) D intercept significantly > A, B or C ($p<0.01$) by Duncan's test. No significant heteroscadasticity.


C.P.M. in 50µl blood
10⁴
10³
10²
10
A. Control i.v.
B. Control i.p.
D. Thyroxine i.p.
C. Thyroxine i.v.
5
10
15
20
25
30
35
40
45
Time from injection (hr.)

This was significant by Student's 't' test at a level of $p<0.01$. The reason for this is not clear. Since no difference in decay rates was noted between the two routes of injection, i.p. injection was chosen subsequently, for convenience.

3.5.5. The effect of T.S.H. on the decay of T_4-^{125}I in the mouse (expts. 3.31-3.32)

If T.S.H. were to make any contribution to its response in the McKenzie assay via an alteration in thyroxine disposal rate it should do so at normal thyroid stimulatory doses. For this reason large doses or repeated injections were not employed. Two experiments were performed, checking the decay rate over 24 hours and 6 hours, respectively, following T.S.H. injection.

Method. In each experiment three groups of 8 randomly selected mice were thyroxinised by preparation for the McKenzie assay (omitting ^{131}I). They were injected with approximately 1μCi T_4-^{125}I and bled (50μl) 6 hours later. Immediately after bleeding, 0.5ml 1% B.S.A. (group A), 1mU b-T.S.H.[1] (group B) or 10mU b-T.S.H. (group C) in 0.5ml 1% B.S.A. were injected. Further blood samples were taken 12 hr. and 24 hr. (expt. 3.31), or 6 hr. later (expt. 3.32). Blood was subjected to amberlite I.R.A. 400 chromatography before counting. In a further experiment

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(1) International Standard b-T.S.H. in 1% B.S.A.

(4.7), described in section 4.4, T_4-^{125}I decay was followed over 2 hours after the injection of 0.5mU T.S.H.

Results. Figure 3.25 shows the decay curves for the first experiment (3.31) which were exponential over the period studied. No difference in decay rate or intercept values could be found. In experiment 3.32 results were expressed as $\frac{\text{c.p.m.}_{12\text{ hr.}}}{\text{c.p.m.}_{6\text{ hr.}}}$ (measuring times from the T_4-^{125}I injection) and, again, no significant difference could be detected between groups (table 3.8). Similarly, in experiment 4.7 T.S.H. failed to alter T_4-^{125}I decay over 2 hours (table 4.2).

3.5.6. The effect of L.A.T.S. or control IgG on T_4-^{125}I disposal rate (expt. 3.33)

Method. In a single experiment three groups of randomly selected mice were thyroxinised and injected with T_4-^{125}I as above. After the first (6 hr.) bleed, 0.5ml of normal saline (group A), 2.9mg N.IgG (L.A.T.S.-negative) in 0.5ml saline (group B) or 2.9mg of R.N.1 IgG (prepared from a potent L.A.T.S. serum) in 0.5ml saline (group C) was injected and further bleeds (50µl) were undertaken at 12 hr., 18 hr. and 30 hr. after the first injection (T_4-^{125}I). Tubes were submitted to amberlite I.R.A. 400 absorption before counting.

Results. Figure 3.26 and the accompanying table show that decay rates were the same for all groups.

3.5.7. Discussion

The regression analysis used here assumes the applicability of single compartment kinetics to iodothyronine

b-T.S.H.	No. of mice	Animal wt.(g)[1]	$t_{\frac{1}{2}}$(hr)[1,2]	Intercept[1,3] (c.p.m.)
Control	8	18.6 ± 0.8	5.42 ± 0.18	25,787 ± 1062
1mU	8	18.5 ± 0.4	5.44 ± 0.20	24,236 ± 691
10mU	7	18.1 ± 0.7	5.09 ± 0.20	28,482 ± 1421

Each point represents the mean P.B.^{125}I of 8 mice (A,B) or 7 mice (C). Injected T_4-^{125}I contained 3.5% ^{125}I by paper chromatography. 2.62×10^6 c.p.m. T_4-^{125}I injected (i.p.) at time zero. T.S.H. injected (i.v.) in 1% B.S.A. immediately after first bleed (6 hr.). All mice thyroxine treated.

(1) Mean ± S.E.M.
(2) No significant difference between groups by analysis of variance.
(3) Intercepts of T.S.H. treated groups not significantly different from control (Duncan's test).

FIGURE 3.25

The effect of T.S.H. on the decay of T_4-^{125}I over 30 hours in the mouse (expt. 3.31)

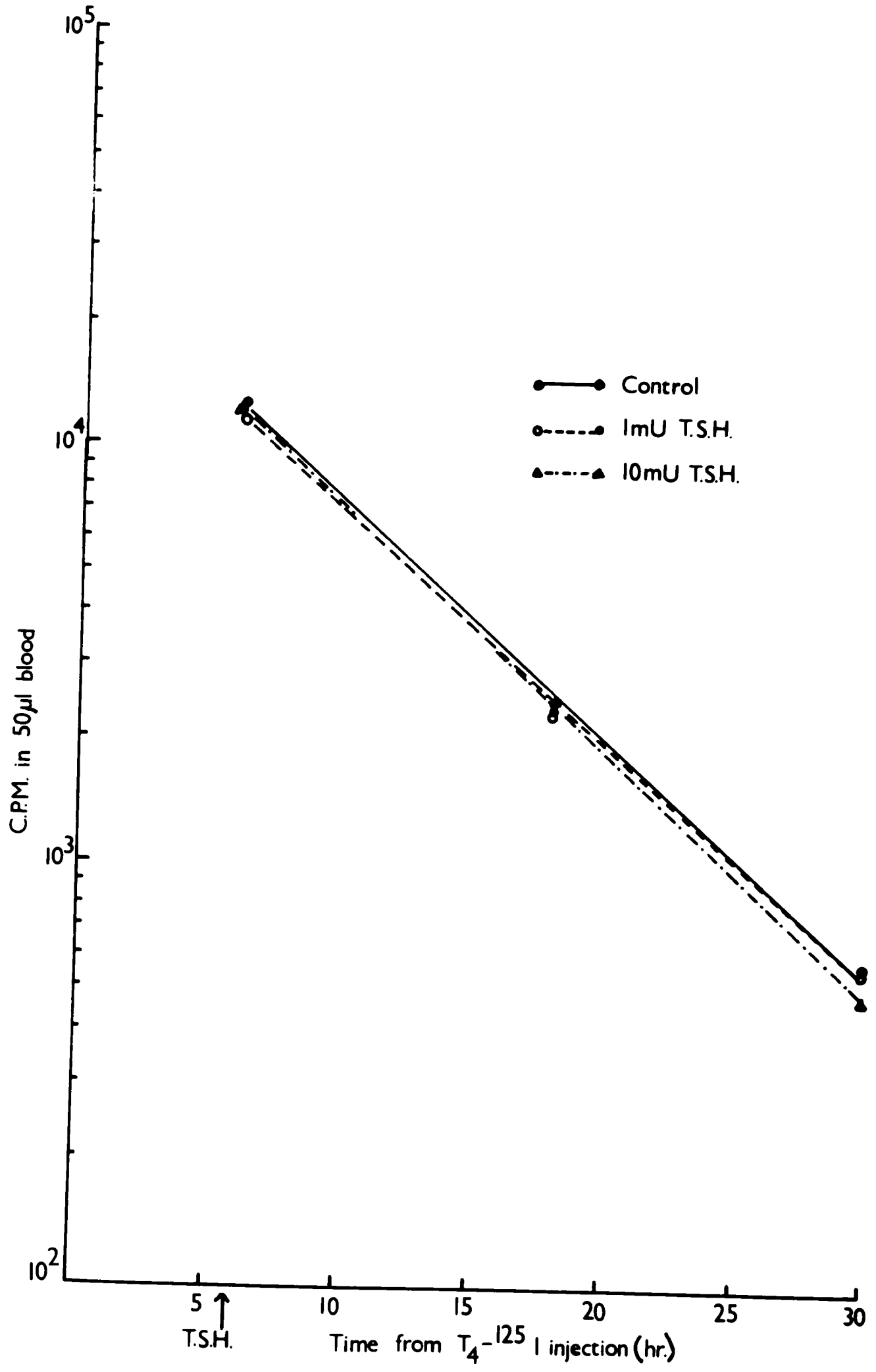

10^5
10^4
10^3
10^2
C.P.M. in 50µl blood
Control
1mU T.S.H.
10mU T.S.H.
5
T.S.H.
10
15
20
25
30
Time from T_4-^{125}I injection (hr.)

TABLE 3.8. The effect of T.S.H. on the decay of T_4-^{125}I over 6 hours in mice (expt. 3.32)

Group	b-T.S.H.	Animal wt.(g)(1)	$\frac{\text{c.p.m. at 12 hr. x 100}}{\text{c.p.m. at 6 hr.}}$(1)	$\frac{\log \text{c.p.m. 12 hr.}}{\log \text{c.p.m. 6 hr.}}$(1)
A	Control	19.8 ± 1.4	45.1 ± 2.4	1.649 ± 0.025
B	1mU	20.8 ± 1.3	44.8 ± 2.2	1.648 ± 0.022
C	10mU	18.8 ± 1.4	45.2 ± 3.5	1.647 ± 0.030

(1) Mean ± S.E.M. for 8 mice. No significant difference between groups by analysis of variances. Mice treated with thyroxine as for McKenzie assay.

Group	Treatment	Mean ± S.E.M.	Range	Significance (2)
A	Control - saline	11.1 ± 1.1	5.37 - 0.23	±
B	R.N.(I.A.E.R. - positive)IgG-1	1.4 ± 1.1	3.12 ± 0.[illegible]	±
C	R.N.(I.A.T.C. positive)IgG-2	7	11.7 ± 1.1	5.1[illegible] ± [illegible]

Each point represents the mean P.B. ^{125}I of 5 mice (A,B) or 3 mice (C). Injected T_{44} ^{125}I corrected [illegible] ^{125}I-by paper chromatography. 18.7 x 10^6 c.p.m. injected (i.p.) at time [illegible] .25ml normal saline. IgG injected (i.v.) in 0.5ml normal saline 11 days after first bleed (6 hr.). All mice thyroxine treated.

(1) Mean ± S.E.M.
(2) No significant difference between groups by analysis of variance.

Fig. 1. Effect of [illegible] positive and [illegible] T_{44} ^{125}I

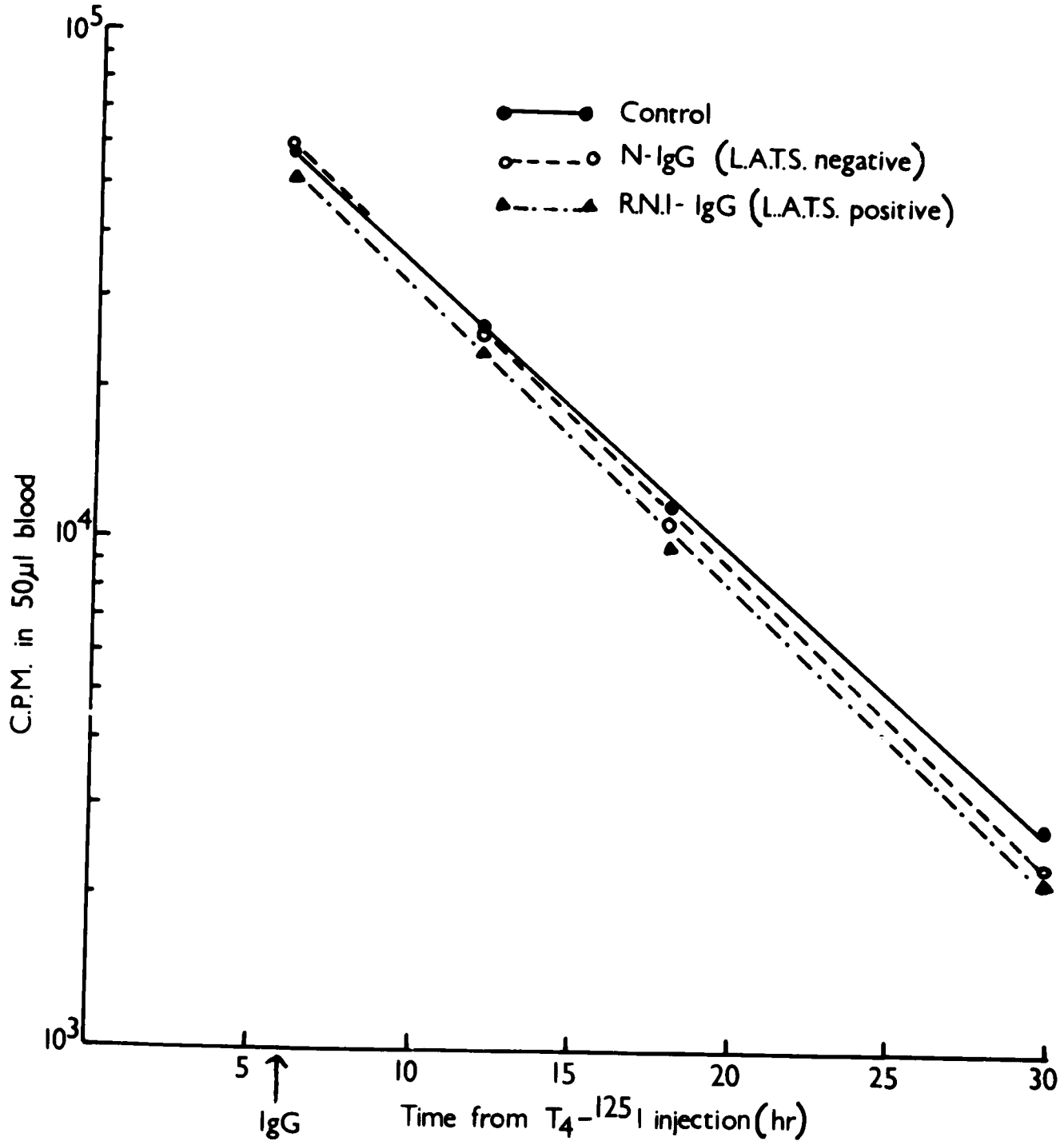
FIGURE 3.26

The effect of L.A.T.S. positive and L.A.T.S. negative IgG on the decay rate of T_4-^{125}I in the mouse (expt. 3.33)

Group	IgG (0.5ml)	No. of mice	Animal wt.(g)(1)	$t_{\frac{1}{2}}$(hr)(1,2)	Intercept (c.p.m.)(1,2)
A	Control - saline	8	21.1 ± 1.1	5.37 ± 0.23	12,261 ± 647
B	N.(L.A.T.S. negative)IgG-2.9mg	8	21.6 ± 1.1	5.13 ± 0.12	13,134 ± 941
C	R.N.1 (L.A.T.S. positive)IgG-2.9mg	7	21.2 ± 1.1	5.16 ± 0.17	11,471 ± 1760

Each point represents the mean P.B. ^{125}I of 8 mice (A,B) or 7 mice (C). Injected T_4-^{125}I contained 1.5% $^{125}I^-$ by paper chromatography. 18.7×10^6 c.p.m. injected (i.p.) at time zero in 0.25ml normal saline. IgG injected (i.v.) in 0.5ml normal saline immediately after first bleed (6 hr.). All mice thyroxine treated.

(1) Mean ± S.E.M.
(2) No significant difference between groups by analysis of variance.


Control
N-IgG (L.A.T.S. negative)
R.N.I- IgG (L.A.T.S. positive)
C.P.M. in 50µl blood
10^5
10^4
10^3
5
10
15
20
25
30
IgG
Time from T4-125I injection (hr)

metabolism. This has been challenged recently and alternative methods have been devised for use in man, involving computer calculation of constants for three or four compartment models (Koutras et al, 1970; Nicoloff and Dowling, 1968) computer integration of decay curves in a non-compartment analysis (Oppenheimer et al, 1975) or a constant infusion technique (Cavalieri et al, 1970). Whilst such techniques yield strikingly different distribution volumes and fractional clearance rates for T_3 the changes for T_4 are less marked (Oppenheimer et al, 1975). However, non-compliance with single compartment kinetics probably accounted for the minor deviations from the exponential in some of the present T_4-^{125}I decay curves, but the approximations made were close enough to allow meaningful comparison of fractional turnover rates. Indeed the figures for thyroxine half life obtained here were in good agreement with those obtained by others, using similar techniques, in mice (Florsheim et al, 1970; Wills and Schindler, 1970). The use of commercially available T_4-^{125}I or T_4-^{131}I as tracer raises certain objections:

1. The specific activity of commercial T_4-^{125}I or T_4-^{131}I (50μCi/mg for T_4-^{125}I) necessitates the injection of physiologically significant doses to obtain workable radioactivity levels. In euthyroid animals, injected label may comprise 10% of the total extra thyroid thyroxine pool (Galton, 1968). However, in experiments on thyroxinised mice the injected label was only a small fraction of administered thyroxine.

2. Commercially available T_4-^{125}I (or T_4-^{131}I) is labelled only in the α ring (3'5' position) whereas biosynthetic labelling introduces radioiodine equally into α and β rings (Plaskett, 1961). Thus, theoretically, commercial T_4-^{125}I and endogenously labelled thyroxine could decay at different rates (Rall et al, 1964). However, for this to be true, endogenously labelled thyroxine metabolites, such as reverse T_3, must accumulate in the circulation. But the absolute levels of other iodinated compounds in blood are small in comparison with thyroxine (Pileggi et al, 1964; Chopra, 1974) and, moreover, chromatography of serum taken from mice with prelabelled thyroid glands reveals only thyroxine and iodine, with traces of triodothyroxine (Pinchera et al, 1965). Therefore, this objection is unlikely to be of consequence (see Rall et al, 1964, for further discussion).

Recirculation of iodine through the thyroid gland could theoretically distort thyroxine decay curves, but since endogenously labelled thyroxine would be subject to the same process, no effort was made to eliminate it. In any case, Wills and Schindler (1970) and Solomon et al (1970) both considered recirculation to be insignificant on the basis of thyroid uptake of iodide derived from labelled thyroxine. Moreover, prevention of recirculation by iodide or methimazole did not alter the distribution space of T_4-^{131}I (Wills and Schindler, 1970). In the present experiments (expt. 3.28) no significant quantities of protein bound radioiodine appeared in the blood of mice over 24 hours following the injection of 1μCi radioiodide. Thus, recirculation was not significant in mice

with unstimulated glands. Recirculation through stimulated glands was also unlikely to be significant since there was no significant change in distribution volume with T.S.H. or L.A.T.S. (expts. 3.31-3.32)

Thyroxine levels were not measured in these experiments. T.S.H. and L.A.T.S. would undoubtedly have increased the blood thyroxine level but such increases would probably have been small in comparison with the high initial levels in McKenzie prepared mice. In one experiment in which protein bound blood radioactivity was measured in mice, treated as for the McKenzie assay with thyroxine containing T_4-^{125}I at a known specific activity, the whole blood T_4 level was calculated to be $63 \pm 11\mu g/100ml$[1] (see appendix 6).

Neither T.S.H. nor L.A.T.S. IgG affected the decay pattern or half life of thyroxine in the thyroxinised mice. Therefore, the response to these stimulators in the McKenzie assay may be taken to represent a direct thyroid action.

3.6. DISCUSSION AND CONCLUSIONS

The experiments above confirm that the longer time course of the response to L.A.T.S. in the McKenzie assay is attributable to the slower decay rate of the stimulator.

Labelled thyrotropin decays very rapidly in the mouse and very little could be detected chromatographically 40 minutes after injection[2]. Despite this the response to T.S.H. in the McKenzie assay peaks at 2 hours and continues for at least 7 hours. The colloid droplet response peaks somewhat earlier (at 30 minutes) but continues for more than 2 hours

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(1) Mean ± S.E.M. of 8 mice
(2) Protein bound counts reduced to <3% of 1 min. value (expt. 3.9)

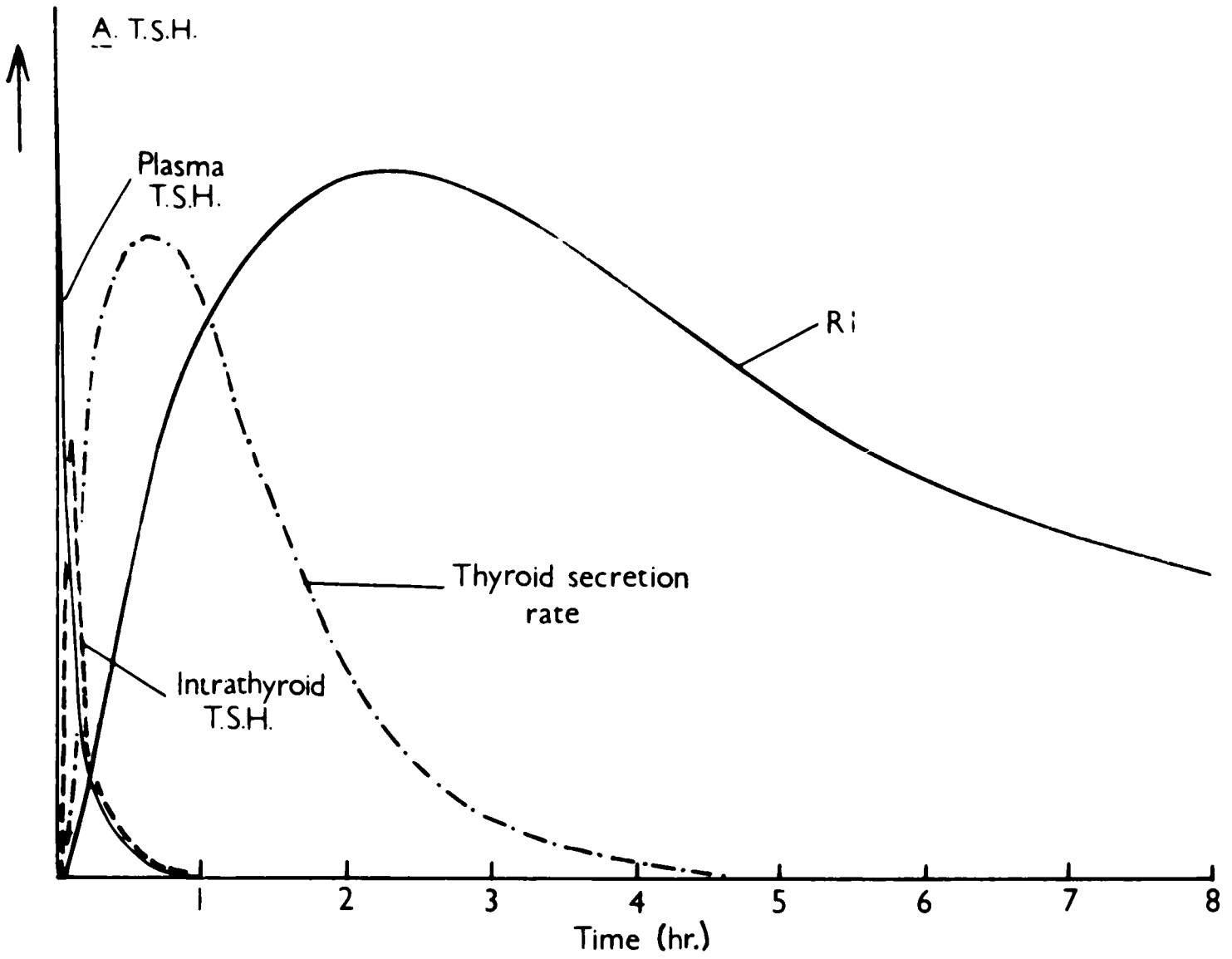
after T.S.H. injection in the mouse (Shishiba et al, 1967). Thus thyroid activation continues for some time after the loss of T.S.H. from the circulation. Since excess anti-T.S.H. antiserum had no effect on the circulating radioiodine response when injected more than 10 minutes after T.S.H., the delayed response peak was unlikely to be due to persistent binding of the stimulator by thyroid receptors. Malan et al (1974) have shown that the persistent response to T.S.H. of rat thyroid lobes in the presence of trypsin in vitro is confined to the secretion process and is not shared by cA.M.P. activation. They suggested that the rate at which thyroid secretion is "switched off" is dependent upon the rate of thyroglobulin hydrolysis within colloid droplets. This could also be the step limiting the rate at which the mouse thyroid is 'switched off' in vivo following a T.S.H. injection.

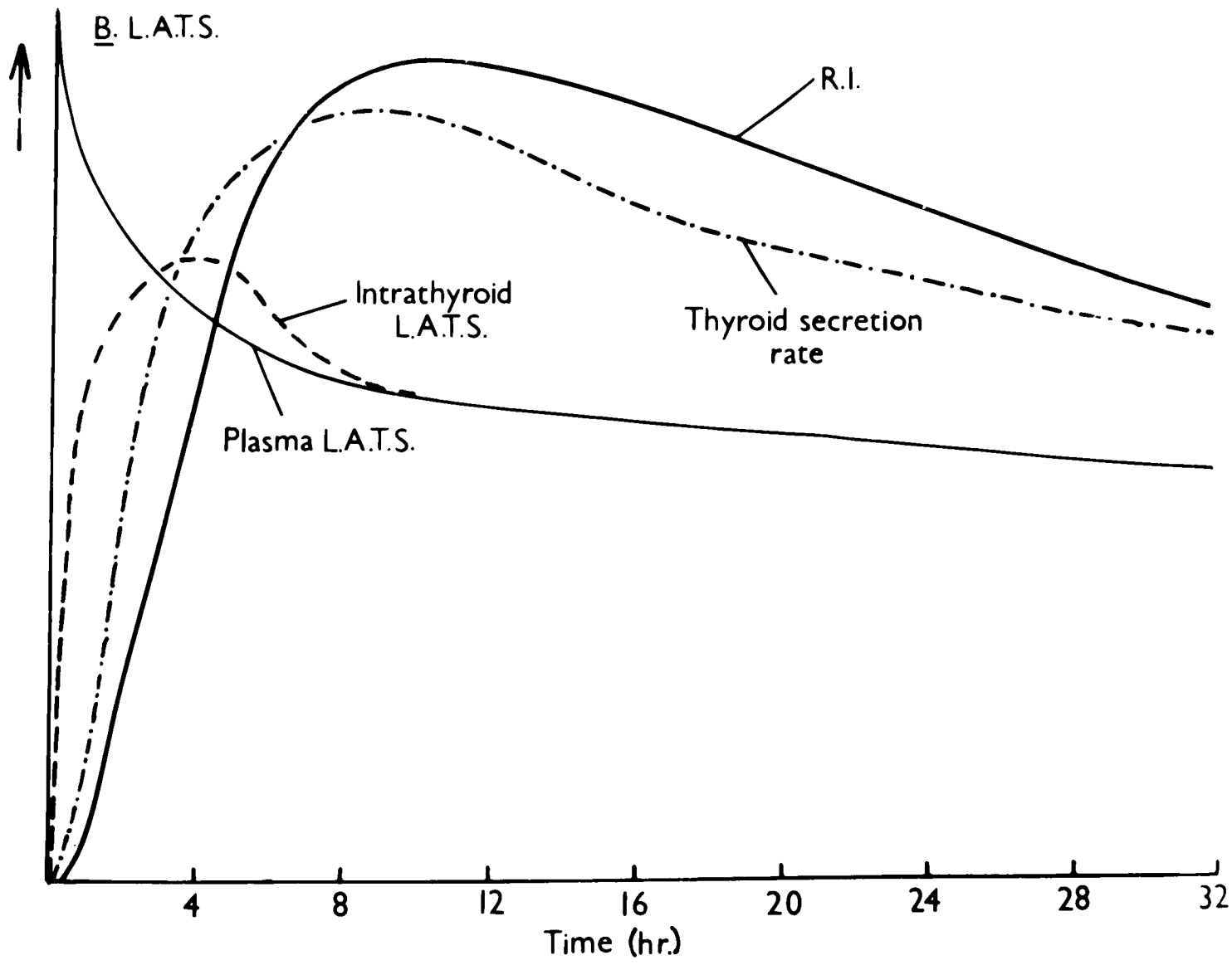
The postulated sequence of events following an intravenous injection of T.S.H. is represented in figure 3.27A. T.S.H. rapidly disappears from the circulation making a transitory appearance in the extracellular fluid. Enhanced thyroid secretion is initiated at this time and persists due to the slow process of thyroglobulin digestion. The response index peaks even later because of the time taken to build up circulating radioiodine but subsequently decays following the cessation of the thyroid response.

In contrast to T.S.H., h-IgG decayed slowly in mice with an initial equilibration phase lasting 12-24 hours and a slower exponential decay phase whose half life was in the order of

FIGURE 3.27

A theoretical analysis of the events leading to stimulation following the injection of T.S.H.(A) or L.A.T.S.(B) in the McKenzie assay


A. T.S.H.
Plasma
T.S.H.
R i
Thyroid secretion
rate
Intrathyroid
T.S.H.
1
2
3
4
5
6
7
8
Time (hr.)


B. L.A.T.S.
R.I.
Intrathyroid
L.A.T.S.
Thyroid secretion
rate
Plasma L.A.T.S.
4
8
12
16
20
24
28
32
Time (hr.)

100 hours. L.A.T.S. IgG decayed at the same rate as normal IgG. In a single experiment L.A.T.S. activity decayed at a similar rate to total IgG. A minor discrepancy between L.A.T.S. activity and h-IgG concentration at 72 hours after injection could be explained by thyroid uptake of L.A.T.S. However, substantial L.A.T.S. activity could still be detected at this time.

Smith and Munro (1972) demonstrated that the continuing response in the McKenzie assay depends on the persistence of circulating L.A.T.S. They injected (i.p.) a human thyroid 4S fraction, which was capable of absorbing L.A.T.S., 8 hours after an injection (i.v.) of L.A.T.S. and noted that the subsequent response was reduced. This constitutes further evidence that L.A.T.S. is long acting because of its slower decay rate(1).

Figure 3.27B shows the postulated sequence of events following L.A.T.S. injection. The activity of the thyroid gland at any time following an injection of L.A.T.S. is assumed to depend on the intrathyroid extracellular fluid L.A.T.S. concentration. However, the validity of this assumption is difficult to assess since on the one hand L.A.T.S. might be persistently bound in an active form or, on the other hand, some degree of adaptation to constant levels might occur(2). Intrathyroid extracellular fluid L.A.T.S. concentration increases during the distribution

---

(1) The elimination of L.A.T.S. IgG by injections of anti-h-IgG antiserum was attempted here but this proved lethal, probably because of a hypersensitivity reaction induced by large quantities of circulating antigen-antibody complexes.

(2) The possibility of adaptation is raised by the demonstration of thyroid refractoriness following T.S.H. exposure in vitro (Kaneko, 1976; Shuman et al, 1976; Takasu et al, 1976)

phase to reach a maximum during the first 24 hours. The subsequent decline in intrathyroid L.A.T.S. concentration probably mirrors plasma concentration. Peak thyroid activity should accompany the maximum intrathyroid L.A.T.S. activity but the peak response index occurs somewhat later because of the time required for the build up of labelled thyroxine in the circulation. The subsequent decay of the response probably depends on the rate of elimination of L.A.T.S. from the circulation and the rate at which thyroid radioiodine stores are depleted.

Conclusion: T.S.H. is short-acting because of its rapid plasma decay rate, the pattern of response being determined by intrathyroid mechanisms. L.A.T.S. is long-acting because of its slow plasma decay rate.

PART 4

RECEPTORS IN THE MOUSE THYROID

Experiments on the actions of various biogenic or synthetic amines on the mouse thyroid in vivo and the effects of receptor blocking agents.

4.1. INTRODUCTION

The extensive work of Melander and his colleagues has established that a variety of amines, including adrenaline, noradrenaline, isopropyl noradrenaline (I.P.N.A.), dopamine and 5-hydroxytryptamine (5-H.T.) are able to stimulate secretion by the mouse thyroid in vivo (Melander, 1971a). The actions of adrenaline and noradrenaline could be blocked by pretreatment with suitable doses of the α-receptor blocking agents, phenoxybenzamine (P.B.A.) and phentolamine (Ph.A.) and therefore would appear to be mediated by α-receptors on the thyroid cell (Ericson et al, 1970; Melander, 1970, 1971a, b). The action of 5-H.T. was also blocked by α-antagonists, as had been noted for other systems (Gyermek, 1966). However, at the doses used, both α-blockers inhibited the actions of T.S.H. and L.A.T.S., whilst the stimulating action of I.P.N.A. was unchanged or mildly enhanced. On the other hand, the action of the predominantly β-agonist, I.P.N.A., was inhibited by a β-blocking agent, dl-propranolol, at doses which failed to affect the response to T.S.H., L.A.T.S., adrenaline, noradrenaline or 5-H.T.

The inhibitory action of α-blocking agents on stimulation by T.S.H. and L.A.T.S. but not I.P.N.A. raised the possibility that α-adrenergic (or 5-H.T.) receptors may be related to or closely linked with receptors for T.S.H. and L.A.T.S. (Melander, 1971a). This hypothesis would appear attractive in the light of the formerly widely held belief that thyrotoxicosis may be precipitated by stress (Dewhurst et al, 1968). Therefore, in the present work, a series of experiments was undertaken in an attempt to clarify the relationship between α- and β-adrenergic, T.S.H. and L.A.T.S. receptors by testing the effects of receptor blocking

agents on the response of the mouse thyroid to various agents in vivo.

4.2. MATERIALS AND METHODS

The McKenzie assay was used as described in part 2 (section 2.1) to test the effects of possible stimulators on the mouse thyroid. Results were analysed using Student's or Dunnett's 't' tests as described in appendix 1.

International Standard b-T.S.H.[1] was used throughout. The characteristics of the L.A.T.S. sera used here (R.N., R.W.) are described in appendix 4.

l-adrenaline[2] and l-isopropyl noradrenaline[2] were stored in the dark at room temperature. 5-hydroxytryptamine creatinine sulphate[2] and l-noradrenaline hydrochloride[2] were stored, tightly capped, in a desiccator at -10°C. dl-propranolol[3] and phenoxybenzamine[4], which were gifts from Dr. P. Bolton Department of Pharmacology, Oxford, were stored in the dark at room temperature. Anti-T.S.H. antibodies were prepared and stored as described in appendix 5.

Substances for intravenous (i.v.) administration were injected in 0.5ml 1% B.S.A., whereas those for intraperitoneal (i.p.) injection were made up in 0.5ml normal saline. Solutions of adrenaline required the addition of 0.05 to 0.1ml 1N HCl per 20ml for dissolution and appropriate controls of 1% B.S.A. acidified to the same degree were employed.

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(1) M.R.C. Standards Division, N.I.M.R., Mill Hill
(2) Sigma Chemical Co., Kingston-upon-Thames, Surrey
(3) 'Inderal', I.C.I. Pharmaceuticals Ltd., Macclesfield, Cheshire
(4) 'Dibenzyline', Smith Kline and French Laboratories, Welwyn Garden City, Hertfordshire

4.3. THE EFFECTS OF ADRENALINE, NORADRENALINE, ISOPRENALINE (I.P.N.A.) AND 5-HYDROXYTRYPTAMINE (5-H.T.) ON MOUSE THYROID SECRETION IN VIVO (expts. 4.1-4.6)

Method and Results. In the first experiments adrenaline, noradrenaline, I.P.N.A. and 5-H.T. were injected intravenously into groups of 8 mice, prepared as for the McKenzie assay. $Log_{10}R.I._{2\ hr.}$ values (mean ± S.E.M.) were calculated for each group and compared by Duncan's test with that of a control group injected with 1% B.S.A. (table 4.1).

Adrenaline and noradrenaline, at doses of 0.05μM per animal as used by Melander (1971a) proved lethal in every case, even with slow injection over 30 seconds (table 4.1A). In another experiment the same dose was lethal when infused over 1 minute into mice which had not received thyroxine. The maximum sublethal dose (0.013μM adrenaline: 0.025μM noradrenaline) failed to give a detectable response in a further experiment (table 4.1B).

5-H.T. and I.P.N.A. consistently gave significant responses at doses similar to those used by Melander (table 4.1A, C, D). The peak occurred at about 2 hours (fig. 4.1). However, the responses to both agents were small (usually <2.3) and the gradients of the dose response lines were shallow (expt. 4.4, table 4.1C; expt. 4.5, fig. 4.2). At all doses used, 5-H.T. caused some degree of distress to the mice as shown by transient tachypnoea and hypokinesia lasting for an hour or more. I.P.N.A. had no obvious effect on the behaviour of the mice other than a transient tachypnoea lasting for less than five minutes after injection.

TABLE 4.1. The effect of adrenaline (A.), noradrenaline (N.A.), 5-H.T. and I.P.N.A. on thyroid iodine release in the mouse in vivo

Experiment	Sex	Injection	No. of mice	Log_{10} R.I.$_{2\ hr.}$ (1)	p (2)
A. 7.3.72. (expt. 4.1)	F	1% B.S.A.	8	1.931 ± 0.037	-
		0.05μM A.	0 (8) (3)	Lethal	
		0.05μM N.A.	0 (8)	Lethal	
		1.1μM 5-H.T.	8	2.204 ± 0.034	<0.01
		0.05μM I.P.N.A.	7	2.099 ± 0.066	<0.05
		0.05mU T.S.H.	8	2.710 ± 0.072	<0.01
B. 20.3.72. (expt. 4.2)	M	1% B.S.A.	8	1.910 ± 0.020	-
		0.025μM N.A.	6 (2)	1.878 ± 0.048	N.S.
		0.013μM N.A.	6 (1)	1.973 ± 0.046	N.S.
		0.5mU T.S.H.	8	2.709 ± 0.041	<0.01
		Acidified B.S.A.	8	1.990 ± 0.022	-
		0.013μM A.	4 (4)	2.002 ± 0.037	N.S.
		0.006μM A.	5 (3)	1.895 ± 0.081	N.S.
C. 13.4.72. (expt. 4.4)	M	1% B.S.A.	8	1.946 ± 0.024	-
		0.05μM I.P.N.A.	8	2.178 ± 0.024	<0.01
		0.15μM I.P.N.A.	8	2.198 ± 0.038	<0.01
D. 10.8.72. (expt. 4.6)	M	1% B.S.A.	8	2.011 ± 0.024	-
		0.30μM I.P.N.A.	7	2.402 ± 0.042	<0.01
		10 mg glucose	8	2.016 ± 0.027	N.S.
		50 mg glucose	8	1.925 ± 0.026	N.S.

(1) Mean ± S.E.M.
(2) p for difference from control (Duncan's test)
(3) Number of deaths in brackets

FIGURE 4.1

The time course of response to 5-H.T. and I.P.N.A.
(Experiment 4.3)

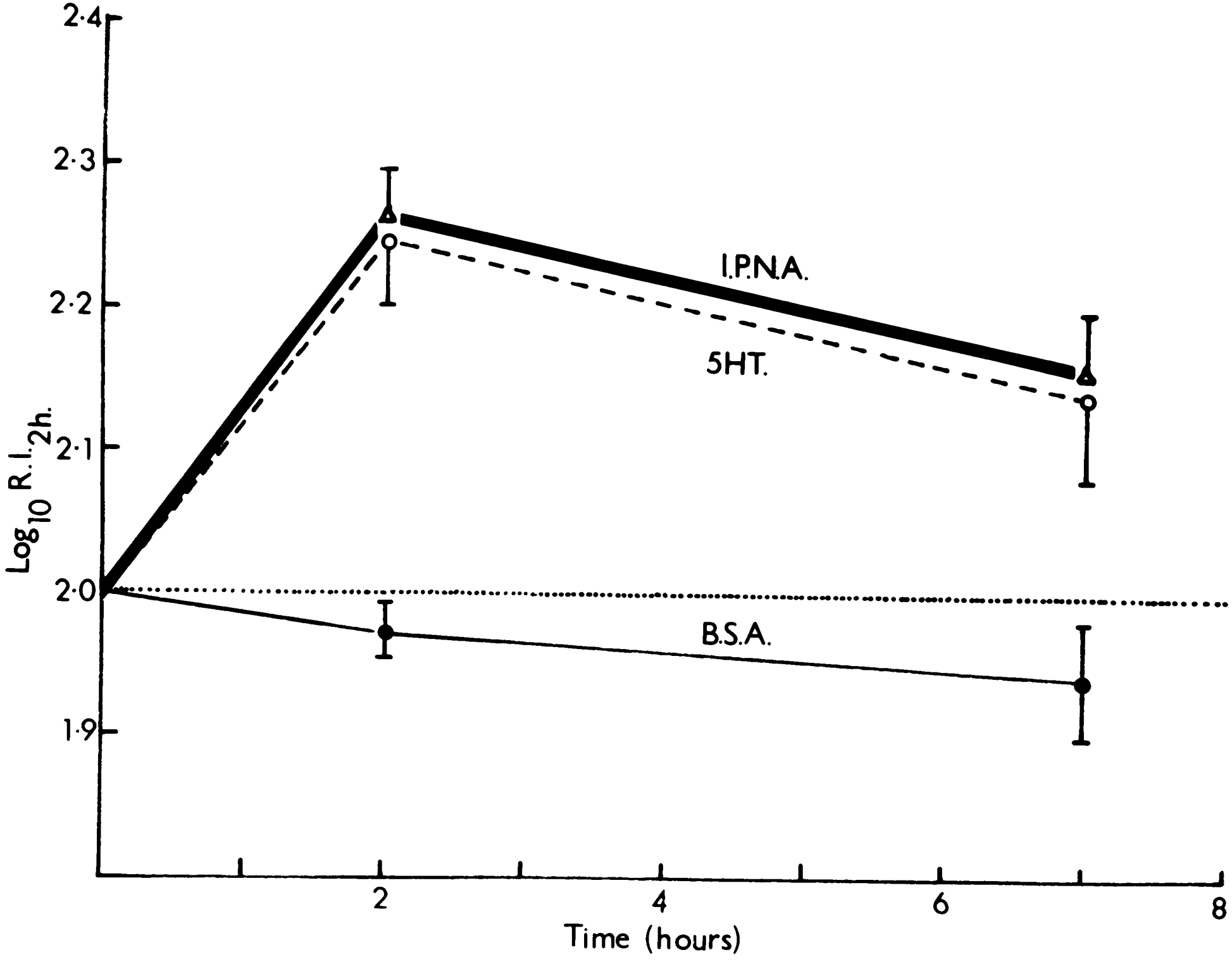
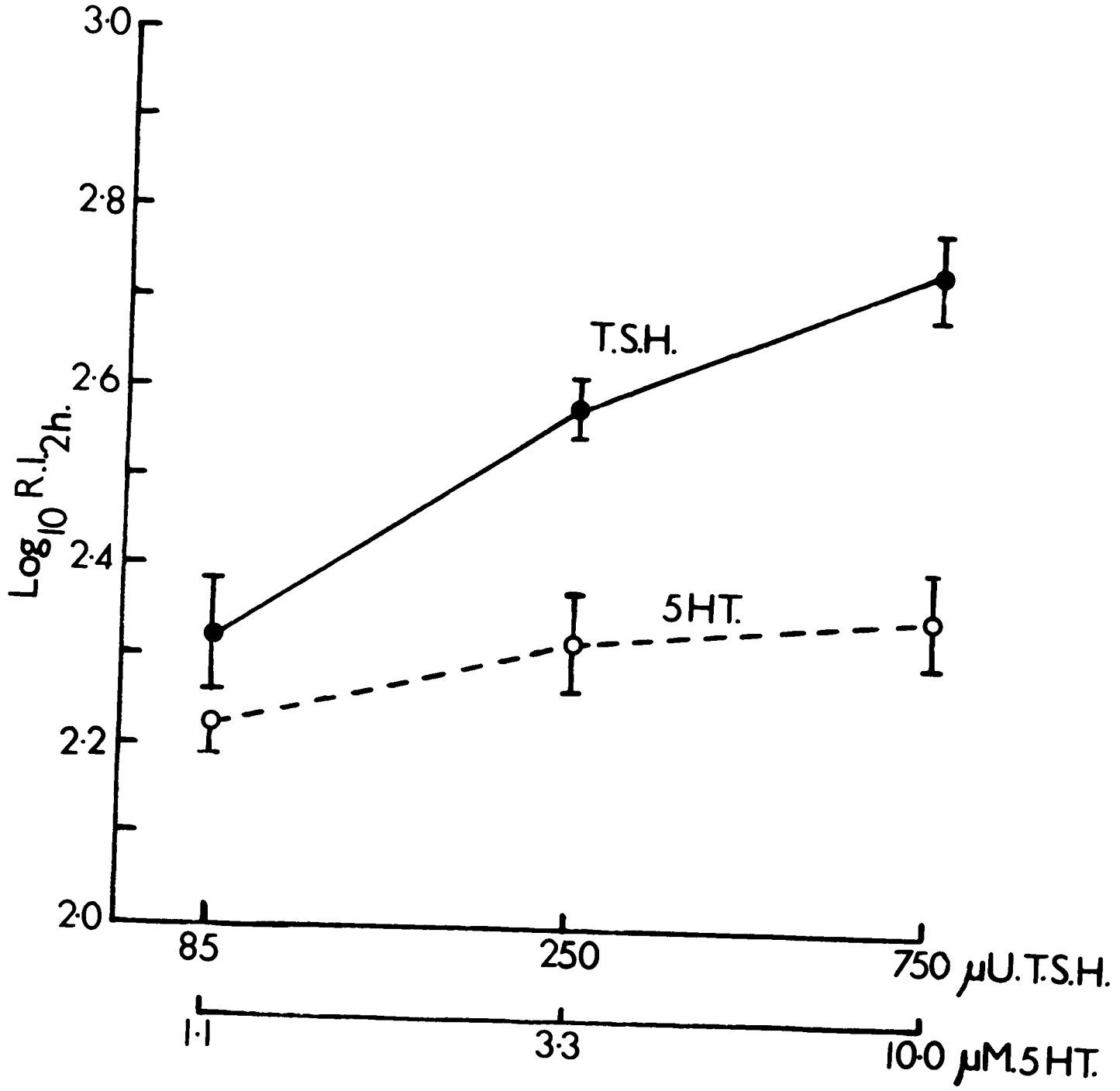

2·4
2·3
2·2
2·1
2·0
1·9
Log_{10} R.I.$_{2h.}$
I.P.N.A.
5HT.
B.S.A.
2
4
6
8
Time (hours)

FIGURE 4.2

$\underline{\text{Log}_{10}}$ dose vs. $\log_{10}$ R.I. 2 hr. curves for 5-H.T. and T.S.H. (expt. 4.5)


3·0
2·8
2·6
2·4
2·2
2·0
Log10 R.I.2h.
T.S.H.
5HT.
85
250
750 μU.T.S.H.
1·1
3·3
10·0 μM.5HT.

