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Assessment of thyroid hormone assays

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SYNOPSIS Four techniques for estimating serum T4 and three for estimating serum T3 have been investigated and found to be satisfactory in routine use. Normal ranges for each technique have been established. Estimation of serum T3 by the commercial kits tested appears to have a high discriminant value in the diagnosis of hyperthyroidism, although the diagnostic definition used inevitably enhances the apparent sensitivity of these techniques. Estimation of serum T4 will identify the majority of patients with symptomatic hypothyroidism. The low sensitivity of T3 in the diagnosis of thyroid failure is confirmed.

There has been a dramatic increase in the number of diagnostic techniques available for the investigation of suspected thyroid disease over the last two decades. The relative merits and limitations of the procedures currently available have been extensively reviewed (Evered, 1974; Evered *et al*, 1976) and a strategy for the investigation of the three common thyroid problems, that is, suspected hyperthyroidism, suspected hypothyroidism, and goitre, has been developed by these and other workers (Britton *et al*, 1975). The increasing use of new *in vitro* tests of thyroid function, the proliferation of commercially available kits, and problems of definition and classification have all led to confusion in the minds of many physicians and biochemists. The objective of this paper is to investigate the reliability and discriminant value of *in vitro* techniques for measuring total serum thyroxine (T4) and total serum triiodothyronine (T3) in the investigation of thyroid disease.

Definitions

HYPERTHYROIDISM

Hyperthyroidism was said to be present in subjects with clinical features of thyrotoxicosis and a raised serum triiodothyronine concentration by an established assay (Hesch and Evered, 1973). It is accepted that this definition is open to question, but it has the merit of specificity although it may be disputed on the grounds of sensitivity.

HYPOTHYROIDISM

Hypothyroidism was said to be present in subjects with clinical features of hypothyroidism and a raised serum thyrotrophin (thyroid stimulating hormone; TSH) concentration (Evered *et al*, 1973).

Asymptomatic (or subclinical) forms of the disease were excluded by definition.

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Materials

NORMAL SUBJECTS

Eighty normal subjects studied using all the techniques investigated were derived from laboratory and other hospital staff. Patients with non-thyroidal illness were not studied in view of the known effect of illness upon circulating thyroid hormone levels (Carter *et al*, 1974). The normal ranges for T4 using the Thyopac-4(R) kit and for T3 using the technique of Hesch and Evered (1973) and the Radiochemical Centre T3 kit were also established on a randomly selected sample of a non-hospital population. Full details of this study—the Whickham Community Survey—have been published elsewhere (Tunbridge *et al*, 1975).

SUBJECTS WITH THYROID DISEASE

Subjects with thyroid disease were seen consecutively in the Newcastle Endocrine Clinic after referral for assessment by one of three consultant endocrinologists (Professor R. Hall, Dr F. Clark or Dr D. C. Evered). Forty hyperthyroid and 40 hypothyroid subjects were studied.

PREGNANT SUBJECTS

Twenty pregnant subjects were drawn from a local antenatal clinic (Mr T. Lind) or were subjects who were pregnant when seen in the Whickham Com-

munity Survey. All were in the second or third trimester of pregnancy.

SUBJECTS ON AN ORAL CONTRACEPTIVE

One hundred and eighteen subjects receiving an oral contraceptive were drawn from the Whickham Community Survey.

Methods

SERUM THYROXINE ESTIMATION

Competitive protein-binding assays (CPBA)

CPBA was carried out using the Thyopac-4(R) kit from the Radiochemical Centre, Amersham. A small comparative study was carried out using a column technique as described by Seligson and Seligson (1972).

Radioimmunoassay (RIA)

Serum T4 was measured by RIA using two techniques:

(a) Newcastle assay: Specific T4 antisera were raised by the serial injection of T4 methyl ester hydrochloride conjugated with bovine serum albumin into New Zealand White rabbits. Standard T4 solutions were prepared by the technique of Murphy (1965). Hormone free serum was prepared by charcoal extraction. The assay technique is as follows:

0·475 ml buffer (with or without standard T4)
Barbital buffer—50 mM pH 8·6
0·025 ml unknown or hormone free serum
0·1 ml ANS (400 μg)
0·1 ml ^{125}I T4 (500 pg/tube)
0·1 ml antiserum
Incubate overnight at 4°C.
Separate with methyl-cellulose/charcoal.
Aspirate supernatant after 10 minutes.

