

Serum protein-bound iodine or total thyroxine

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My brief is to discuss the serum protein-bound iodine (PBI) and total thyroxine (T_4) as tests of thyroid function.

I should like first to emphasize that when we measure serum PBI or T_4 we are looking at only one aspect of circulating thyroid hormone. There is the additional factor of the unsaturated sites on the thyroxine-binding proteins and it is the relationship between the two which defines the concentration of circulating free hormone, the determinant of

thyroid status. This is shown in diagrammatic fashion in fig 1 which illustrates the changes that occur in thyrotoxicosis and hypothyroidism and in those situations where there are alterations in thyroxine-binding protein capacity, such as the increase in thyroxine-binding globulin (TBG) that accompanies pregnancy. The PBI and T_4 and their variants define merely one aspect of thyroid hormone secretion and there are other tests available which may be better for evaluating thyroid status, for example, free thyroxine in serum (FT₄) (Ingbar, Braverman, Dawber, and Lee, 1965; Sterling and Brenner, 1966), total and free serum triiodothyronine (Sterling, Bellabarba, Newman, and Brenner, 1969; Chopra, Solomon, and Beall, 1971; Mitsuma, Nikei, Gershengorn, and Hollander, 1971; Jacobs, Mackie, Eastman, Ellis, Ekins, and McHardy-Young, 1973), and urinary thyroxine and triiodothyronine (Chan and Landon, 1972; Chan, Besser, Landon, and Ekins, 1972; Burke, Shakespear, and Fraser, 1972) (table I).

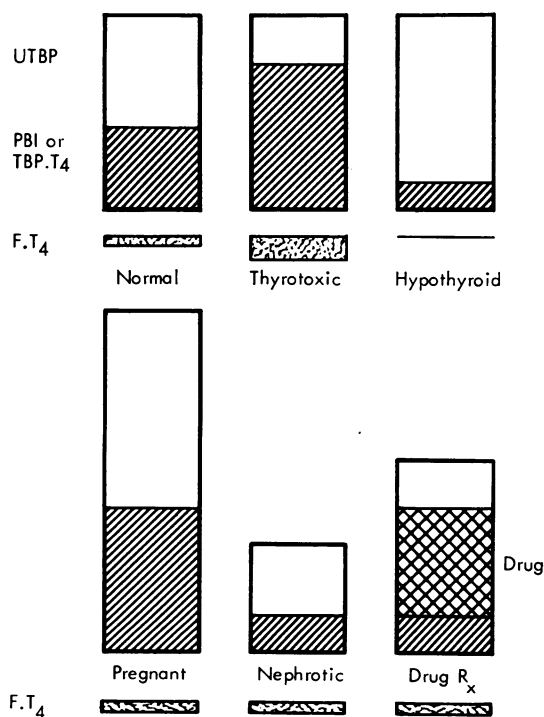


Fig 1 Diagrammatic representation of T_4 transport in blood in various conditions showing unsaturated sites on thyroxine-binding proteins (UTBP), T_4 bound to thyroxine-binding proteins (PBI or TBP.T₄) and free thyroxine (FT₄).

- 1 Serum PBI^{1,2,7} (BEI, thyroxine—by column, CPBA—saturation analysis)
- 2 Serum PBI^{1,2,1}.
- 3 T_3/T_4 uptake tests (RBC, resin, dextran gel, charcoal, dialysis)
- 4 Free thyroxine (dialysis, gel filtration, free thyroxine index—factor, effective thyroxine ratio—ETR)
- 5 Urinary T_4 , T_3
- 6 Serum T_3

Table I Thyroid hormone release tests

Serum Protein-bound Iodine

The PBI is usually accepted as a satisfactory indicator of thyroid status (Acland, 1971), but until the advent of the Technicon AutoAnalyzer, methodology was rather complex and involved essentially two alternative techniques of liberating the organic iodine; acid digestion/distillation or alkaline incineration (an acid digestion stage is used in the automated system). Recovery of iodine varies between 85 and 90% from acid distillation and 89 to 97% from alkaline ashing. The iodine liberated by digestion is estimated by its catalytic action on the

reduction of ceric sulphate by arsenious acid.