In the last experiment of this series (expt. 4.6, table 4.1D) injections of 10 and 50mg glucose[(1)] were also included. These were without effect, whereas I.P.N.A. gave a good response, suggesting that the action of I.P.N.A. on blood radioiodine was unlikely to have been mediated by hepatic glucose release.

Comment. Thus 5-H.T. and I.P.N.A. were shown to cause a response in the McKenzie assay. However, adrenaline and noradrenaline proved lethal at doses previously used by Melander (1971a) and failed to affect blood radioiodine at sublethal doses.

4.4. THE EFFECT OF 5-H.T. AND I.P.N.A. ON PROTEIN BOUND ^{131}I (P.B.^{131}I) AND T_4 ^{125}I DECAY RATE (expt. 4.7)

In order to confirm that thyroid stimulation had taken place, it was necessary to show that the increase in circulating radioiodine was, at least partly, organic and not attributable to a reduction in the decay rate of circulating labelled thyroid hormone (as discussed in part 3). Therefore, in one experiment the effects of 5-H.T. and I.P.N.A. on P.B.^{131}I and T_4 ^{125}I decay rate were measured concurrently.

Method. Four groups of 8 mice were prepared as before, except that each received an injection of T_4 ^{125}I[(2)] (approximately 2μCi in 0.25ml saline i.p.) 10 hours before the experiment. The following test substances were administered in 0.5ml i.v.

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(1) dl-Glucose, B.D.H. analytical reagent
(2) I.M.14, The Radiochemical Centre, Amersham

1. 1% B.S.A.
2. 1.1μM 5-H.T.
3. 0.3μM I.P.N.A.
4. 0.5mU T.S.H.

Blood samples (100μl) were taken into 0.5ml dialysed(1) human plasma before and 2 hr. after injection. The tubes were counted, subjected to amberlite I.R.A. 400 chromatography as described in appendix 6 (to remove iodide) and recounted for 10 minutes in a twin channel Nuclear Chicago autogamma spectrometer at 12.5-75kev (^{125}I) and 314-414kev (^{131}I). Those which failed to reach twice background on the second count were excluded from the analysis. ^{125}I counts were adjusted for ^{131}I crossover and results were expressed as $\log_{10}$R.I.$_{2\ hr.}$ for both ^{131}I and ^{125}I counts.

Results. (table 4.2) 5-H.T., I.P.N.A. and T.S.H. significantly increased P.B.^{131}I in parallel with the total ^{131}I. $\log_{10}$R.I.$_{2\ hr.}$ values for ^{125}I or P.B.^{125}I in control, I.P.N.A. or T.S.H. groups were similar,(although significant heteroscadascity precluded the use of Dunnett's 't' test) implying that they did not affect T_4 decay rate. $\log_{10}$R.I.$_{2\ hr.}$ (P.B.^{125}I) for mice treated with 5-H.T. was significantly less than for the controls when compared using Student's 't' test with correction of degrees of freedom for unequal variances (appendix 1). This implied an increased peripheral degradation of thyroxine which would have tended to reduce the observed response to 5-H.T. rather than to give rise to a false response.

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(1) Dialysed against distilled water for 24 hr.

TABLE 4.2. The effect of 5-H.T. and I.P.N.A. on P.B.^{131}I release and T_4 ^{125}I decay in the mouse (expt. 4.7) 31.7.73.Males.

A. ^{131}I counts

	Total ^{131}I		P.B.^{131}I(1)	
Injection	$\log_{10}$ R.I.$_{2\ hr.}$ (2)	p (3)	$\log_{10}$ R.I.$_{2\ hr.}$ (2)	p (3)
1% B.S.A.	2.002 ± 0.025	-	1.946 ± 0.042	-
1.1μM 5-H.T.	2.284 ± 0.021	<0.01	2.289 ± 0.045	<0.01
0.3μM I.P.N.A.	2.403 ± 0.039	<0.01	2.380 ± 0.048	<0.01
0.5mU T.S.H.	2.742 ± 0.041	<0.01	2.705 ± 0.036	<0.01

B. ^{125}I counts

	Total ^{125}I		P.B.^{125}I(1)	
Injection	$\log_{10}$ R.I.$_{2\ hr.}$ (2)	p (4)	$\log_{10}$ R.I.$_{2\ hr.}$ (2)	p (4)
1% B.S.A.	1.881 ± 0.016	N.S. (5)	1.879 ± 0.015	<0.05 (5)
1.1μM 5-H.T.	1.840 ± 0.024		1.831 ± 0.006	
0.3μM I.P.N.A.	1.893 ± 0.011		1.863 ± 0.020	
0.5mU T.S.H.	1.883 ± 0.006		1.872 ± 0.006	

(1) After amberlite I.R.A. 400 chromatography
(2) Mean ± S.E.M. of 8 mice
(3) p for difference from control by Dunnett's 't' test
(4) Heteroscadasticity ($p<0.001$) precluded Dunnett's 't' test
(5) Comparison by Student's 't' test with corrected d.f. for unequal variances

Comments. Since 5-H.T. and I.P.N.A. both caused an increase in P.B.^{131}I without slowing T_4 ^{125}I decay rate, they must have enhanced thyroid secretion.

4.5. DID 5-H.T. AND I.P.N.A. STIMULATE THE THYROID DIRECTLY OR VIA THE RELEASE OF T.S.H. BY THE PITUITARY GLAND?

(expts. 4.8-4.10)

In order to rule out possible pituitary mediation of the actions of 5-H.T. and I.P.N.A., their effects on hypophysectomised mice can be studied (Ericson et al, 1970; Melander, 1970). However, hypophysectomy is difficult, the thyroid gland is often traumatised in the process and the animals are subsequently more susceptible to the stresses of the experiment. An alternative method, used here, is to prevent any circulating T.S.H., released by the pituitary, from acting on the thyroid gland by an intravenous loading dose of anti-T.S.H. antiserum.

Method. 1% B.S.A. (control) or appropriate dilutions of anti-b-T.S.H. (guinea pig 2, appendix 5) were injected intravenously (in 0.25ml) into each mouse 5 minutes before the stimulator (in 0.25ml) and the effect on the $\log_{10}$R.I.$_{2\ hr.}$ ascertained.

Results. In the first experiment (expt. 4.8) the effect of anti-T.S.H. was tested at dilutions of 1 in 500 and 1 in 50 on the response to a homogenate of mouse pituitaries (1 in 8ml 1% B.S.A.). Table 4.3 shows that the response was substantially reduced by 1 in 500 antiserum and abolished by 1 in 50 antiserum.

TABLE 4.3. The effect of previous anti-T.S.H. injection on the responses to 5-H.T. and I.P.N.A.

Experiment	Sex	1st Injection(0.25ml)	2nd Injection(0.25ml)	No. of Mice	Log_{10}R.I. 2 hr.(1)	p(2)
A. 8.2.72. (expt. 4.8)	F	1% B.S.A.	m-T.S.H.(1pit/8ml)(3)	8	2.518 ± 0.027	
		1/500 Anti-T.S.H.	" "	7	2.181 ± 0.059	
		1/50 Anti- T.S.H.	" "	8	1.972 ± 0.052	
B. 13.4.72. (expt. 4.9)	M	1% B.S.A.	1% B.S.A.	8	1.924 ± 0.035	) N.S.
		1/5 Anti-T.S.H.	1% B.S.A.	6	1.971 ± 0.041	)
		1% B.S.A.	3.3µM 5-H.T.	8	2.172 ± 0.051	) N.S.
		1/5 Anti-T.S.H.	3.3µM 5-H.T.	7	2.262 ± 0.036	)
C. 20.4.72. (expt. 4.10)	M	1% B.S.A.	1% B.S.A.	8	1.921 ± 0.025	) N.S.
		1/5 Anti-T.S.H.	1% B.S.A.	8	1.968 ± 0.036	)
		1% B.S.A.	0.10µM I.P.N.A.	8	2.294 ± 0.048	) N.S.
		1/5 Anti-T.S.H.	0.10µM I.P.N.A.	8	2.238 ± 0.056	)

(1) Mean ± S.E.M.

(2) p for difference by Student's 't' test.

(3) Pituitary glands, taken from freshly killed mice, homogenised with saline (2ml/gland) in small glass homogenisers, spun in a bench centrifuge for 20 minutes and stored frozen at between -10 and -20°C in aliquots. On the day of assay one aliquot removed and made up to 1 pituitary/8ml with 1% B.S.A.

In the subsequent experiments (expts. 4.9-4.10) the effects of a high concentration of antiserum, 1 in 5, which would have been sufficient to neutralise the thyrotropic activity of 2-3 mouse pituitaries in vitro (derived from the data of table A3, appendix 5), were tested on the response to 3.3μM 5-H.T. and 0.10μM I.P.N.A. In neither case was the response significantly reduced, nor was there any significant alteration in the 'response' to 1% B.S.A.

Comments. Since the response to the mouse pituitary extract was considerably greater than the response to 5-H.T. or I.P.N.A., and since the responses to these amines were unaffected by a dose of antiserum ten times greater than was required to abolish the response to the pituitary extract, the responses to the amines could not have been mediated by the release of pituitary T.S.H. These results are also against the possibility that 5-H.T. or I.P.N.A. act by increasing the delivery of T.S.H. to thyroid cells by a direct action on thyroidal blood flow, since circulating T.S.H. should have been completely neutralised by anti-T.S.H. antibodies. This confirms the results of Ericson et al (1970) and Melander (1970), who found that 5-H.T. and I.P.N.A. were able to produce a response in surgically hypophysectomised mice.

4.6. THE EFFECT OF P.B.A. ON THE RESPONSES TO T.S.H., L.A.T.S., 5-H.T. AND I.P.N.A. (expts. 4.11-4.14)

In an attempt to detect any relationship between the receptors for the various thyroid stimulators, experiments were undertaken to test the effects of the α-adrenergic antagonist, phenoxybenzamine (P.B.A.), on thyroid stimulation.

Method. In the initial experiments (expts. 4.11-4.12) the dosage regime for P.B.A. was that of Melander (1971b). Groups of mice were prepared for the McKenzie assay but, in addition, each mouse received i.p. injections of either 0.4mg P.B.A. or 0.2ml saline[(1)] or 0.2ml saline 18 hr. and 2 hr. before the injection of stimulator. In subsequent experiments (expts. 4.13-4.14) the second dose of P.B.A. or saline was omitted, the remaining one being given at -16 hr. The mice were bled for determination of response at 0 hr., 2 hr., and in the case of L.A.T.S. or 1% B.S.A., also at 7 hr. and 24 hr.

Results. Table 4.4 shows that in the first two experiments P.B.A. abolished the response to 5-H.T., significantly reduced the responses to T.S.H., L.A.T.S. and I.P.N.A. but had no significant effect on that to 1% B.S.A. These results agree with those of Melander (1971a, b) except that in his experiments the response to I.P.N.A. was unchanged or, in some cases, increased by P.B.A. However, in the present experiments P.B.A. caused generalised signs of distress in all mice which received it (hypokinesia, sweating and tachypnoea). These signs were present for 4-6 hours following the injection. In the second experiment 15 out of 48 mice died in the P.B.A. treated group.

Since P.B.A. is known to be a long acting α-blocking agent (Nickerson, 1970) it seemed possible that the second injection was unnecessary. This was borne out by experiment 4.13 in which a single i.p. injection of 0.4mg P.B.A. at -16 hr. abolished the response to 1.1μM 5-H.T. (table 4.5A). In

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(1) At this concentration P.B.A. formed a suspension. This was made up immediately before use and shaken before each aliquot was removed

TABLE 4.4. The effect of two doses of P.B.A. on the response to thyroid stimulators in the mouse

Experiment	Sex	Stimulator (0.5ml iu)	Control i.p. at -18hr. & -2hr. (0.2ml saline)		P.B.A. (0.4mg i.p. at -18hr. & -2hr.)		p(2)
			No. of Mice	Log_{10}R.I.(1)	No. of Mice	Log_{10}R.I.(1)	
A. 4.7.72. (expt. 4.11)	M	Int.Std. T.S.H. 0.25mU	7	2.498 ± 0.042	7	2.386 ± 0.031	<0.05
		Int.Std. T.S.H. 0.75mU	7	2.689 ± 0.042	7	2.359 ± 0.042	<0.001
		5-H.T. 1.1μM	7	2.249 ± 0.048	6	1.960 ± 0.046	<0.001
		I.P.N.A. 0.1μM	8	2.266 ± 0.035	7	2.078 ± 0.033	<0.002
		L.A.T.S.(1/3 R.N.)(3)	7	2.581 ± 0.039(2 hr) 2.885 ± 0.021(7 hr) 2.868 ± 0.053(24hr)	7	2.245 ± 0.077(2 hr) 2.454 ± 0.117(7 hr) 2.464 ± 0.152(24hr)	<0.01 (7 hr)
		1% B.S.A.	8	1.972 ± 0.021(2 hr)		1.939 ± 0.031(2 hr)	N.S. (2 hr)
		1% B.S.A.	8	1.941 ± 0.041(7 hr) 2.088 ± 0.046(24hr)	7	1.860 ± 0.045(7 hr) 1.856 ± 0.071(24hr)	N.S. (7 hr) <0.02 (24hr)
B. 11.7.72. (expt. 4.12)	F	Int.Std. T.S.H.0.085mU	8	2.232 ± 0.043	6	2.054 ± 0.045	<0.02
		Int.Std. T.S.H. 0.25mU	8	2.397 ± 0.019	7	2.053 ± 0.032	<0.01
		Int.Std. T.S.H. 0.75mU	8	2.565 ± 0.055	5	2.267 ± 0.058	<0.01
		5-H.T. 1.1μM	7	2.144 ± 0.015	5	2.004 ± 0.012	<0.001
		I.P.N.A. 0.05μM	8	2.112 ± 0.06	5	1.918 ± 0.039	<0.05
		0.15μM	8	2.161 ± 0.049	5	1.993 ± 0.068	<0.1

(1) 2 hr. response except where specified. Mean ± S.E.M.
(2) Student's 't' test with adjustment of p for unequal variances (where necessary)
(3) 1 part R.N. serum: 2 parts 5% B.S.A.

TABLE 4.5. The effect of a single injection of P.B.A. on the response to thyroid stimulation in the mouse

Experiment	Sex	Stimulator	Control (0.2ml saline i.p. at -16 hr) No. of Mice	Control Log_{10}R.I.(1)	P.B.A. (0.4mg i.p. at -16 hr) No. of Mice	P.B.A. Log_{10}R.I.(1)	p(2)
A. 4.8.72. (expt. 4.13)	F	5-H.T. 1.1μM	5	2.381 ± 0.054	8	1.988 ± 0.023	<0.001
B. 11.8.72. (expt. 4.14)	F	Int.Std. T.S.H. 0.5mU	8	2.656 ± 0.037	7	2.656 ± 0.043	N.S.
		5-H.T. 1.1μM	8	2.199 ± 0.024	8	1.910 ± 0.023	<0.001
		I.P.N.A. 0.15μM	8	2.168 ± 0.029	7	2.141 ± 0.052	N.S.
		L.A.T.S. (½ R.W.)(3)	8	2.540 ± 0.043(2 hr) 2.790 ± 0.058(7 hr) 2.900 ± 0.043(24hr)	8	2.464 ± 0.045(2 hr) 2.762 ± 0.069(7 hr) 2.797 ± 0.071(24hr)	N.S.(7 hr)
		1% B.S.A.	8	1.996 ± 0.029(2 hr) 1.854 ± 0.036(7 hr) 1.943 ± 0.041(24hr)	8	1.964 ± 0.020(2 hr) 1.681 ± 0.055(7 hr) 1.815 ± 0.029(24hr)	N.S.(2 hr) <0.05(7 hr) <0.05(24hr)

(1) 2 hr. response except where specified. Mean ± S.E.M.
(2) Student's 't' test.
(3) 1 part R.W. serum: 1 part 5% B.S.A.

experiment 4.14 the response to 1.1μM was yet again abolished, whereas those to T.S.H., L.A.T.S. and I.P.N.A. were not significantly affected by a single dose of P.B.A. The 'response' to 1% B.S.A. was unchanged at 2 hr. but reduced significantly at 7 hr. and 24 hr. (an effect which had also been noted in expt. 4.11). In these experiments the mice showed only transient signs of distress, which disappeared long before the injection of stimulator.

Comments. In all experiments P.B.A. completely abolished the response to 5-H.T. Since P.B.A. is known to interact not only with α-receptors but also with those for 5-H.T. (Gyermek, 1966), it is assumed that P.B.A. acted via 5-H.T. receptors rather than 5-H.T. via α-receptors. The same reasoning probably applies to the abolition of the thyroid effects of 5-H.T. by phentolamine (Melander, 1971a, b) since this is also a weak 5-H.T. antagonist (Gyermek, 1966). It is notable that P.B.A. only reduced the response to other thyroid stimulators with the double dose regime and had no effect with the single dose regime. The possible reasons for this are:

1. Firstly, P.B.A. may have non-specific inhibitory or possibly toxic action on the mouse thyroid, lowering its responsiveness to any stimulator. If such an action were exerted at higher doses than required for α-adrenergic or 5-H.T. receptor blockade, the lack of effect on the response to T.S.H., L.A.T.S. and I.P.N.A. when the single dose P.B.A. regime was used (expt. 4.14) would be explained. That a

generalised toxic effect might have indeed occurred was suggested by the signs of distress noted in the animals receiving the double dose regime.

2. P.B.A. is not a highly specific receptor blocking agent since it is known to block α-receptors, 5-H.T. receptors and, at higher doses, also acetyl choline receptors (Nickerson, 1970). Therefore, it may block others such as β-adrenergic or T.S.H. receptors at even higher doses.

3. P.B.A. could itself produce a response in the mouse, thus increasing the baseline counts and possibly also depleting thyroid radioiodine significantly, leading to a reduction of the observed response index following injection of a stimulator. In favour of this possibility was the reduction of the 24 hr. 'response' to 1% B.S.A. in P.B.A. treated mice (expt. 4.11 and 4.14), as if this represented the recovery phase from a response which was already in progress at the time of the initial bleed. Furthermore, calculation of the initial counts for these experiments (excepting those of expt. 4.11, which were rendered unsuitable by the use of separate counters for the control and P.B.A. groups and expt. 4.13 in which too few animals were used) showed that these were significantly raised when two doses of P.B.A. were used (in expt. 4.12), but this increase did not reach significance when a single dose of P.B.A. had been given (table 4.6). Given that P.B.A. does increase initial counts, is this sufficient to account for the reduction in response to T.S.H., L.A.T.S., I.P.N.A. or even 5-H.T.? In order to make the comparison it is necessary to return to the use of the untransformed response index and, taking the null hypothesis

TABLE 4.6. Initial counts following administration of P.B.A. in mice with prelabelled thyroid glands

Experiment	P.B.A. doses	Control		P.B.A.		$C_{P.B.A.}/C_{Control}$	p (2)
		No.	Initial counts (1)	No.	Initial counts (1)		
11.7.72. (expt. 4.12)	2	46	1043 ±± 58	33	1761 ± 175	1.65	<0.01
11.8.72. (expt. 4.14)	1	40	1302 ± 101	38	1500 ± 101	1.13	N.S.

(1) Mean ± S.E.M. 10 min. counts, 314-414 kev Nuclear Chicago autogamma. Corrected for background. No correction made for radioactive decay. P.B.A. groups counted later than, but within 12 hours of, control groups.

(2) Student's 't' test employing correction of f for unequal variances where necessary.

that the increase in counts, R, in response to a stimulator is unchanged by P.B.A., then:

$$R.I._{Control} = \frac{(C_{Control} + R) \times 100}{C_{Control}}$$

and $$R.I._{P.B.A.} = \frac{(C_{P.B.A.} + R) \times 100}{C_{P.B.A.}}$$

(where $C_{Control}$ and $C_{P.B.A.}$ are the initial counts for control and P.B.A. groups) solving these equations for $C_{P.B.A.}/C_{Control}$ we get

$$\frac{C_{P.B.A.}}{C_{Control}} = \frac{(R.I._{Control} - 100)}{(R.I._{P.B.A.} - 100)}$$

$\frac{C_{P.B.A.}}{C_{Control}}$, obtained from the data of table 4.6 is compared in table 4.7 with the $\frac{(R.I._{Control} - 100)}{(R.I._{P.B.A.} - 100)}$ values calculated from the data of experiments 4.12 and 4.14. In experiment 4.12 in which P.B.A. had a marked effect on all responses the $\frac{(R.I._{Control} - 100)}{(R.I._{P.B.A.} - 100)}$ values for T.S.H., I.P.N.A. and 5-H.T.(1) (table 4.7) were far in excess $\frac{C_{P.B.A.}}{C_{Control}}$ (1.65) suggesting that the reduction in response with P.B.A. may be due only in part to an effect on the initial count, the major part being possibly attributable to a toxic action or a specific inhibitory effect as discussed above in (1).

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(1) L.A.T.S. was not included in this experiment

TABLE 4.7. $(R.I._{Control}-100)/(R.I._{P.B.A.}-100)$ for expts. 4.12 and 4.14 (see text)

Expt. 4.12. 2 doses P.B.A.

Stimulator	$\frac{(R.I._{Control}-100)}{(R.I._{P.B.A.}-100)}$ (1)	
Int.Std. T.S.H. 0.085mU	76/17	= 4.5
Int.Std. T.S.H. 0.25mU	151/15	= 10.1
Int.Std. T.S.H. 0.75mU	289/92	= 3.1
5-H.T. 1.1μM	38/-16	= -2.4
I.P.N.A. 0.05μM	52/4.3	= 12.1
I.P.N.A. 0.15μM	40/1.06	= 38

Expt. 4.14. 1 dose P.B.A.

Stimulator	$\frac{(R.I._{Control}-100)}{(R.I._{P.B.A.}-100)}$ (1)	
Int.Std. T.S.H. 0.5mU	364/367	= 0.99
5-H.T. 1.1μM	60/-18	=-3.3
I.P.N.A. 0.15μM	49/44	= 1.11
L.A.T.S. (½R.W.)	530/556	= 0.95
1% B.S.A.	0.6/-7	=-0.09

(1) 2 hr. response except for L.A.T.S. where 7 hr. response used.

4.7. THE EFFECT OF P.B.A. ON BLOOD RADIOIODINE LEVEL IN THE "McKENZIE" MOUSE (expts. 4.15-4.16)

Because of the evidence above suggesting that P.B.A. itself may cause a response in the system, this possibility was tested directly in two experiments.

Method. In the first experiment (4.15) 2 hr., 7 hr. and 24 hr. responses were determined after a single injection of 0.4mg P.B.A. i.p. (at 0 hr.). In the second experiment (4.16) 0.4mg P.B.A. i.p. was given at 0 hr. and repeated at 16 hr., responses being determined at 3 hr., 16 hr. (immediately preceding the 16 hr. injection) and 19 hr.

Results. In both experiments significant responses of long durations were obtained (table 4.8). The second injection appeared not to enhance the response in experiment 4.16.

Comments. These results show that P.B.A. is able to produce a response in the McKenzie system which would account for the increase in initial counts seen in P.B.A. treated mice in previous experiments. In experiment 4.16, there appeared to be substantial differences between the response 16 hr. after a single dose of P.B.A. and that 3 hr. after the second of the two doses. Thus, the single dose regime would be expected to produce an increase in initial counts as indeed happened in experiment 4.14, although this failed to reach statistical significance. The cause of the response following P.B.A. was not investigated, but it would be of interest to determine whether P.B.A. has a direct effect on the thyroid or whether it produces the response via an effect on the peripheral degradation

TABLE 4.8. The effect of P.B.A. alone on the blood radioiodine levels of 'McKenzie prepared' mice

A. The effect of 0.4mg P.B.A. i.p. on the response at 2, 7 and 24 hr. (expt. 4.15) 10.8.72. Females.

Injection	No. of Mice	Log_{10} R.I.(1) 2 hr.	7 hr.	24 hr.
Control (0.2ml saline)	8	1.968 ± 0.020	1.964 ± 0.024	1.852 ± 0.021
P.B.A. (0.4mg)	7	2.025 ± 0.034	2.101 ± 0.047	2.017 ± 0.026
	p(2)	N.S.	<0.05	<0.001

B. The effect of injections of 0.4mg P.B.A. i.p. at 0 hr. and 16 hr. on the responses at 3 hr., 16 hr. and 19 hr. (expt. 4.16) 17.8.72. Males.

Injection	No. of Mice	Log_{10} R.I.(1) 3 hr.	16 hr.	19 hr.
Control (2x0.2ml saline)	8	1.891 ± 0.022	1.878 ± 0.028	1.866 ± 0.029
P.B.A. (2 x 0.4mg)	8	2.057 ± 0.047	2.203 ± 0.035	2.095 ± 0.065
	p(2)	<0.01	<0.001	<0.01

(1) Mean ± S.E.M.

(2) Student's 't' test.

of thyroid hormone. This could be studied by the method described in section 4.4.

4.8. THE EFFECT OF dl-PROPRANOLOL ON THE RESPONSES TO T.S.H., L.A.T.S., 5-H.T. AND I.P.N.A. (expts. 4.17-4.19)

Since I.P.N.A., a β-adrenergic agonist, was capable of thyroid stimulation, it was of interest to test the effects of the β-blocking agent, dl-propranolol, on the response to this and other stimulators.

Method. The treatment regime was similar to that of Melander (1971a, b). Groups of mice were prepared as usual. Following the initial (0 hr.) bleed each mouse was given immediately an i.p. injection of either 0.4mg dl-propranolol in 0.2ml saline or 0.2ml saline alone. 15 min. later the stimulator was injected and the mice were bled again after a further 2 hr. and, in the case of L.A.T.S. or 1% B.S.A. injected animals, at 7 hr. and 24 hr. Three such experiments were performed and the calculated $\log_{10}$R.I. values compared for propranolol treated and control troups.

Results. Propranolol alone had no obvious toxic effect on the mice but the combination of 5-H.T. and propranolol caused obvious distress resulting in one death in the first experiment and eight in the second. For this reason 5-H.T. was not included in the third experiment. A further problem encountered in the first two experiments was the lower responses to 5-H.T. and I.P.N.A. than had been encountered previously. However, they were still highly significant ($p<0.01$) when compared with the control groups (1% B.S.A.).

As table 4.9 shows, propranolol abolished the response to I.P.N.A. in all experiments without affecting the responses to 5-H.T. (1 expt.) or T.S.H. (3 expts.). The response to L.A.T.S. was significantly lower in the propranolol treated mice of experiment 4.17 but this was probably a spurious result in view of the lack of a similar trend in subsequent experiments.

The design of this experiment was such that if propranolol itself were able to produce a response it would be manifest as a significant rise in the control (1% B.S.A.) response of the propranolol treated mice compared with that of the saline treated mice. Indeed in the first two experiments (4.17 and 4.18) small but significant increases in response occurred, peaking at between 2 hr. and 7 hr. In experiment 4.19, however, the difference was less obvious although a significant increase in response was found at 24 hr. with propranolol. Thus it seems likely that propranolol also may cause a small and variable, but significant, response in this system. However, this was not sufficient to interfere with the main object of the experiment, which was to test its effects on the responses to proven thyroid stimulators.

Comments. These results suggest that I.P.N.A. acts on the thyroid gland by combination with β-adrenergic receptors, of which 5-H.T., T.S.H. and L.A.T.S. act independently.

4.9. DISCUSSION

The results of these experiments support the arguments put forward by Melander (1971a) that 5-H.T. and I.P.N.A. stimulate thyroid secretion in mice and that this action is

TABLE 4.9. The effect of dl-propranolol on the response to thyroid stimulators in the mouse

Experiment	Sex	Stimulator	Control (0.2ml saline i.p. at -15 min.) No. of Mice	Control (0.2ml saline i.p. at -15 min.) Log_{10} R.I.(1)	dl-Propranolol (0.4mg i.p. at -15 min.) No. of Mice	dl-Propranolol (0.4mg i.p. at -15 min.) Log_{10} R.I.(1)	p(2)
A. 18.7.72. (expt. 4.17)	M	Int.Std. T.S.H. 0.5mU	8	2.479 ± 0.038	8	2.461 ± 0.049	N.S.
		5-H.T. 1.1μM	8	2.086 ± 0.028(4)	7	2.078 ± 0.027	N.S.
		I.P.N.A. 0.15μM	7	2.095 ± 0.035(4)	6	1.964 ± 0.044	<0.01
		L.A.T.S. (1/3 R.N.)(3)	8	2.439 ± 0.023(2 hr) 2.849 ± 0.032(7 hr) 2.768 ± 0.041(24hr)	7	2.322 ± 0.038(2 hr) 2.698 ± 0.033(7 hr) 2.717 ± 0.066(24hr)	<0.05(2 hr) <0.01(7 hr) N.S.(24hr)
		1% B.S.A.	8	1.861 ± 0.022(2 hr) 1.838 ± 0.032(7 hr) 1.876 ± 0.053(24hr)	7	1.959 ± 0.011(2 hr) 1.950 ± 0.033(7 hr) 1.835 ± 0.038(24hr)	<0.01(2 hr) <0.02(7 hr) N.S.(24hr)

(1) 2 hr. response except where indicated. Mean ± S.E.M.
(2) Student's 't' test.
(3) 1 part R.N.serum: 2 parts 5% B.S.A.
(4) Responses significant at $p<0.01$ cf. 1% B.S.A. (Duncan's test)

/Cont'd.

TABLE 4.9. The effect of dl-Propranolol on the response to thyroid stimulators in the mouse (Cont'd.)

Experiment	Sex	Stimulator	Control (0.3ml saline) i.p. at -15 min) No. of Mice	Control Log_{10} R.I.(1)	dl-Propranolol (0.4mg i.p. at -15 min) No. of Mice	dl-Propranolol Log_{10} R.I.(1)	p(2)
B. 27.7.72. (expt. 4.18)	F	Int.Std. T.S.H. 0.5mU	6	2.539 ± 0.051	6	2.568 ± 0.044	N.S.
		5-H.T. 1.1μM	7	2.192 ± 0.043	All died	-	
		I.P.N.A. 0.15μM	6	2.076 ± 0.029(4)	7	1.924 ± 0.038	<0.01
		L.A.T.S. (½ R.W.)(3)	7	2.292 ± 0.067(2 hr) 2.538 ± 0.072(7 hr) 2.571 ± 0.086(24hr)	7	2.351 ± 0.084(2 hr) 2.723 ± 0.081(7 hr) 2.731 ± 0.091(24hr)	N.S.(2 hr) N.S.(7 hr) N.S.(24hr)
		1% B.S.A.	6	1.809 ± 0.065(2 hr) 1.842 ± 0.052(7 hr) 1.848 ± 0.036(24hr)	6	1.929 ± 0.015(2 hr) 1.989 ± 0.014(7 hr) 1.777 ± 0.044(24hr)	N.S.(2 hr) <0.05(7 hr) N.S.(24hr)
C. 4.8.72. (expt. 4.19)	F	Int.Std. T.S.H. 0.5mU	8	2.631 ± 0.052	7	2.669 ± 0.030	N.S.
		I.P.N.A. 0.3μM	6	2.225 ± 0.061	6	2.038 ± 0.018	<0.05
		L.A.T.S. (½ R.W.)(3)	7	2.507 ± 0.042(2 hr) 2.911 ± 0.040(7 hr) 2.799 ± 0.070(24hr)	7	2.487 ± 0.056(2 hr) 2.925 ± 0.066(7 hr) 2.798 ± 0.070(24hr)	N.S.
		1% B.S.A.	8	1.943 ± 0.047(2 hr) 1.857 ± 0.031(7 hr) 1.814 ± 0.031(24hr)	6	1.934 ± 0.029(2 hr) 1.964 ± 0.041(7 hr) 1.938 ± 0.032(24hr)	N.S.(2 hr) N.S.(7 hr) <0.05(24hr)

(1) 2 hr. response except where specified. Mean ± S.E.M.
(2) Student's 't' test with adjustment of f for unequal variances (where necessary).
(3) 1 part R.W. serum: 1 part 5% B.S.A.
(4) Responses significant at $p<0.01$ cf. 1% B.S.A. (Duncan's test)

not mediated by the pituitary gland. The finding that adrenaline and noradrenaline in concentrations used by Melander were lethal, even when injected slowly into normal mice not treated with thyroxine, is not easily explained without postulating an inherent difference in susceptibility to catecholamines between the strain of mice used here and that used by the Swedish group. Nevertheless, this effect prevented the confirmation or contradiction of Melander's conclusions regarding these catecholamines. However, the evidence which he has put forward, based on the effects on ^{131}I release and colloid droplet formation in hypophysectomised mice, is strong. Furthermore, the evidence that sympathetic stimulation activates the thyroid adds further weight to the hypothesis that thyroid cells may be stimulated by catecholamines (Melander et al, 1972, 1974).

That these biologically active amines act directly on the thyroid is strongly favoured by work on isolated calf thyroid cells in which stimulation of iodide uptake and organification has been observed in the presence of adrenaline, noradrenaline, I.P.N.A., dopamine and 5-H.T. (Maayan and Ingbar, 1968, 1970; Maayan et al, 1971; Melander et al, 1973b). The experiments of Ericson et al (1970) and, in the case of I.P.N.A. and 5-H.T., the present experiments confirm that this is also the case in the mouse in vivo. The present experiments also emphasise that the effect on blood radioiodine levels in mice is independent of any action on the peripheral degradation of labelled thyroid hormone.

Since these amines are vasoactive it is possible that the increased release of radioiodine from the prelabelled thyroids of mice was secondary to effects on thyroidal blood flow, either by increased delivery of T.S.H. to the cells or by more rapid removal of secreted thyroid hormone from the gland. Certainly 5-H.T., like histamine, has been shown to be vasodilator in the rat or rabbit thyroid (Melander et al, 1975c; Söderberg, 1958) and I.P.N.A. might also be expected to be vasodilator if β-receptors are present in thyroid blood vessels. Adrenaline and noradrenaline, on the other hand, are both vasoconstrictor in the dog or rabbit thyroid (Ahn et al, 1969; Mobray and Peart, 1960; Söderberg, 1958) and therefore would not be expected to act via blood flow, although small doses of noradrenaline were vasodilator in one report (Mobray and Peart, 1960). However, several lines of evidence are against this mechanism for 5-H.T. and I.P.N.A. action:

1. 5-H.T. and I.P.N.A. are active in anti-T.S.H. pretreated animals (as described above) or surgically by hypophysectomised mice (Ericson et al, 1970; Melander, 1970, 1971a) which demonstrates that T.S.H. is not required for their action.
2. Both 5-H.T. and I.P.N.A. have been shown to increase colloid droplet formation in mice in vivo (Ericson et al, 1970; Melander, 1971a) which suggests that secretion itself is stimulated rather than the removal of secretory products.
3. 5-H.T. and I.P.N.A., like adrenaline and noradrenaline, have been shown to act on isolated thyroid cells in vitro (Maayan and Ingbar, 1968, 1970; Maayan et al, 1971; Melander et al, 1973b).

In the present experiments the maximal effects which could be achieved with I.P.N.A. and 5-H.T. were small compared with those obtainable with T.S.H. or L.A.T.S. This would be against their having prime importance in the physiological control of thyroid secretion. However, it is notable that stimulation of the cervical sympathetic trunk in mice has given quite impressive responses, comparable with those to T.S.H. (Melander et al, 1972). Therefore, local release of amines by physiological mechanisms may achieve higher local concentrations in the region of the receptors.

Phenoxybenzamine (P.B.A.), an α-receptor blocking agent (Nickerson, 1970), inhibited the action of 5-H.T. on the thyroid, as found by other workers (Ericson et al, 1970; Melander, 1970, 1971a, b). From this evidence it is uncertain whether 5-H.T. acts on α-receptors or whether P.B.A. (and phentolamine, which was also used by Melander, 1971a, b) also acts on 5-H.T. receptors, although the latter would seem likely in view of the known action of P.B.A. on 5-H.T. receptors. Perhaps the use of specific 5-H.T. antagonists (e.g. methysergide) may help to clarify this further.

In the present experiments, whereas P.B.A. reduced the responses to T.S.H., L.A.T.S. and (in disagreement with Ericson et al, 1970, and Melander, 1971a, b) I.P.N.A., this effect could be eliminated by altering the dose regime. Thus a clear dissociation between the action of 5-H.T. and those of other stimulators was achieved. On this basis the possibility raised by Melander (1971a) that T.S.H. and α-adrenergic or 5-H.T.

receptors were closely linked seems unlikely. A new finding was that P.B.A. itself caused a response in the McKenzie assay. Whereas the possibility of an effect on the metabolism of thyroid hormone was not eliminated, it seems reasonable to suggest that P.B.A. may have a slight stimulatory effect on the thyroid, mediated by those receptors which it blocks.

The results of experiments on the action of the β-receptor antagonist, dl-propranolol (Nickerson, 1970), were in accord with previous work (Ericson et al, 1970; Melander, 1971a, b) in that a specific blockade of I.P.N.A. action was achieved. This suggests that I.P.N.A. acts via specific β-adrenergic receptors on the thyroid cell[(1)].

Thus it appears that there are several pathways to the stimulation of the thyroid, at least in mice. They are summarised in figure 4.3. Whereas each of these stimulators has been shown to act in more than one species, some species appear to be less responsive than others to stimuli other than T.S.H. For instance, rabbit thyroid secretion is only mildly affected by catecholamines (Söderberg, 1958) and rat thyroid secretion is stimulated less dramatically than that of the mouse following an injection of L.A.T.S. (Purves and Adams, 1960).

The most challenging aspect of this subject is that it has been impossible to separate convincingly the actions of T.S.H. and L.A.T.S. on the thyroid either in this system or in a great variety of others (section 1.2.2.). Thus the hypothesis has arisen that T.S.H. and L.A.T.S. act on the same receptor

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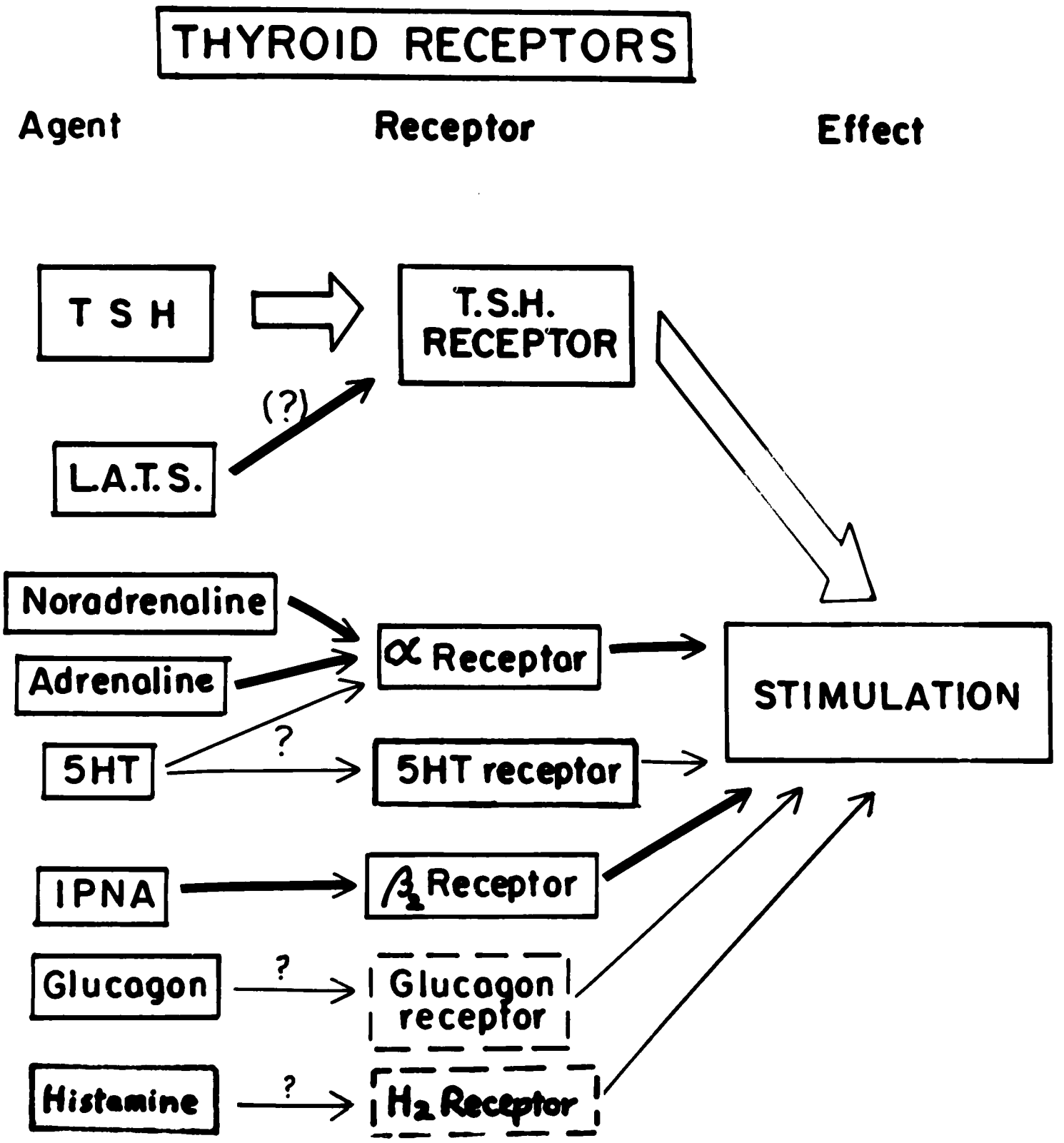
(1) It has recently been shown that the mouse thyroid has specific β_2 receptors since the cardioselective β_1 antagonist I.T.P. (1-isopropylamine-3-(2-thiazoloxy)-2-propranol) did not modify the response to I.P.N.A. in mice in vivo. Furthermore, the β_2 selective agonist, terbutaline, was able to stimulate the thyroid (Melander et al, 1975a)

FIGURE 4.3

Receptors and stimulators in the thyroid gland

(see text)


THYROID RECEPTORS
Agent
Receptor
Effect
T S H
T.S.H. RECEPTOR
(?)
L.A.T.S.
Noradrenoline
Adrenoline
α Receptor
STIMULATION
5HT
?
5HT receptor
IPNA
β2 Receptor
Glucagon
?
Glucagon receptor
Histamine
?
H2 Receptor

(Burke, 1971). However, proof must come from two types of experiment.