(b) Commercial assay: T4 RIA was also carried out using a commercially available kit supplied by the Radiochemical Centre, Amersham.

SERUM TRIIODOTHYRONINE ESTIMATION

Serum T3 was measured by RIA using three techniques.

Newcastle assay

T3 RIA was measured using a modification of the technique described by Hesch and Evered (1973). The only differences from the original assay lay in the use of barbital buffer (50 mM pH 8·6) and ANS as a blocking agent.

Commercial assays

T3 RIA was carried out using the kits which are marketed by the Radiochemical Centre, Amersham and Hoechst Pharmaceuticals.

THYROID HORMONE BINDING TEST (THBT)

THBT was performed using a ^{125}I-T3 Sephadex uptake (Thyopac-3, Radiochemical Centre, Amersham).

TSH

Serum TSH was measured by a double-antibody RIA (Hall *et al*, 1971).

Analysis of data

The data derived from these studies were analysed using standard statistical techniques. The distribution of thyroid hormone concentrations within the Community is not significantly skewed, and thus it was possible to calculate means and standard deviations without transformation of the data. The sensitivity and specificity of each technique was assessed. Sensitivity is assessed by the number of affected subjects detected in relation to the number affected in the population tested. Specificity is evaluated as 1 minus the number of false positives as a proportion of the total number of non-affected subjects.

Thus, sensitivity is calculated:

$$\text{Sensitivity } (\%) = \frac{a}{a+c} \times 100$$

and specificity as:

$$\text{Specificity } (\%) = \frac{d}{b+d} \times 100$$

Where a = diseased subjects abnormal by the test (true positives)
b = non-diseased subjects abnormal by the test (false positives)
c = diseased subjects normal by the test (false negatives)
d = non-diseased subjects normal by the test (true negatives)
(Holland and Whitehead, 1974)

Results

TECHNICAL FACTORS

All the techniques gave acceptable degrees of precision and reproducibility. The intra-assay coefficient of variation ranged between 3·4 and 6·3% and interassay coefficient of variation between 5·0 and 9·0% for T4 and T3 assays (table I). The Thyopac-3 was shown to be highly precise and reproducible.

Technique	Precision (within assay variation: coefficient of variation)	Reproducibility (between assay variation: coefficient of variation)
T4		
Thyopac-4	4·2	5·0
CPBA—column	6·3	9·0
RIA—Newcastle	6·0	6·9
RIA—Amersham	4·6	7·7
T3		
RIA—Newcastle	5·5	8·7
RIA—Amersham	3·4	8·1
RIA—Hoechst	6·3	—
THBT		
Thyopac-3	0·7	1·9

Table I *Precision and reproducibility (per cent)*

	n	Mean	SD	Percentiles 2·5	Percentiles 97·5	
Thyroxine						
CPBA (Thyopac-4)	80	99·00	32	57	147·00	
	1685	105·00	22	63	153·00	WS[1]
CPBA (Column)	20	95·00	26	52	148·00	
RIA (NC)	80	108·00	23	67	149·00	
RIA (RCC)	80	103·00	19	64	141·00	
Triiodothyronine						
RIA (NC)	80	1·75	0·51	0·95	2·70	
	255	1·66	0·54	0·92	2·61	WS[1]
RIA (RCC)	80	2·67	0·55	1·77	3·77	
	255	2·69	0·60	1·84	4·15	WS[1]
RIA (Hoechst)	80	1·77	0·41	1·08	2·38	

Table II *Normal values (nM/l)*

Normal ranges derived from 80 hospital personnel. [1]Additional data for T4-CPBA (Thyopac-4) and T3—RIA (NC) and RIA (RCC) derived from the Whickham Survey (WS).

NORMAL SUBJECTS

The mean values obtained for T4 ranged between 95 and 108 nM/l. The limits of normality were assumed to be between the 2·5 and 97·5 percentile points for the population studied (table II). These ranges match closely those established by using 2SD above and below the mean. The values obtained are in close agreement with those derived from 1685 normal subjects in the Whickham Survey.

There was good agreement between the mean values for T3 using the Newcastle and Hoechst assays, although a narrower range was observed with the latter. The Newcastle values were closely matched by those obtained in the Whickham Survey population. The RCC assay consistently measured mean values 0·9-1·0 nM/l higher than the other two assays. The values obtained in the study of 80 laboratory personnel were closely matched by values obtained from a random subsample of 255 subjects from the survey population assayed over a six-month period (table II).