The major constituents of the PBI (table II) are the iodothyronines, ie, thyroxine and to a much smaller extent triiodothyronine. Variable amounts of the mono- and di-iodotyrosines, MIT and DIT (O'Halloran and Wellby, 1966) and probably some

Iodothyronines (thyroid hormones): T₃, T₄
 Iodotyrosines: MIT, DIT
 Inorganic iodide
 Iodoproteins: thyroglobulin, iodoalbumin

Table II *Constituents of PBI*

inorganic iodide are also measured, and if there are circulating iodoproteins, for example, thyroglobulin or iodoalbumin these may be detected by this technique, as may iodine-containing drugs. Specificity is therefore potentially poor and proves so in practice; Murphy, Pattee, and Gold (1966) stated that some 13% of PBI results in their laboratory were invalid because of contamination with non-hormonal iodine and Griffiths, Martin, and Spinella (1972) found 8% of grossly contaminated specimens with PBI values of over 20 $\mu\text{g}/100\text{ ml}$. Other workers (Lee, Tietz, and Martinez, 1972) claim that 25% of all samples reaching the laboratory were invalidated by iodine contamination from one source or another. Even when contamination is excluded there remain certain situations where the PBI may not correctly indicate thyroid status. These include alterations in thyroxine-binding protein capacity (so that the PBI is deviated in the same direction), the administration of drugs interfering with the chemical analysis (particularly gold and mercurials) and secretion of abnormal iodinated compounds from the thyroid (table III).

Altered TBG capacity
 Administration of iodinated drugs
 Administration of drugs interfering with chemical analysis
 Secretion of abnormal iodinated compounds

Table III *Causes of misleading PBI*

The drugs affecting the PBI include agents interfering with thyroxine synthesis or transport, and certain of the large group of iodinated compounds which are becoming increasingly implicated as a cause for invalid results. An example of the lack of specificity of the PBI is seen in figure 2 (Sönksen, Ekins, Stevens, Williams, and Nabarro, 1968). It shows the changes in PBI produced by the commonly taken drug cloioquinol together with its lack of effect on serum thyroxine measured by the displacement technique. The very high PBI took some three months to return to normal after cessation of cloioquinol while the serum thyroxine remained unchanged.

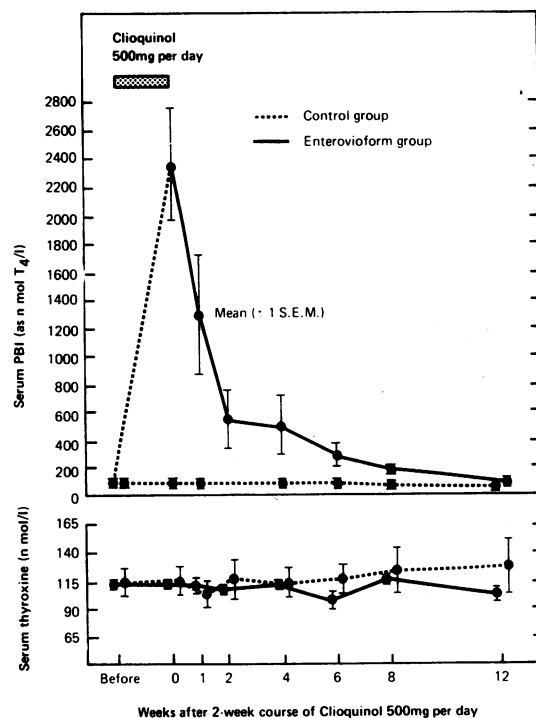


Fig 2 *Sequential serum levels of PBI and T₄ (mean \pm SEM) in healthy volunteers given cloioquinol (Enterovioform).*

The reproducibility (precision) of the PBI assay is fairly high. With acid distillation techniques the coefficient of variation (CV) is about 8% and with alkaline ashing it is about 6%. The CV is reduced markedly by automation (Horn, personal communication): at low levels of PBI (mean 3.5 $\mu\text{g}/100\text{ ml}$ or 70 nmol/l as T₄) the within-batch CV was 1.59%, at intermediate levels (mean 7.6 $\mu\text{g}/100\text{ ml}$ or 152 nmol/l as T₄) 0.77% and at high levels (mean 10.3 $\mu\text{g}/100\text{ ml}$ or 206 nmol/l as T₄) 1.19%. The values for the CV between batches were slightly higher (3.03%, 0.78%, and 1.38% respectively). Our own experience in Newcastle at two university hospital laboratories indicates a within-batch CV of between 2 and 3% overall.

If contaminated specimens are excluded the diagnostic efficiency of the PBI appears quite satisfactory and the overall accuracy is between 80 and 95% for this technique. However, up to 20% of patients with hypothyroidism can have a normal PBI.