1. Testing the effect of inhibitory analogues of T.S.H. (or L.A.T.S.) on the response of the thyroid to T.S.H. and L.A.T.S. Two reports of the modification of T.S.H. to form an antagonist have emerged. Sonnenberg and Money (1957) acetylated a crude pituitary extract and found that the resulting preparation was devoid of T.S.H. activity but could offset the increase in chick thyroid weight and uptake of radioiodine produced by an unmodified pituitary preparation. El Kabir et al (1964) carbamylated a purified b-T.S.H. preparation (5IU/mg) to produce a preparation which had no intrinsic T.S.H. activity but which could antagonise the activity of up to four times the quantity of unaltered T.S.H. in the McKenzie assay or an in vitro assay. In one experiment this material reduced the response to an L.A.T.S. serum in the McKenzie assay, suggesting a common site of action for T.S.H. and L.A.T.S.

2. The investigation of binding of labelled T.S.H. to thyroid slices, homogenates or cell fractions and the effects of L.A.T.S. on such binding. Recent evidence from American laboratories concerning the binding of labelled L.H., h-C.G., insulin and A.C.T.H. to their target glands suggested that it was indeed possible to study hormone-receptor interactions with such techniques.

Therefore investigations along these lines were mounted. Preliminary attempts were made to produce a T.S.H. antagonist by carbamylation, succinylation or acetylation but these

proved unsuccessful. On the other hand, studies on the binding of T.S.H.-^{125}I to thyroid homogenates met with greater reward. The results of the latter experiments are presented in part 5.

Conclusion: 5-H.T. and I.P.N.A. stimulate the mouse thyroid via receptors which are different from each other and from the receptors for T.S.H. and L.A.T.S.

PART 5

STUDIES ON THE BINDING OF T.S.H. BY THYROID GLAND HOMOGENATES

5.1. INTRODUCTION

It is generally accepted that many drugs and all hormones act by combination with certain receptor groups or molecules within their target tissues. Whilst the theoretical analysis of these interactions has contributed greatly to the understanding of tissue responses to humoral agents (Furchgott, 1964), direct evidence of drug-receptor or hormone-receptor combination has been sadly lacking.

Recently, the widespread availability of radiolabelled hormones has permitted the demonstration of specific binding to target organ components. In this way specific steroid receptor proteins have been identified and used in sensitive competitive binding assays (Corker et al, 1970; Korenman et al, 1970; Robinson and Fanestil, 1970; Shutt, 1968; Thomas, 1971). Similarly, receptors have been characterised for a variety of polypeptide hormones, including insulin (Cuatracasas et al, 1972; Gavin et al, 1972), glucagon (Pohl et al, 1971; Rodbell et al, 1971a, b, c), A.C.T.H. (Lefkowitz et al, 1971a, b), L.H. and h-C.G. (Catt et al, 1971; Kammerman et al, 1972; De Krester et al, 1971; Leidenberger and Reichert, 1972a, b; Tsuruhara et al, 1972) and F.S.H. (Batt et al, 1973; Means and Vaitukaitis, 1972; Vaitukaitis et al, 1971).

At the outset of the work described in this part, there was little direct evidence of T.S.H.-receptor combination in

the thyroid gland. Pastan et al (1966) had proposed that T.S.H. acts by rapid binding to the cell membrane on the basis of the persistent effects of T.S.H. on glucose-1-^{14}C oxidation in canine thyroid slices, which had been exposed to the hormone and washed thoroughly in hormone free medium. The abolition by phospholipase C of T.S.H. stimulated phospholipogenesis in bovine (Larsen and Wolff, 1967), porcine or ovine thyroid slices (Burke, 1969; Haye and Jacquemin, 1970) and phospholipogenesis or glucose-1-^{14}C oxidation in bovine or porcine thyroid slices (Macchia et al, 1970), and the presence of T.S.H. sensitive adenyl cyclase activity in purified bovine thyroid plasma membranes (Wolff and Jones, 1971; Yamashita and Field, 1970) add weight to this argument.

In an early attempt to characterise specific binding sites for T.S.H., Pastan et al (1966) found that the uptake of labelled T.S.H. by canine thyroid slices was no greater than that of labelled bovine gamma globulin. Lowenstein and Greenspan (1971) found a greater proportion of label in the thyroids of iodide-treated rats or perchlorate-treated mice after an injection of T.S.H.^{131}I than after albumin^{131}I. However, their results could have been explained by the rapid release of iodide-^{131}I from T.S.H.-^{131}I and subsequent concentration by an incompletely blocked thyroid. Schell-Frederick and Dumont (1970) gave brief mention to some unpublished studies demonstrating the binding of labelled T.S.H. to isolated bovine thyroid cells. 50% of their bound label was displaced by excess carrier hormone, suggesting

reversible attachment to a limited number of binding sites. Haye and Jacquemin (1971) reported saturable binding of labelled T.S.H. to pig thyroid slices which parallelled the increase in ^{32}P incorporation into phospholipid.

During the work described in this part there appeared two further reports demonstrating specific interactions of labelled T.S.H. with thyroid tissue. Manley et al (1972) used bovine T.S.H. labelled with ^{125}I by the chloramine T method (Greenwood et al, 1963) and thyroid slices from cattle or P.T.U.-treated guinea-pigs, whereas Lissitzky et al (1973) studied the binding of T.S.H. labelled with ^{125}I by the lactoperoxidase method (Thorell and Johansson, 1971) to cultured porcine thyroid cells. Since the completion of this work several other papers have appeared on the subject (e.g. Amir et al, 1973, 1976; Field, 1975; Hall et al, 1975; Manley et al, 1974a, b; Mehdi et al, 1973; Mehdi and Nussey, 1975; Moore and Wolff, 1974; Smith and Hall, 1974; Tate et al, 1975; Verrier et al, 1974). These will be discussed in relation to the present work.

5.2. THE AIMS OF THE STUDY

This project was an attempt to demonstrate specific binding of labelled T.S.H. to thyroid receptors and to characterise some of the factors affecting the initial reaction of thyrotropin with its target gland. It was also hoped that it would throw further light upon the way in which other thyroid stimulators interact with thyroid cells. In particular it offered a way of testing directly the hypothesis

that L.A.T.S. acts through the same receptors as T.S.H., as suggested by Burke (1971).

5.3. THE CHOICE OF SYSTEM

Receptor binding studies have usually been performed by incubating a labelled hormone with a preparation of its target tissue in vitro, separating the bound hormone from the free, and measuring the proportion of the total radioactivity retained by the tissue. This requires (1) a hormone labelled to a sufficient specific activity but which retains substantial, and preferably full, biological activity and (2) a tissue preparation which allows easy and efficient separation of bound and free label.

5.3.1. Labelling procedure

Highly purified bovine T.S.H.[(1)] was labelled with ^{125}I by the chloramine T method as described in part 2 (section 2.2). Following initial experiments this method of labelling was found to reduce biological activity by 50-70% (see section 2.3). However, its use was continued since useful results were forthcoming.

5.3.2. Tissue preparation

A particulate fraction of thyroid homogenates offered several advantages over tissue slices:

1. Particulate preparations do not concentrate iodide to the same degree.

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(1) 40IU/mg

2. Greater concentrations of the receptor may be achieved.
3. Removal of unbound label is easier and more efficient.
4. Homogenisation should reduce variability.

All these advantages should increase the "signal to noise ratio" of experimental results.

Since fractions sedimenting between 120 and 2000g had been used successfully to study the binding of L.H. and h-C.G. by testicular (de Krester et al, 1971; Leidenberger and Reichert, 1972a, b) and ovarian homogenates (Tsuruhara et al, 1972), a similar fraction of thyroid tissue was used here. Such a fraction should contain large fragments of cell membrane, leaving the microsomes in the supernatant. A greater yield of receptors would have been achieved with faster centrifugation but practical considerations precluded this[1]. The method chosen could be performed using a general purpose refrigerated centrifuge[2] which could spin up to 192 1-2ml samples at 2,300g in a single run. This capacity was later to prove an asset.

5.4. HOMOGENISATION PROCEDURE

Bovine thyroids were obtained from a slaughterhouse. They were removed as soon as possible after death (usually within 1 hr.) and transported on ice to the laboratory. They were trimmed free of fat, cut into approximately 1mm thick slices and left in ice cold phosphate buffered saline (0.05M sodium phosphate[3] pH7.5 and 0.1M sodium chloride[3] containing 0.1g/l sodium merthiolate[4]) for up to four hours. During this time the buffer was changed three or four times to remove

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(1) M.S.E. Superspeed 65 was kindly offered by Dr. G. Fink but a suitable rotor was not available.
(2) M.S.E. Mistral 6L, generously made available by Drs. K. Brown-Grant, G. Fink and F. Grief
(3) 'Analar' grade, B.D.H. Chemicals Ltd., Poole
(4) Laboratory reagent, B.D.H. Chemicals Ltd., Poole

as much thyroglobulin as possible, since this has been found to interfere with the preparation of some thyroid cell fractions (Swettenham K.,[1] personal communication). Following this the slices were blotted on filter paper, weighed and homogenised with two volumes of buffer for 30-60 seconds with a Silverson Emulsifier[2]. This crude homogenate was centrifuged[3] at 140g for 10 minutes at 4°C to bring the fat to the top and sediment heavy tissue debris. The middle layer was removed with a Pasteur pipette and filtered through a glass wool plug. The filtrate was collected on ice and centrifuged at 2000g[4] for 25 minutes. The supernatant was discarded and the pellet resuspended in phosphate buffered saline (P.B.S.) to give a final concentration of 5g equivalents of thyroid tissue per ml. This 2000g fraction (subsequently referred to as the homogenate) was stored in screw-topped vials at 4°C until use. In this way up to 300g of thyroid tissue could be processed in one day. Light microscopy of haematoxylin and eosin-stained smears of the homogenate revealed much cell debris, some fibres and some degenerating intact cells. The homogenisation procedure is outlined in figure 5.1.

5.5. FIRST ATTEMPTS TO DEMONSTRATE BINDING OF T.S.H. BY THYROID HOMOGENATES

At the time of the first experiments little data on the biological activity of iodinated T.S.H. preparations had

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(1) Senior Technician to Prof. I. Doniach, The London Hospital
(2) Silverson Emulsifier, Silverson Machines Ltd., London S.E.1
(3) 500 r.p.m. M.S.E. Mistral 6L with general purpose head no.62303 8 x 50ml buckets
(4) 2800 r.p.m. M.S.E. Mistral 6L with general purpose head no.62303 8 x 50ml buckets

FIGURE 5.1

Method for preparation of bovine thyroid homogenates

PREPARATION OF A BOVINE THYROID HOMOGENATE

BUFFER – 0·05M Phosphate pH 7·5 0·1M NaCl with 10^{-4} merthiolate
(All procedures at 0-4°C)

FRESH BOVINE THYROIDS
Dissected out, trimmed of fat,
cut into 1 mm slices.

↓

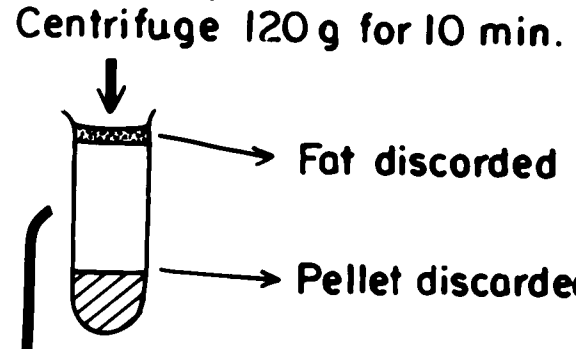
Washed in buffer and
stood in buffer 3-4 hr.

↓

Homogenised with 2 vol.
buffer (30-60 secs top speed
Silverson homogeniser.)

↓

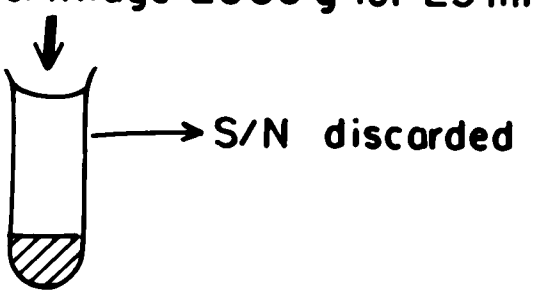
Centrifuge 120 g for 10 min.



S/N filtered through glass wool to remove
excess fat

↓

Centrifuge 2000 g for 25 min



Pellet resuspended in buffer to 5gm.equiv./ml
and stored at 4°C until use

been accumulated. It was assumed that iodination by method B (section 2.2) (45 second reaction on ice) would be less likely to damage the hormone and was therefore used in the initial studies.

5.5.1. First experiment (expt. 5.1)

Method. Fresh bovine thyroids(1) were divided into two lots, one consisting of pale tissue from young animals and the second consisting of darker, leaner tissue from an old cow. Each lot was processed as described above but the resulting homogenate was stored frozen in 2ml aliquots at -20°C for three days.

On thawing, both of the homogenates were found to have formed gels. They were therefore resuspended as completely as possible by vigorous agitation on a vortex mixer. T.S.H.-^{125}I(2) was prepared freshly on the day of experiment and diluted to give 35,000 c.p.m. in 10µl. Tubes(3) were set up in duplicate as shown in table 5.1 containing T.S.H.-^{125}I and varying quantities of either homogenate with or without 100mU carrier T.S.H.(4) in a total volume of 2ml. The homogenate was added last, after counting (10^4 counts, 12.5-75kev Nuclear Chicago autogamma), and the tubes were individually agitated before incubating for three hours on a water bath at 37°C. The sedimenting homogenate was resuspended every 30 minutes throughout the incubation. After three hours the tubes were spun at approximately 2300g(5) for 30 minutes at 4°C. The

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(1) The identity of the tissue was confirmed by histology
(2) Specific activity approximately 40mCi/mg
(3) L.P.3, Luckham Ltd., Burgess Hill, Sussex
(4) 'Thytropar', Armour Pharmaceuticals, Chicago, USA
(5) M.S.E. Mistral 6L 3100 r.p.m. general purpose head 62303 multi-tube carriers

TABLE 5.1. Design for experiment 1 (Section 5.5). See text.

Tube	T.S.H. ^{125}I (1) (µl)	1% B.S.A. (2) (ml)	200mU/ml T.S.H. (3) (ml)	P.B.S. 1% B.S.A. (4) (ml)	Homogenate (ml)
1 + 7	10	0.5	-	1.4	0.1
2 + 8	10	0.5	-	1.3	0.2
3 + 9	10	0.5	-	1.0	0.5
4 + 10	10	-	0.5	1.4	0.1
5 + 11	10	-	0.5	1.3	0.2
6 + 12	10	-	0.5	1.0	0.5

Tubes 1-6, homogenate 1; Tubes 7-12, homogenate 2
All tubes made up in duplicate. Three hour incubation at 37°C.

(1) 35,000 c.p.m. in 10µl
(2) Cohn fraction V (Sigma Chemicals Ltd.), 1g/100ml normal saline
(3) 'Thytropar' (Armour Pharmaceuticals Ltd.), 200mU/ml 1% B.S.A.
(4) P.B.S. pH7.5 containing 1g/100ml B.S.A.

supernatants were aspirated and the tubes counted. The pellets were then washed twice by resuspension in 2ml ice cold P.B.S.-1% B.S.A. on a vortex mixer[1] followed by recentrifugation and aspiration of the supernatants. The tubes were recounted after each wash.

Results. Figure 5.2 shows the percentage of label retained by each pellet after a single wash. This bore a roughly linear relationship to the concentration of homogenate. The radioactivity remaining in the pellet was greatly reduced in the presence of 50mU/ml carrier T.S.H., suggesting the presence of saturable binding sites within the pellet. The effect of washing the pellet is shown in figure 5.3. 'Saturable' T.S.H.-^{125}I binding (B_s) was reduced by successive washes but the 'unsaturable' T.S.H.-^{125}I binding (B_c, binding in the presence of excess carrier T.S.H.) was also reduced (fig. 5.3A) resulting in an increase in the ratio of saturable to total binding (B_s/B_t) from 0.6 to 0.8 with the first wash (fig. 5.3B). Since no further increase in the B_s/B_t ratio was achieved, a single wash was performed in subsequent experiments.

5.5.2. Second experiment (expt. 5.2)

Method. Two further bovine thyroid homogenates (3 and 4) were prepared and the above experiment repeated with the following modifications:

1. The homogenates were used after overnight storage at 4°C. T.S.H. was iodinated by method B[2] on the day of the experiment.

2. The concentration of carrier T.S.H. was increased from 50mU/ml to 125mU/ml.

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(1) Complete resuspension was not achieved in this experiment
(2) Specific activity 70mCi/mg

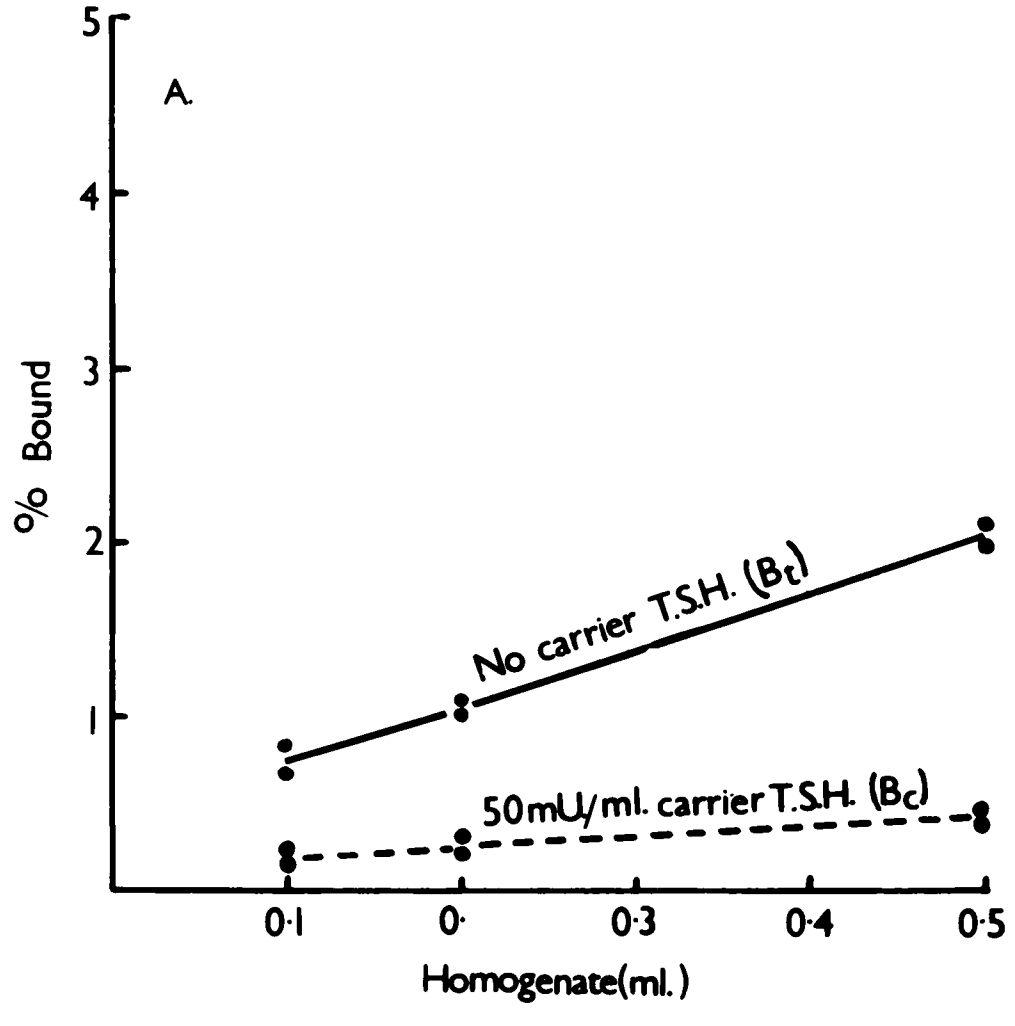
FIGURE 5.2

A preliminary experiment on the binding of T.S.H.-^{125}I to bovine thyroid homogenates (expt. 5.1)

Total volume: 2ml. Three hour incubation at 37°C. Ordinate represents percent bound after a single wash. Abscissa represents volume of homogenate added (5g. equiv. per ml.).

A: Homogenate 1

B: Homogenate 2


A.
5
4
3
2
1
% Bound
No carrier T.S.H. (Bt)
50mU/ml. carrier T.S.H. (Bc)
0·1
0·
0·3
0·4
0·5
Homogenate(ml.)

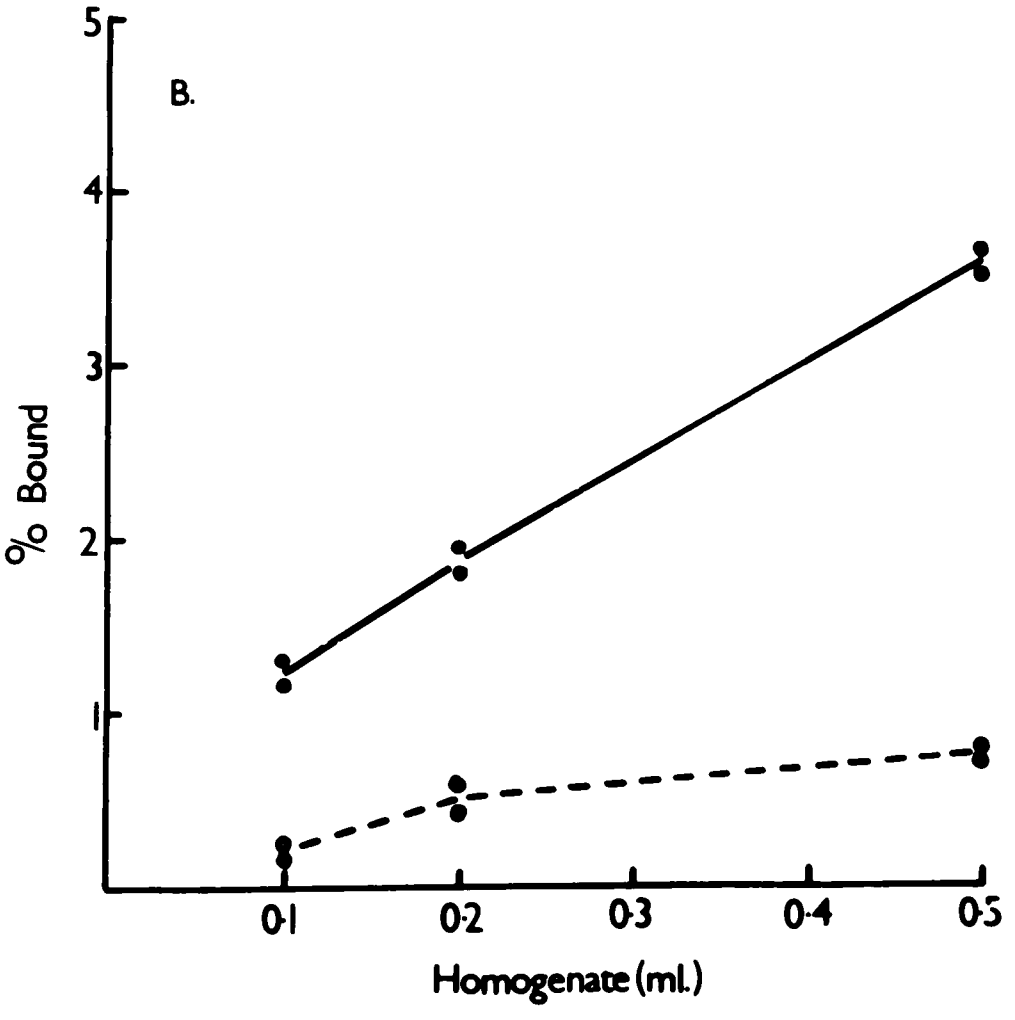

B.
5
4
3
2
1
% Bound
0·1
0·2
0·3
0·4
0·5
Homogenate (ml.)

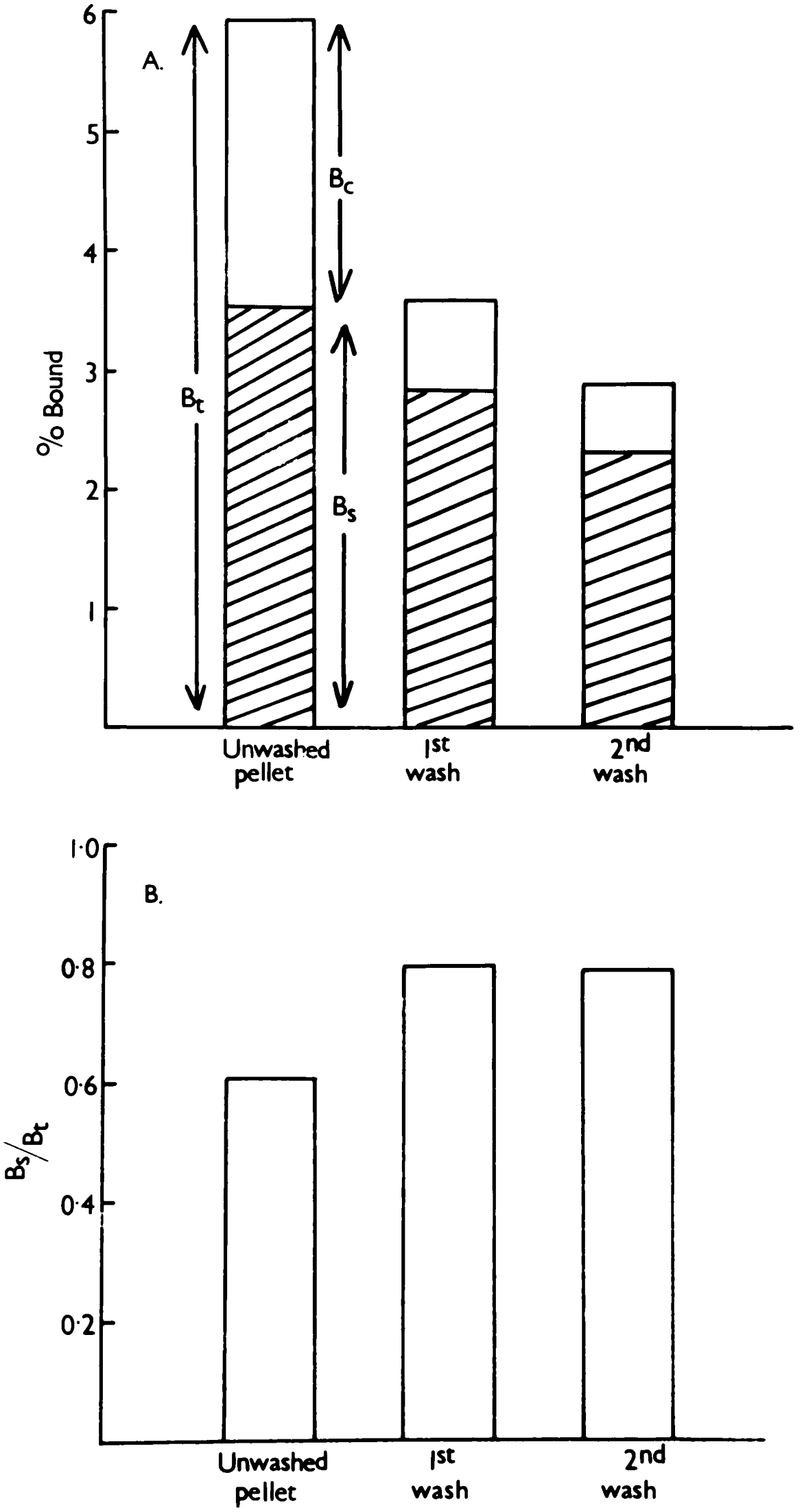
FIGURE 5.3

The effect of washing on the retention of T.S.H.-^{125}I by the homogenate (expt. 5.1)

0.5ml homogenate 2. Total volume: 2ml. Three hour incubation at 37^{o}C.

A. Unshaded area represents binding in presence of 50mU/ml., carrier T.S.H. (B_c). Shaded area represents "saturable" binding ($B_s = B_t - B_c$)

B. Same data plotted in terms of B_s/B_t.


A.
6
5
4
3
2
1
% Bound
B_t
B_c
B_s
Unwashed pellet
1st wash
2nd wash
B.
1·0
0·8
0·6
0·4
0·2
B_s/B_t
Unwashed pellet
1st wash
2nd wash

3. Following incubation, centrifugation was performed as above and the pellets were washed once before counting.

In this experiment there was no macroscopic evidence of precipitation of the homogenate during the incubation and the pellets were easily resuspended.

Results. The results for both homogenates were similar (fig. 5.4). The magnitude of the T.S.H.-^{125}I uptake was similar to that of experiment 1 and 7-8% of this was 'saturable'. The relationship between percent bound and the volume of the homogenate added was a straight line, passing through the origin.

5.5.3. Comments

These results suggested that the 2000g fraction of bovine thyroid contained saturable T.S.H. binding sites which possibly corresponded to T.S.H. receptors. However, the uptake of label was rather low, being less than 5% at homogenate concentrations of 1.25g equiv./ml. In contrast, Leidenberger and Reichert (1972a) achieved 30% binding of labelled h-C.G. to rat testicular homogenates at a concentration of 33mg equiv./ml. Similarly, Tsurahara et al (1972) reported up to 30% uptake of h-C.G. at a very low concentration of rat ovarian homogenate (in the order of 3-4mg equiv./ml). If these binding sites did indeed represent true receptors, three possible factors could have accounted for the poor binding:

1. The supply of biologically active T.S.H.-^{125}I may have been limiting, that remaining in solution being biologically inactive and incapable of binding to receptors. Since there

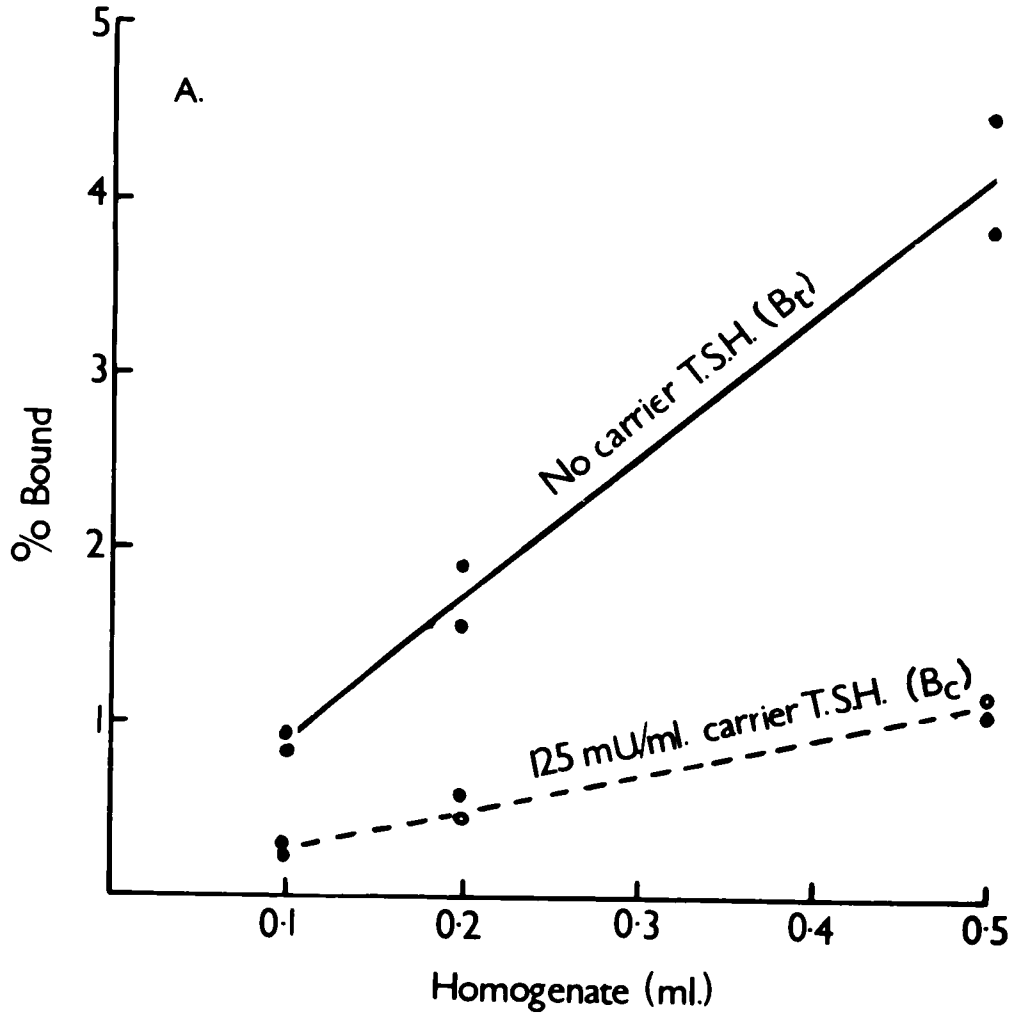
FIGURE 5.4

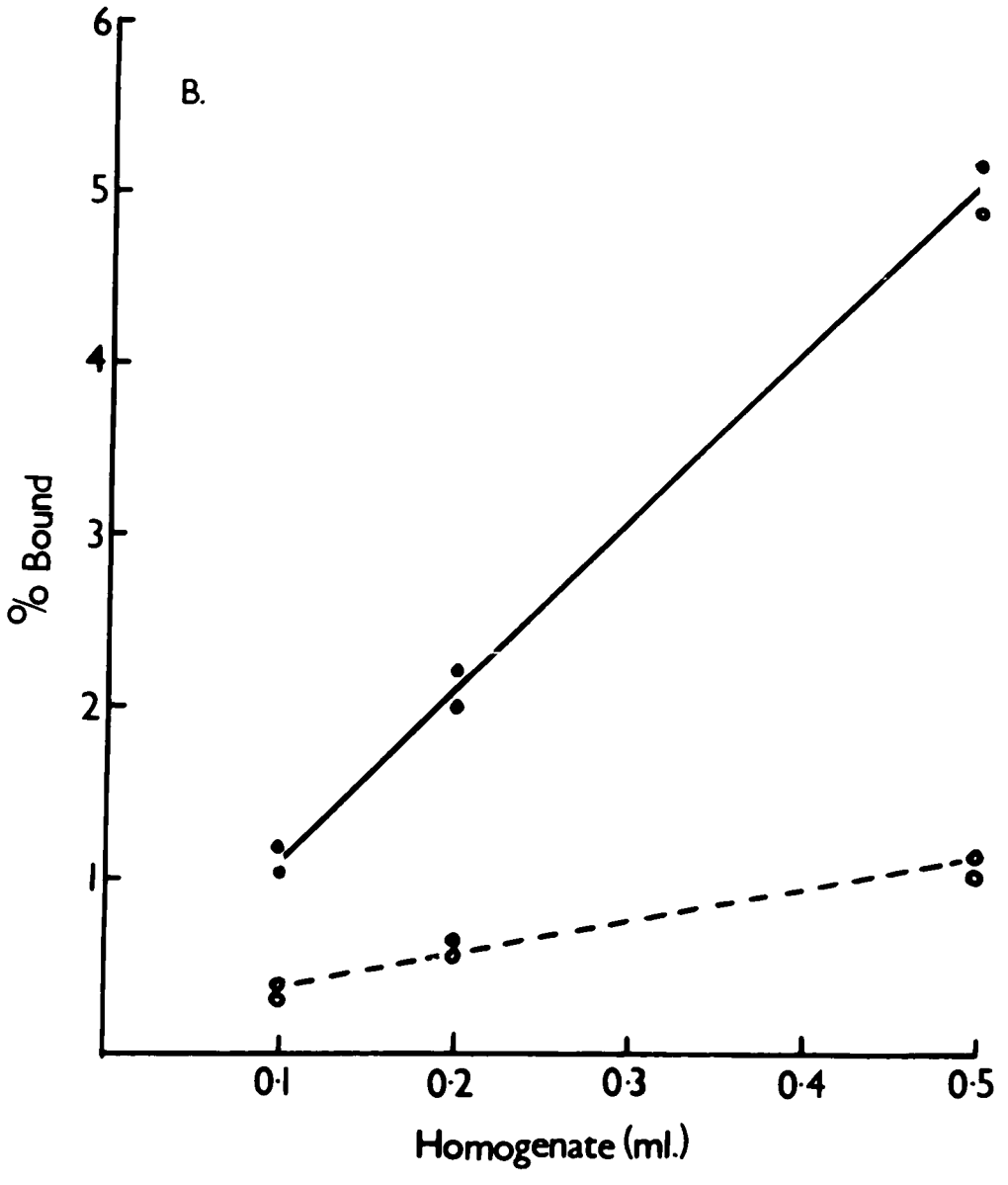
A preliminary experiment on the binding of T.S.H. ^{125}I to bovine thyroid homogenates (expt. 5.2)

Total volume: 2ml. Three hour incubation at 37°C.

A: Homogenate 3

B: Homogenate 4


A.
5
4
3
2
1
% Bound
No carrier T.S.H. (Bc)
125 mU/ml. carrier T.S.H. (Bc)
0·1
0·2
0·3
0·4
0·5
Homogenate (ml.)


B.
6
5
4
3
2
1
% Bound
0·1
0·2
0·3
0·4
0·5
Homogenate (ml.)

a linear increase in percent bound with homogenate concentration in experiment 5.2, the receptor concentration must have been rate limiting rather than the supply of biologically active T.S.H.-^{125}I. Furthermore, up to 25% binding has been obtained in subsequent experiments at greater homogenate concentrations.

2. There may have been a low receptor concentration: (a) in the original bovine thyroid tissue, and/or (b) in the homogenate, there having been a substantial loss of receptors in the homogenisation procedure.

With regard to (a) Manley et al (1972, 1974a) were impressed by the relative dearth of receptors in bovine thyroid tissue in comparison with hyperplastic guinea-pig thyroid. Moreover, in later experiments (section 5.15) human thyroid homogenates appeared to contain much higher concentrations of binding sites than bovine thyroid homogenates. Thus, the receptor concentration in bovine thyroid tissue is probably low. Considering (b), the loss of receptors on preparation was probably significant, but not of prime importance since, as will be shown in section 5.12, binding by the microsomal fraction was of similar magnitude but no soluble receptors were detected.

3. T.S.H.-^{125}I may have a low affinity for the binding sites. Possible reasons for this are: (a) T.S.H. itself may have a relatively low affinity for its receptors. As will be shown in section 5.14, the affinity constant for the present system was comparable with those for other T.S.H.-receptor systems but relatively low in comparison with that for h-C.G.-receptor

interactions. This suggests that the lower affinity of T.S.H., per se, for its receptor may be another factor contributing to the poor binding here.

(b) The affinity of T.S.H. for its receptors may be reduced by iodination.

(c) The reaction conditions may have been suboptimal.

One important difference between the reaction conditions of the present system and those of the h-C.G.-receptor systems was in the use of sodium merthiolate as an antibacterial in the buffers here. It was possible that merthiolate somehow inhibited T.S.H.-^{125}I uptake, since it is known to affect the activity of certain proteins (Esplin, 1965). In three experiments where merthiolate was omitted, T.S.H.-^{125}I uptake was similar, but carrier T.S.H. caused little reduction in binding (table 5.2A). In a later experiment bovine thyroids were cut in two and the halves homogenised separately in the presence or absence of sodium merthiolate (table 5.2B). Preparation in the presence of merthiolate was clearly beneficial but addition at the incubation stage had less effect on the saturable T.S.H.-^{125}I uptake. This suggested that merthiolate somehow prevents deterioration of the homogenate. In support of this, the pellets of merthiolate-free homogenates were found to be gelled, as noted with merthiolate containing homogenates after 3-4 days storage. How merthiolate acts is unclear but may be related to the impurity of the fraction, since in subsequent experiments Mehdi and Nussey (1975) successfully

TABLE 5.2. The effect of sodium merthiolate (100mg/l) on T.S.H.^{125}I uptake by bovine thyroid homogenates

A. Uptake of T.S.H.^{125}I by bovine thyroid homogenates prepared and incubated in the absence of merthiolate

Expt.(1)	% bound(2)	
	No carrier T.S.H.	Excess carrier T.S.H.(3)
5.5	2.75 (2.66, 2.85)	1.60 (1.62, 1.59)
5.6	5.37 (5.32, 5.41)	4.54 (4.56, 4.51)
5.7	3.40 (3.22, 3.58)	2.04 (2.11, 1.97)

(1) Total volume 2ml.; 1.25g. equiv./ml homogenates; 3 hour incubation at 37°C
(2) Mean (of duplicates)
(3) 250mU 'Thytropar'

B. The effect of preparation and for incubation in the presence or absence of merthiolate on the uptake of T.S.H.^{125}I(2) (expt. 5.8)

Preparation	Incubation	% bound(3)	
		No carrier T.S.H.	Excess carrier T.S.H.(4)
Merthiolate	Merthiolate	8.58 (8.67, 8.48)	1.52 (1.45, 1.59)
No merthiolate	Merthiolate	5.90 (6.23, 4.98)	1.97 (2.11, 1.82)
No merthiolate	No merthiolate	3.93 (3.96, 3.90)	2.97 (2.92, 3.06)

(1) Total volume 1.025ml.; 2.5g. equiv. homogenate; 8 hour incubation at 20°C
(2) Iodinated by method A (S.A. 150mCi/mg)
(3) Mean (of duplicates)
(4) 500mU 'Thytropar'

used merthiolate-free particulate fractions sedimenting at 25,000g or 100,000g. One possible explanation is that merthiolate may somehow counter the actions of lysosomal enzymes freed during homogenisation.

The major cause of low binding was probably the use of P.B.S.as buffer since subsequent investigators have found that binding is decreased as the ionic strength increases from 0.01M to 0.15M (Amir et al, 1973; Bashford et al, 1975; Tate et al, 1975). Moreover, the pH of the medium (7.5) may have been suboptimal since other investigators have reported pH optima of 5.5 to 6.5 for the binding to thyroid plasma membranes (Amir et al, 1976;Bryson et al, 1976; Moore and Wolff, 1974; Tate et al, 1975).However, Manley et al (1974a) found a wide tolerance with maximum binding between pH6 and pH8 for guinea-pig thyroid 10,000g fraction.

5.6. THE TIME COURSE OF T.S.H.-^{125}I UPTAKE AND THE EFFECT OF TEMPERATURE (expt. 5.9)

Methods. In order to develop a reproducible and convenient system for the investigation of T.S.H. binding to bovine thyroid homogenates, further modifications were introduced:

1. T.S.H. iodinated by method B[1] five days previously was used.
2. The homogenate concentration was increased from 1.25 to 2.5g equiv./ml by reducing the volume from 2ml to approximately 1ml.
3. In this and subsequent experiments (unless otherwise stated)

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(1) Specific activity approximately 40mCi/mg.

reactants other than the homogenate were added in 1% B.S.A. giving a final B.S.A. concentration in the incubation mixture of 5.1 to 5.3g/l (constant within each experiment).

4. Since pilot studies had suggested that thyroid binding sites were incompletely saturated at a carrier T.S.H.(1) concentration of 125mU/ml, this was increased to approximately 500mU/ml.