PREGNANCY AND OESTROGEN PREPARATIONS

Serum T4 and T3 values were significantly raised in subjects taking an oral contraceptive (n = 118) and during pregnancy (n = 20) (table III). Calculation of a free thyroxine or a free triiodothyronine index corrected the majority (96%) of values to within the previously established ranges for normality.

	Mean	SD	Observed range
T4			
CPBA (Thyopac-4)	121	31	71-252
FT_4T	96	26	51-174
RIA (NC)	162	30	123-226
FT_4T	117	21	93-163
T3			
RIA (NC)	2·24	0·64	1·12-4·15
FT_3T	1·85	0·55	0·95-3·04
RIA (RCC)	3·36	0·71	2·07-5·53
FT_3T	2·68	0·60	1·76-4·56
RIA (Hoechst)	2·00	0·32	1·24-2·38
FT_3T	1·43	0·22	0·83-1·81

Table III *Pregnancy and oestrogen preparations (all values in nM/l)*

SUBJECTS WITH THYROID DISEASE

Hyperthyroidism

The range of serum T4 and T3 values observed in hyperthyroidism using the techniques listed is shown in table IV. Estimation of the serum T4 discriminated well between euthyroid and hyperthyroid subjects when this variable was measured by RIA. The CPBA (Thyopac-4), however, proved to be a poor discriminant. A small comparative study using a column technique for the estimation of T4 yielded values comparable to those obtained by RIA. It was clear (see figure) that the Thyopac-4 technique progressively underestimated T4 values greater than 125 nM/l. Estimation of serum T3, however, by all techniques discriminated absolutely between euthyroid and hyperthyroid subjects in the present study (table V).

	Hyperthyroidism (40)	Hypothyroidism (40)
Thyroxine		
CPBA (Thyopac-4)	114->260	<10-63
RIA (NC)	150-420	<10-67
RIA (RCC)	129->300	<10-75
Triiodothyronine		
RIA (NC)	2·76-> 7·5	<0·15-1·72
RIA (RCC)	4·45->15·0	<0·15-3·02
RIA (Hoechst)	2·76-> 9·0	<0·15-1·90

Table IV *T4 and T3 values (nM/l) in thyroid disease*

Hypothyroidism

The range of serum T4 and T3 values seen in hypothyroidism is shown in table IV. Serum T4 levels were low in approximately 90% of subjects using all the techniques studied in patients with symptomatic

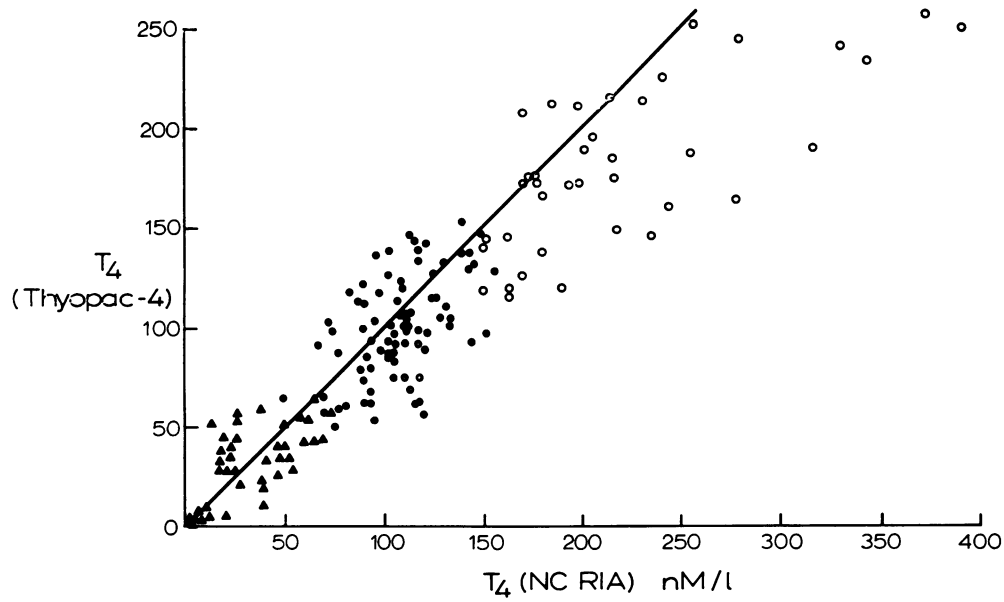

Figure *Serum T4 measured by RIA (NC) and CPBA (Thyopac-4) in 80 normal, 40 hypothyroid, and 40 hyperthyroid subjects. Line at 45°.*