The effect of temperature was investigated as follows (expt. 5.9):

Tubes were set up in duplicate each containing 30,000 c.p.m. T.S.H.-^{125}I in 25µl and either 0.5ml P.B.S.-1% B.S.A. or 0.5ml 1000 mU/ml 'Thytropar' in P.B.S.-1% B.S.A. The tubes were counted and 0.5ml (2.5g equiv.) of a thyroid homogenate (prepared the previous day) were added. Incubation was then performed in a refrigerator at 4°C, or on a water bath at 20°C or 37°C for 1, 3, 8 or 24 hours. At the end of this time the tubes were centrifuged, washed and counted.

Results. The experiment enabled curves representing the time course of T.S.H.-^{125}I binding to be drawn (fig. 5.5). Four important points emerged from this:

1. T.S.H. binding rose to a maximum but declined thereafter. This fall in binding may have been due to destruction (or solubilisation) of the receptor sites, perhaps by lysosomal enzymes released from the tissue during homogenisation and incubation. At 37°C, the homogenate tended to precipitate after 8-24 hours and, on centrifugation, formed a gel which resisted

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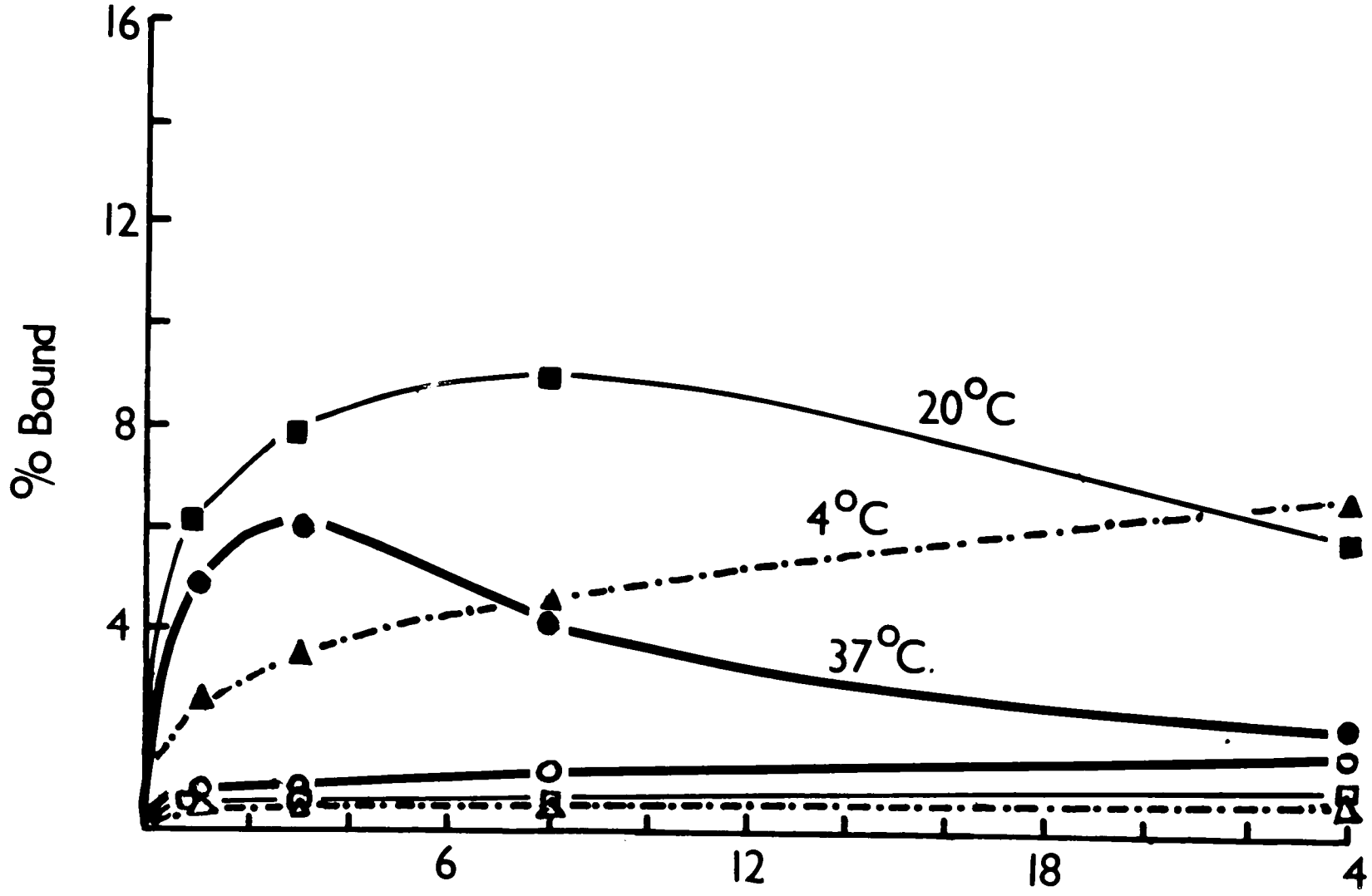
(1) 'Thytropar', Armour Pharmaceuticals Ltd., Chicago, U.S.A.

FIGURE 5.5

The effect of temperature on the time course of T.S.H.-^{125}I uptake by a bovine thyroid homogenate (expt. 5.9)

Total volume: 1.025ml.

30,000 c.p.m. T.S.H.^{125}I (Method B). Specific activity 40mCi/mg.; 2.5g. equiv. bovine thyroid homogenate per tube. Each point represents the mean of duplicates.



Temp. °C	No carrier T.S.H.	500 mU. T.S.H.
4	▲–·–·–·–▲	△–·–·–·–△
20	■———■	□———□
37	●———●	○———○

resuspension. These changes were similar to those found after freezing and thawing or storage for 3-4 days at 4°C. A further possibility was that T.S.H. lost its receptor combining capacity in the presence of thyroid homogenate. This could be tested by comparing the receptor uptake of T.S.H.-^{125}I, previously incubated in the presence of thyroid homogenate, with that of untreated T.S.H.-^{125}I.

2. The rates of rise and fall of binding were increased by raising the temperature.

3. The maximum binding at 20°C was about 50% greater than at 37°C.

4. In the presence of excess carrier T.S.H. the uptake was low and largely unaffected by the temperature or duration of incubation.

Comments. These results suggested that an 8 or 10 hour incubation at 20°C would provide the optimum uptake of label. In later experiments, however, the temperature was increased to 22°C to allow for higher room temperatures.

5.7. THE EFFECT OF IODINATION METHOD (expt. 5.10)

Method. T.S.H. iodinated by method A(1) and B(2) (section 2.2) were compared for their ability to bind to thyroid homogenate. In order to confirm the time course of T.S.H.-^{125}I binding at 20°C, the experiment was performed using various incubation times (1, 3, 10 and 24 hr.).

Results. A similar time course of uptake was found for both T.S.H.-^{125}I preparations, peaking at 8-10 hours as in the

---

(1) Specific activity 110mCi/mg
(2) Specific activity 40mCi/mg

previous experiment (fig. 5.6). The maximum uptake of T.S.H. labelled by method B was slightly greater than that of T.S.H. labelled by method A (19.4% cf. 17.6%). The uptakes in the presence of excess carrier T.S.H. were 1.4% (B) and 1.5% (A).

Comments. Harsher iodination conditions reduced the subsequent uptake of T.S.H.-^{125}I by thyroid preparations. However, the reduction in binding was small and the advantage was outweighed by those of higher specific activity material. Method A allowed the use of smaller quantities of labelled T.S.H. per experiment, thus conserving 'pure' T.S.H. which was in short supply. It was therefore used for all subsequent receptor binding studies. In future experiments 25,000-50,000 c.p.m. were added to each tube, corresponding to 10-20μU unlabelled hormone.

5.8. THE EFFECT OF INCUBATION DAMAGE ON T.S.H.-^{125}I UPTAKE

(expts. 5.11-5.12)

During initial experiments it was found that the non-saturable binding (B_c) of a given T.S.H.^{125}I preparation to different homogenates tended to increase with time, reaching 50% of the total uptake (B_t) two to three weeks after iodination (by method A). Therefore, to increase its shelf life, T.S.H.-^{125}I was purified by gel filtration on a 23 x 1cm column of Sephadex G-100 as described in section 2.2.5, when used more than one week after iodination.

Methods. In two experiments the effect of rechromatography was tested by comparing the binding of rechromatographed or untreated T.S.H.-^{125}I, 15 (expt. 5.11) or 7 days (expt. 5.12) after iodination.

FIGURE 5.6

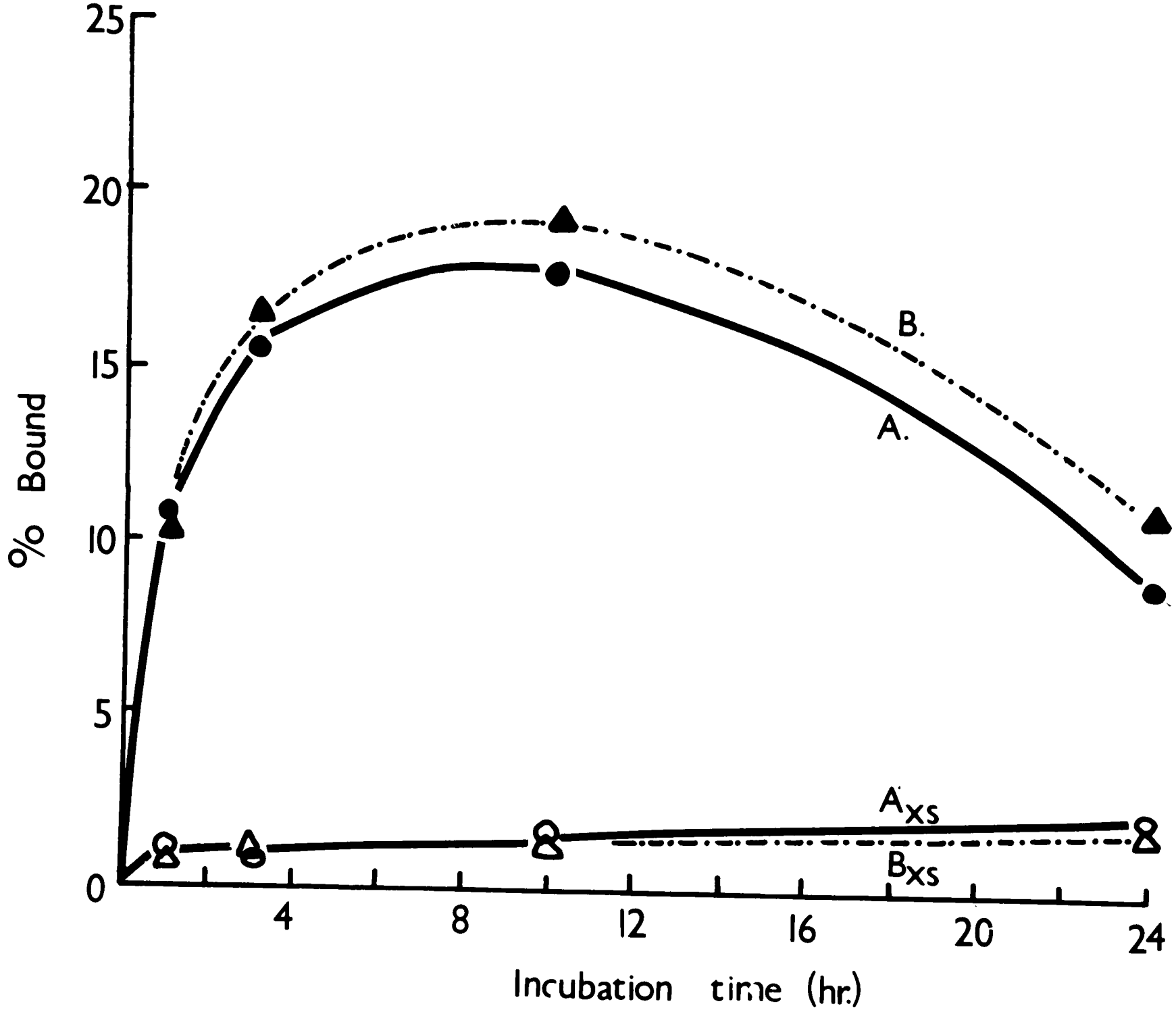
The effect of iodination method on the T.S.H.-^{125}I uptake by a bovine thyroid homogenate (expt. 5.10)

Total volume 1.025ml.; 2.5g equiv. bovine thyroid homogenate per tube; 20°C incubation.

A: 40,000 c.p.m. T.S.H.^{125}I iodinated by method A (S.A. 110mCi/mg)

B: 35,000 c.p.m. T.S.H.^{125}I iodinated by method B (S.A. 40mCi/mg)

A_{XS} and B_{XS}: as for A and B respectively with the addition of 500mU carrier T.S.H.('Thytropar')


25
20
15
10
5
0
% Bound
B.
A.
A_{xs}
B_{xs}
4
8
12
16
20
24
Incubation time (hr.)

Results. The improvement with rechromatography is shown in table 5.3. This took the form of an increased saturable to total uptake ratio (B_s/B_t) with or without an increased saturable uptake (B_s).

Comments. Much of the improvement with rechromatography may be attributable to the removal of iodide, which is taken up by the homogenate (section 5.9), but molecular aggregates may also be involved. In this context it would be interesting to study the binding of aggregated T.S.H.^{125}I to thyroid homogenates.

In further experiments, T.S.H.-^{125}I was used within two weeks of iodination (by method A) or within one week of rechromatography. Labelled T.S.H. was discarded 4 weeks after iodination.

5.9. WAS THE BOUND LABEL IODIDE OR T.S.H.?

Since thyroid homogenates can concentrate and organify iodide (Doniach and Logothetopoulos, 1955; Wyngaarden et al, 1951), it was possible that the bound label was iodide released by degradation of T.S.H.-^{125}I. This was tested in two ways.

5.9.1. Was T.S.H.^{125}I deiodinated during the incubation?

(expt. 5.13)

Method. Approximately 300,000 c.p.m. freshly rechromatographed T.S.H.-^{125}I were incubated with 2.5g equiv. bovine thyroid homogenate in 1ml P.B.S.-0.5% B.S.A. for 10 hours at 20°C. The tube was centrifuged and 0.3ml of the 2000g supernatant was passed down a 100 x 1cm column of

TABLE 5.3. The effect of incubation damage and rechromatography on T.S.H. ^{125}I binding by bovine thyroid homogenates

Expt.	Total volume (ml)	Homogenate (g.equiv.)	TSH ^{125}I S.A. mCi/mg	Days since iodination	%I[3]	Rechromatography	Carrier T.S.H. (mU)	% bound	B_s	B_s/B_t
5.11[1]	1.05	2.5	150	15	15	-	-	11.97(12.01,11.57)[4]	5.36	0.46
						-	500	6.43(6.27,6.59)		
						+	-	10.06(10.21,9.91)	8.31	0.81
						+	500	1.75(1.69,1.82)		
5.12[2]	0.425	1.0	120	7	3.5	-	-	9.41 ± 0.66[5]	6.59	0.70
						-	200	2.82 ± 0.05		
						+	-	8.26 ± 0.10	6.54	0.79
						+	200	1.72 ± 0.08		

(1) 8 hour incubation at 20°C
(2) 8 hour incubation at 22°C
(3) Determined from elution pattern on 23, x 1cm Sephadex G-100 column
(4) Mean (of duplicates)
(5) Mean ± S.E.M. (of triplicates)

Sephadex G-100 as described in section 2.3.1.

Results. The pattern of radioactivity in the eluate did not differ significantly from that of the original T.S.H.-^{125}I on the same column (24 hours beforehand) except for a slight increase in iodide contamination (from 0.9 to 2.0%).

5.9.2. The effects of goitrogens and carrier T.S.H. on T.S.H.-^{125}I and iodide-^{131}I ($^{131}I^-$) uptake by bovine thyroid homogenates (expts. 5.14-5.15)

Since perchlorate (ClO_4^-) prevents iodide uptake (Stanbury and Wyngaarden, 1952; Wyngaarden et al, 1952, 1953) and propylthiouracil (P.T.U.) prevents iodide organification by the thyroid (Astwood, 1945, 1954) a combination of these drugs should inhibit any iodide uptake by the homogenate. The concentrations were those used by Manley et al (1972) to prevent the uptake of iodide by guinea-pig thyroid slices.

Method. In two experiments the effects of T.S.H. (∿500mU/ml) and a combination of P.T.U.(1) (1mM/l) and ClO_4^-(2) (10mM/l) on the binding of T.S.H.-^{125}I and $^{131}I^-$(3) were tested. The experimental plan (shown in table 5.4) was similar for both experiments but in the second the tubes were triplicated rather than duplicated. Also, the total volume was reduced from 1.025ml to 0.525ml and the temperature increased to 22°C for the second experiment.

Results. These are shown in table 5.4 and figure 5.7. Their essential features are the same for both experiments:

1. Excess carrier T.S.H. inhibited T.S.H.-^{125}I uptake without

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(1) 4(6) Propyl-2-thiouracil, Koch-Light Laboratories Ltd., Colnbrook, Bucks.
(2) Sodium perchlorate, B.D.H. laboratory reagent
(3) ^{131}I, I.B.S. 1P., The Radiochemical Centre, Amersham

TABLE 5.4. The effect of T.S.H. and goitrogens on T.S.H.-^{125}I and $^{131}I^-$ binding to bovine thyroid homogenates

A. Expt. 5.14

Label	Carrier T.S.H.(mU)(2)	P.T.U.(mM)(3)	ClO_4^-(mM)(4)	% bound(5)
T.S.H.^{125}I	-	-	-	4.29 (4.31, 4.27)
	500	-	-	1.23 (1.27, 1.18)
	-	10^{-3}	10^{-2}	4.33 (4.24, 4.41)
	500	10^{-3}	10^{-2}	1.25 (1.38, 1.11)
$^{131}I^-$	-	-	-	4.32 (4.30, 4.34)
	500	-	-	3.93 (3.73, 4.13)
	-	10^{-3}	10^{-2}	1.09 (1.08, 1.09)
	500	10^{-3}	10^{-2}	1.01 (1.01, 1.01)

Total volume 1.025ml.; 2g. equiv. homogenate; 8 hour incubation at 20°C

/Cont'd.

TABLE 5.4. Cont'd.

B. Expt. 5.15

Label[1]	Carrier T.S.H.(mU)[2]	P.T.U.(mM)[3]	ClO_4^-(mM)[4]	% bound[6]	p[7]
T.S.H.^{125}I	-	-	-	8.34 ± 0.22	-
	250	-	-	1.32 ± 0.10	<0.01[8]
	-	5×10^{-4}	5×10^{-3}	5.66 ± 0.13	<0.01
	250	5×10^{-4}	5×10^{-3}	1.06 ± 0.01	<0.01
$^{131}I^-$	-	-	-	21.52 ± 1.63	-
	250	-	-	19.85 ± 0.88	N.S.
	-	5×10^{-4}	5×10^{-3}	1.09 ± 0.23	<0.01
	250	5×10^{-4}	5×10^{-3}	1.17 ± 0.14	<0.01

Total volume 0.525ml.; 1g. equiv. homogenate; 8 hour incubation at 22°C

(1) Approx. 30,000 c.p.m.
(2) 'Thytropar', Armour Pharmaceuticals Ltd., Chicago, USA.
(3) 4(6)propyl-2-thoracil, Koch-Light Laboratories Ltd., Colnbrook, Bucks.
(4) Sodium perchlorate, Laboratory reagent, B.D.H. Chemicals Ltd., Poole.
(5) Mean (of duplicates)
(6) Mean ± S.E.M. (of triplicates)
(7) p for difference from first member of group (Dunnett's 't' test)
(8) Heteroscadasticity $p<0.05$

FIGURE 5.7

The effect of T.S.H. and goitrogens on T.S.H.-^{125}I and $^{131}I^-$ binding to bovine thyroid homogenates

A. Expt. 5.14

Total volume 1.025ml; 2g. equiv. thyroid homogenate; 8 hour incubation at 20°C.

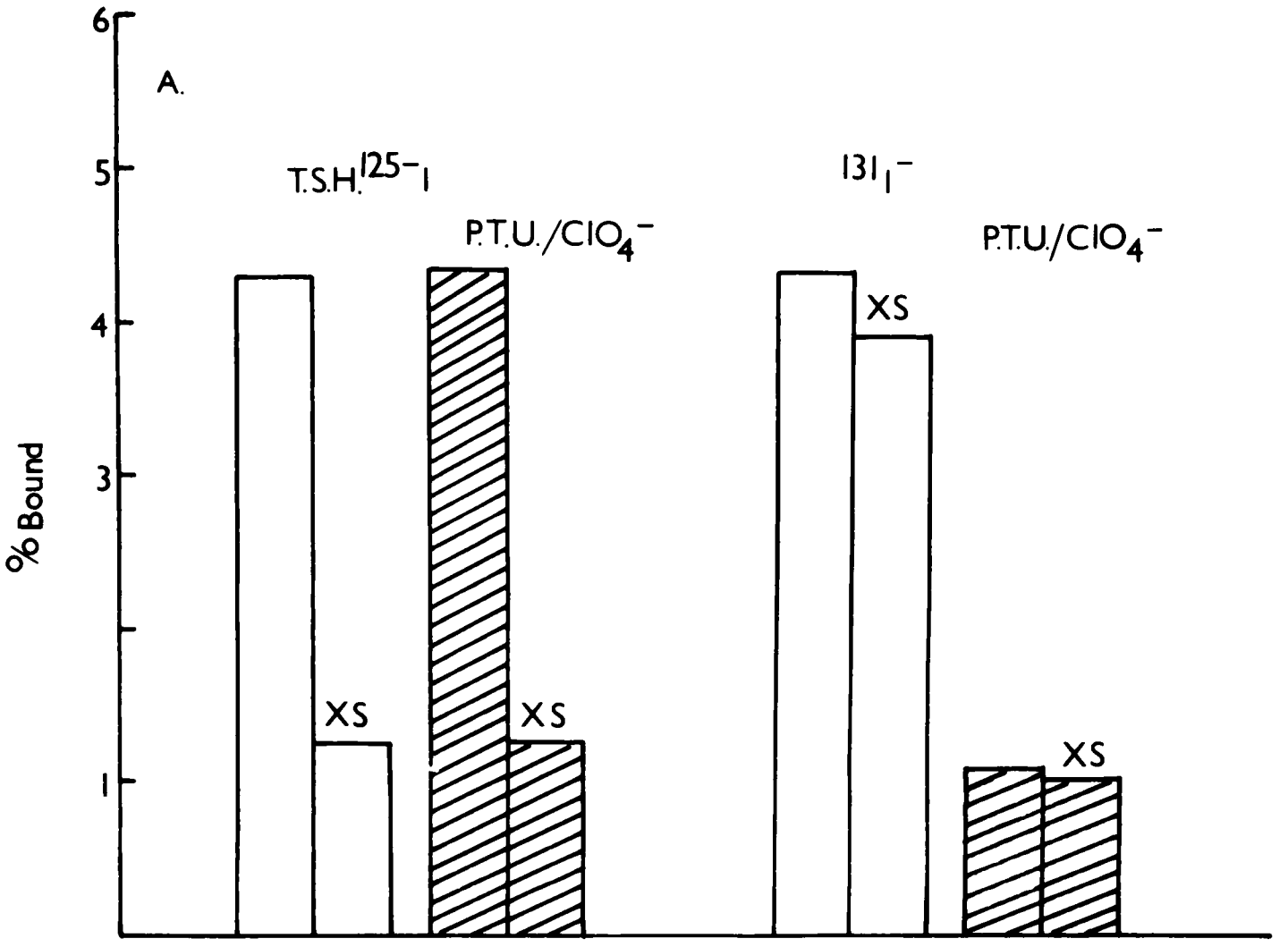
Each bar represents the mean of duplicates.

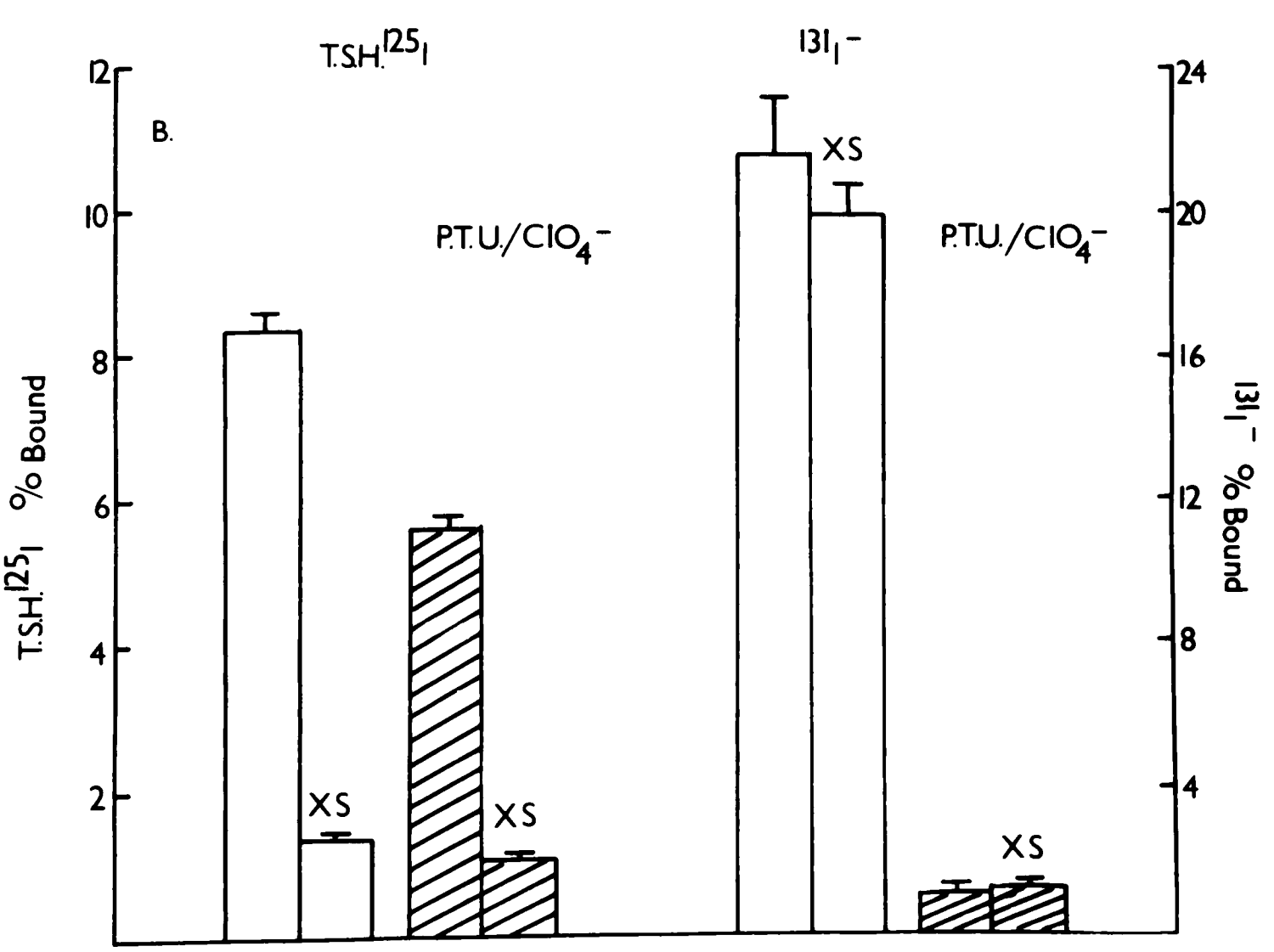
B. Total volume 0.525ml; 1g. equiv. thyroid homogenate; 8 hour incubation at 22°C.

Each bar represents mean ± S.E.M. of duplicates.

XS: Excess carrier T.S.H. (approximately 500mU/ml 'Thytropar').

Shaded bars: In presence of 1mM P.T.U. + 10mM Na ClO_4.


A.
T.S.H.$^{125}I^-$
P.T.U./ClO_4^-
$^{131}I^-$
P.T.U./ClO_4^-
XS
XS
XS
XS
% Bound
6
5
4
3
1


B.
T.S.H.^{125}I
$^{131}I^-$
P.T.U./ClO_4^-
P.T.U./ClO_4^-
XS
XS
XS
XS
T.S.H.^{125}I % Bound
$^{131}I^-$ % Bound
12
10
8
6
4
2
24
20
16
12
8
4

effect on $^{131}I^-$ uptake.

2. The P.T.U./ClO_4^- combination inhibited $^{131}I^-$ uptake almost totally but had no effect (expt. 5.14) or a slight inhibitory effect (expt. 5.15) on T.S.H.-^{125}I uptake.

5.9.3. Comments

Thus, little T.S.H.-^{125}I deiodination took place during the incubation and, moreover, T.S.H.-^{125}I and $^{131}I^-$ uptakes differed in their susceptibilities to the effects of carrier T.S.H. or goitrogens. Therefore, the label taken up could not have been iodide derived from T.S.H.-^{125}I. Furthermore, the magnitude of the binding achieved in this system suggested that T.S.H.-^{125}I, per se, was bound, although molecular alteration on binding could not be excluded.

5.10. WAS THE BINDING OF T.S.H.-^{125}I REVERSIBLE? (expt. 5.16)

In order to investigate the reversibility of binding, the displacement of bound T.S.H.-^{125}I by excess carrier T.S.H. was studied.

Method. 16 tubes containing fresh bovine thyroid homogenate (2.5g equiv.) and approximately 80,000 c.p.m. of recently rechromatographed T.S.H.-^{125}I were incubated for 4 hours at 20°C in the presence (control) or absence (test) of 500mU carrier T.S.H. in a total volume of 1ml. At the end of this time, two "control" and two "test" tubes were centrifuged and the resultant pellets washed. To each of the remaining tubes was added either 500mU T.S.H. in 50µl P.B.S.-1% B.S.A. (test) or 50µl P.B.S.-1% B.S.A. (control). Two "test" and two

"control" tubes were subsequently removed 1 hour later and again at 3 and 5 hours, centrifuging and washing immediately.

Results. These are summarised in figure 5.8. Approximately 70% of the saturably bound label (B_s) was displaced by excess carrier T.S.H. within 3 hours but no further displacement was achieved at 5 hours. Thus the reaction of T.S.H.-^{125}I with the homogenate was only partially reversible.

Comments. The reason for this partial reversibility is not clear. Other workers have since reported rapid and almost completely reversible binding of labelled T.S.H. to thyroid membranes (Amir et al, 1973; Manley et al, 1974a; Verrier et al, 1974), although in each case dissociation failed to follow a simple exponential course and showed a marked reduction in dissociation rate with time. Also, Manley et al found that dissociation was slower following a 2 hour preincubation than after a 30 minute preincubation with T.S.H.-^{125}I.

It seemed possible that irreversible binding was related to the labelled T.S.H. used in the present experiments since similar results were obtained with identically labelled T.S.H. and human thyroid membranes (Mehdi and Nussey, 1975). Such results could be explained by irreversible binding by partially damaged hormone to the receptors. However, Field (1975) has also reported persistent incomplete dissociation (50-60%) of T.S.H.-receptor complexes whether measured by T.S.H.-^{131}I binding to bovine thyroid membranes or by the effects of

FIGURE 5.8

The reversibility of binding of T.S.H.-^{125}I to a bovine thyroid homogenate (expt. 5.16)

First incubation: Total volume 1.00ml.; 2.5g. equiv. bovine thyroid homogenate; 4 hour incubation at 20°C.

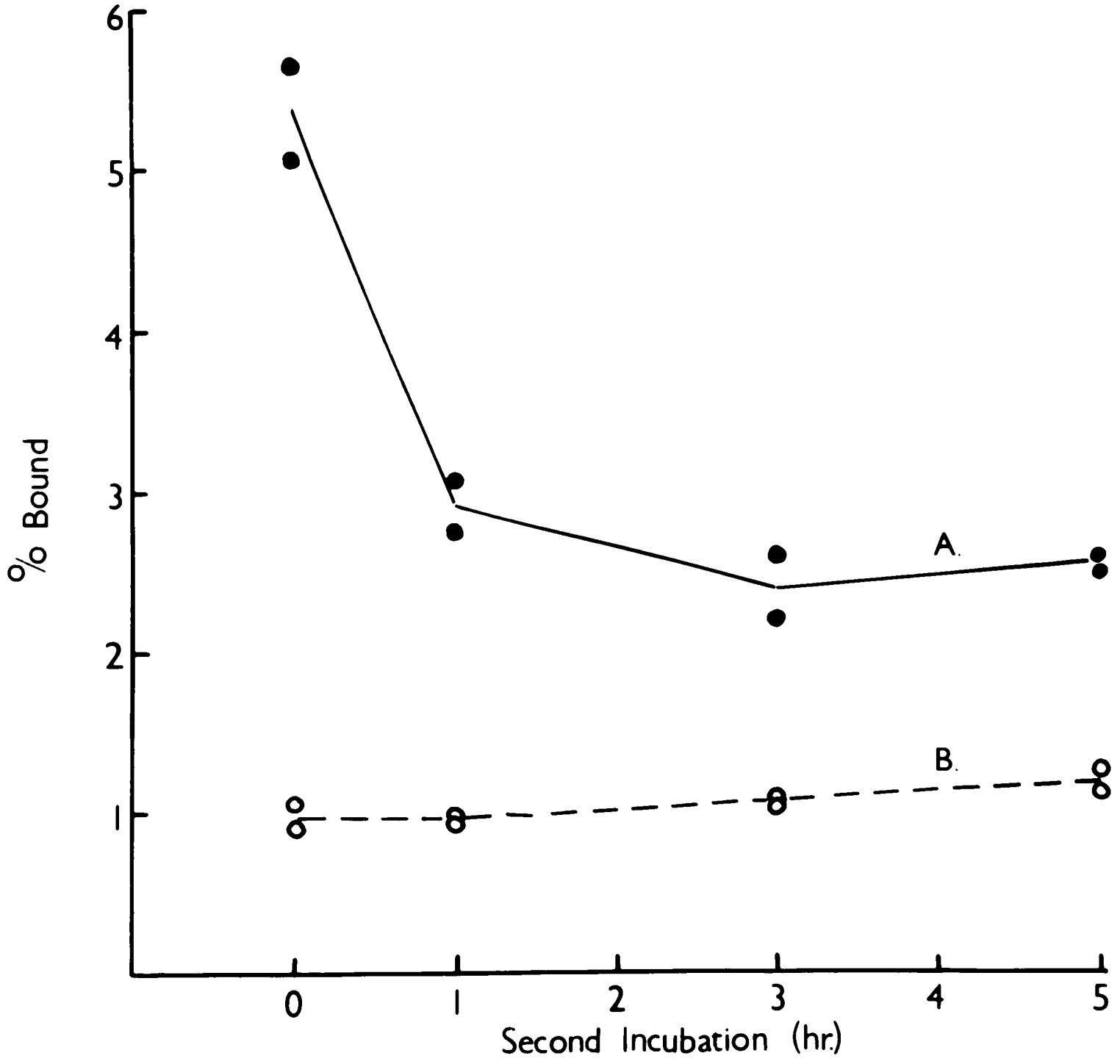
A: No carrier T.S.H.

B: 500mU carrier T.S.H. ('Thytropar')

Second incubation: A, 500mU T.S.H. added in 0.05ml.; B, 0.05ml. P.B.S.-1% B.S.A. added; incubation at 20°C.

A: Test incubation.

B: Control incubation.


% Bound
6
5
4
3
2
1
A.
B.
0
1
2
3
4
5
Second Incubation (hr.)

unlabelled T.S.H. on the generation of cA.M.P. by beef thyroid slices. Therefore this effect may be a feature of the T.S.H.-receptor reaction, per se, at least under certain experimental conditions.

Rodbell et al (1971a) showed that the dissociation of glucagon from rat liver cell membranes was dependent on a supply of G.T.P. It would therefore seem possible that G.T.P. is involved in the dissociation of the T.S.H.-receptor complex especially since it is known to enhance T.S.H. stimulation of adenylcyclase (Manley et al, 1974a; Wolff and Cook, 1973). However, the failure of G.T.P. to affect T.S.H. binding in the experiments of Moore and Wolff (1974) and Manley et al (1974a) is against this suggestion.

5.11. DOES T.S.H.-^{125}I BIND TO OTHER TISSUES?

(expts. 5.17-5.19)

If the phenomenon of T.S.H.-^{125}I binding is evidence of a hormone-receptor interaction, then it should be demonstrable only in those tissues on which thyrotropin acts. Indeed, Manley et al (1972) failed to detect T.S.H. binding activity in slices from a variety of tissues other than thyroid (including kidney, liver, adrenal, testis and salivary gland). Therefore, experiments were designed to test whether T.S.H.-^{125}I binds to homogenates of other tissues.

Method. In the first experiment (expt. 5.17) homogenates of thyroid and liver were prepared as described in section 5.4. In the case of the liver homogenate, the 2000g pellet was difficult to define since it formed a thick slurry at the

bottom of the tube. As much as possible of this was saved and made up to approximately 5g equiv./ml with a small quantity of buffer. The uptake of T.S.H.-^{125}I by each homogenate was determined after an 8 hour incubation at 20^{o}C in the presence or absence of carrier T.S.H. (table 5.5A).

In the second experiment (expt. 5.18) adrenal and kidney homogenates were also included, but to reduce the volume of the pellet obtained after incubation and centrifugation, the concentrations of non-thyroid tissue homogenates were reduced (table 5.5B).

In the third experiment (expt. 5.19) graded doses of carrier T.S.H. were employed and the temperature increased to 22^{o}C (fig. 5.9).

Results. In all three experiments T.S.H.^{125}I bound to tissues other than thyroid (table 5.5, fig. 5.9). In the case of liver and adrenal all experiments showed a partial inhibition of T.S.H.^{125}I uptake by carrier T.S.H. and experiment 5.19 (fig. 5.9) showed that this could be achieved with relatively small doses of carrier T.S.H. Experiment 5.18 showed a slight inhibition by carrier T.S.H. of binding to kidney homogenate but this was not confirmed in experiment 5.19.

Comments. These results are clearly at variance with the previous data of Manley et al (1972) on guinea-pig tissues. Furthermore, Winand and Kohn (1972) found a much smaller uptake of tritiated T.S.H. by cell membranes prepared from guinea-pig liver or muscle than by those from harderian gland or thyroid, although they did not test the effect of carrier T.S.H. in their experiments. There are several possible explanations

TABLE 5.5. The binding of T.S.H. ^{125}I to bovine thyroid, liver adrenal and kidney homogenates

A. Expt. 5.17

Tissue	Conc^{n} g.equiv./ml	% bound(1) No carrier T.S.H.	% bound(1) 500mU Carrier T.S.H.	Saturable binding (B_s)
Thyroid	2.5	6.47 (6.28, 6.67)	2.14 (2.02, 2.26)	4.33
Liver	2.5	6.50 (6.75, 6.24)	3.78 (4.22, 3.34)	2.72

Total volume 1.05ml.; 8 hour incubation at 20°C

(1) Mean (of duplicates)

B. Expt. 5.18

Tissue	Conc^{n} g.equiv./ml	% bound(1) No carrier T.S.H.	% bound(1) 500mU Carrier T.S.H.	Saturable binding (B_s)
Thyroid	2.5	7.31 (7.43, 7.19)	2.13 (2.22, 2.05)	5.18
Liver	0.95	3.49 (3.46, 3.52)	2.25 (2.31, 2.19)	1.24
Adrenal	0.95	4.21 (4.13, 4.30)	3.23 (3.20, 3.26)	0.98
Kidney	0.95	2.95 (3.11, 2.79)	2.59 (2.61, 2.56)	0.36

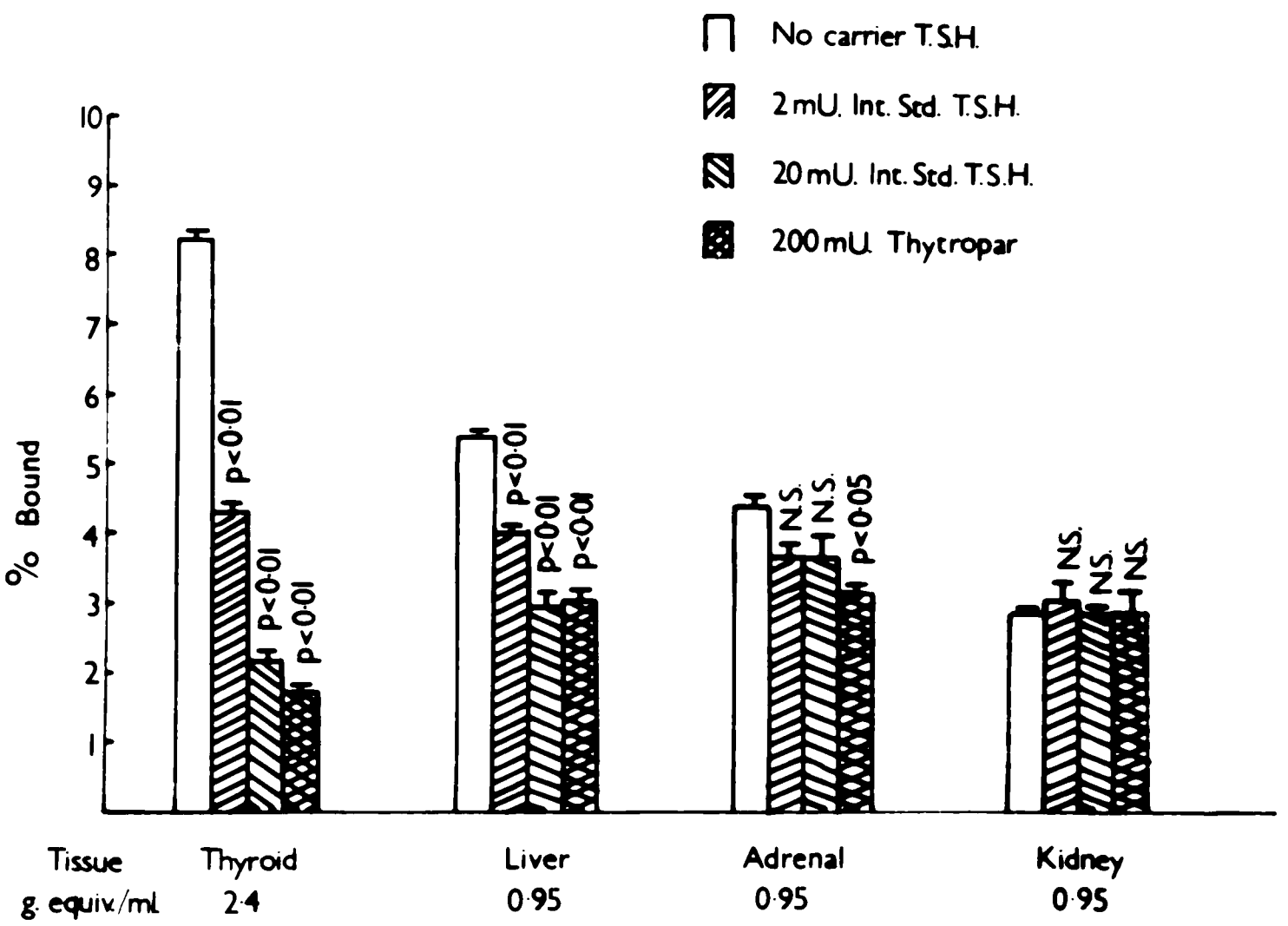
Total volume 1.05ml.; 8 hour incubation at 20°C

(1) Mean (of duplicates)

FIGURE 5.9

The effect of carrier T.S.H. on the uptake of T.S.H.-^{125}I by homogenates of various bovine tissues (expt. 5.19)

Total volume 0.425ml.; 8 hour incubation at 22°C. Each bar represents mean ± S.E.M. of triplicates. p Values represent probability for difference from % bound in absence of carrier T.S.H. by Dunnett's 't' tests (no significant heteroscadasticity).


No carrier T.S.H.
2 mU. Int. Std. T.S.H.
20 mU. Int. Std. T.S.H.
200 mU. Thytropar
% Bound
10
9
8
7
6
5
4
3
2
1
p<0·01
p<0·01
p<0·01
p<0·01
p<0·01
p<0·01
N.S.
N.S.
p<0·05
N.S.
N.S.
N.S.
Tissue
Thyroid
Liver
Adrenal
Kidney
g. equiv./ml.
2·4
0·95
0·95
0·95

for the findings of the present work:

1. The binding to other tissues may be accounted for by the presence of significant numbers of adipocytes, which are known to respond to T.S.H. (Freinkel, 1961; Hart and McKenzie, 1971; Rodbell, 1964; Rudman et al, 1963; White and Engel, 1958), and therefore might be expected to have binding sites for T.S.H. However, very few adipocytes could be found in frozen sections of bovine liver stained with oil red O.

2. The binding may be interpreted as evidence for the existence of T.S.H. receptors in other tissues. These could either be 'redundant' or could mediate some hitherto unknown actions of T.S.H. on the control tissues.

3. The binding sites in liver and adrenal may represent sites at which T.S.H. degradation takes place. However, if this were the case a greater number of sites would be expected in kidney, which is known to take up T.S.H. in vivo (Bakke, 1963; Fontaine, 1970; Ridgeway et al, 1974).

4. The association of T.S.H.-^{125}I with (a) other tissues or (b) all tissues may be artefactual.

On the present evidence a distinction between these possibilities cannot be made.

5.12. THE INTERACTION OF T.S.H.-^{125}I WITH OTHER THYROID CELL FRACTIONS

5.12.1. Microsomal fraction (expt. 5.20)

Method. In one experiment the 2000g supernatant from the preparation of a bovine thyroid homogenate was retained and stored frozen at -20^{o}C for 6 days. After thawing, a

small amount of insoluble material was removed by centrifuging at 2000g for 10 minutes. The supernatant was then centrifuged for 60 minutes at 105,000g[(1)]. The soluble fraction was decanted and stored frozen at -20°C. The pellet was resuspended in P.B.S. to give a concentration of approximately 3.5g equiv./ml and stored overnight at 4°C. 2ml aliquots of this were incubated for 4 hours at 20°C with 2ml P.B.S.-1% B.S.A. and 350,000 c.p.m. T.S.H.-^{125}I in the presence or absence of 2IU of a commercial T.S.H. preparation (Thytropar) in 14ml polycarbonate tubes. The tubes were capped, filled to the top with ice cold P.B.S.-B.S.A. and centrifuged for 60 minutes at 105,000g. The supernatants and pellets were then counted separately in plastic counting vials.

Results. The uptake of T.S.H.-^{125}I by the pellet was 7.9% in the absence of carrier T.S.H. and 2.5% in its presence (table 5.6).

Comments. This shows that the fraction sedimenting between 2000g and 105,000g contained T.S.H. binding activity. The different incubation conditions (made necessary by the use of the high speed centrifuge) make the results difficult to compare with those for the 2000g sediment. However, the binding was of similar magnitude at a similar homogenate concentration. Since these early experiments, Mehdi et al (1973) and Mehdi and Nussey (1975) have shown that T.S.H. labelled[(2)] in the Department of Human Anatomy by method A binds to thyroid fractions sedimenting between 2000g and 25,000g or between 2000g and 105,000g. Furthermore, they have also demonstrated saturable binding to human and bovine

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(1) 40,000 r.p.m. M.S.E. Superspeed 65 with 8 x 14ml angle head, no.59115

(2) By the present author

TABLE 5.6. The uptake of T.S.H. ^{125}I by bovine thyroid microsomal fraction

	% bound
No carrier T.S.H.	7.92 (7.76, 8.07)
2 IU Commercial T.S.H.	2.49 (2.64, 2.33)
Saturable binding	5.43

For conditions see text (section 5.12.1)

thyroid membranes prepared by discontinuous sucrose density gradient centrifugation.

5.12.2. Soluble fraction (expt. 5.21)

The assessment of binding to soluble receptors is complicated by the added difficulty in separating bound label from free. In the first experiments attempts were made to use albumin coated charcoal to absorb free T.S.H.^{125}I. However, these proved unsuccessful since the addition of carrier T.S.H. or thyroid soluble fraction was sufficient to prevent the absorption of free T.S.H.^{125}I by the charcoal. Gel filtration has been used successfully to separate receptor-bound and free insulin (Cuatrecasas, 1972; Gavin et al, 1972) or A.C.T.H. (Lefkowitz et al, 1970). Therefore this technique was also tried.

Method. 0.2ml freshly rechromatographed T.S.H.-^{125}I was incubated with 0.4ml 105,000g supernatant from the previous experiment for 6 hours at 20^{o}C and then overnight at 4^{o}C. 0.2ml of this solution was layered (with added sucrose) on a 50 x 1cm column of Sephadex G-200 and eluted with P.S.A.-0.1% B.S.A. at 4^{o}C.

Results. The label travelled as a single peak corresponding to that obtained with untreated T.S.H.-^{125}I.

Comment. No high molecular weight fraction capable of binding T.S.H. could be detected in the cytosol by this method.

5.13. THE EFFECTS OF GRADED DOSES OF T.S.H. AND OTHER PITUITARY HORMONES ON THE BINDING OF T.S.H.-^{125}I TO BOVINE THYROID HOMOGENATES (expts. 5.22, 5.24, 5.27)

In an attempt to determine the specificity of the inhibition of T.S.H.-^{125}I binding by carrier hormone, the effects of various pituitary hormones were tested. An early experiment had suggested that carrier T.S.H. inhibited the binding of T.S.H.-^{125}I to bovine thyroid homogenates at concentrations in the range 0.33 to 100mU/ml. This was used as a basis to select carrier hormone concentrations for the experiments below.

Methods. Three experiments were performed to compare the ability of the following hormone preparations to inhibit T.S.H.-^{125}I binding:

1. Experiment 5.22: International Standard T.S.H., commercial T.S.H. (Thytropar), bovine L.H. (N.I.H.-L.H.-B8)(1), bovine G.H. (N.I.H.-G.H.-B17)(1) and bovine prolactin (N.I.H.-P.-B3)(1).
2. Experiment 5.24: Int. Std. T.S.H. and 'pure' b-T.S.H. (40IU/mg)(2)
3. Experiment 5.27: Int. Std. T.S.H., 'pure' b-T.S.H.(2), b-L.H.(1), b-G.H.(1) and b-prolactin(1).

Relevant experimental details are presented with figures 5.10-5.12.

Results. Inhibition of binding curves were plotted (figs. 5.10-5.12), giving roughly parallel curves for all pituitary hormones except prolactin. This was the only

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(1) Generously supplied by Dr. L. Reichert, N.A.M.D., Bethesda
(2) Generously supplied by Prof. J. G. Pierce, University of California

FIGURE 5.10

The effect of graded doses of pituitary hormones on the binding of T.S.H.-^{125}I to a bovine thyroid homogenate (expt. 5.22)

Total volume 1.05ml.; 2.5g. equiv. thyroid homogenate; 8 hour incubation at 20°C.
Each point represents the mean of duplicates except where stated.

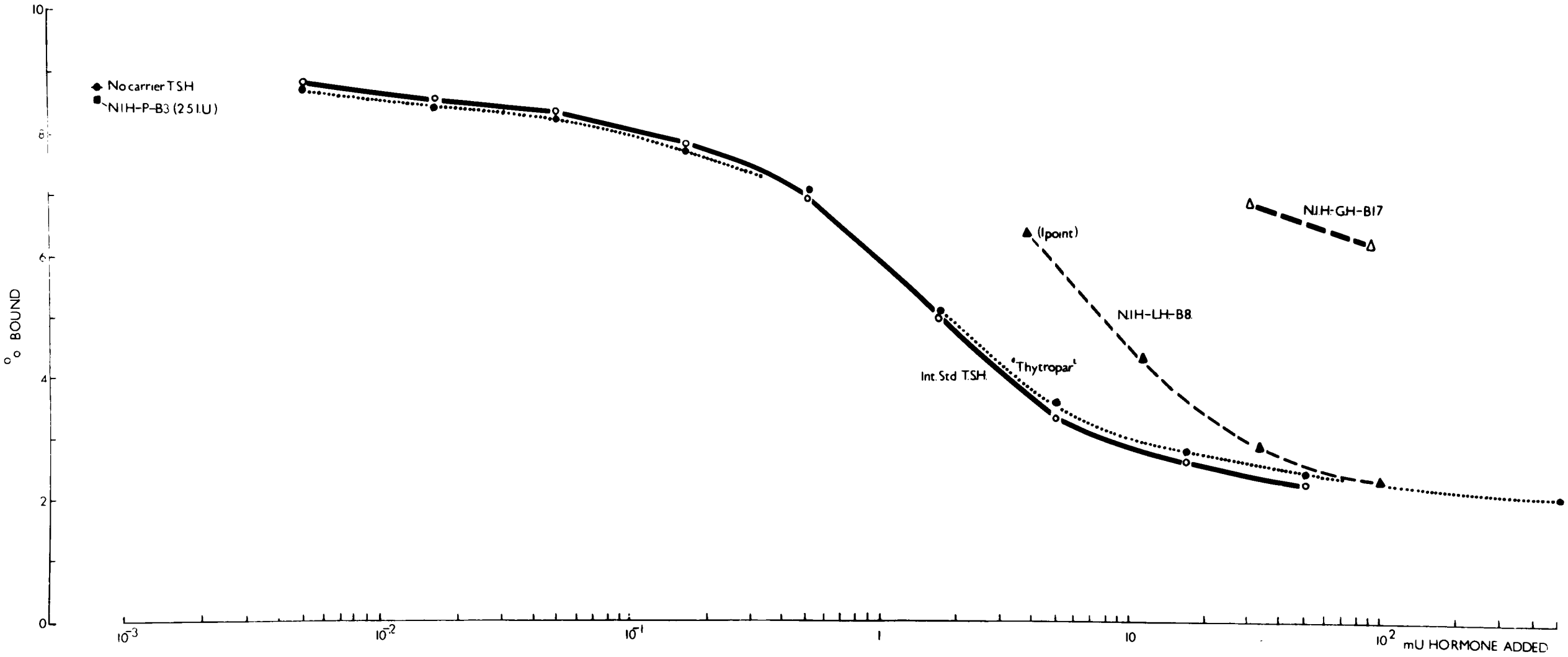

No carrier TSH
NIH-P-B3 (2.5 I.U)
(1point)
NIH-GH-B17
NIH-LH-B8
'Thytropar'
Int. Std T.S.H.
% BOUND
10
8
6
4
2
0
10^{-3}
10^{-2}
10^{-1}
1
10
10^{2}
mU HORMONE ADDED

FIGURE 5.11

The effect of pure T.S.H. on the binding of T.S.H.-^{125}I to a bovine thyroid homogenate (expt. 5.24)

Total volume 1.025ml.; 2.5g. equiv. thyroid homogenate; 8 hour incubation at 20°C.

Each point represents the mean of duplicates except where stated.

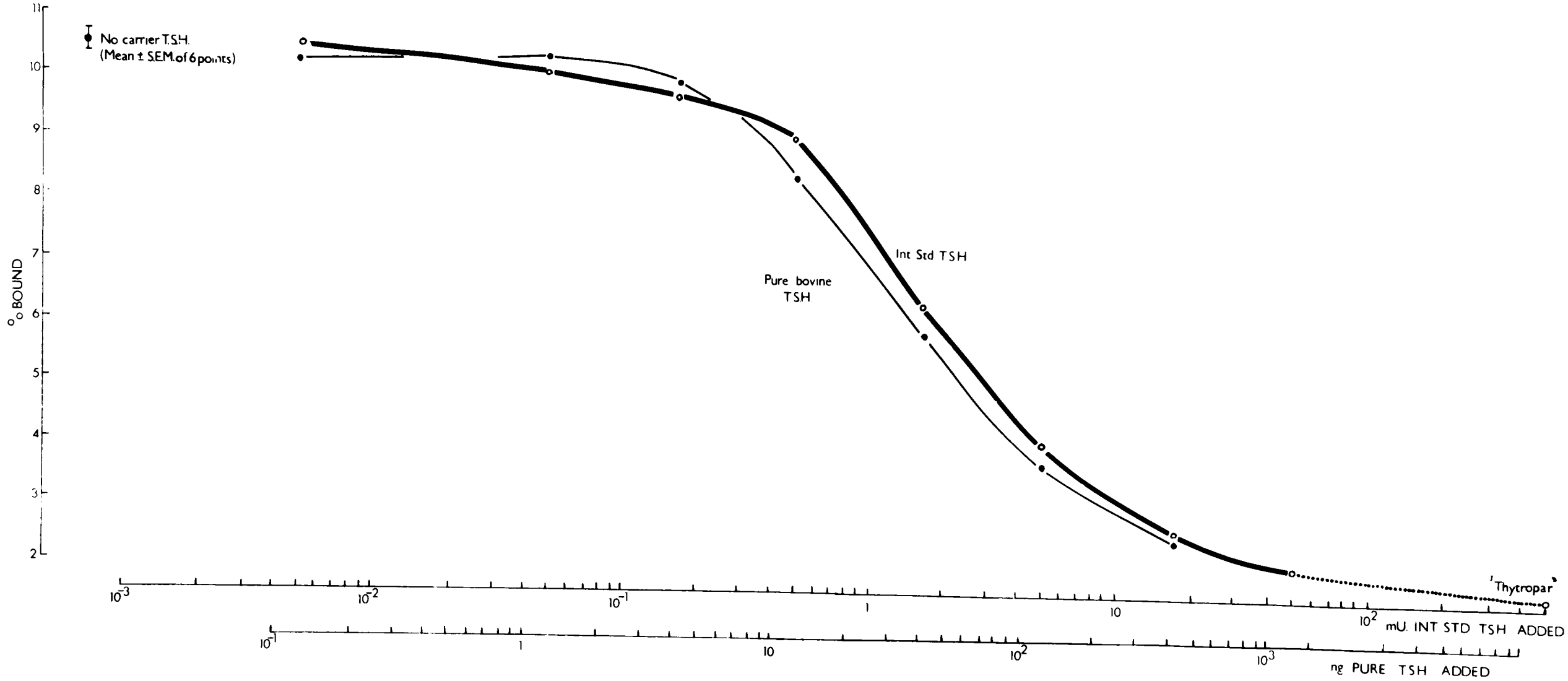
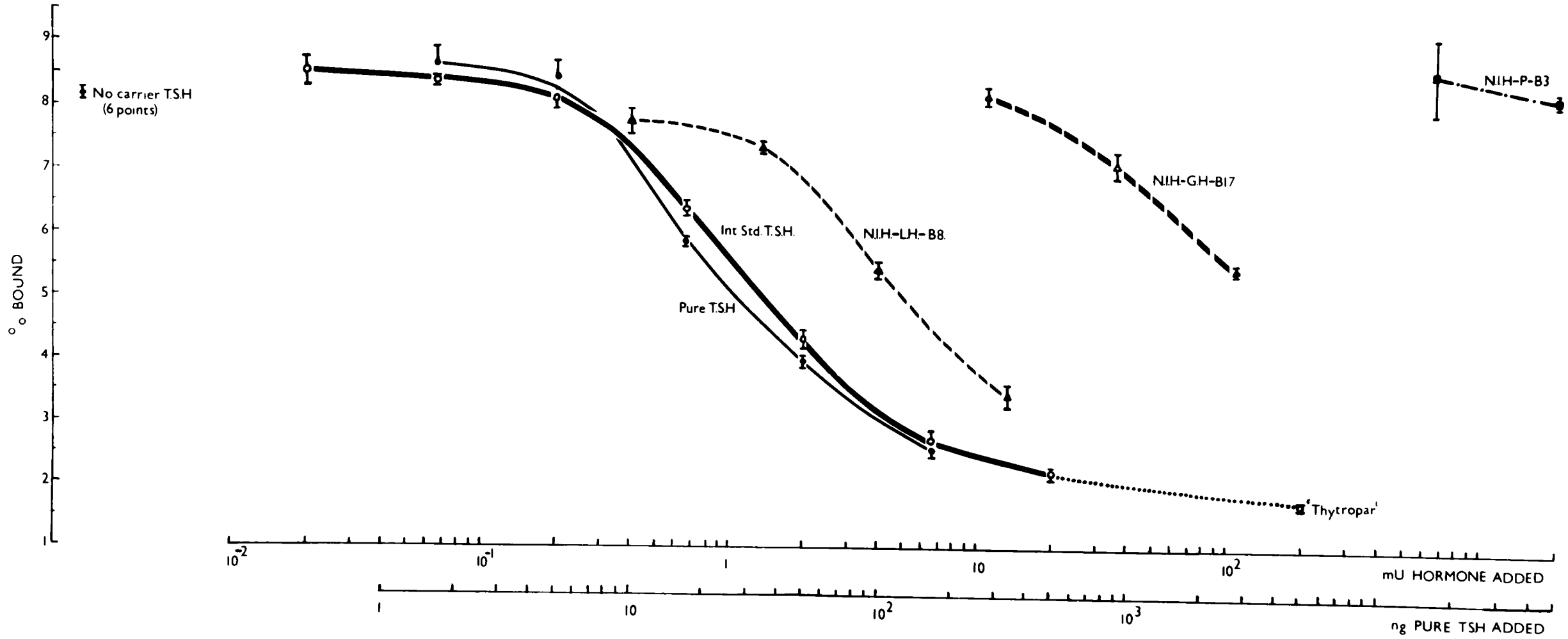

No carrier T.S.H.
(Mean ± S.E.M. of 6 points)
% BOUND
11
10
9
8
7
6
5
4
3
2
Int Std T.S.H
Pure bovine
T.S.H
'Thytropar'
10^{-3}
10^{-2}
10^{-1}
1
10
10^{2}
mU. INT STD TSH ADDED
10^{-1}
1
10
10^{2}
10^{3}
ng PURE TSH ADDED

FIGURE 5.12

The effect of graded doses of pituitary hormones on the binding of T.S.H.-^{125}I to a bovine thyroid homogenate (expt. 5.27)

Total volume 0.425ml.; 1.0g. equiv. thyroid homogenate; 8 hour incubation at 22°C.
Each point represents the mean ± S.E.M. of triplicates except where stated.


No carrier T.S.H
(6 points)
Int Std. T.S.H.
Pure T.S.H
N.I.H.-LH.-B8.
N.I.H.-G.H.-B17
N.I.H.-P-B3
'Thytropar'
% BOUND
9
8
7
6
5
4
3
2
1
10^{-2}
10^{-1}
1
10
10^{2}
mU HORMONE ADDED
1
10
10^{2}
10^{3}
ng PURE TSH ADDED

preparation essentially free of T.S.H. activity (10^{-5}IU T.S.H./IU prolactin). It is notable that 'pure' T.S.H. was as active as Int. Std. T.S.H.

The points giving 20-80% of maximum binding were adjusted for unsaturable uptake (B_c) and replotted as logits ($\ln \frac{B_s}{B_{s(max)}-B_s}$) in figures 5.13-5.15. The data from each of these experiments was subjected to analysis of variance and the potency ratios and their confidence limits were calculated for each hormone preparation in terms of International Standard units for T.S.H. by the method of Bliss (1952), as described in appendix 1. These are presented in table 5.7. The lines were generally parallel within each experiment except for the L.H. line in experiment 5.27, which did diverge from the Int. Std. T.S.H. line. However, this divergence ($p<0.05$) could be eliminated by omitting the low dose of L.H., whose results were somewhat higher than the others in the experiment. Despite this, nonlinearity was insignificant. Heteroscadasticity was undetectable by Bartlett's test.

Comments. Comparison of the calculated potency for each hormone with the activity on bioassay gives a reasonably good fit, although a tendency towards higher values is seen with the present system. A small diminution in biological activity of our standard T.S.H., which had been stored for several years, might explain this result.

These results strongly suggest that the inhibition of binding by pituitary hormone preparations is attributable to their T.S.H. activity.

FIGURE 5.13

The effect of graded doses of pituitary hormones on the binding of T.S.H.-^{125}I to a bovine thyroid homogenate -logit curves (expt. 5.22)

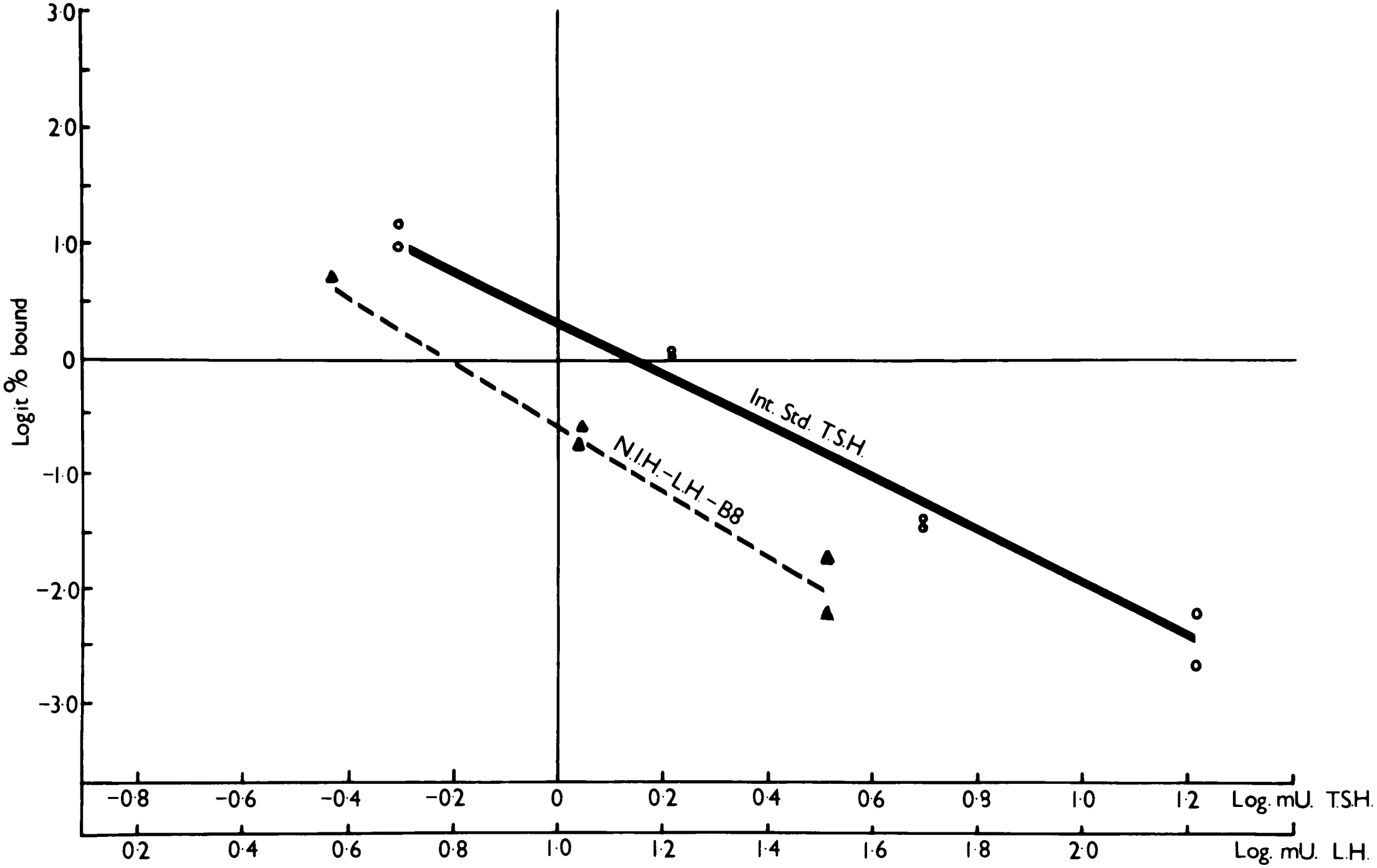

3·0
2·0
1·0
0
-1·0
-2·0
-3·0
Logit % bound
Int. Std. T.S.H.
N.I.H.-L.H.-B8
-0·8
-0·6
-0·4
-0·2
0
0·2
0·4
0·6
0·8
1·0
1·2
Log. mU. T.S.H.
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
1·8
2·0
Log. mU. L.H.

FIGURE 5.14

The effect of pure T.S.H. on the binding of T.S.H.-^{125}I to a bovine thyroid homogenate -logit curves

(expt. 5.24)

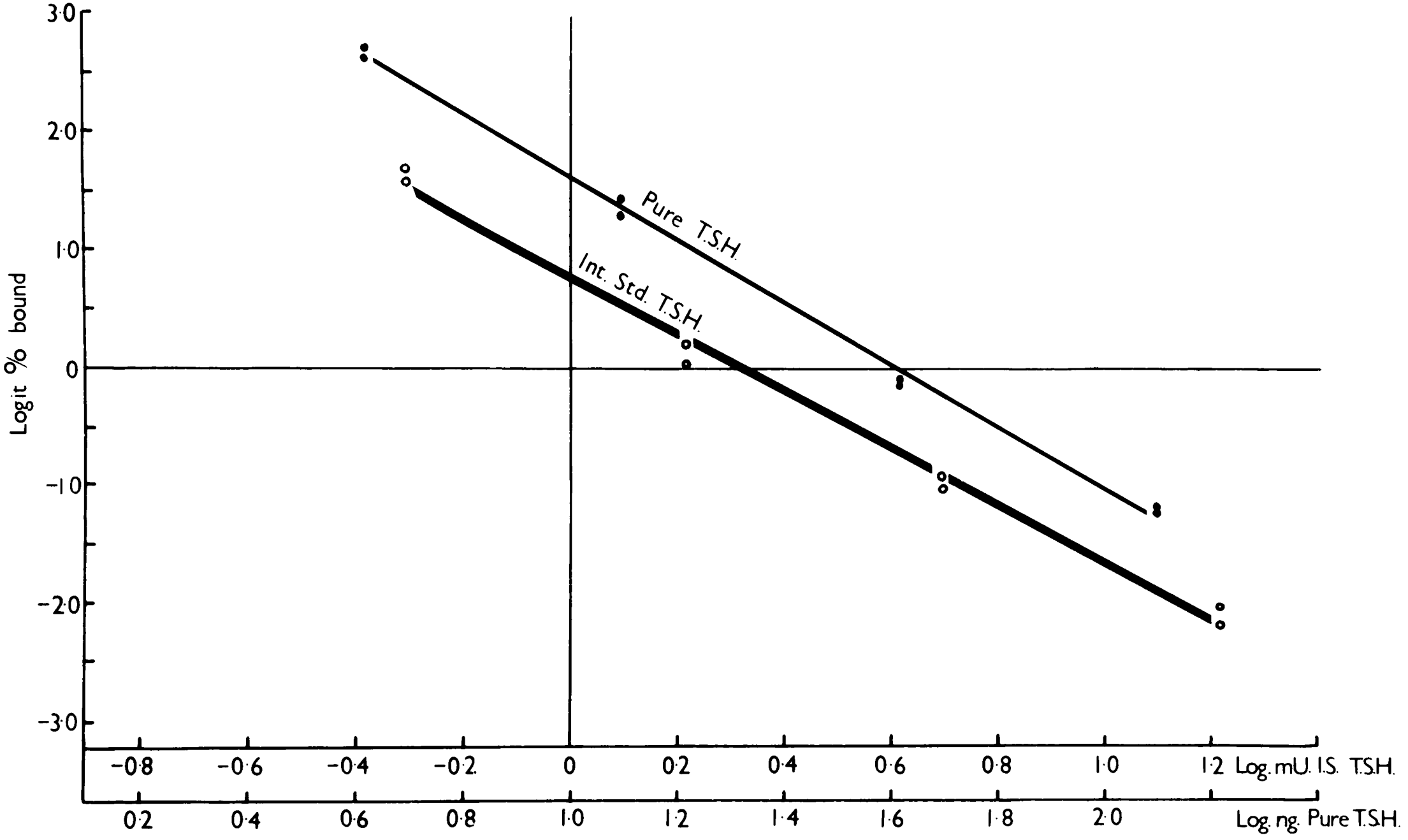

3·0
2·0
1·0
0
-1·0
-2·0
-3·0
Logit % bound
Pure T.S.H.
Int. Std. T.S.H.
-0·8
-0·6
-0·4
-0·2
0
0·2
0·4
0·6
0·8
1·0
1·2
Log. mU. I.S. T.S.H.
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
1·8
2·0
Log. ng. Pure T.S.H.

FIGURE 5.15

The effect of graded doses of pituitary hormones on the binding of T.S.H.-^{125}I to a bovine thyroid homogenate -logit curves (expt. 5.27)

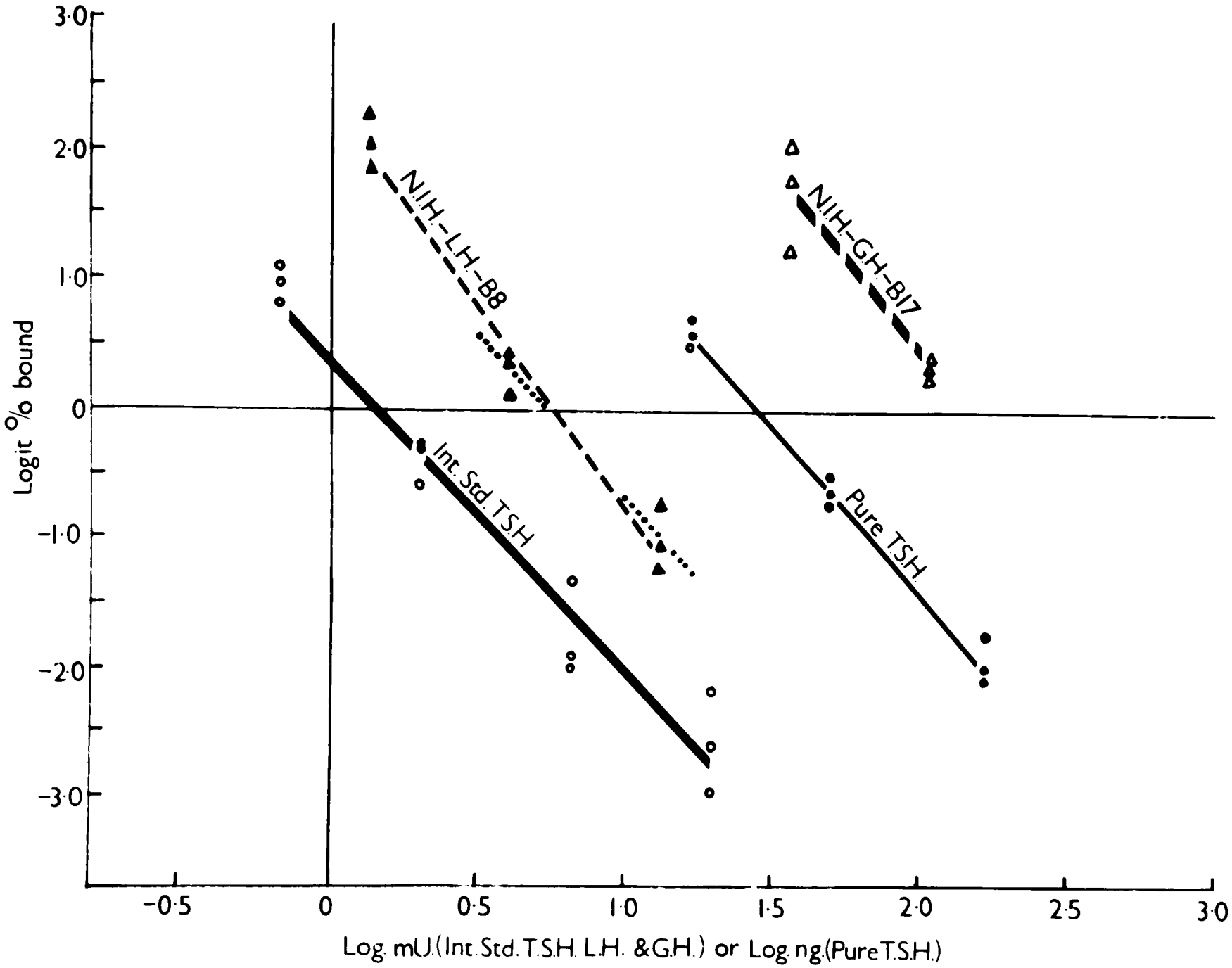

3·0
2·0
1·0
0
-1·0
-2·0
-3·0
Logit % bound
NIH-LH-B8
NIH-GH-B17
Int. Std. TSH
Pure TSH
-0·5
0
0·5
1·0
1·5
2·0
2·5
3·0
Log. mU.(Int. Std. T.S.H. L.H. &G.H.) or Log. ng.(Pure T.S.H.)

TABLE 5.7. T.S.H. potency of pure bovine T.S.H., bovine L.H. (N.I.H.-L.H.-B8) and bovine G.H. (N.I.H.-G.H.-B17) in terms of Int.Std.T.S.H. (see section 5.13 for details)

Expt.	Stimulator	λ	Sig. of slope	Divergence	Potency (95% Conf. limits)	Bioassay potency
5.22	Int.Std.T.S.H.	0.080	p<0.001	-	-	-
(2.2.73)	N.I.H.-L.H.-B8	0.087	p<0.01	N.S.	248 (206-300) mU/IU L.H.	190mU/IU L.H.(1)
5.24	Int.Std.T.S.H.	0.058	p<0.001	-	-	-
(18.5.73)	Pure T.S.H.	0.035	p<0.001	N.S.	50.7(44.6-57.4) IU/mg	40 IU/mg(2)
5.27	Int.Std.T.S.H.	0.124	p<0.001	-	-	-
(5.9.73)	Pure T.S.H.	0.051	p<0.001	N.S.	53.0(42.6-65.7) IU/mg	40 IU/mg(2)
	N.I.H.-L.H.-B8 (3 points)	0.078	p<0.001	p<0.05	-	-
	N.I.H.-L.H.-B8 (2 points)	0.083	p<0.001	N.S.	298 (221-395) mU/IU L.H.	190mU/IU L.H.(1)
	N.I.H.-G.H.-B17	0.086	p<0.001	N.S.	9.46 (6.36-13.29)mU/IU G.H.	<54mU/IU G.H.(1)

(1) As specified by Dr. L. Reichert, N.I.A.M.D. - Assay by chick thyroid p^{32} uptake method of Lamberg (1953) No confidence limits given.

(2) As assayed by Prof. J. G. Pierce (method unspecified). Reassayed by McKenzie method on arrival. Assay (i) 38.5 (19.1-77.3) IU/mg. (ii) 44.5 (28.9-71.7) IU/mg

5.14. ESTIMATES OF THE ASSOCIATION CONSTANT (K_A)

(expts. 5.22-5.27)

Method. The data of section 5.13, together with the results of three other experiments, were used to assess the association constant (K_A) of the T.S.H.-receptor interaction by the method of Scatchard (1949). This is based on the law of mass action and plots bound/free (B/F) against bound (B). For a second order reaction the graph should be a straight line whose slope is K_A. In the present experiments bound hormone was adjusted for unsaturable binding so that B/F was calculated as $^{B}s/100-B_s$ (where B_s = saturable binding) and B was estimated as B_s x added T.S.H. The slope of the graph was estimated by the method of least squares, omitting any obviously deviant values. K_A was calculated from the slope on the assumptions that the molecular weight of b-T.S.H. is 28,500 (Pierce, 1971) and that the activity of pure b-T.S.H. is 40IU/mg.

Results. All plots were approximately linear apart from a reduction in slope("tail-off") at high T.S.H. concentrations in three cases. Two plots are illustrated in figure 5.16. The lower one (B) demonstrates the "tail-off". The slopes and K_A values are presented in table 5.8 and ranged from 0.41 to 0.79ml/mU and 4.7 to 9.0 x 10^8 M^{-1}, respectively.

Comments. These values for K_A compare surprisingly well with most other estimations (table 5.9), considering the diverse conditions employed. Indeed Moore and Wolff (1974) have since found that changing their standard conditions for T.S.H. binding to those of their adenyl cyclase assay resulted

FIGURE 5.16

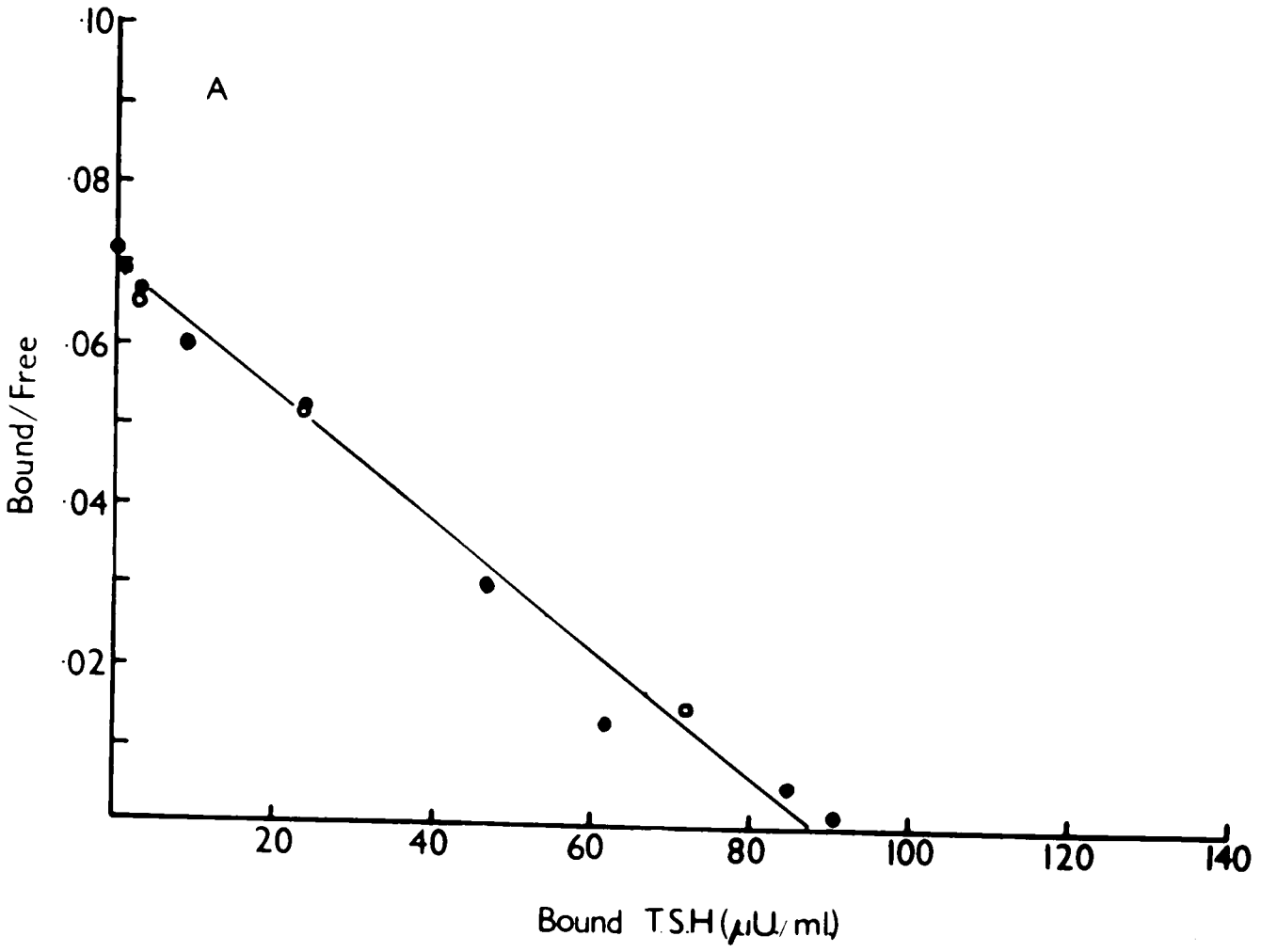
Scatchard plots for the binding of bovine T.S.H. to bovine thyroid homogenates

A. Experiment 5.22: Slope 0.788ml/mU; K_A 8.98 x 10^8 M^{-1}.

B. Experiment 5.25: Slope 0.534ml/mU; K_A 6.06 x 10^8 M^{-1}. "Tail-off" marked.

Closed circles: Int. Std. T.S.H.; open circles: 'Thytropar'.

Int. Std. points only included in calculation of K_A (by method of least squares).


A
·10
·08
·06
·04
·02
Bound/Free
20
40
60
80
100
120
140
Bound T.S.H (μU/ml)

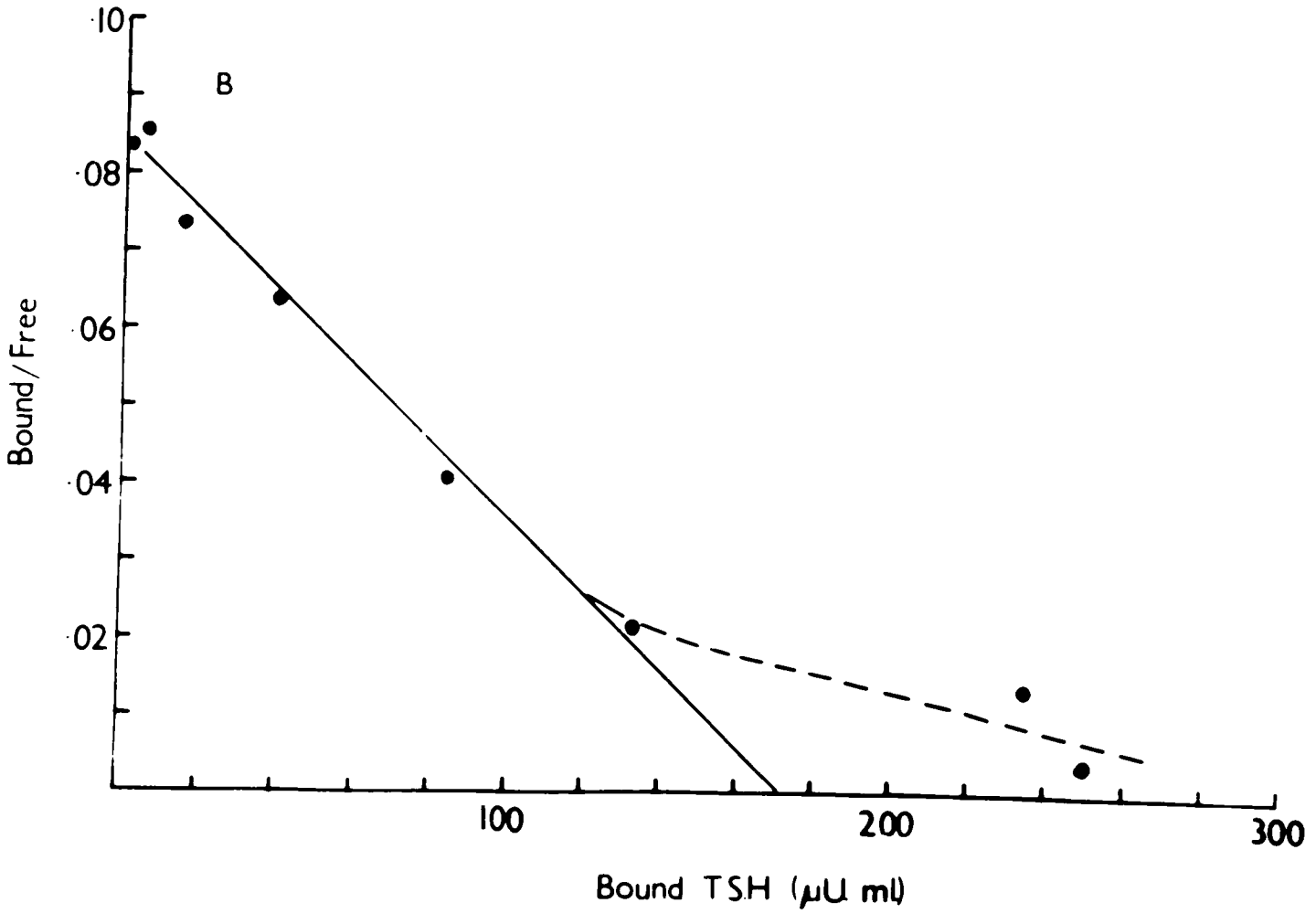

B
·10
·08
·06
·04
·02
Bound/Free
100
200
300
Bound TSH (μU ml)

TABLE 5.8. Estimation of the association constant (K_A) for the T.S.H.-bovine thyroid receptor reaction

Experiment[1]	temp °C	r[2]	Slope (ml.mU)	$K_A(M^{-1})$	"Tail-off"
5.22 (2.2.73)	20	0.993	0.788	8.98×10^8	-
5.23 (31.3.73)	20	0.992	0.523	5.96×10^8	+
5.24 (18.5.73)	20	0.997	0.595	6.64×10^8	-
5.25[3] (22.6.73)	22	0.992	0.534	6.06×10^8	+ +
5.26[3] (28.6.73)	22	0.999	0.507	5.78×10^8	+ +
5.27 (5.9.73)	22	0.986	0.412	4.70×10^8	-

(1) 8 hour incubation

(2) r = correlation coefficient for points used in estimation of K_A

(3) Homogenates washed before use to remove microsomal contaminants and centrifugation prolonged to 60 min. (expts. 5.25 and 5.26 only)

TABLE 5.9. Comparison of K_A values for T.S.H.-receptor interactions obtained by various investigators

Authors	Method	K_A (M^{-1})
Gibbons	b-T.S.H.-^{125}I binding to bovine thyroid homogenates at 20-22°C	4.7-9.0 x 10^8
Pastan et al (1966)	Persistent effects of b-T.S.H. on canine thyroid slice G-1-^{14}C oxidation at 1°C	3 x 10^8
Manley et al (1972)	b-T.S.H.-^{125}I binding to hyperplastic guinea pig thyroid slices at 25°C (2 orders of binding sites)	(1) 3.8 x 10^8 (2) 8 x 10^3
Lissitsky et al (1973)	p-T.S.H.-^{125}I binding to porcine thyroid cells at 35°C	1 x 10^9
Verrier et al (1974)	p-T.S.H.-^{125}I binding to: (1) porcine thyroid cells at 35°C (2) porcine thyroid cell membranes at 27°C	 1.9 x 10^9 5.8 x 10^8
Manley et al (1974a)	b-T.S.H.-^{125}I binding to 10,000g particulate fractions of: (1) guinea pig thyroid at 37°C (2) murine thyroid at 37°C (3) bovine thyroid at 37°C (4) ovine thyroid at 37°C	 1.8 x 10^9 0.8 x 10^9 1.0 x 10^9 0.2 x 10^9
Smith and Hall (1974b)	b-T.S.H.-^{125}I and guinea pig thyroid membranes at 37°C	2.6 x 10^{10}
Moore and Wolff (1974)	b-T.S.H.-^{125}I and b-T.S.H.-^{3}H and bovine thyroid membranes (1) at 0°C (1st order sites) (2) at 23°C (2nd order sites)	 (1) 2.2-3.1 x 10^8 (2) 0.7-1.1 x 10^6
Bashford et al (1975)	b-T.S.H.-^{125}I and human thyroid membranes at 25°C or 37°C	3 x 10^9 - 10^{10}
Tate et al (1975)	b-T.S.H.-^{125}I and bovine thyroid membranes at 37°C	2.5 x 10^9

in a 10-20 fold reduction in K_A. However, the figures of the present experiments are lower than those obtained for the L.H. of h-C.G.-receptor systems upon which the present system was modelled: 3.3×10^9 (Leidenberger and Reichert, 1972a) and 1.5×10^{10} M^{-1} (Tsuruhara et al, 1972).