	Sensitivity				*Specificity*
	Hyperthyroidism		*Hypothyroidism*		
	>97·5 percentile	*>2SD above mean*	*<2·5 percentile*	*>2SD below mean*	
T4					
CPBA (Thyopac-4)	65	65	88	91	93
RIA (NC)	100	100	97	97	96
RIA (RCC)	95	95	89	89	94
T3					
RIA (NC)	100 (by definition)	100	56	45	99
RIA (RCC)	100	100	54	51	98
RIA (Hoechst)	100	100	55	47	96

Table V *Sensitivity and specificity (per cent)*

hypothyroidism. The sensitivity of these techniques was not improved by calculating the free thyroxine index. Estimation of serum T3 proved to be an insensitive index of hypothyroidism. The sensitivity of these techniques was not materially increased by calculating a free triiodothyronine index (table V).

The specificity of all the techniques tested was high (93-99%) by comparison with the methods used for definition.

Discussion

The development of supraregional and regional radioimmunoassay services has placed hormone assays within the reach of every physician, and the availability of hormone assay kits has made it possible for radioimmunoassay techniques to be performed in nearly all hospital laboratories. No significant technical problems were encountered with any of the commercial kits used, and the precision and reproducibility were acceptable with all the assay techniques used.

The selection of an appropriate normal (reference) population poses many problems and no universally acceptable criteria have been established. The traditional use of blood donors, hospital personnel or the relatives of patients has many practical advantages, but the use of these groups does involve processes of selection which yield subjects who are frequently unrepresentative of the general population

in terms of age, sex, and socioeconomic group distribution. The normal ranges in this study have been established on a group of hospital personnel, but these results appear to be applicable to the general population since the ranges established using three of the techniques matched closely those seen in the Whickham Survey (Tunbridge *et al*, 1975). The sample seen in this survey was a 1 in 6 random sample taken from the electoral role in Whickham, County Durham. The response rate in the survey was 82·4%, and the sample closely matched the whole population of that community and the UK, in age, sex, and socioeconomic group structure. The range for normality was established on those subjects without any marker of thyroid disease or on any medication which would alter thyroid function tests (that is, subjects with thyroid antibodies, a raised TSH, a goitre, a personal or family history of thyroid disease, and those taking an oral contraceptive). There was good agreement between assays on the range of T4 in the reference population (60-150 nM/l). There was, however, one important and major difference in T3 values measured. The RCC T3 kit consistently measured higher values than the other two assays used which gave comparable results. The pack leaflet supplied with this kit acknowledges this difference and also indicates that quite markedly different values were obtained from reference populations in different centres. The explanation for these differences is not apparent, but it seems improbable that they can be attributed to regional variations in the light of the results of other assays of T3, but it is possible that they may represent differences in the selection of normal subjects. It is well established that non-thyroidal illness or cold surgery can lead to a significant reduction in measured T3 levels (Carter *et al*, 1974; Burr *et al*, 1975). The importance of each laboratory establishing its own normal range and appropriate care in the selection of a reference population (Holland and Whitehead, 1974) cannot be stressed too highly.

The expected increase in T4 and T3 levels was seen in pregnant subjects and those taking an oral contraceptive. The calculation of a free thyroxine or triiodothyronine index corrected the majority of values into the normal range. This calculation will not fully correct the thyroid hormone values in those subjects with the highest thyroxine binding globulin (TBG) capacities.