The "tail-off" at high T.S.H. concentrations is reminiscent of the results of Manley et al (1972) who concluded that a second population of binding sites existed. There appears to be some disagreement in subsequent literature over the existence of low affinity binding sites, evident in some reports (Bashford et al, 1975; Field, 1975; Moore and Wolff, 1974) but not in others (Lissitzky et al, 1973; Manley et al, 1974a; Smith and Hall, 1974b; Verrier et al, 1974). In the present experiments "tail-off" was greatest in those cases in which resuspension of the pellet formed on centrifugation was more difficult. As previously mentioned, this phenomenon seemed to be associated with deterioration of the homogenate. Therefore low affinity binding sites could arise by enzymic degradation or spontaneous alteration of high affinity receptors.

An alternative explanation of the "tail-off" is that the intrinsic assumptions of the Scatchard plot were not satisfied:

1. The reaction must be of the second order (i.e. A + B = AB). It would seem possible that the system was more complex than originally envisaged. For example, cofactors may be required for the binding or release of the hormone or, as suggested by Tate et al (1975), there may be negative cooperativity between receptors at high occupancy.

2. The reaction must be reversible. Evidence presented earlier (section 5.10) showed that the binding of T.S.H.-^{125}I was not completely reversible.

3. The system must be at equilibrium. Since the uptake of T.S.H.-^{125}I was biphasic (section 5.6), the maximum binding did not necessarily reflect an equilibrium between bound and free, but may merely have represented the point at which the rate of T.S.H.-receptor attachment equalled the rate at which receptors are inactivated.

4. The label employed must be a perfect tracer. Clearly, this was not so here since iodination reduced the biological activity of the T.S.H. (section 2.3). It is likely that this was due, at least in part, to a reduced affinity for the receptors of some or all of the T.S.H. molecules. However, since the percentage bound was low, the effect on the slope of the Scatchard plot would have been small because the estimated B/F and B values would have been reduced roughly in proportion.

5.15. THE EFFECT OF WASHING AND PROLONGATION OF CENTRIFUGATION ON T.S.H.-^{125}I BINDING BY BOVINE THYROID HOMOGENATES

Incompletely sedimented receptors could provide a source of inaccuracy and reduce observed binding. These could be: (a) contaminating soluble or microsomal receptors, (b) incompletely sedimented 2000g fraction receptors, or (c) receptors solubilised during incubation. No direct attempt was made to assess the contribution of soluble receptors. However, indirect evidence that these did not present a serious problem comes from experiment 5.21 (section 5.12.2)

in which T.S.H.-^{125}I ran essentially as a single peak on Sephadex G-200 chromatography after incubation in the presence of thyroid cytosol. Therefore, attempts were made to assess the contribution of other sources of incompletely sedimented receptors.

An effect equivalent to that of unsedimented receptors could be produced by elution of T.S.H.-^{125}I from the homogenate during washing. This factor was also investigated.

5.15.1. The effect of washing the homogenate before use

(expt. 5.28)

Method. A fresh bovine thyroid homogenate was divided into two portions, one of which was washed by suspending in 10 volumes of ice cold P.B.S., centrifuging at 2000g for 25 minutes at 4^{o}C and resuspending in the original volume of ice cold P.B.S. The uptake of T.S.H.-^{125}I by each homogenate was determined under the usual conditions in the presence of absence of carrier T.S.H.

Results. Table 5.10 shows that the uptake was unchanged when a washed homogenate was used.

Comments. No advantage was gained by the extra step of washing the homogenate. However, in the absence of knowledge of the efficiency of centrifugation this gives little information about the degree of microsomal or cytosol contamination of the unwashed homogenates.

5.15.2. The effect of prolongation of centrifugation

(expts. 5.29-5.30)

Method. In two experiments homogenates were incubated

TABLE 5.10. Comparison of T.S.H.-^{125}I uptake by washed and unwashed homogenates (expt. 5.28)

Homogenate	% bound (1)	
	No carrier T.S.H.	200mU Carrier T.S.H.
Unwashed	11.65 ± 0.40	1.11 ± 0.05
Washed	11.98 ± 0.38 N.S.(2)	1.11 ± 0.005 N.S.(2)

Total volume 0.425ml.; 1.0g. equiv. thyroid homogenate; 8 hour incubation at 22°C

(1) Mean ± S.E.M. of triplicates

(2) Student's 't' test

with P.B.S.-1% B.S.A. in the presence or absence of carrier T.S.H. and, in the first experiment, also in the absence of B.S.A. The tubes were then centrifuged either in the usual way (2 x 30 min, 2300g) or for a longer period (2 x 60 min, 2300g).

Results. Table 5.11 shows that in both experiments a slight increase in sedimentation of bound label was achieved with longer centrifugation, although statistical significance was not reached. The effect appeared to be linked to the addition of B.S.A. to the medium since no change was observed when B.S.A. was omitted (expt. 5.29).

Comments. Despite the slight increase in sedimentation of label, the more prolonged centrifugation was not adopted as a standard technique since the increase in binding was small and the pellets became difficult to resuspend after the first centrifugation, increasing the variability of results. Furthermore, in two experiments washed homogenates and prolonged centrifugation were used (expts. 5.25, 5.26); the K_A values were similar to the others in the series but the "tail-off" was marked in both cases (table 5.8).

5.15.3. Estimation of the loss of label on washing the pellet (expts. 5.10, 5.31, 5.32)

Method. In three experiments the tubes were counted following aspiration of the supernatant after the first and again after the second centrifugation.

Results. The losses of saturable binding (B_s) of label

TABLE 5.11. The effect of prolonging centrifugation time on T.S.H.-^{125}I binding to bovine thyroid homogenate

A. Expt. 5.29

Centrifugation (2300g)	% bound (1) P.B.S.	P.B.S.-B.S.A. (2)	500mU Carrier TSH
2 x 30 min	9.10(9.19,9.02)	10.86(10.71,11.01)	2.13(2.24,2.01)
2 x 60 min	9.09(9.30,8.87)	11.54(11.81,11.27)	2.12(2.06,2.17)

Total volume 1.05ml.; 2.5g. equiv. bovine thyroid homogenate; 8 hour incubation at 22°C

(1) Mean of duplicates
(2) Final B.S.A. concentration 5.2g/l

B. Expt. 5.30

Centrifugation (2300g)	% bound (1) P.B.S.-B.S.A. (2)	200mU Carrier T.S.H.
2 x 30 min	8.26 ± 0.10	1.72 ± 0.05
2 x 60 min	9.38 ± 0.49 N.S. (3)	1.65 ± 0.08 N.S. (3)

Total volume 0.425ml.; 1.0g. equiv. bovine thyroid homogenate; 8 hour incubation at 22°C

(1) Mean ± S.E.M. of triplicates
(2) Final B.S.A. concentration 5.3g/l
(3) Student's 't' test

on washing were 11% (expt. 5.10), 12% (expt. 5.31) and 34% (expt. 5.32)(table 5.12).

Comments. The large loss of label in the last experiment may be related, at least in part, to the larger relative volume used to wash the pellet and to the longer time spent in resuspension of the pellet, since this was done in conjunction with another experiment with a larger number of tubes (expt. 5.27). In order to minimise losses, ice cold buffer was used for washing, the tubes being kept on ice, and mixing was performed as rapidly as possible immediately before centrifuging. Despite these precautions, some elution of label from binding sites was inevitable.

5.16. THE UPTAKE OF T.S.H. ^{125}I BY HUMAN THYROID HOMOGENATES (expts. 5.33-5.35)

Method. Because of difficulties in obtaining fresh tissue pathological human thyroids, taken at thyroidectomy and stored frozen at -10 to -20°C, were used(1). Homogenates were prepared as for bovine tissue but were always used within 12 hours of thawing the stored tissue. Incubations were performed as before using a total volume of approximately 1ml per tube, but a lower concentration of homogenate was found to be sufficient (about 1g equiv./ml).

During the incubation the homogenate tended to gel. Anticoagulants (10mM E.D.T.A. or heparin) failed to prevent this change, which was analgous to the gelling observed with frozen bovine thyroid homogenates. The gel made washing difficult and this was therefore omitted in most experiments.

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(1) Kindly supplied by Prof. I. Doniach, The London Hospital, and Dr. J. Harris, Stoke Mandeville Hospital

TABLE 5.12. Estimation of the loss of receptor bound label on washing

Expt.	Incubation temp. (°C)	Total volume (ml)	Carrier T.S.H. (mU)	% bound(1) Before wash	% bound(1) After wash(2)	% loss of binding
5.10	20	1.05	-	28.0 (28.4, 27.5)	19.4 (19.8, 18.9)	11%
			500	7.7 (7.4, 8.0)	1.4 (1.5, 1.3)	
5.31	22	1.05	-	15.4 (15.6, 15.1)	7.3 (7.5, 7.0)	12%
			500	8.5 (8.1, 8.9)	1.2 (1.4, 1.0)	
5.32	22	0.425	-	21.26 ± 0.98	8.26 ± 0.11	34%
			200	11.45 ± 0.08	1.72 ± 0.07	

(1) Mean of duplicates (expts. 5.10, 5.31) or mean ± S.E.M. of triplicates (expt. 5.32)

(2) All washes performed with 2.0ml ice cold P.B.S.-1% B.S.A. per tube.
8 hour incubations.
Bovine thyroid homogenate concentrations: 2.5g. equiv. (expts. 5.10, 5.31) or 1.0g. equiv. (expt. 5.32).

In any case, the improved binding of T.S.H.-^{125}I to human homogenates reduced the necessity for this. During these experiments it was found that the addition of B.S.A. reduced binding to human thyroid homogenates (in contrast to the increased binding seen with bovine homogenates). Therefore in later experiments B.S.A. was kept to a minimum, being present at a concentration of 0.05g/1 in the final incubation mixture.

Results. 10-30% binding was achieved in most experiments. In three experiments the effects of different doses of carrier T.S.H. were tested. The inhibition of binding curve for one of them is shown in figure 5.17. Scatchard plots were also performed (fig. 5.18) and the K_A was calculated at 4.5×10^8 to 1.2×10^9 (table 5.13). "Tail-off" was particularly marked in these experiments, rendering the K_A determination somewhat imprecise.

Comments. These results were in many ways similar to those obtained with bovine tissue although the receptor concentration appeared to be higher. It seems likely that the nonlinearity of the Scatchard plots and the clotting of the homogenate were attributable to the use of frozen tissue. Unfortunately, fresh tissue was not available to test this.

5.17. THE EFFECTS OF L.A.T.S. ON T.S.H.-^{125}I BINDING TO THYROID HOMOGENATES

It has been proposed that L.A.T.S. exerts its effects on the thyroid by acting on T.S.H. receptors (Burke, 1971). If this is so then L.A.T.S. might be expected to compete with T.S.H.-^{125}I for binding sites on thyroid homogenates.

FIGURE 5.17

The effect of graded doses of b-T.S.H. (Int.Std.) on the uptake of T.S.H.-^{125}I by a human thyroid homogenate (expt. 5.33)

Total volume 1.05ml.; 1.0g. equiv. human thyroid homogenate (S.K. thyrotoxic); B.S.A. concentration 5.25mg/ml; 8 hour incubation at 20^{o}C.

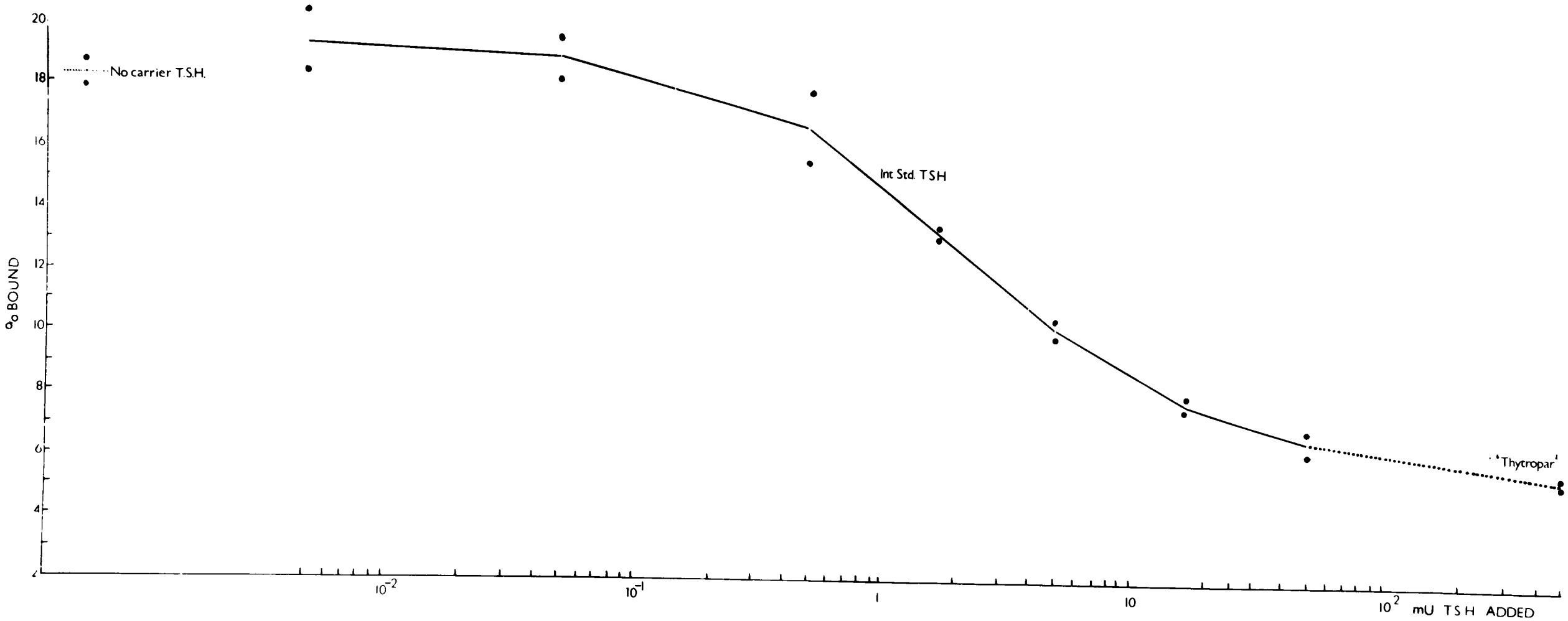
20
18
16
14
12
10
8
6
4
% BOUND
No carrier T.S.H.
Int Std. TSH
'Thytropar'
10^{-2}
10^{-1}
1
10
10^{2} mU TSH ADDED

FIGURE 5.18

Scatchard plot for the binding of b-T.S.H. to a human thyroid homogenate (expt. 5.33)

Slope of line 0.425ml/mU; K_A 4.85 x 10^8 M^{-1}.

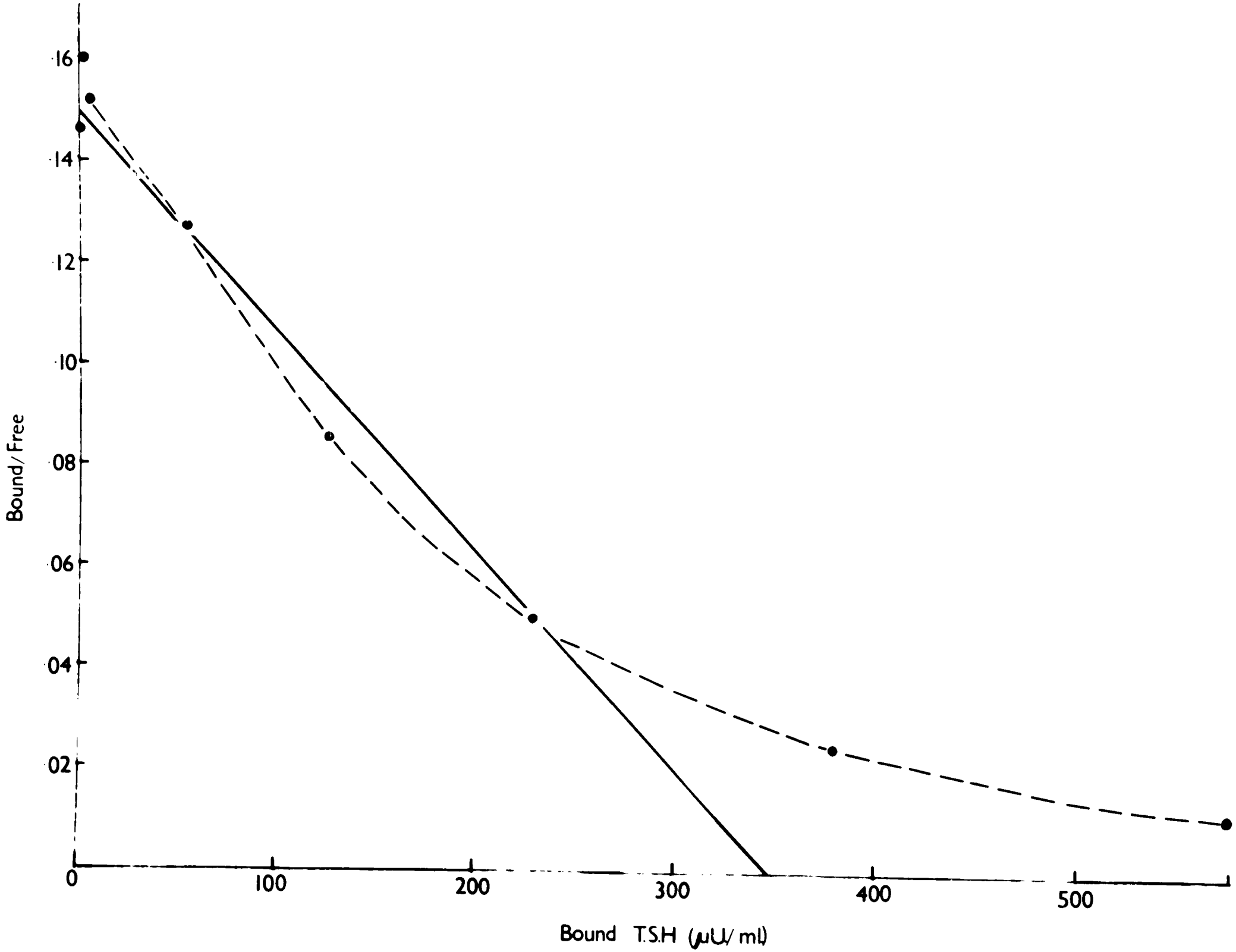
·16
·14
·12
·10
·08
·06
·04
·02
0
100
200
300
400
500
Bound/Free
Bound T.S.H (μU/ml)

TABLE 5.13. Estimation of the association constant (K_A) for the interaction between b-T.S.H. and human thyroid homogenates

Expt.	Temp (°C)	r	Slope (ml/mU)	K_A (1/M)	"Tail-off"
5.33 (8.2.73)	20	0.989	0.425	4.85×10^8	+ + +
5.34 (28.3.73)	20	0.960	1.08	12.3×10^8	+ + +
5.35 (16.6.73)	22	0.950	0.393	4.48×10^8	+

All thyroids taken at operation from thyrotoxic patients

B.S.A. concentrations: expt.5.33, 5.25mg/ml

expt.5.34, 0.155mg/ml

expt.5.35, o.125mg/ml

5.17.1. Preliminary experiments (expts. 5.36-5.37)

Methods. IgG was prepared as described previously (section 2.4) from a high L.A.T.S. serum taken from a thyrotoxic patient (R.N.1) and a L.A.T.S.-negative plasma (N) obtained from the blood transfusion service(1) (appendix 4).

In the first experiment (expt. 5.36) triplicated tubes were made up to a total volume of 0.70ml containing 1.0g equiv. human thyroid homogenate (prepared from the thyroid of a patient with thyrotoxicosis) and 2.6mg L.A.T.S. IgG, 2.6mg NIgG, 2.6mg B.S.A. or 2.6mg B.S.A. plus 500mU carrier T.S.H. 30,000 c.p.m. T.S.H.-^{125}I were added (in 50µl P.B.S.-1% B.S.A.) after an initial one hour preincubation and the tubes were incubated for a further 7 hours at 20°C.

In the second experiment (expt. 5.37) extra controls were used. Tubes were made up in triplicate to a total volume of 0.75ml, containing 1.0g equiv. of human thyroid homogenate (T.S., thyrotoxic) and 2.6mg B.S.A., L.A.T.S. IgG or N.IgG or no protein, in the presence or absence of 500mU carrier T.S.H. After one hour, 30,000 c.p.m. T.S.H.-^{125}I in 50µl P.B.S.-1% B.S.A. were added and the tubes were incubated for a further 7 hours at 20°C.

In both experiments 'gelling' occurred, but despite this, the pellets were washed as before.

Results. Table 5.14 shows the results of both experiments in terms of percent bound before and after washing. The results of experiment 5.37 are also illustrated in figure 5.19. In both experiments L.A.T.S. IgG significantly reduced the

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(1) Radcliffe Infirmary, Oxford

TABLE 5.14. The effect of L.A.T.S. on T.S.H.-^{125}I binding to human thyroid homogenates

A. Expt. 5.36

Protein added (2.6mg)	% bound(1) Before washing	After washing
B.S.A.	19.47 ± 0.07	10.50 ± 0.61
B.S.A. - 500mU T.S.H.	7.43 ± 0.12	1.93 ± 0.13
N IgG (L.A.T.S. -ve)	26.37 ± 0.45	14.74 ± 0.67
R.N.1 IgG (L.A.T.S. + ve)	16.93 ± 0.15	8.27 ± 0.50(2)

Total volume 0.70ml.; 1.0g. equiv. human thyroid homogenate (S.K., thyrotoxic); 1 hour preincubation in absence of label, 7 hour subsequent incubation with T.S.H.-^{125}I at 20°C.

(1) Mean ± S.E.M. of triplicates

(2) p for difference between N.IgG and R.N.1 IgG <0.001 (Student's 't' test)

/Cont'd.

TABLE 5.14. Cont'd.

B. Expt. 5.37

Protein added (2.6mg)	Carrier T.S.H. (mU)	% bound(1) Before washing	% bound(1) After washing	p(2)
Nil	-	21.0 ± 0.21	9.67 ± 0.06	-
B.S.A.	-	18.5 ± 0.51	7.74 ± 0.11	<0.01
B.S.A.	500	10.1 ± 0.23	1.00 ± 0.04	-
N. IgG (L.A.T.S.-ve)	-	21.6 ± 0.20	9.30 ± 0.11	N.S.
N. IgG (L.A.T.S.-ve)	500	9.97 ± 0.09	0.98 ± 0.01	-
R.N.1 IgG (L.A.T.S.+ve)	-	14.1 ± 0.15	3.78 ± 0.12	<0.01
R.N.1 IgG (L.A.T.S.+ve)	500	9.80 ± 0.23	1.05 ± 0.07	-

Total volume 0.75ml.; 1.0g. equiv. human thyroid homogenate (T.S., thyrotoxic); 1 hour preincubation in absence of label, 7 hour subsequent incubation with T.S.H.-^{125}I at 20°C.

(1) S.E.M. of triplicates

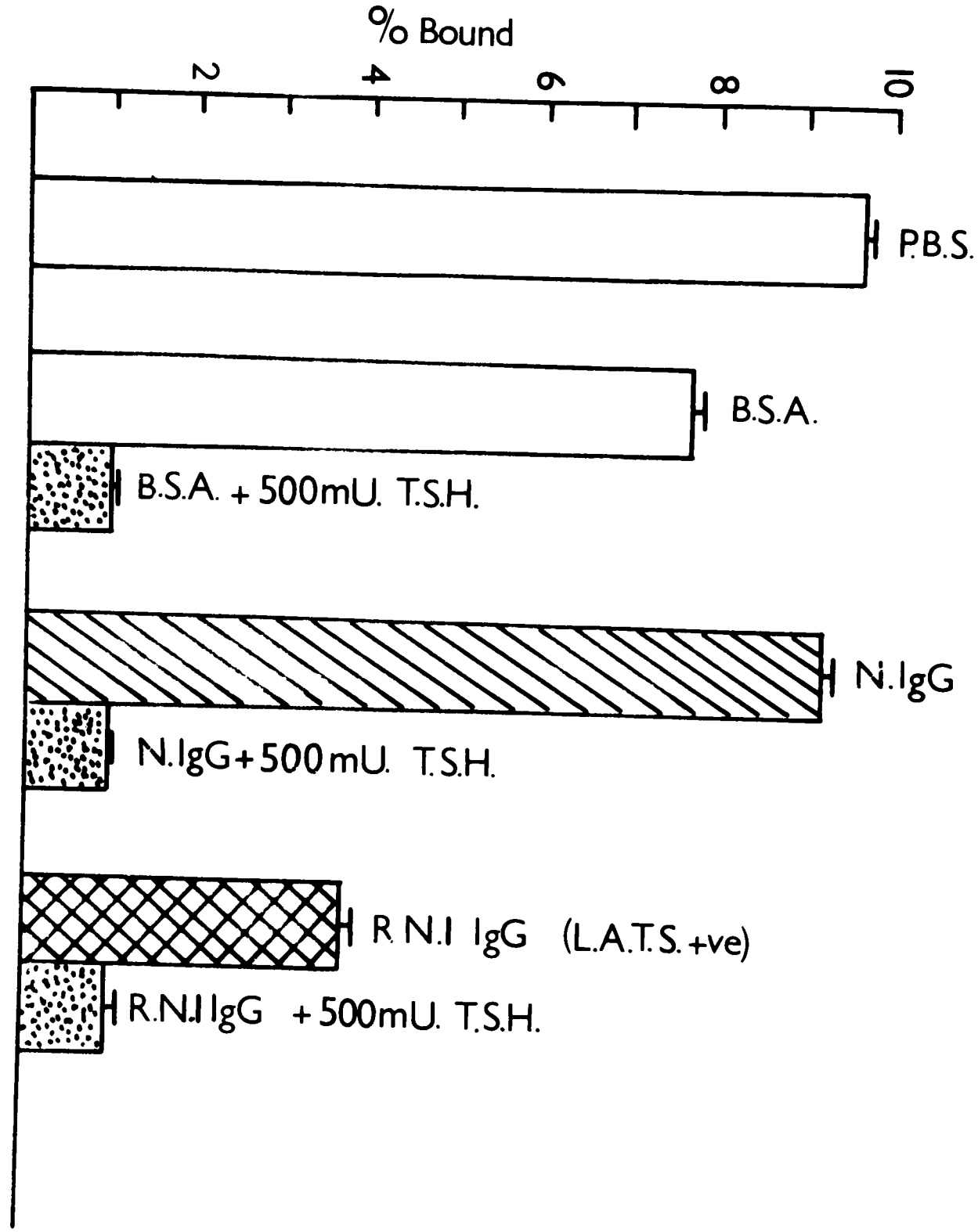
(2) p for difference from control counts (after washing) by Dunnett's 't' test. (Results in presence of Carrier T.S.H. excluded from calculation.)

FIGURE 5.19

The effect of L.A.T.S. on T.S.H.-^{125}I binding to a human thyroid homogenate (expt. 5.37)

Total volume 0.75ml; 1.0g. equiv. human thyroid homogenate (T.S. thyrotoxic); 1 hr. preincubation in absence of label; 7 hr. subsequent incubation with T.S.H.^{125}I at 20^{o}C with or without 2.6mg IgG or B.S.A.

Each bar represents mean ± S.E.M. of triplicates.


% Bound
2
4
6
8
10
P.B.S.
B.S.A.
B.S.A. + 500mU. T.S.H.
N.IgG
N.IgG+500mU. T.S.H.
R N.I IgG (L.A.T.S.+ve)
R.N.I IgG +500mU. T.S.H.

binding of T.S.H.-^{125}I in comparison with NIgG. The second experiment showed that B.S.A. was also capable of reducing the 5% bound in comparison with observations in the absence of added protein. N.IgG has no such effect.

Comment. The reduction of binding by L.A.T.S. supports the hypothesis that L.A.T.S. acts on T.S.H. receptors. However, whilst L.A.T.S. negative IgG failed to show this action, B.S.A. inhibited T.S.H.-^{125}I binding. Both of these binding inhibitors affected the saturable binding only.

5.17.2. Further experiments on the effect of L.A.T.S. IgG on T.S.H.^{125}I binding by human and bovine thyroid homogenates (expts. 5.38-5.40)

Methods. In order to investigate this effect further, IgG was prepared from the sera of two euthyroid subjects (C.P.G., M.J.)(1), two L.A.T.S.-negative sera from patients with thyrotoxic Graves disease (J.B., Y.V.), three high L.A.T.S. sera from patients with thyrotoxic Graves' disease (R.C., R.N., R.W.)(1) and a pool of serum (C.F.T.) containing high titres of anticytoplasmic thyroid antibodies (as measured by the thyroid complement fixation test(2))(3). The effects of the IgGs were tested in two experiments using bovine homogenates and one experiment using a human homogenate. Eight hour incubations were performed in which T.S.H.^{125}I was added after one hour, except in experiment 5.38 where all reagents were added at the start of an eight hour incubation.

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(1) Serum was taken on two occasions from C.P.G., M.J. and R.N. and IgG separated
(2) Prof. D. Doniach, The Middlesex Hospital, London
(3) See appendix 4 for details of sera

Results. These are shown in tables 5.15 and 5.16. Experiments 5.39 and 5.40 are also shown in figure 5.20. In all three experiments only L.A.T.S. positive IgG inhibited T.S.H.-^{125}I binding(1). Other IgGs, whether or not they contained antithyroid antibodies or were prepared from sera from patients with Graves' disease, had no effect on T.S.H.^{125}I binding(2). These experiments also clearly demonstrated that B.S.A. enhanced T.S.H.-^{125}I binding to bovine homogenates whilst it inhibited binding to human homogenates.

Comments. Whilst these early experiments were not extensive enough to enable definitive conclusions to be made, they suggested that L.A.T.S., per se, was responsible for the inhibition of binding. This activity could not be related to the presence of other antithyroid antibodies (e.g. antimicrosomal antibodies) since the antimicrosomal antibody was without effect, and neither R.N. nor R.W. L.A.T.S. IgG contained other antithyroid antibodies (see appendix 4). That L.A.T.S.-containing IgGs can reduce the binding of T.S.H. to receptor preparations has since been confirmed by several workers (Manley et al, 1974b; Mehdi and Nussey, 1975; Smith and Hall, 1974a, b) although one group reported the failure of a single moderately potent L.A.T.S. IgG to affect binding of tritiated T.S.H. to bovine thyroid plasma membranes (Amir et al, 1973).

The failure of L.A.T.S.-negative IgGs from patients with Graves' disease to affect binding to human homogenates came as a surprise in view of the suggestion by Adams and Kennedy (1971), which was corroborated by subsequent observations (Onaya et al, 1973; Shishiba et al, 1973), that immunoglobulins capable of stimulating the human thyroid were present in the sera of most,

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(1) $p<0.01$ for all L.A.T.S. IgGs in experiments 5.39 and 5.40 (Dunnett's 't' test)
(2) N.S. from control (Dunnett's 't' test)

TABLE 5.15. The effect of L.A.T.S. on T.S.H.^{125}I binding to bovine thyroid homogenates (expts. 5.38, 5.39)

Protein	Thyroid status	L.A.T.S.	% bound(2) Expt. 5.38	Expt. 5.39
Nil	-	-	7.66 (7.70, 7.62)	7.54 ± 0.08(3)
B.S.A.	-	-	10.53(10.91,10.16)	9.19 (9.16, 9.22)
C.P.G.1 IgG	Euthyroid	-	7.28 (7.38, 7.18)	7.21 (7.33, 7.10)
C.P.G.2 IgG		-	-	7.61 (7.56, 7.66)
M.J.1 IgG		-	8.61 (8.49, 8.73)	8.22 (8.22, -)
M.J.2 IgG		-	-	7.69 (7.54, 7.85)
C.F.T. +ve IgG	?	-	8.23 (8.30, 8.17)	7.85 (7.85, 7.85)
Y.V. IgG	Thyrotoxic	-	7.15 (6.98, 7.32)	7.82 (7.58, 8.06)
J.B. IgG		-	7.42 (7.67, 7.17)	7.89 (7.91, 7.88)
R.N.2 IgG	Thyrotoxic	+	3.93 (3.83, 4.03)	4.25 (4.15, 4.34)
R.C. IgG		+	-	6.88 (6.85, 6.91)
R.W. IgG		+	4.76 (4.72, 4.79)	4.62 (4.70, 4.54)
500mU T.S.H.	-	-	1.76 (1.77, 1.75)	1.38 (1.36, 1.40)
B.S.A. + 500mU T.S.H.	-	-	1.70 (1.66, 1.74)	-
C.P.G.1 IgG + 500mU TSH	Euthyroid	-	1.84 (1.93, 1.74)	-
R.N.2 IgG + 500mU T.S.H.	Thyrotoxic	+	1.85 (1.97, 1.72)	-

Total volume 1.025ml.; 2.5g. equiv. bovine thyroid homogenate; 1 hour preincubation in absence of label plus 7 hour incubation with T.S.H.^{125}I (expt. 5.38) or 8 hour incubation (expt. 5.39) at 22°C.

(1) 5mg B.S.A. or IgG per tube.
(2) Mean of duplicates except where specified.
(3) Mean ± S.E.M. of 6 tubes.

TABLE 5.16. The effect of L.A.T.S. on T.S.H.^{125}I binding to human thyroid homogenates (expt. 5.40)

Protein[1]	Thyroid status	L.A.T.S.	% bound[2][3]
Nil	-	-	25.49 ± 0.99[4]
B.S.A.	-	-	18.83 (19.26, 18.40)
C.P.G.1 IgG	Euthyroid	-	23.95 (24.25, 23.85)
C.P.G.2 IgG	Euthyroid	-	25.07 (25.16, 24.99)
M.J.1 IgG	Euthyroid	-	22.79 (22.53, 23.05)
M.J.2 IgG	Euthyroid	-	25.02 (24.97, 25.07)
C.F.T. + ve IgG	?	-	23.80 (23.62, 23.98)
Y .V. IgG	Thyrotoxic	-	24.73 (24.68, 24.79)
J.B. IgG	Thyrotoxic	-	24.93 (25.36, 24.51)
R.N.2 IgG	Thyrotoxic	+	11.46 (10.64, 12.28)
R.C. IgG	Thyrotoxic	+	18.22 (18.47, 17.96)
R.W . IgG	Thyrotoxic	+	10.35 (10.97, 9.73)
500mU T.S.H.	-	-	7.67 (7.74, 7.60)

Total volume 1.025ml.; 0.75g. equiv. human thyroid homogenate (colloid goitre); 1 hour preincubation in absence of label, 7 hour incubation with T.S.H.^{125}I at 22°C.

(1) 5mg B.S.A. or IgG added per tube.
(2) Mean of duplicates except where specified.
(3) Unwashed precipitate.
(4) Mean ± S.E.M. of 4 tubes.

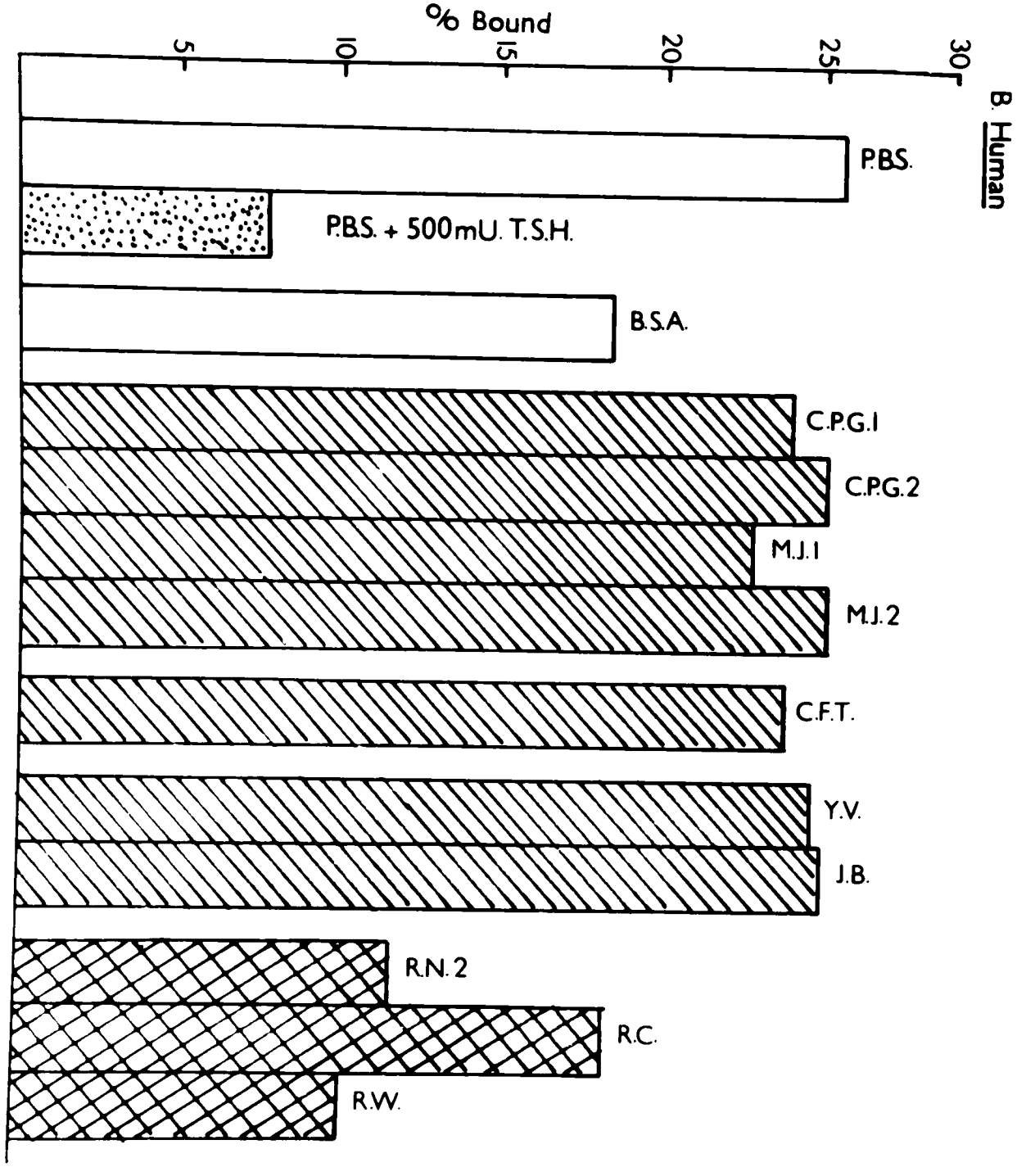
FIGURE 5.20

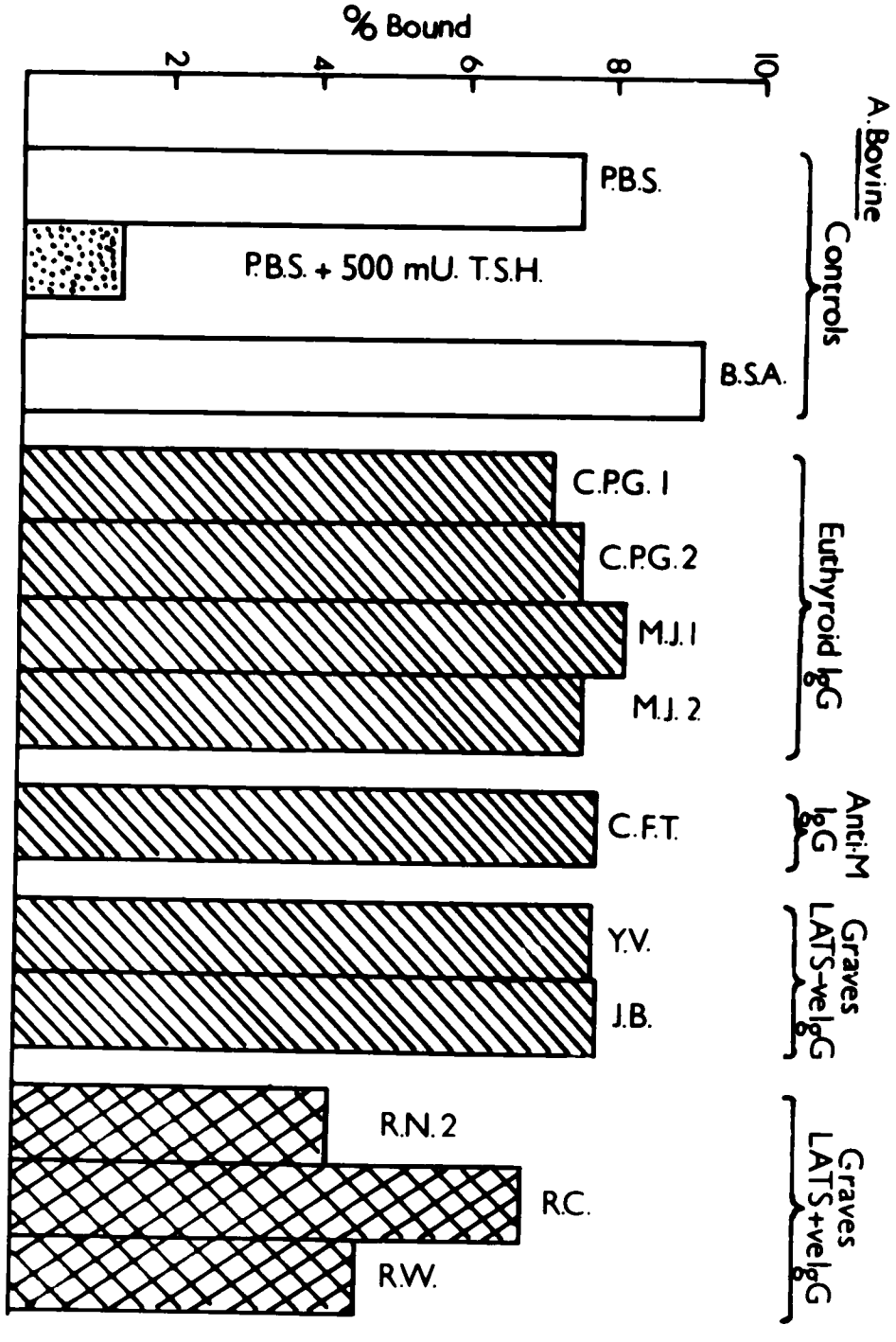
The effect of L.A.T.S. on T.S.H.-^{125}I binding to bovine and human thyroid homogenates

A. Bovine (expt. 5.30)

B. Human (expt. 5.40)

5mg. protein per tube. Other conditions described with Tables 5.15 and 5.16.


% Bound
5
10
15
20
25
30
B. Human
P.B.S.
P.B.S. + 500mU. T.S.H.
B.S.A.
C.P.G.1
C.P.G.2
M.J.1
M.J.2
C.F.T.
Y.V.
J.B.
R.N.2
R.C.
R.W.


% Bound
2
4
6
8
10
A. Bovine
P.B.S.
P.B.S. + 500 mU. T.S.H.
B.S.A.
C.P.G. 1
C.P.G.2
M.J.1
M.J. 2
C.F.T.
Y.V.
J.B.
R.N.2
R.C.
R.W.
Controls
Euthyroid IgG
Anti-M IgG
Graves LATS-ve IgG
Graves LATS+ve IgG

if not all, patients with thyrotoxic Graves' disease. Indeed, subsequent investigators have found that IgGs capable of inhibiting T.S.H.-^{125}I uptake by human thyroid cell fractions may be detected in L.A.T.S.-negative sera from patients with Graves' disease (Hall et al, 1975; Smith and Hall, 1974a, b). It can only be concluded that the IgGs from L.A.T.S.-negative thyrotoxics were insufficiently active to affect the binding in the present system(1).

5.18. THE EFFECT OF GRADED DOSES OF L.A.T.S. (expts. 5.41-5.42)

Methods. In two experiments the effect of graded doses of L.A.T.S. IgG was tested on T.S.H.-^{125}I uptake by human thyroid homogenates. The experimental details are presented with figures 5.21 and 5.22.

Results. (figs. 5.21 and 5.22) In both experiments the inhibition of binding by L.A.T.S. IgG was dose related. In experiment 5.42 B.S.A. also inhibited binding in a dose related fashion but the necessary dose was greater (mg/ml or molar concentration). Control IgG was without effect.

Comments. The dose related inhibition of binding by B.S.A. in the human system, which has since been confirmed by Mehdi and Nussey (personal communication) using purified plasma membranes, might appear to weaken the case for a specific effect of L.A.T.S. IgG. However, that these effects are qualitatively different is suggested by the fact that B.S.A. has opposite effects on T.S.H.-^{125}I binding to bovine

(1) The patients from whom the L.A.T.S. negative sera were taken were undergoing courses of neomercazole for persistent thyrotoxicosis at the time they were bled. It has been shown recently that thyroid stimulating immunoglobulins may disappear during neomercazole treatment (Hall et al, 1975; Mukhtar et al, 1975)

FIGURE 5.21

The effect of graded doses of L.A.T.S. IgG on T.S.G.-^{125}I binding to a human thyroid homogenate (expt. 5.41)

Total volume 0.75ml.; 1.0g. equiv. human thyroid homogenate (J.B. thyrotoxic); 8 hour incubation at 22^{o}C (no preincubation); L.A.T.S. - R.W. IgG.

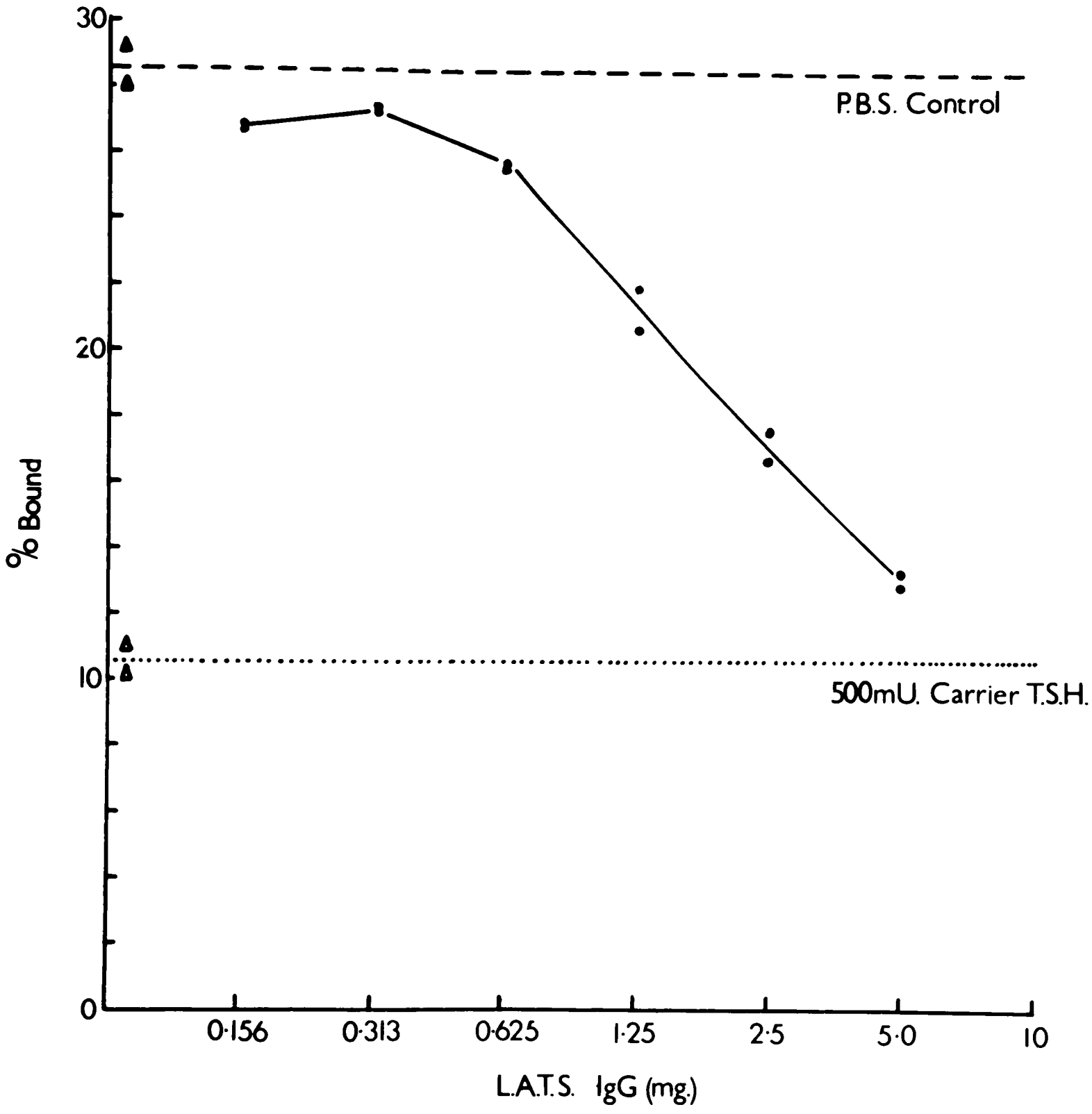

30
20
10
0
% Bound
P.B.S. Control
500mU. Carrier T.S.H.
0·156
0·313
0·625
1·25
2·5
5·0
10
L.A.T.S. IgG (mg.)

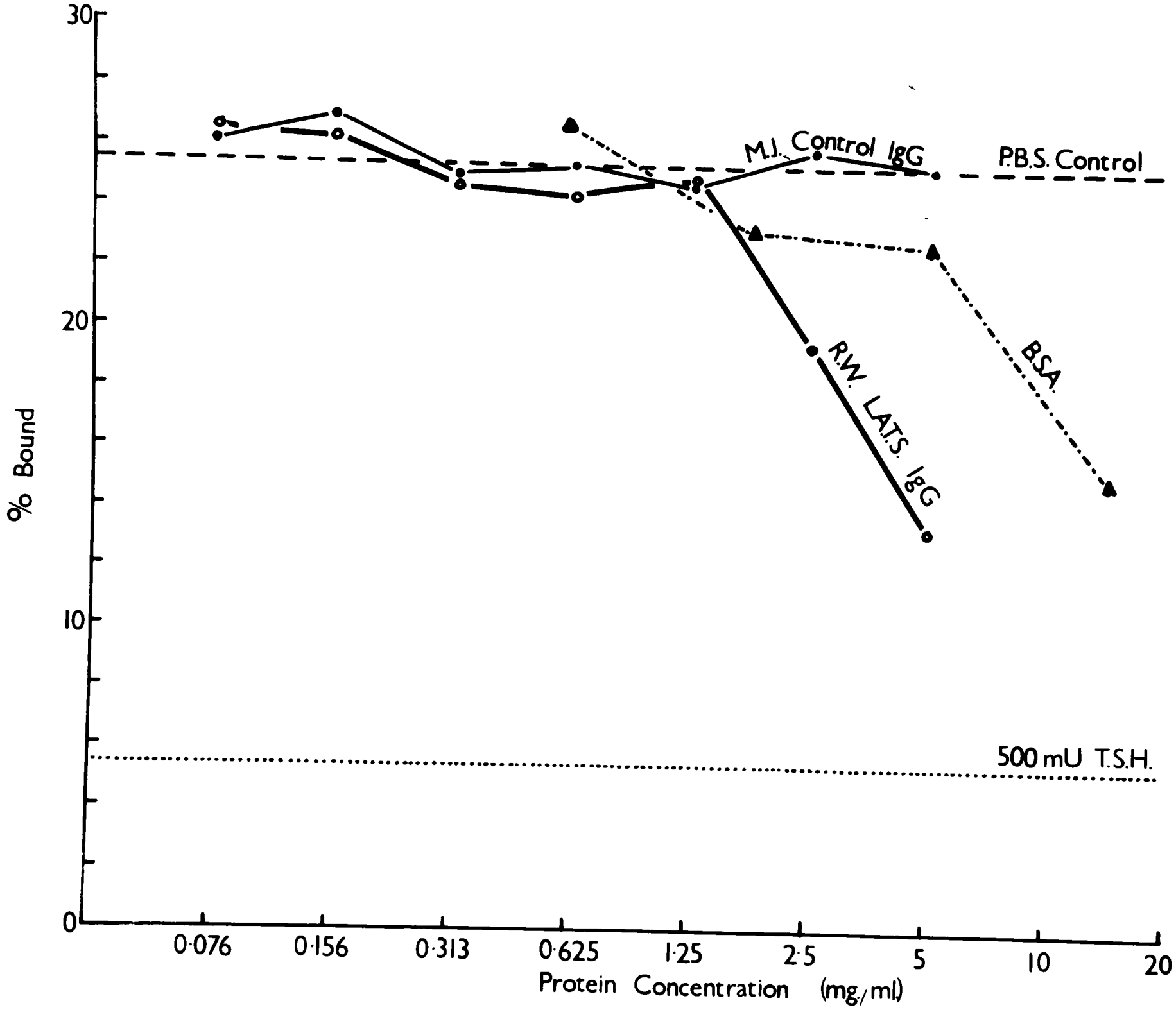
FIGURE 5.22

The effects of graded doses of IgG and B.S.A. on T.S.H.-^{125}I binding to a human thyroid homogenate (expt. 5.42)

Total volume 0.4ml.; 0.2g. equiv. human thyroid homogenate (X, thyrotoxic); 8 hour incubation at 20°C (no preincubation).

Each point represents the mean of duplicates.

(N.B. M.J. control IgG represents M.J. 2IgG.)


30
20
10
0
% Bound
M.J. Control IgG
P.B.S. Control
R.W. LATS. IgG
BSA
500 mU T.S.H.
0·076
0·156
0·313
0·625
1·25
2·5
5
10
20
Protein Concentration (mg./ml.)

and human homogenate whereas L.A.T.S. IgG has similar effects. Moreover, L.A.T.S.-negative IgG has little effect in either system.

5.19. THE EFFECT OF L.A.T.S. ON THE SLOPE OF THE SCATCHARD PLOT

The way in which L.A.T.S. affects the Scatchard plot may be of interest from a mechanistic point of view. For instance, if L.A.T.S. were to inhibit T.S.H. binding by an allosteric action on the T.S.H. receptor, then the number of available sites may be unchanged, but K_A will be diminished. If, on the other hand, L.A.T.S. were to combine noncompetitively with T.S.H. receptors, 'blocking' them, then the result would be a reduction in the maximum bound without any effect on the observed association constant.

Method. Because of the gross nonlinearity of Scatchard plots with human homogenates, bovine thyroid homogenates only were used. In two experiments L.A.T.S. IgG, control IgG or no protein addition were compared in their effects. The experimentsl detail is presented with figure 5.23.

Results. In both experiments marked "tail-off" occurred and in one this reached such proportions as to prevent any conclusions being drawn onto the slopes.

The results of the other experiment (expt. 5.44) are shown in figure 5.23. L.A.T.S. produced an apparent lowering of K_A from 5.24 x 10^8 to 3.39 x 10^8 M^{-1}(1).

Comments. The results of this experiment must be regarded cautiously because of the marked "tail-off" which would tend to reduce the slope of plots in which the maximum bound is low. Furthermore, Manley et al (1974b) have since found that L.A.T.S. IgG reduces the number of available receptors in guinea-pig thyroid cell fractions without affecting the K_A, suggesting that L.A.T.S. combines in a noncompetitive manner with the T.S.H. receptors to inactivate them.

5.20. DOES L.A.T.S. BIND T.S.H.?

The reduction of T.S.H.-^{125}I binding to thyroid homogenates by L.A.T.S. has thus far been considered to result from an action of the latter on receptors. However, it also appeared conceivable that L.A.T.S. acted by combination with T.S.H.-^{125}I. A receptor directed action seemed more likely in view of the T.S.H. independent stimulatory action of L.A.T.S. on the thyroid, and the lack of interference of L.A.T.S. with h-T.S.H. radioimmunoassay (Akande, 1972). However, direct evidence relevant to the present system was lacking. Therefore, two experiments were designed to test whether L.A.T.S. is able to bind b-T.S.H.-^{125}I.

5.20.1. Direct approach (expt. 5.45)

In the first experiment T.S.H.^{125}I was incubated with

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(1) N.S. by Student's 't' test (as described in appendix 1)

FIGURE 5.23

The effect of L.A.T.S. on the Scatchard plot for binding of T.S.H.^{125}I to a bovine thyroid homogenate

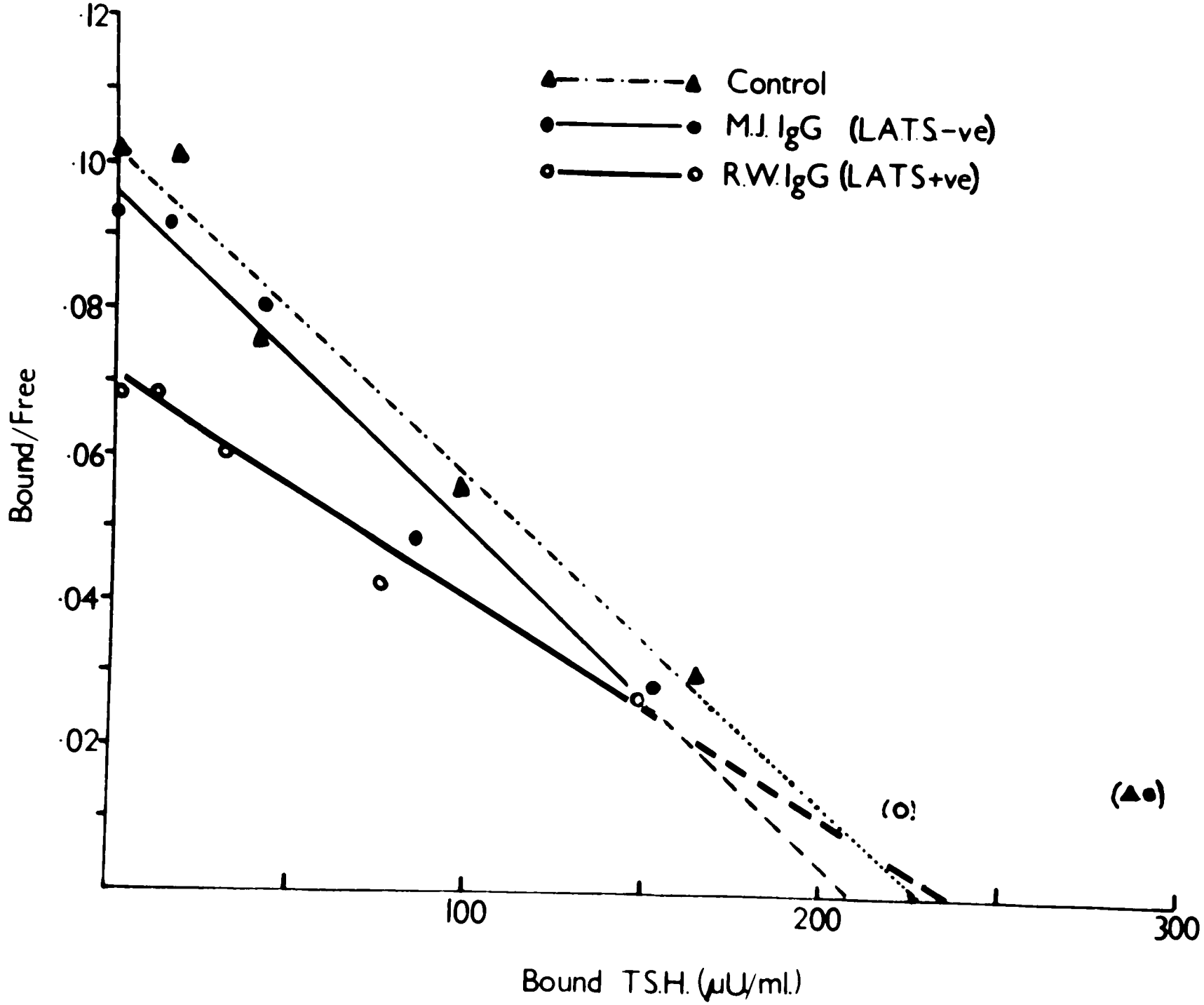
Total volume 0.45ml; B.S.A. concentration 0.1%; 1.0g. equiv. bovine thyroid homogenate; 8 hour incubation at 22°C.

Each point represents mean of duplicates.

Protein[2]	L.A.T.S.	r[1]	Slope (ml/mU)	K_A (M^{-1})
Control	-	0.982	0.447	5.09×10^8
M.J. 2IgG	-	0.984	0.459	5.29×10^8
R.W. 2IgG	+	0.984	0.298	3.39×10^8

(1) r = correlation coefficient of points used for line (points in brackets omitted).

(2) 2mg protein added per tube.


Control
M.J. IgG (LATS-ve)
R.W. IgG (LATS+ve)
Bound/Free
·12
·10
·08
·06
·04
·02
100
200
300
(○)
(▲●)
Bound T.S.H. (μU/ml.)

L.A.T.S. or control IgG and which was subsequently precipitated by the 'double antibody' method.

Method. 50,000 c.p.m. T.S.H.^{125}I were incubated for 8 hours at 22^{o}C alone or with 2mg L.A.T.S. or control IgG in a total volume of 0.4ml P.B.S. Aliquots of 25µl were incubated with 100µl sheep anti-human gamma globulin(1) (a known excess determined by a pilot experiment using IgG^{125}I) overnight at 4^{o}C. The tubes were made up to 1ml with ice cold P.B.S.-0.1% B.S.A. and centrifuged for 60 minutes at 2300g at 4^{o}C and the supernatants aspirated before counting.

Results. The percentages of initial radioactivity retained in the tubes are shown in table 5.17. No differences were evident between tubes, indicating no uptake of T.S.H.-^{125}I by L.A.T.S. IgG.

Comments. A possible criticism of this experiment was that T.S.H.-^{125}I may dissociate from L.A.T.S. IgG on precipitation by anti-IgG antibodies. This seemed unlikely in view of the failure of other antibody-antigen complexes to dissociate when precipitated by the double antibody technique.

5.20.2. Indirect approach: The effect of preincubation with L.A.T.S. IgG on the subsequent uptake of T.S.H.-^{125}I in the absence of L.A.T.S. homogenate by a bovine thyroid (expt. 5.46)

If L.A.T.S. acted by combination with T.S.H.-^{125}I, then a preincubation with L.A.T.S. and its subsequent removal by washing should have no effect on the percent bound in a subsequent incubation of the homogenate with T.S.H.-^{125}I. In

(1) M.R.54, Wellcome Reagents Ltd., Beckenham, Kent

TABLE 5.17. The failure of L.A.T.S. or control IgG to bind T.S.H.^{125}I (expt. 5.45)

Protein	L.A.T.S.	% bound(1)
-	-	3.13 (2.12, 4.14)
R.N.2 IgG	+	3.67 (4.41, 2.93)
M.J.1 IgG	-	3.44 (3.43, 3.44)
C.P.G.2 IgG	-	3.20 (2.45, 2.94)

(1) Mean of duplicates

See section 5.20.1 for experimental details

this experiment a bovine homogenate was used because the gelling of human homogenates would have prevented the resuspension necessary for the second incubation.

Method. Tubes were set up in triplicate containing 1g equiv. bovine thyroid homogenate and 2mg L.A.T.S. IgG, 2mg control IgG or no IgG in 0.4ml P.B.S. and incubated for 2 hours at 22°C. The tubes were centrifuged and washed with 2ml 1% B.S.A. The pellets were resuspended in 0.4ml 1% B.S.A. and 25µl T.S.H.-^{125}I and the tubes incubated for a further 8 hours before recentrifuging, washing and counting.

Results. As can be seen from table 5.18 , L.A.T.S. IgG was capable of reducing significantly the observed binding even though it was not present in the second incubation in which T.S.H.-^{125}I was added.

Comments. Although the effect of L.A.T.S. was small in this experiment, it was sufficient to show that L.A.T.S. has a lasting effect on bovine thyroid homogenates which can be exerted in the absence of T.S.H.-^{125}I. The amount of unbound L.A.T.S. carried over from the first incubation into the second must be small and would be insufficient to account for the effect since in a concomitant experiment on the same homogenate 12.5% of the quantity of the same L.A.T.S. IgG failed to affect T.S.H.-^{125}I binding when present throughout a 10 hour incubation (table 5.18). Thus it would seem highly probable that L.A.T.S. acts on the homogenate and not by binding T.S.H.-^{125}I.

TABLE 5.18. The effect of preincubation with L.A.T.S. IgG on the subsequent uptake of T.S.H.-^{125}I in the absence of L.A.T.S. by a bovine thyroid homogenate (expt. 5.46)

Protein added (2mg) 1st incubation	Carrier T.S.H. (mU)	% bound[1]	p[2]
Nil (control)	-	10.74 ± 0.26	-
C.P.G.2 IgG	-	10.22 ± 0.28	N.S.
R.W. IgG	-	8.17 ± 0.17	$p<0.01$
Nil	500	2.39 (2.42, 2.35)[3]	-

Total volume 0.4ml. (1st incubation), 0.425ml. (2nd incubation); 1.0g. equiv. thyroid homogenate; 2 hour 1st incubation at 22°C in presence of IgG followed by 8 hour 2nd incubation in presence of T.S.H.-^{125}I.

(1) Mean ± S.E.M. of triplicates except where specified

(2) p for difference from control by Dunnett's 't' test

(3) Mean (of duplicates). (Tube lost during experiment.)

5.21. THE EFFECTS OF 5-H.T., I.P.N.A. AND DL-PROPRANOLOL ON T.S.H.-^{125}I BINDING BY BOVINE THYROID HOMOGENATES

(expts. 5.47-5.50)

Since several biologically active amines have been shown to cause thyroid stimulation by acting on receptor sites distinct from those for T.S.H. (see part 4), it was of interest to test the effects of these compounds and their antagonists in the present system. 5-H.T. (5-hydroxytryptamine) and I.P.N.A. (Isopropylnoradrenaline) were chosen since these had been shown to act on separate receptors in the mouse in the experiments of part 4. dl-propranolol was also of special interest since this had been shown to have, in addition to its β-blocking activity, an action on various aspects of thyroid metabolism (Levey, Roth and Pastan, 1969; Onaya and Solomon, 1969).

Methods. In two experiments (expts. 5.47 and 5.48) the effects of 5-H.T.(1) were tested at concentrations ranging from 0.5-10μM/ml and of I.P.N.A.(2) at 0.05-1μM/ml. In order to detect 'non-specific' effects, controls containing excess carrier T.S.H. were used. In two further experiments (expts. 5.49 and 5.50) the effects of dl-propranolol(3) at concentrations of 0.2-200μg/ml were tested in the presence or absence of excess carrier T.S.H.

Results. The results of all experiments are shown in tables 5.19 and 5.20. It is evident that none of the amines

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(1) 5-hydroxyptamine creatinine sulphate, Sigma Chemical Co., Kingston-upon-Thames
(2) 1-Isopropyl noradrenaline hydrochloride, Sigma Chemical Co., Kingston-upon-Thames
(3) 'Inderal', I.C.I. Pharmaceuticals Ltd., Macclesfield, Cheshire

TABLE 5.19. The effects of 5-H.T. and I.P.N.A. on T.S.H.-^{125}I binding by bovine thyroid homogenates

A. Expt. 5.47

Amine	Concentration mg/l	Concentration mM/l	% bound[1] No carrier T.S.H.	% bound[1] 500mU carrier T.S.H.	B_s%[2]
Nil	-	-	6.17 (6.17, 6.16)	1.79 (1.80, 1.78)	4.38
5-H.T.	195	0.5	6.11 (6.11, -)	-	-
5-H.T.	1950	5	8.42 (8.38, 8.47)	3.99 (3.97, 4.01)	4.43
I.P.N.A.	12.5	0.05	6.12 (6.15, 6.09)	-	-
I.P.N.A.	125	0.5	8.15 (8.15, 8.14)	3.30 (3.41, 3.20)	4.85

Total volume 1.05ml.; 2.5g. equiv. thyroid homogenate; 8 hour incubation at 20°C.

(1) Mean (of duplicates)

(2) B_s = saturable binding (see section 5.5)

B. Expt. 5.48

Amine	Concentration mg/l	Concentration mM/l	% bound[1] No carrier T.S.H.	p[2]	% bound[1] 500mU carrier T.S.H.[3]	B_s%
Nil	-	-	8.31 ± 0.40	-	1.08 ± 0.09	7.23
5-H.T.	390	1	9.77 ± 0.17	<0.05	-	-
5-H.T.	3900	10	16.12 ± 0.09	<0.01	6.91 ± 0.18	9.21
I.P.N.A.	25	0.1	8.74 ± 0.17	N.S.	-	-
I.P.N.A.	250	1	9.47 ± 0.47	N.S.	1.76 ± 0.02	7.71

Total volume 0.4ml.; 0.75g. equiv. thyroid homogenate; 8 hour incubation at 22°C.

(1) Mean ± S.E.M. of triplicates.

(2) p for difference from control by Dunnett's 't' test

(3) Heteroscadasticity ($p<0.05$) precluded comparison by Dunnett's 't' test.

TABLE 5.20. The effects of dl-propranolol (a β-blocking agent) on T.S.H.-^{125}I binding by bovine thyroid homogenates

A. Expt. 5.49

Concentration of dl-propranolol mg/l	μM/l	% bound(1) No carrier T.S.H.	500mU carrier T.S.H.
0	0	11.52 (11.47, 11.57)	3.64 (3.86, 3.41)
0.1	0.39	11.03 (10.94, 11.12)	-
1.0	3.9	11.13 (11.15, 11.12)	3.85 (4.03, 3.60)
200	770	10.77 (10.86, 10.69)	3.37 (3.48, 3.25)

Total volume 1.05ml.; 2.5g. equiv. thyroid homogenate; 8 hour incubation at 20°C.

(1) Mean of duplicates

B. Expt. 5.50

Concentration of dl-propranolol mg/l	μM/l	% bound(1) No carrier T.S.H.	p(2)	500mU carrier T.S.H.	p(2)
0	0	7.85 ± 0.02	-	1.10 ± 0.03	-
2	7.7	7.91 ± 0.13	N.S.	1.00 ± 0.03	N.S.
200	770	7.33 ± 0.19	N.S.	1.06 ± 0.03	N.S.

Total volume 0.4ml.; 0.75g. equiv. thyroid homogenate; 8 hour incubation at 22°C.

(1) Mean ± S.E.M. of triplicates.
(2) p for difference from control by Dunnett's 't' test.

tested reduced saturable binding, although higher concentrations of 5-H.T. and I.P.N.A. increased the non-saturable binding.

Comments. These results are consistent with the existence of T.S.H. and amine receptors as separate entities. The concentrations of 5-H.T. and I.P.N.A. used were far in excess of the 1-10mg/l required to stimulate the organification of iodine in isolated calf-thyroid cells (Maayan and Ingbar, 1968; Maayan et al, 1971). Propranolol was similarly without effect, both at 1-2mg/l which is sufficient to produce β-blockade (Maayan and Ingbar, 1968, 1970; Maayan et al, 1971), and at 200mg/l which is capable of inhibiting the response to T.S.H. of adenylate cyclase activity, glucose oxidation or ^{32}P incorporation into phospholipid (Levey, Roth and Pastan, 1969; Onaya and Solomon, 1969). The latter action of propranolol has been attributed to its lysosomal stabilising (Onaya and Solomon, 1969) or local anaesthetic actions (Marshall et al, 1975a, b). These results suggest that the action of higher concentrations of propranolol is directed at some point between the receptor and adenylate cyclase within the cell membrane(1).

5.22. DISCUSSION

These results show conclusively that thyroid homogenates are capable of binding T.S.H.-^{125}I labelled by the chloramine T method. T.S.H. binding to thyroid tissue has been confirmed by a number of other authors using different thyroid preparations and labelling methods (Amir et al, 1973; Field, 1975;

(1) A further effect on thyroid secretion at a later step seems likely since propranolol inhibits cA.M.P.-induced mouse thyroid secretion in vitro (Kendall-Taylor and Munro, 1970b)

Lissitzky et al, 1973; Manley et al, 1972, 1974a, b; Mehdi et al, 1973; Mehdi and Nussey, 1975; Moore and Wolff, 1974; Smith and Hall, 1974; Verrier et al, 1974).

The evidence that these binding sites represent T.S.H. receptors is, however, largely circumstantial:

1. The sites were specific for T.S.H. since binding could be inhibited by small quantities of pure T.S.H., whereas inhibition by other pituitary hormone preparations failed to exceed that expected from their bioassayable T.S.H. activity.
2. The calculated association constant (K_A) was in reasonable agreement with the figures obtained by other workers (table 5.9), considering the diversity of conditions employed. In agreement with some investigators (Bashford et al, 1975; Field, 1975; Moore and Wolff, 1974; Tate et al, 1975) but not others (Lissitzky et al, 1973; Manley et al, 1974a; Smith and Hall, 1974b; Verrier et al, 1974) the Scatchard plot "tailed-off" at high T.S.H. concentrations. It was suggested that this effect was an artefact of the system, perhaps resulting from partially inactivated receptors (section 5.14). However, others have suggested the existence of a second low affinity T.S.H. receptor (Manley et al, 1972; Moore and Wolff, 1974) or 'negative cooperativity' between receptors (Tate et al, 1975).

There was also a reasonable agreement of the K_D for T.S.H.-^{125}I binding in the present work (K_D = dissociation constant = $1/K_A$ = 1.1-2.1 x 10^{-9}M) and the K_M for adenyl cyclase activation in bovine thyroid homogenates (3 x 10^{-9}M)

(Pastan and Katzen, 1967) considering differences in conditions[1]. In more specific comparisons, Moore and Wolff (1974) and Verrier et al (1974) have found excellent correlations between the K_D for labelled T.S.H. binding and the T.S.H. concentration producing half maximal stimulation of adenyl cyclase under the same conditions[1]. Manley et al (1974a) have also reported a remarkable correlation between T.S.H. binding and adenyl cyclase activity.

Interestingly, Moore and Wolff (1974) also found a second peak in adenyl cyclase activation at lower concentrations of T.S.H., corresponding to their low affinity binding sites. Therefore a second set of T.S.H. receptors may have been linked to adenyl cyclase in their bovine thyroid membranes. These could have accounted for the "tail-off" of the Scatchard plot in the present experiments. However, whether these receptors are present in vivo or whether they represent high affinity receptors partially inactivated during preparation of the cell fraction remains open.

3. T.S.H.-^{125}I binding was also inhibited by L.A.T.S. containing IgG. L.A.T.S. can produce thyroid stimulation qualitatively identical with that of T.S.H. in almost every system studied and has been postulated to act via the same receptor (see part 1). The most attractive explanations for these facts are (a) that the receptors for L.A.T.S. and T.S.H. are, at least, closely linked, if not identical, and (b) that the T.S.H.-^{125}I binding sites observed here were true receptors.

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(1) From the law of mass action for a receptor (R) and hormone (H) reaction:
$K_D = [R] \times [H] / [RH]$
If the response α RH , at ½ max. response $[R] = [RH]$
$\therefore K_D = [H]$ (= K_M for response)

Another possibility was that the phenomenon of T.S.H.-^{125}I binding resulted from stimulation, per se, but this seemed unlikely since the thyroid stimulators, 5-H.T. and I.P.N.A., which probably act via receptors distinct from those for T.S.H. (see part 3), did not affect binding.

4. T.S.H.-^{125}I iodinated by the same method has been shown to bind to thyroid plasma membranes (Mehdi et al, 1973; Mehdi and Nussey, 1975), upon which the receptors are probably situated (Pastan et al, 1966). T.S.H. labelled with tritium (Amir et al, 1973, 1976; Moore and Wolff, 1974) or iodinated by the lactoperoxidase method (Amir et al, 1976; Field, 1975; Moore and Wolff, 1974; Tate et al, 1976; Verrier et al, 1974) have similarly been shown to bind to thyroid plasma membranes. T.S.H.-^{125}I or T.S.H.-^{3}H also bind to cell membranes from guinea-pig harderian glands (Bolonkin et al, 1975; Winand and Kohn, 1972) which also respond to T.S.H. (Lamberg et al, 1956; Pochin, 1952; Singh and McKenzie, 1969, 1970; Winand and Kohn, 1975a).

There were, however, certain unexpected findings:

1. The time course of T.S.H.-^{125}I binding observed here was very slow in comparison with the rapid uptake of T.S.H. inferred from the experiments of Pastan et al (1966). These investigators studied the effects of exposure of canine thyroid slices to T.S.H. pulses of increasing duration on subsequent glucose-1-^{14}C oxidation. They found that maximum effect was achieved almost instantaneously at 37^{o}C and within 15 minutes at 0^{o}C. Therefore, T.S.H. binding to receptors may

be maximal within seconds or minutes of exposure. In the present experiments maximum uptake of T.S.H.-^{125}I was not achieved for several hours. Slow binding was also a feature of the h-C.G.-^{125}I-receptor systems upon which the method was based (Catt et al, 1971; Leidenberger and Reichert, 1972a) and even slower binding was reported by Manley et al (1972) for T.S.H.-^{125}I labelled by the chloramine T method and guinea pig thyroid slices. Mehdi and Nussey (1975) reported that the uptake of T.S.H.-^{125}I (iodinated using method A) by human thyroid membranes, reached a plateau after 30-60 minutes (at 0-4^{o}C and 25^{o}C) or after 1-2 hours (at 35^{o}C), although a continued rise after this time is evident from their data. A similar time course has been noted by Manley et al (1974a) for chloramine T labelled T.S.H. and guinea pig thyroid homogenates although they reported that, as in the present work, binding was slower at lower temperatures. In contrast, Amir et al (1973) and Moore and Wolff (1974) found that maximal binding of T.S.H.-^{3}H or T.S.H.-^{125}I (labelled by the lactoperoxidase method) to bovine thyroid membranes occurred within 5 minutes at 35^{o}C. These are more consistent with the results of Pastan et al (1966) described earlier. Precise comparison of all these data is difficult because of the diverse experimental conditions, but there seems to be a general tendency towards slower binding with T.S.H.-^{125}I labelled by the chloramine T method. This would be consistent with the reduced biological activity of such T.S.H. Whilst this implies a reduced rate constant for binding and (unless the rate constant for dissociation is proportionately reduced) a reduced K_A for

the T.S.H.-^{125}I-receptor reaction, it would not greatly affect the experimentally determined K_A value for the T.S.H.-receptor reaction (see section 5.14).

2. The temperature sensitivity of the reaction appears to vary from one report to another. In most, increasing the temperature from 0 to 37^{o}C increased the maximum bound (Lissitzky et al, 1973; Manley et al, 1974a; Mehdi and Nussey, 1975). This pattern of temperature sensitivity was eliminated (Amir et al, 1973; Bashford et al, 1975) or even reversed (Moore and Wolff, 1974) when low ionic strength (<0.01M) medium was used. The present experiments were carried out in P.B.S. and therefore should have conformed to the former pattern. In agreement with this, binding was markedly increased at 20^{o}C compared with 4^{o}C. However, binding was decreased again at 37^{o}C. A possible explanation for this comes from the finding that binding of T.S.H.-^{125}I decreased steadily after attainment of the maximum bound, which was taken as evidence of deterioration of the homogenate. Therefore the apparent reduction in binding at 37^{o}C could have resulted from a disproportionate acceleration of binding site inactivation, overriding the increased binding capacity. Field (1975) has since reported a similar pattern of temperature sensitivity for T.S.H.-^{131}I and bovine plasma membranes and reached similar conclusions. Amir et al (1976) have also found that binding sites were inactivated at 37^{o}C but noted that the addition of Ca^{2+} could prevent this. They suggested that a change in the plasma membrane was responsible.

3. T.S.H.-^{125}I binding was incompletely reversible in the present experiments. This has also been reported by some investigators using T.S.H. iodinated by the lactoperoxidase

method (Field, 1975) but not by others (Amir et al, 1973; Manley et al, 1974a; Verrier et al, 1974). This effect may therefore depend on the experimental conditions and the question of whether or not this obtains in vivo is open to debate. In this connection, the recent observations of De Rubertis et al (1975) may be relevant. They have confirmed and extended the original observations of Pastan et al (1966) in demonstrating that stimulation continues for at least 3 hours following removal of T.S.H. from the medium bathing thyroid slices by extensive washing. The fact that this persistent effect of T.S.H. could be abolished by trypsin or reduced by anti-T.S.H. antiserum was taken as evidence of adenyl cyclase activation by persistently membrane-bound T.S.H. These results also favour the occupancy theory of receptor activation (that activation occurs with receptor occupation) rather than the rate theory[1] (that activation occurs on combination with the receptor).

4. The binding of T.S.H. was not restricted to thyroid homogenates. Bovine liver and adrenal, but not kidney, also contained saturable binding sites for T.S.H.-^{125}I. The explanation for this is not clear but, as discussed earlier (section 5.11), these sites may represent 'redundant' receptors or mediate hitherto unsuspected actions of T.S.H.

Despite these inconsistencies, the weight of evidence is in favour of the proposition that T.S.H.-^{125}I binding sites on homogenates were T.S.H. receptors. If this was the case, the inhibition of binding by L.A.T.S., but not by 5-H.T., I.P.N.A. or propranolol, constitutes further evidence that L.A.T.S. acts via T.S.H. receptors whereas amines exert

(1) Furchgott (1964)

their effects by a different route.

Hormone-receptor binding systems may have several practical applications. Firstly, they are useful in the elucidation of the mechanisms involved in hormone action. Secondly, they offer a means of hormone assay combining precision with the measurement of biological activity.

Radio-receptor assays have been developed for several other hormones (e.g. Corker et al, 1970; Korenman et al, 1970; Leidenberger and Reichert, 1972b; Robinson and Fanestil, 1970; Shutt, 1968) but the present system was not suitable for T.S.H. assay because of its low sensitivity and interference by added proteins (e.g. serum albumin). However, L.A.T.S.-containing IgG competed with T.S.H.-^{125}I for the receptors and this offered a new method for detection of thyroid stimulating antibodies in thyrotoxicosis. The present project was terminated short of this development, but other investigators have subsequently extended the work using techniques derived from the present system. Mehdi and Nussey (1975) found that T.S.H. binding inhibitors were present in 6 out of 6 L.A.T.S.-positive sera but not in 3 L.A.T.S.-negative thyrotoxic sera. However, the L.A.T.S. levels did not correlate with the inhibitory activity. In contrast, Hall et al (1975) found inhibitors in all 40 sera from untreated thyrotoxics irrespective of L.A.T.S. levels, but the yield of positive results was reduced to 50% on treatment. This method is almost certainly valid for thyroid stimulating antibody determinations since the inhibition of T.S.H.-^{125}I binding was directly related to the stimulation of adenylate cyclase

activity in human thyroid membranes (Hall et al, 1975).

These results are in keeping with those of Onaya et al (1973), Shishiba et al (1973) and McKenzie and Zaharija (1976) who showed that human specific thyroid stimulating IgGs were present in L.A.T.S.-negative thyrotoxic sera.

Improved T.S.H. receptor binding assays are now being used in many parts of the world (e.g. Bryson et al, 1976; Hall et al, 1975; Ichikawa et al, 1976; Smith, 1976). One of the modifications introduced by other workers has been the use of purified plasma membranes. This has led to improved receptor yields but has imposed technical limitations, by increasing the complexity and expense of equipment required and reducing the number of samples per assay. Other modifications have included the use of T.S.H. labelled by the lactoperoxidase method and improvements in reaction conditions (e.g. the use of buffers of low ionic strength). These techniques have already proved their usefulness and will probably continue to be of value in the further elucidation of the aetiology and pathogenesis of Graves' disease. The possibility of clinical application has also been raised by the good correlation of thyroid stimulating immunoglobulin levels with other thyroid function tests (Clague et al, 1976) and the observation that thyroid stimulating antibodies may disappear on treatment (Hall et al, 1975; Mukhtar et al, 1975). However, this aspect remains to be explored.

Conclusions: Thyroid homogenates contain specific T.S.H. binding sites which probably represent thyroid receptors.

L.A.T.S., but not 5-H.T., I.P.N.A. or propranolol, prevents binding of T.S.H. to the receptors. These results are consistent with the concept that L.A.T.S. acts by combination with the T.S.H. receptor whereas amines act independently.

APPENDICES

APPENDIX 1. STATISTICAL METHODS

Standard deviation (S.D.) of a group of n observations was calculated as $s = \frac{\Sigma(y - \bar{y})^2}{(n - 1)} = \frac{\{y^2\}}{(n - 1)}$ where y represents an individual observation and $\bar{y}$ is the mean of observations.

Standard error of the mean (S.E.M.) of a group of n observations was calculated as $\frac{s}{\sqrt{n}}$.