Estimation of the serum T4 by radioimmunoassay proved an effective discriminant of hyperthyroidism in the majority of cases. Estimation using the Thyopac-4 kit was, however, less satisfactory, and if this technique is used (together with a T3 assay) the prevalence of T3 thyrotoxicosis will be overestimated. This assay progressively underestimated the T4 value by comparison with the column technique and radioimmunoassay at T4 concentrations greater than 125 nM/l. The major difference between this technique and the column method lies in the extraction procedure, and it seems probable that this step accounts for the differences. This underestimation of higher T4 values has been reported before using a CPBA technique with an extraction procedure. The estimation of T3 appears to be a highly sensitive index of hyperthyroidism, and this view is consistent with earlier reports (Britton *et al*, 1975; Shalet *et al*, 1975). It must, however, be admitted that the diagnostic definition used inevitably enhances the apparent sensitivity of these techniques. It is unfortunate that there is no other acceptable, independent, and sensitive laboratory criterion against which these techniques can be assessed—as with TSH in the diagnosis of primary hypothyroidism. T3 may also be raised in some clinically euthyroid subjects and these groups have been reviewed elsewhere (Evered, 1975). It is possible that hyperthyroidism could exist with a normal T3 in a patient with a reduced TBG capacity or in one with a serious illness depressing the T3 concentration. Calculation of a free triiodothyronine index should correct the T3 value in the former situation and a thyrotrophin releasing hormone (TRH) test would be required to confirm the diagnosis in the latter case (Evered, 1974).

Serum T4 estimation appears to be an adequate discriminant of symptomatic hypothyroidism in the majority of patients. The low discriminant value of T3 estimation observed confirms earlier reports (Evered *et al*, 1973). Estimation of TSH remains the most sensitive index of primary hypothyroidism (Evered *et al*, 1973).

The diagnostic specificity of these techniques is satisfactory for routine purposes. The development of routine radioimmunoassay procedures for TSH estimation and the availability of synthetic TRH have made it possible to resolve diagnostic problems adequately.

References

Britton, K. E., Quinn, V., Brown, B. L., and Ekins, E. P. (1975). A strategy for thyroid function tests. *Brit. med. J.*, **3**, 350-352.

Burr, W. A., Griffiths, R. S., Black, E. G., Hoffenberg, R., Meinhold, H., and Wenzel, K. W. (1975). Serum triiodothyronine and reverse triiodothyronine concentrations after surgical operation. *Lancet*, **2**, 1277-1279.

Carter, J. N., Eastman, C. J., Corcoran, J. M., and Lazarus, L. (1974). Effect of severe chronic illness on thyroid function. *Lancet*, **2**, 971-974.

Evered, D. C. (1974). Diseases of the thyroid gland. In *Investigation of Endocrine Disorders. Clin. Endocr. Metab.*, **3**, 425-450.

Evered, D. C. (1975). The interrelationship between tri-

iodothyronine and thyroxine in thyroid disease. In *Thyroid Hormone Metabolism*, edited by W. A. Harland and S. J. Orr, pp. 335-345. Excerpta Medica. Amsterdam. Academic Press, London.

Evered, D. C., Ormston, B. J., Smith, P. A., Hall, R., and Bird, T. (1973). Grades of hypothyroidism. *Brit. med. J.*, **1**, 657-662.

Evered, D. C., Vice, P. A., and Clark, F. (1976). The investigation of thyroid disease. Radiochemical Centre. Medical Monographs No. 9.

Hall, R., Amos, J., and Ormston, B. J. (1971). Radioimmunoassay of human serum thyrotrophin. *Brit. med. J.*, **1**, 582-585.

Hesch, R. D., and Evered, D. C. (1973). Radioimmunoassay of triiodothyronine in unextracted human serum. *Brit. med. J.*, **1**, 645-648.

Holland, W. W., and Whitehead, T. P. (1974). Value of new laboratory tests in diagnosis and treatment. *Lancet*, **2**, 391-394.

Murphy, B. P. (1965). The determination of thyroxine by competitive protein-binding analysis employing an anion-exchange resin and radiothyroxine. *J. Lab. clin. Med.*, **66**, 161-167.

Seligson, H., and Seligson, D. (1972). Measurement of thyroxine by competitive protein binding. *Clin. chim. Acta.*, **38**, 199-205.

Shalet, S. M., Beardwell, C. G., Lamb, A. M., and Gowland, E. (1975). Value of routine serum-triiodothyronine estimation in diagnosis of thyrotoxicosis. *Lancet*, **2**, 1008-1010.

Tunbridge, W. M. G., Evered, D. C., Hall, R., Appleton, D., Brewis, M., Clark, F., Evans, J. G., Young, E., Bird, T., and Smith, P. (1975). Thyroid disease in an English Community. In *7th International Thyroid Conference, Boston.* (Excerpta Medica, Int. Congr. ser., 361). American Elsevier, New York.