Student's 't' test was used to compare two groups of observations taken under different treatment conditions. The value of 't' was calculated as

$$t = \frac{\bar{y}_1 - \bar{y}_2}{s\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \qquad \text{where } s^2 = \frac{\{y_1^2\} + \{y_2^2\}}{n_1 + n_2 - 2}$$

where s_1 and s_2 represent the S.D. of n_1 and n_2 observations, respectively, whose means are $\bar{y}_1$ and $\bar{y}_2$. Provided the variances (s^2) did not differ significantly by an F ratio test[1] (where $F_1 = \frac{s_1^2}{s_2^2}$ or $F_2 = \frac{s_2^2}{s_1^2}$) the number of degrees of freedom in 't', f, were calculated as $f = n_1 + n_2 - 2$. Where the variances differed significantly by comparison of F_1 or F_2 with the values given by a set of statistical tables (Fisher and Yates, 1963), t was calculated as

$$t = \frac{\bar{y}_1 - \bar{y}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$, and f was corrected as described by Bailey

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(1) Two-tailed

(1959):

$$f = \frac{1}{\frac{u^2}{(n_1 - 1)} + \frac{(1 - u)^2}{(n_2 - 1)}} \qquad \text{where } u = \frac{{s_1}^2/n_1}{{s_1}^2/n_1 + {s_2}^2/n_2}$$

Probability for difference (p) was obtained by using statistical tables (Fisher and Yates, 1963).

Dunnett's 't' test (Dunnett, 1964) was used for the comparison of the mean of observations from more than one treatment group with that of a control, in cases where all groups were equally replicated. For k groups of observations the mean within-group variance (${s_m}^2$) was calculated as $\frac{\{y_1^2\} + \{y_2^2\} + \ldots + \{y_k^2\}}{nk - k}$ and f calculated as nk - k. t was then calculated for the comparison of the mean, $\bar{y}_i$, of group i with the mean, $\bar{y}_1$, of the control group as:

$$t = \frac{\bar{y}_i - \bar{y}_1}{s_m\sqrt{2/n}}$$

The probability (p) for the difference between the mean of each group and that of the standard was obtained by direct comparison of 't' with the control values in tables II and III of Dunnett (1964)(1).

In this form, Dunnett's test is strictly valid only in the absence of heteroscadasticity (i.e. heterogeneity of variances)(1). Therefore heteroscadasticity was tested for by the method of Bartlett, as described by Bailey (1959). The figure for $\chi^2_{(k-1)}$ was calculated as M/C where $M = (N - k)\ln {s_m}^2 - \Sigma(n - 1)\ln {s_i}^2$ and $C = 1 + \frac{1}{3(k - 1)}\left(\Sigma\frac{1}{(n - 1)} - \frac{1}{N - k}\right)$ where

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(1) When the number of observations or the variance of the control group differs from that of the treatment groups the calculation of t must be modified and the critical values for t adjusted as described by Dunnett (1964).

$N = n_1 + n_2 + ... + n_k$ and s_i^2 = the variance of each individual group. Heterogeneity of variance was deemed significant if $\chi^2_{(k-1)}$ exceeded that found in statistical tables (Fisher and Yates, 1963) for k - 1 degrees of freedom and p = 0.05. Where heteroscadasticity was significant this is stated in the text.

Duncan's multiple range test (Duncan, 1955, 1957) was used for multiple comparisons and for comparison of observations (y) from k unequally replicated treatment groups (n_i observations/group: $N = \Sigma n_i$) with a control. The steps in the calculation are laid out in table A.1. Firstly, an analysis of variance was performed (a) and the 'between-group' and mean 'within-group' variances (M and s^2) calculated. Further analysis was undertaken if $M/_{s}2$ exceeded the critical value for F in the tables for k - 1 and N - k degrees of freedom(1).

Critical values for adjusted significant ranges (R_p') were calculated from the 'Studentized significant ranges' (Z_p) given by tables II ($p<0.05$) or III ($p<0.01$) in the paper by Duncan (1955) for subsets of p means from 2 to k as $R_p' = SZ_p$ (as in b). Treatment means were ranked (in c) with their replication numbers below. Adjusted subset ranges (e.g. (E-B)') were calculated by multiplying the difference between means ($\bar{y}_i - \bar{y}_j$) by a correction factor $a_{ij} = \sqrt{2n_in_j/(n_i + n_j)}$. These were compared with the critical values for R_p^1 testing in the sequences described by Duncan (1957)(see also table A.1).

The slope of the regression line for a series of observations (y) at a series of treatment levels (e.g. times) (x) was calculated by the method of least squares. If the fitted regression line was taken as $y = a + bx$ then the

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(1) One-tailed

TABLE A.1. Duncan's multiple range test for several unequally replicated means

a) Analysis of variance for k doses with n_i observations (y_i) each. $N = \Sigma n_i$, $T_i = \Sigma y_i$.

Variation assigned to:	D.F.	S.S.	M.S.	F ratio
Between treatments	k-1	$\Sigma T_i^2/n_i - \Sigma T_i/n_i$	M	M/s^2
Within treatments	N-k	by subtraction	s^2	
Total	N-1	$\{y^2\}$		

b) Critical values $R'_p = SZ_p$

e.g. for 5 means A-E.

p	(2)	(3)	(4)	(5)
Z_p	Z_2	Z_3	Z_4	Z_5
R'_p	R'_2	R'_3	R'_4	R'_5

c) Ranked treatment means

e.g.	Group	B	<	A	<	C	<	D	<	E
	Mean	$\bar{y}_b$		$\bar{y}_a$		$\bar{y}_c$		$\bar{y}_d$		$\bar{y}_e$
	Replication no.	n_b		n_a		n_c		n_d		n_e

d) Test sequences $a_{ij} = \sqrt{2n_i n_j/(n_i + n_j)}$

Sequence	Steps
(1) $(E - B)' = (E - B)\ a_{e,b}$	vs. R'_5; $(E - A)'$ vs. R'_4, etc.
(2) $(D - B)'$	vs. R'_4, etc.
(3) $(C - A)'$	vs. R'_3, etc.
(4) $(A - B)'$	vs. R'_2.

N.B. Testing continues in each sequence until adjusted range of subset fails to exceed R'_p. Further comparisons made only if replication numbers within each subset > either of extreme means. In this case value of p remains the same until R'_p exceeded. p then diminishes by 1 for each significant difference obtained in sequence.

slope $b = \frac{\Sigma(x - \bar{x})(y - \bar{y})}{\Sigma(x - \bar{x})^2} = \frac{\{xy\}}{\{x^2\}}$ and the intercept $a = \bar{y} - b\bar{x}$.

Correlation coefficients were calculated as described in Bailey (1959) as $r = \frac{\{xy\}}{\{x^2\}\{y^2\}}$. The presence of significant correlation was confirmed where necessary by calculating Student's 't' as $\frac{r\sqrt{n - 2}}{\sqrt{1 - r^2}}$ with $n - 2$ degrees of freedom for a total of n observations.

The significance of slopes was tested by calculating 't' as $t = \frac{b}{s\sqrt{\frac{1}{\{x^2\}}}}$ where $s^2 = \frac{1}{n - 2}\left\{\{y^2\} - \frac{\{xy\}^2}{\{x^2\}}\right\}$

and comparing with the critical values for t in tables for $f = n - 2$. Slopes could be compared by estimating

$$t = \frac{b_1 - b_2}{s_m\sqrt{\frac{1}{\{x_1^2\}} + \frac{1}{\{x_2^2\}}}} \quad \text{where } s_m^2 = \frac{(n_1 - 2)s_1^2 + (n_2 - 2)s_2^2}{n_1 + n_2 - 4}$$

In experiments described in part 2, where the exponential decay of labelled IgG or thyroxine in the blood was followed and the effects of various treatments on the decay rate were tested, several animals were used per treatment. In this case, the slope and intercept of the $\log_{10}$ radioactivity vs. time regression was calculated for each animal as above and the resultant values combined to give an estimate of mean ± S.E.M. for each treatment. The effects of various treatments were compared using Student's, Dunnett's or Duncan's tests as described above.

Analysis of variance for bioassays. This was performed using the method of Bliss (1952).

1. Calculation of the log dose-response line. For a bioassay in which the response y (in the case of the McKenzie assay,

$\log_{10}$R.I.) is measured at various log dose levels x. k is the number of doses used in the standard curve and n is the number of observations for each dose. N is the total number of observations and T_d is the dose total ($=n\bar{y}$ for a given dose x_i). The slope of the dose-response line b was calculated as $\frac{\{xy\}}{\{x^2\}}$

where $\{xy\} = \Sigma(x\ T_d) - \frac{\Sigma(nx)\ T_d}{N}$ and $\{x^2\} = \Sigma n\ x^2 - \frac{\Sigma^2(nx)}{N}$.

The intercept (a) was calculated as $\bar{y} - b\bar{x}$.

2. Analysis of variance was performed with the aid of a work table similar to that described by Bliss (1952). This is shown in table A.2. The significance of the slope was given by the F ratio B^2/s^2 and the significance of any curvature was given by $A/s_m^{2(1)}$. The index of precision (λ) for the assay was calculated as s/b. Bartlett's test was used to detect heterscadasticity as described above.

3. Comparison of standard and unknown potencies. Where practicable more than one dose of unknown was employed. In this case the combined slope $b_c = \frac{\Sigma\{xy\}}{\Sigma\{x^2\}} = \frac{\{xy\}_s + \{xy\}_u}{\{x^2\}_s + \{x^2\}_u}$

where subscripts s and u denote standard and unknown, respectively. The combined variance due to slope $b_c^2 = \frac{\Sigma^2\{xy\}\tilde{}}{\Sigma\{x^2\}}$

Heterogeneity of variance was detected by Bartlett's test (see above) where $s_m^2 = \frac{\Sigma y^2_s + \Sigma y^2_u - \Sigma T_d^2/n_i}{N_u - k_u + N_s - k_s}$. Divergence

of unknown and standard regression lines was detected by an F ratio test where $F = \frac{\{y^2\}b}{s_c^2}$, where $\{y^2\}b = B_s^2 + B_u^2 - B_c^2$

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(1) One-tailed

TABLE A.2. Work table for analysis of variance of bioassays (after Bliss, 1952)

Variation assigned to	D.F.	S.S.	M.S.	F ratio
Slope of line b	1	$B^2 = \frac{\{xy\}^2}{\{x^2\}}$	B^2	$B^2/_{s}{}^2$
Scatter of dose means about the line	k-2	$\Sigma(T_d{}^2/n) - \Sigma^2 \frac{T_d}{N} - B^2$	A	$A/_{s_m}{}^2$
Deviation about dose means	N-k	$\Sigma y^2 - \Sigma(T_d{}^2/n)$	$s_m{}^2$	
Total scatter about line	N-2	$\{y^2\} - B^2$	s^2	
Total scatter about mean	N-1	$\{y^2\} = \Sigma y^2 - \Sigma^2 \frac{T_d}{N}$		

D.F. = degrees of freedom
S.S. = sum of squares
M.S. = mean square = S.S./D.F.

and $s_c^2 = \frac{\{y^2\}_s - B_s^2 + \{y^2\}_u - B_u^2}{N_s + N_u - 4}$. The index of precision of the combined slope $\lambda_c = \frac{s_c}{b_c}$. The potency of the unknown sample M was calculated as $M = \bar{x}_s - \bar{x}_u + \frac{(\bar{y}_u - \bar{y}_s)}{b_c}$.

The 95% confidence limits were $X_L = \bar{x}_s - \bar{x}_u - C^2 \frac{(\bar{y}_s - \bar{y}_u)}{b_c} \pm t(s_m)$.

Where $C^2 = \frac{B_c^2}{B_c^2 - s_c^2 t^2}$, t = Student's 't' statistic for p = 0.05 and $N_s + N_u - 4$ degrees of freedom, and

$s_m = \lambda_c \frac{1}{N_u} \frac{1}{N_s} + \frac{(\bar{y}_u - \bar{y}_s)}{B_c^2 - s_c t^2}$. In those cases where a single dose of the unknown was employed the calculation was performed in a similar manner but substituting b_s for b_c and B_s^2 for B_c^2.

s_c^2 was calculated as $\frac{\{y^2\}_s - B_s^2 + \{y^2\}_u}{N_s + N_u - 3}$.

All calculations were performed with the aid of a desk computer (Olivetti 'Programma 101' or Sumlock Compucorp 342 'Statistician').

APPENDIX 2. STOCK SOLUTIONS

A. Solutions for the McKenzie assay

1. Normal saline - 9g sodium chloride[1] dissolved in 500ml distilled water and made up to 1000ml.

2. Thyroxine infection (20mg/l) - 2mg thyroxine[2] added to 20ml normal saline. 1N NaOH was added dropwise until the solution was clear. It was then made up to 100ml with normal saline.

3. Thyroxine drinking water (0.33mg/l) - 1mg dissolved in 20ml distilled water as above and made up to 3 litres with distilled water. Solutions (2) and (3) were stored in the dark at room temperature for up to 10 days.

4. 1% B.S.A. was made up by dissolving 1g bovine serum albumin[3] in 100ml normal saline. Storage was at 0-4°C.

5. International standard bovine thyrotropin tablets (as supplied by the M.R.C. Standards Division, Mill Hill) were stored in a dessicator at -10 to -20°C. Standard solutions of 100mU/ml in 1% B.S.A. were made up at intervals of less than 3 months by weighing a tablet on a tortion balance and dissolving it in 1% B.S.A. to a concentration of 1.16g/l, as described in the accompanying literature. Aliquots of 0.5ml were stored frozen in stoppered vials at -10 to -20°C.

B. Buffers

1. Stock sodium phosphate solutions

(a) 0.5M NaH_2PO_4:- 69.005g $NaH_2PO_4.H_2O$[4] or 78.005g $NaH_2PO_4.H_2O$[4] were dissolved in approximately 700ml distilled

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(1) Analytical reagent, B.D.H. Chemicals Ltd., Poole
(2) Sodium thyroxine hexahydrate, Sigma Chemicals Ltd., Kingston-upon-Thames
(3) Cohn fraction V, Sigma Chemical Co., Kingston-upon-Thames
(4) Analytical reagent, B.D.H. Chemicals Ltd., Poole

water on a treater stirrer and made up to 1 litre.

(b) 0.5M Na_2HPO_4:- 179.8g $Na_2HPO_4.12H_2O$[1] were made up to 1 litre similarly. Both were stored at room temperature.

2. 0.5M phosphate buffer pH7.5. 16ml 0.5M NaH_2PO_4 were added to 84ml Na_2HPO_4 and the pH checked using a meter[2]. 1-2ml aliquots were stored frozen for use in protein iodination.

3. Phosphate buffered saline pH7.5 (P.B.S.). 100ml 0.5M phosphate buffer pH7.5 plus 5.844g sodium chloride were made up to 1 litre with distilled water. 100mg sodium mertholate (thiomersal)[1] were added to inhibit bacterial growth, except where specified. The solution was stored at room temperature.

4. P.B.S.-0.1% B.S.A. and P.B.S.-1% B.S.A. were prepared by dissolving 1g and 10g B.S.A., respectively, in 1 litre P.B.S.

5. 0.01M phosphate buffer pH6.5. 5.8ml 0.5M Na_2HPO_4 and 14.2ml 0.5M NaH_2PO_4 were made up to 1 litre with distilled water, checking the pH and storing at room temperature.

6. 0.07M veronal acetate buffer pH8.6 for electrophoresis. 34.363g sodium barbitone[1] and 6.140g barbitone[1] plus 13.672g sodium acetate[1] were each dissolved in 1 litre distilled water. The three solutions were combined and made up to 5 litres with the addition of 0.5g sodium mertholate. The pH was checked and the solution was stored at room temperature out of direct sunlight.

7. 0.012M sodium glycinate for polyacrylamide gel electrophoresis 1.8g sodium glycinate[1] were dissolved in 200ml distilled water. 6N NaOH[1] was added dropwise to pH9.5 with the aid of a pH meter, the solution made up to

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(1) Analytical reagent, B.D.H. Chemicals Ltd., Poole
(2) 'Titrator', Radiometer Ltd., Copenhagen

2 litres and stored at room temperature.

8. 0.2M NaCl - 0.1M tris/HCl buffer pH8.0. 21.15g tris(1) were dissolved in approximately 500ml distilled water. 1N HCl(1) was added to pH8.0 and the solution made up to 1 litre. 58.44g NaCl were dissolved in distilled water and made up to 1 litre. 100ml of the tris/HCl solution and 200ml of the NaCl solution were mixed and made up to 1 litre with distilled water to give the NaCl-tris/HCl buffer pH8.0.

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(1) Analytical reagent, B.D.H. Chemicals Ltd., Poole

APPENDIX 3. PREPARATION OF SEPHADEX COLUMNS

Sephadex[1] G-100 or G-200 was allowed to swell in distilled water at room temperature for at least 72 hr. Fines were decanted repeatedly and the medium changed to the final buffer by three times allowing the gel to settle, decanting the medium and adding buffer.

The column consisted of 1cm diameter glass tubing 7-10cm longer than the desired column length. The lower end of the column was carefully drawn out[2], fitted with narrow plastic tubing and packed with glass wool. The tubing was fitted with a suitable clip.

Columns were wetted with buffer and positioned vertically. A funnel was fitted to the top and with the clip on, buffer was poured to a depth of 5cm. An 80% (v/v) suspension of gel in buffer was slowly poured in and the outflow tube opened. The funnel was kept full of gel and stirred intermittently as the column packed. When the column was a few centimetres above the desired length the funnel was removed and buffer was passed through the column for 24 hr. to allow it to settle.

The void volume (v_o) was measured by applying 100µl of a solution of dextran blue 2000[1] in buffer (with added sucrose) under a head of buffer and co-lecting the effluent in 1ml fractions. V_o(ml) was taken to be the fraction at maximal dye intensity.

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(1) Pharmacia (G.B.) Ltd., Ealing, London
(2) Kindly performed by the University glassblower

APPENDIX 4. HUMAN SERA

Sera from thyrotoxic patients were obtained from various hospitals (see preface). Control sera were obtained from two healthy subjects working in the Department of Human Anatomy and from outdated plasma kindly supplied by the blood transfusion service, Radcliffe Infirmary, Oxford. They were assayed for L.A.T.S. by the McKenzie method as described in section 2.1 and the results expressed in terms of response index ± S.E.M.[1]. Some of the sera were also kindly assayed for antimicrosomal antibodies by both complement fixation and immunofluorescence tests and for antithyroglobulin antibodies by the tanned red cell agglutination test by the Department of Immunology, Middlesex Hospital. Clinical details were not generally available but the information received is included below, along with L.A.T.S. assay values and antibody levels.

L.A.T.S. positive sera

R.N. A middle aged (precise age not stated) manager of a Belgian cycle racing team who suffered from long standing recurrent thyrotoxicosis. Sera were repeatedly strongly positive for L.A.T.S. over 3 years. At the time the two serum samples were taken he was euthyroid on carbimazole but had suffered recent exacerbations of his ophthalmopathy and pretibial myxoedema.

R.N.1. (June 1971) L.A.T.S.: 851 ± 118% (2 hr.), 2150 ± 265% (7 hr.), 1650 ± 166% (24 hr.), no other antithyroid antibodies detectable.

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(1) Since each serum was assayed separately values are not directly comparable, but are a guide to their relative potencies

R.N.2. (January 1973) L.A.T.S.: 1171 ± 126% (2 hr.), 2631 ± 292% (7 hr.), 2132 ± 307% (24 hr.), no other antithyroid antibodies detectable.

R.W. The father (age not stated) of identical twins both suffering from Graves' disease. He presented with overt hyperthyroidism, atrial fibrillation and cardiac failure. He responded to a course of carbimazole, followed by radio-iodine therapy, after which the serum was taken. At this time he was euthyroid (July 1972). L.A.T.S.: 927 ± 112% (2 hr.), 2364 ± 291% (7 hr.), 2103 ± 326% (24 hr.), anti-microsomal and antithyroglobulin antibodies undetectable, weakly positive for thyroid antinuclear antibodies by immunofluroescence.

R.C. and L.L. Sera taken in 1971 from female thyrotoxic patients with high L.A.T.S. titres. No other history available. R.C. L.A.T.S.: 626 ± 58% (2 hr.), 2082 ± 165% (7 hr.), 2679 ± 200% (24 hr.). L.L. L.A.T.S. 660 ± 98% (2 hr.), 1624 ± 265% (7 hr.), 1396 ± 268% (24 hr.).

L.A.T.S. negative thyrotoxic sera

Y.V. A 26 year old female with thyrotoxicosis of 4 years duration which was apparently active at the time the serum was taken despite ongoing carbimazole therapy (February 1973). L.A.T.S.: 109 ± 10% (2 hr.), 96 ± 15% (7 hr.), 89 ± 6% (24 hr.).

J.B. A 43 year old female with persistent thyrotoxicosis. Treated with carbimazole from 1956-1960. In 1964 she underwent subtotal thyroidectomy but received several further courses of carbimazole for recurrences. She was on carbimazole

at the time the serum was taken (February 1973). L.A.T.S.: 124 ± 11% (2 hr.), 110 ± 10% (7 hr.), 84 ± 8% (24 hr.).

Control sera

C.P.G. A 24 year old healthy male with no family history of endocrine disease. C.P.G.1 (June 1971) L.A.T.S.: 109 ± 7% (2 hr.), 118 ± 10% (7 hr.), 100 ± 14% (24 hr.), no antithyroid antibodies detectable. C.P.G.2 (March 1973) L.A.T.S.: 120 ± 24% (2 hr.), 118 ± 16% (7 hr.), 67 ± 5% (24 hr.).

M.J. A 30 year old healthy male with no family history of endocrine disease. M.J.1 (June 1972) L.A.T.S.: 87 ± 9% (2 hr.), 83 ± 7% (7 hr.), 74 ± 9% (24 hr.), no antithyroid antibodies detected. M.J.2 (March 1973) L.A.T.S.: 93 ± 12% (2 hr.), 88 ± 17% (7 hr.), 78 ± 12% (24 hr.).

N. Outdated plasma from the blood transfusion laboratory, Radcliffe Infirmary, Oxford (June 1971). L.A.T.S.: 77 ± 8% (2 hr.), 119 ± 28% (7 hr.), 111 ± 23% (24 hr.), no anticytoplasmic antibodies detected, antithyroglobulin T.R.C. test positive at 1 in 40 dilution.

C.F.T. Pool of serum taken from patients with high titres (>1 in 256) of antimicrosomal antibodies. L.A.T.S.: 76 ± 9% (2 hr.), 66 ± 9% (7 hr.), 63 ± 11% (24 hr.).

APPENDIX 5. THE PREPARATION OF ANTI-T.S.H. ANTIBODIES

Method. Antibodies were raised against commercial bovine T.S.H.(1) in guinea pigs. An emulsion of 10IU T.S.H.(1) in 5ml normal saline and 5ml Freund's adjuvant(2) was freshly prepared before innoculations. Two groups of guinea pigs were treated as follows:

Group 1 comprised 4 animals which were each given four intradernnal injections of 0.2ml of the emulsion in each ear at weekly intervals exsanguinating the animals by cardiac puncture under pentobarbitone anaesthesia(3) two weeks after the last injection.

Group 2 comprised 6 animals which had been prepared using the above regime by Dr. R. Newcomer 12 months previously, but which had not been sacrificed. These were given a single booster injection of 0.2ml emulsion in each ear and exsanguinated one week later, at which time they showed marked local reactions giving rise to irritation, bleeding and tissue breakdown.

The blood samples were allowed to clot and spun separately in a bench centrifuge, taking off the sera, which were stored at -10 to -20^{o}C until use. The presence of anti-T.S.H. antibodies was detected by comparison of the McKenzie assay responses of a standard solution of 1mU/ml T.S.H. (Thytropar), incubated with samples of each serum in dilutions of 1 in 10 to 1 in 10,000 for 24 hours at 4^{o}C with the responses of the untreated T.S.H. solution.

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(1) 'Thytropar', Armour Pharmaceuticals Ltd., Chicago, U.S.A.
(2) Wellcome Reagents Ltd., Beckenham, Kent
(3) 'Nembutal' (0.067ml/100g)

Results. Table A.3 shows the results for each serum. The sera from guinea pigs of group 2 were more potent, suggesting that the standard 5 week course of injections used in group 1 was insufficient to allow an optimal secondary immune response. The serum from guinea pig no. 2 was chosen for use in all further work involving anti-T.S.H. antibodies (parts 2, 3 and 4). This was capable of complete neutralisation of 1mU/ml Int. Std. T.S.H. at 1 in 1000 dilution (table A.4) and was found to be usable in radio-immunoassay at a dilution of 1 in 30,000 (section 2.3.3).

TABLE A.3. The titre of antibodies to T.S.H. in the sera of guinea pigs innoculated with T.S.H. in Freund's adjuvant (see appendix 5)

Group	Animal no.	Dilution for $p<0.001$ decrease in $R.I._{2hr.}$ to 1mU/ml T.S.H.	Dilution for 'neutralisation' of 1mU/ml T.S.H. ($R.I._{2hr.}$ <120%)
1	7	1 in 100	1 in 100
	8	1 in 100	1 in 100
	9	1 in 100	1 in 10
	10	1 in 100	1 in 100
2	1	1 in 1000	1 in 1000
	2	1 in 10,000	1 in 1000
	3	1 in 1000	1 in 100
	4	Not assayed	
	5		
	6	1 in 1000	1 in 1000

Student's 't' test

TABLE A.4. The effect of anti-T.S.H. antiserum (guinea pig 2) on the activity of bovine and mouse T.S.H. assayed by the McKenzie method

Experiment	Sex	Hormone	No. of mice	Antiserum dil.	R.I.$_{2\ hr.}$ (1)
A.		1mU/ml 'Thytropar'	8	-	445 ± 48
12.8.71.			8	1 in 10	96 ± 4
			8	1 in 100	96 ± 4
			8	1 in 1000	105 ± 6
			8	1 in 10,000	179 ± 15
B.		1mU/ml Int. Std.	8	-	441 ± 49
27.1.72.		T.S.H.	8	1 in 100	83 ± 10
			8	1 in 1000	63 ± 7
			8	1 in 10,000	205 ± 50
C.		Mouse pituitaries (2)	7	-	378 ± 40
3.2.72.		1 pituitary/16ml	8	1 in 100	76 ± 9
			8	1 in 1000	115 ± 11
			7	1 in 10,000	332 ± 28

(1) Mean ± S.E.M. (log data not available).
(2) Taken from freshly killed mice, homogenised with saline (2ml/pituitary) in small glass homogenisers, spun in a bench centrifuge for 20 min. to remove debris, made up to 1 pituitary/16ml with 1% B.S.A. and stored at -10 to -20°C until use.

APPENDIX 6. A METHOD FOR REMOVAL OF IODIDE FROM SMALL SAMPLES OF BLOOD OR PLASMA BY CHROMATOGRAPHY ON MICRO-COLUMNS OF AMBERLITE I.R.A. 400 ION EXCHANGE RESIN

A.6.1. Introduction

In experiments on the decay rates of T.S.H.-^{125}I or thyroxine-^{125}I (part 3) in the mouse it was necessary to remove contaminating radioiodide from small samples of blood or plasma. Trichloracetic acid precipitation of protein bound iodine is commonly used for the purpose but this method leaves a significant proportion of radioiodide in the 'protein bound' fraction, especially when whole blood is used (see section 3.2.4). In one experiment more than 15% of the contaminating radioiodide in blood and 4% of that in plasma remained in the protein fraction following T.C.A. precipitation. Furthermore, losses of up to 25% of the protein bound iodine were incurred (table 3.1). However, ion exchange resins have been found to be very effective agents for iodothyronine purification (Blanquit et al, 1955; Fields et al, 1956; Galton and Pitt-Rivers, 1959; Pileggi et al, 1961; Scott and Reilly, 1954; Wynn et al, 1959; Zieve et al, 1955). Slade (1956) described a method for P.B.I. measurement in which plasma was passed through small columns of Dowex 1 or 2 from a syringe removing more than 99.6% of the iodide from human serum. Florsheim et al (1970) employed columns of amberlite I.R.A. 400 which were able to remove 99.8% of the iodide from 2ml samples of mouse blood. In the method described below small columns of amberlite I.R.A. 400 were used to absorb iodide from large numbers (up to 150) of 50µl blood samples.

A.6.2. The preparation of microcolumns of amberlite I.R.A. 400

Small plastic tubes[1] were selected to house amberlite columns so that standard plastic syringes could fit snugly into the upper end. The lower ends of the tubes were packed with small glass wool plugs and the tips cut off. A suspension of amberlite I.R.A. 400[2] was introduced with the aid of a syringe into the upper end of each tube which was then closed with a further glass wool plus. Columns were stored under water until use.

A.6.3. Absorption of iodide from blood samples

Method. In order to determine the optimum conditions, initial experiments were performed using mouse blood to which had been added fresh T_4-^{125}I[3] and iodide-^{131}I[4] to yield approximately 10^5 c.p.m. in 50µl. 50µl samples of this blood were pipetted into 0.5ml aliquots of heparinised normal human plasma (to add extra binding protein) in small plastic counting vials[5] and counted on a twin channel automatic gamma counter[6] at between 12.5-75kev (^{125}I and 314-414kev ^{131}I). Two duplicated standard tubes containing T_4-^{125}I (S) and ^{131}I (T), respectively, were set up simultaneously to allow correction for ^{131}I "crossover" counts onto the ^{125}I channel, radioactive decay and geometrical changes caused by the increased volume following the use of the amberlite columns.

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(1) Microfuge tubes, Beckmann-R.I.I.C. Ltd., Glenrothes, Fife
(2) Koch-Light Laboratories Ltd.
(3) I.M.14, The Radiochemical Centre, Amersham
(4) I.B.S.5, The Radiochemical Centre, Amersham
(5) 3ml flat bottomed tubes, P.T.10944, Luckham Ltd., Burgess Hill, Sussex
(6) Nuclear Chicago autogamma

An empty 2ml plastic syringe was fitted into the upper end of an amberlite column and the contents of a tube were drawn back and forth through the column, taking 5-10 seconds for each stroke. The optimum number of strokes was determined by the experiment described below (section A.6.4). Finally, the contents of the tube were drawn up into the syringe followed by 2 x 0.5ml distilled water pipetted into the vial and drawn through the column. The contents of the syringe were then expelled into the vial and the syringe washed with 2 x 0.5ml distilled water, the washings being added to the contents of the vial. The standard vials were also diluted to 2.5ml before recounting. Columns were discarded after use.

The percentage yield of T_4-^{125}I and iodide-^{131}I was calculated with the aid of a programmed desk computer(1).

	^{125}I channel counts(2)	^{131}I channel counts(2)
^{125}I standard initial count	$^{125}S_1$	-
^{131}I standard initial count	$^{125}T_1$	$^{131}T_1$
Blood initial count	$^{125}C_1$	$^{131}C_1$
Corrected initial count	$^{125}C_1 - \left\{ \frac{^{131}C_1 \times ^{125}T_1}{^{131}T_1} \right\}$	$^{131}C_1$

(1) Olivetti programma 101
(2) All counts background subtracted

	^{125}I channel counts	^{131}I channel counts
^{125}I standard final count	$^{125}S_2$	-
^{131}I standard final count	$^{125}T_2$	$^{131}T_2$
Blood final count	$^{125}C_2$	$^{131}C_2$
Corrected final count	$\frac{^{125}S_1}{^{125}S_2}\left\{^{125}C_2-\left(\frac{^{131}C_2 \times {}^{125}T_2}{^{131}T_2}\right)\right\}$	$\left\{\left(\frac{^{131}C_2 \times {}^{131}T_1}{^{131}T_2}\right)\right\}$

$$\% \text{ yield} = \frac{\text{Corrected final count x 100}}{\text{Corrected initial count}}$$

Iodide-^{125}I content of the T_4-^{125}I was checked by ascending chromatography on Whatmann 3MM paper(1) in n-butanol(2) -glacial acetic acid(2)-water (38:5:6 by volume) for 16-20 hours at room temperature(3) (Robbins and Rall, 1967; Smith, 1960). Strips of 1cm were cut and counted in a scintillation spectrometer at 12.5-75kev. The pattern of radioactivity in one such experiment is shown in figure A.1. Rf values for thyroxine and iodide were 0.75 and 0.1, respectively. In some cases a small percentage (<2%) of material remained at the origin. This was assumed to represent iodoprotein and was disregarded.

A.6.4. Investigation of the column characteristics

A.6.4.1. The number of elutions through the column

Method. In a single experiment the effect of varying the number of passages through the column (1-33) on T_4-^{125}I and iodide-^{131}I yield was tested using groups of 6 tubes per treatment.

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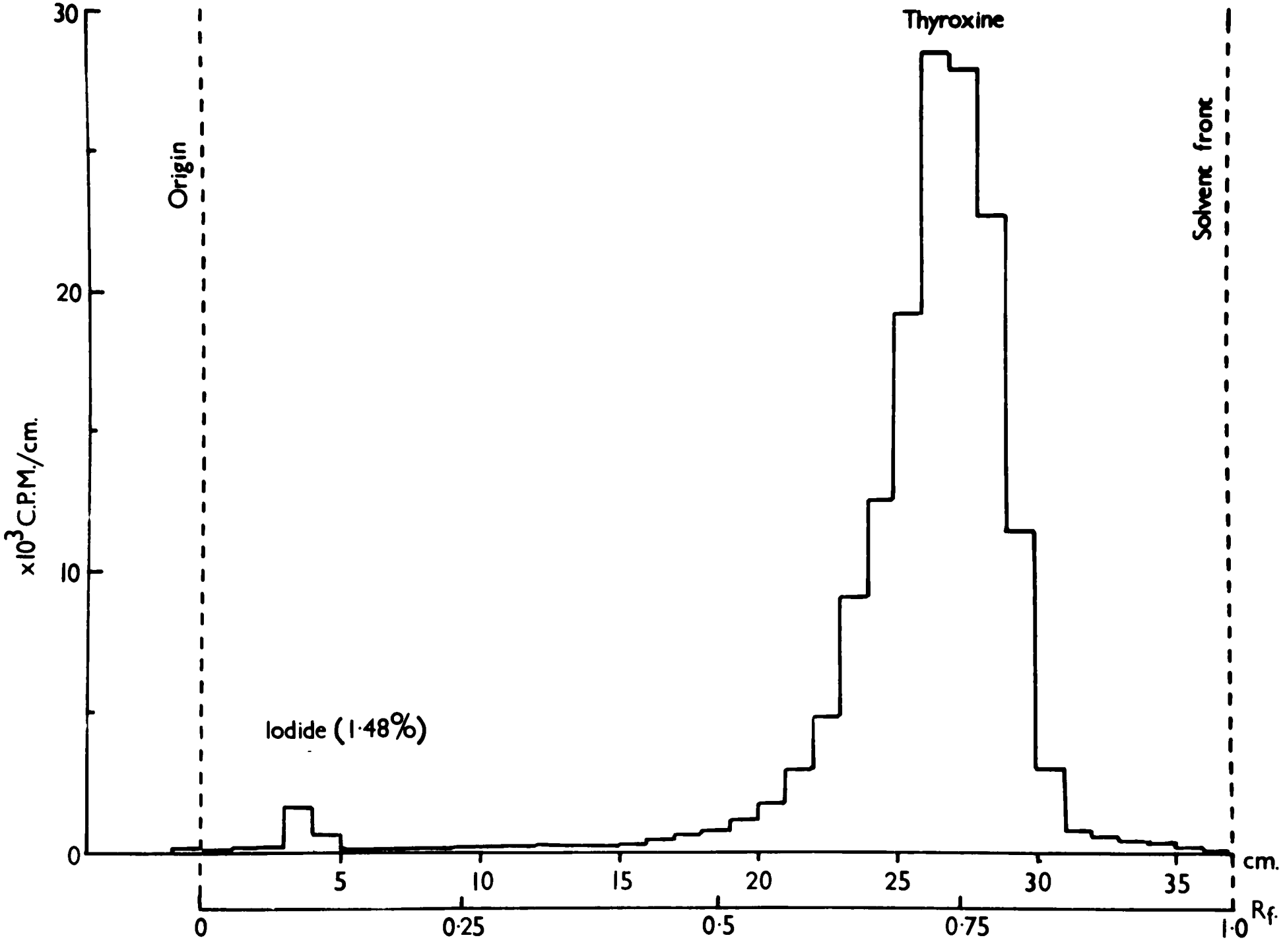
(1) Reeves Ltd.
(2) Laboratory reagent, B.D.H. Chemicals Ltd., Poole
(3) Label applied to paper in human serum to minimise deiodination on drying (Taurog, 1963)

FIGURE A.1

Ascending chromatography of thyroxine-^{125}I on Whatmann 3.M.M. paper in n-butanol:glacial acetic acid:water (38:5:6)


30
20
10
0
×10³C.P.M./cm.
Origin
Thyroxine
Solvent front
Iodide (1·48%)
5
10
15
20
25
30
35
cm.
0
0·25
0·5
0·75
1·0
Rf.

Results. Table A.5 shows that the quantity of thyroxine absorbed was small and constant, but there was a progressive loss of iodide reaching more than 97% at 33 elution. In order to minimise effort, only 17 elutions were performed subsequently.

A.6.4.2. The effect of plasma proteins, ions and red cells

Method. Because of the possibility that saline could compete with iodide for the ion-exchange resin and reduce binding, the effect of minimising the salt concentration by using human plasma, dialysed against distilled water for 12 hours (30ml in 2 litres), as carrier was tested.

The efficiency of absorption under these conditions was compared with that using undialysed human plasma and distilled water or saline washes. In the same experiment the need for carrier plasma was checked by including tubes with saline as replacement. Tubes containing mouse plasma samples were also included. Groups of 18 tubes were allotted to each treatment and these were subdivided into groups of 6 receiving 9, 17 or 33 elutions each.

Results. (table A.6) Removal of ions by dialysis of the carrier plasma decreased the remaining iodide from 3.26 to 0.87% (17 elutions). The presence of carrier plasma was important since this increased the yield of T_4-^{125}I from 87.2% to 99.0% (17 elutions). The method appeared equally effective for mouse plasma and blood. Future experiments were therefore performed using carrier human serum dialysed against distilled water for 12 hours.

TABLE A.5. The effect of varying the number of elutions on the percentage yield of iodide-^{131}I and T_4-^{125}I following amberlite I.R.A. 400 chromatography

Number of elutions	T_4-^{125}I yield (%) (1)	Iodide-^{131}I yield (%) (1)
1	99.2 ± 0.2	17.30 ± 2.37
3	98.9 ± 0.5	9.90 ± 1.25
5	99.1 ± 0.4	6.46 ± 0.58
9	98.5 ± 0.3	3.78 ± 0.28
17	99.6 ± 0.2	3.23 ± 0.31
33	99.7 ± 0.7 (2)	2.86 ± 0.36 (2)

(1) Mean ± S.E.M. of 6 observations except where specified.

(2) Mean ± S.E.M. of 5 observations.

T_4-^{125}I contained 0.8% iodide-^{125}I by ascending paper chromatography in butanol:acetic acid:water (38:5:6)

TABLE A.6. The effect of plasma ions and plasma proteins on amberlite column efficiency

Mouse blood or plasma (50µl)	Carrier medium (0.5ml)	Wash (0.5ml x 2)	T_4-^{125}I yield (%)(1) 9 elutions	17 elutions	33 elutions	Iodide-^{125}I yield (%)(1) 9 elutions	17 elutions	33 elutions
Blood	Saline	Distilled water	88.6±1.3	87.2±1.0	83.9±0.5	2.42±0.11	1.69±0.10	1.51±0.21
Blood	Water-dialysed human plasma	Distilled water	99.5±0.2	99.0±0.5	97.7±0.4	1.16±0.13	0.87±0.16	0.66±0.09
Blood	Human plasma (undialysed)	Distilled water	100.0±0.2	99.6±0.2	99.8±0.2	3.42±0.40	3.26±0.33	2.72±0.55
Blood	Human plasma (undialysed)	Normal saline	98.5±0.2	98.9±0.4	97.5±0.3	8.60±0.31	6.52±0.33	5.69±0.10
Plasma	Water-dialysed human plasma	Distilled water	100.6±0.8	100.0±0.5	100.4±0.3	1.94±0.51	0.84±0.07	0.84±0.07
Plasma	Human plasma (undialysed)	Normal saline	100.6±0.3	99.9±0.4	100.2±0.7	5.95±0.24	4.07±0.19	4.04±0.42

(1) Mean ± S.E.M. of 6 observations.

T_4-^{125}I contained 0.8% iodide-^{125}I by B.A.W. chromatography.

A.6.4.3. The effect of a high blood thyroxine or iodine level

Method. In order to assess the suitability of the columns for use on blood taken from mice treated with thyroxine or iodide, three groups of three mice were each treated as follows:

1. Distilled water to drink for three days,
2. 33mg/l thyroxine solution to drink for three days and injected with 100µg thyroxine in 0.5ml saline i.p. 6 hours before exsanguination.
3. 33mg/l iodide solution to drink for three days and injected with 100µg iodide 30 minutes before exsanguination.

The mice were exsanguinated by orbital puncture under ether anaesthesia and the heparinised blood in each group pooled. Thyroxine-^{125}I and iodide-^{131}I were added to each blood pool to give 50,000 c.p.m. of each in 50µl. Six 50µl blood samples were taken from each pool into vials each containing 0.5ml human plasma dialysed against distilled water. Tubes were subsequently treated as above.

Results. (table A.7) Whereas there was no difference between 'iodised' or control blood, with thyroxinised blood the yield of T_4-^{125}I was reduced from 97.7 to 90.8%, whilst the yield of ^{125}I increased from 1.4 to 2.5%.

The total thyroxine level in this blood was not known, but some guide is available from another experiment (performed for a different purpose). In this, mice were prepared as for the McKenzie assay but giving either (a) 3.3mg/l T_4 in the drinking water with 10µg T_4 injections i.p. on days one and three or (b) 33mg/l T_4 drinking water and 100µg T_4 injections

TABLE A.7. The effect of thyroxine and iodide on column efficiency for removal of iodide from blood by amberlite I.R.A. 400 chromatography

Blood (mouse)	T_4-^{125}I yield (%)(1)	Iodide-^{131}I yield (%)(1)
Normal blood	97.7 ± 0.3	1.38 ± 0.04
Thyroxinised blood	90.8 ± 0.8(2)	2.50 ± 0.08(2)
Iodised blood	97.2 ± 0.5	1.23 ± 0.08

(1) Mean ± S.E.M. of 6 observations.

(2) Significantly different from control by Student's 't' test $p<0.001$

T_4-^{125}I contained 2.7% iodide by ascending paper chromatography in butanol:acetic acid:water (38:5:6)

i.p. on days one and three. Administered thyroxine was trace labelled to a known specific activity with T_4-^{125}I. Blood taken on the fourth day was counted before and after being subjected to amberlite chromatography. In both cases approximately 72% of counts were protein bound and the blood thyroxine levels calculated from the known specific activity of administered T_4 were 63 ± 11[(1)] µg/100ml for the lower dose, but 172 ± 25[(1)] µg/100ml for the higher dose. Therefore, in the thyroxinised blood of the present experiment T_4 may have been in excess of 200µg/100ml.

This high level was sufficient to reduce the yield of thyroxine from the column. This finding raised anxieties over the applicability of this method for use on blood samples taken from mice prepared for the McKenzie assay. However, in an experiment comparing the yield of T_4-^{125}I following amberlite chromatography of 50µl samples of blood from normal mice and mice prepared for the McKenzie assays, the yields were 100.5 ± 0.7 and 101.3 ± 0.6%[(2)], respectively. Therefore, the method would appear to be applicable for blood taken from mice treated for the McKenzie assay but not for blood taken from very highly thyroxinised animals.

A.6.5. Discussion

The method developed here allows rapid removal of contaminating iodide from large numbers of small blood samples using expensive equipment. The method was ideal for the needs of work in part 3, when it was necessary to remove radioiodide from mouse blood samples containing T.S.H.-^{125}I or T_4-^{125}I. The maximum absorption of iodide was in the region of 99%, which was adequate for most purposes.

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(1) Mean ± S.E.M. of 8 mice
(2) Mean ± S.E.M. of 6 mice. N.S. by Student's 't' test

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