There is virtually no information in the literature regarding the sensitivity of the PBI, but the speed of

assay (20 samples per hour by the AutoAnalyzer technique) and the relatively low cost are considerable advantages (Boss, Goolden, Gore, Lucas, and Owen, 1973).

Serum Butanol-extractable Iodine (BEI)

The butanol-extractable iodine (BEI) (Man, Kydd, and Peters, 1951) was introduced as an improvement over the PBI by reason of the greater specificity which resulted from the elimination of non-hormonal iodine from measurement. Unfortunately many of the organic iodinated compounds used in contrast radiography are not removed and the method does not appear to offer any great advantage over the PBI.

Serum Thyroxine (T_4)

A variety of methods are available for determining total T_4 in serum. (Total T_3 may also be measured by certain of these techniques.) They can be divided into two groups (table IV).

1 CHEMICAL

These methods involve column chromatography with ion-exchange resin (Pileggi, Lee, Golub, and

1 Chemical¹

- a Chromatographic isolation
 - Column (ion exchange resin; gel, silicad extran)
 - TLC
- b Solvent partition

2 Radioisotopic: ligand specific proteins (saturation analysis)²

- a Naturally occurring
 - Competitive protein-binding assay, radiostereo assay, displacement analysis
- b Induced (antibodies)
 - Radioimmunoassay

Table IV Measurement of serum total T_4 (T_3)

¹²⁵I content of separated material measured by conventional techniques therefore influenced by non-hormonal iodine
²Extraction usually necessary. Separation B/F by electrophoresis, ion exchange resin, dextran gel, charcoal, dialysis

Henry, 1961), silica (West, Chavré, and Wolfe, 1965), or dextran gel (Jones and Shultz, 1967), or thin-layer chromatography (West *et al*, 1965), or solvent partition (West, Chavré, and Wolfe, 1966). In all cases the hormonal iodine content of the appropriate fraction of serum is measured by conventional chemical means. They are therefore subject to the possibility of invalidation by non-hormonal iodine.

2 RADIOISOTOPIC

Use is made of ligand-specific proteins (saturation

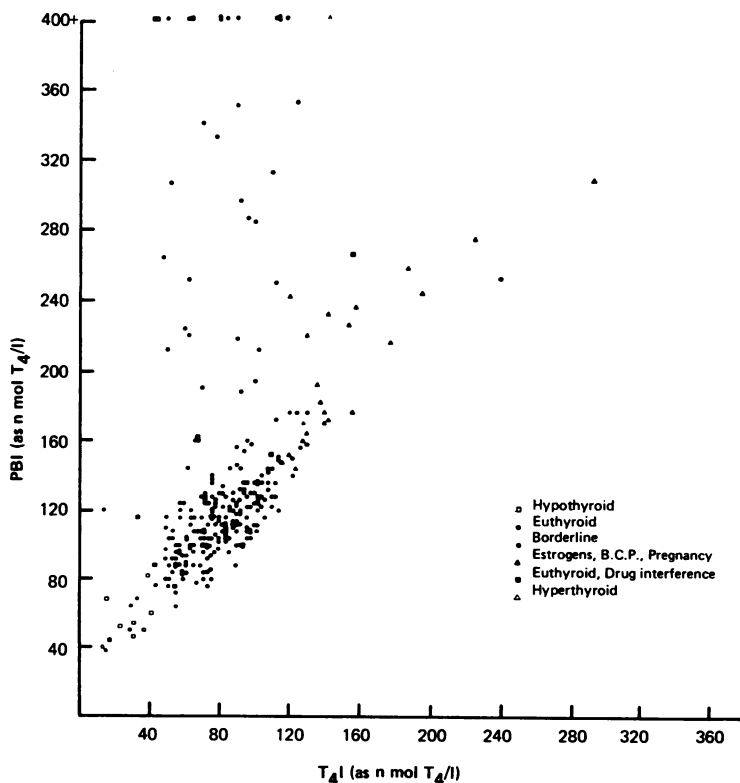


Fig 3 Relationship between values obtained with a modified T_4 -by-column and the PBI.

analysis). The proteins may be naturally occurring (TBG) and as such are used in competitive protein-binding assay (CPBA) (displacement assay). Extraction of hormone from serum is usually necessary and the eventual separation of the bound from the free fractions may be carried out by ion-exchange resin, dextran gel, charcoal (Murphy *et al*, 1966; Ekins, Williams, and Ellis, 1969; Maclagan and Howorth, 1969; Braverman, Vagenakis, Foster, and Ingbar, 1971) or automated dialysis (Griffiths *et al*, 1972). Several commercial kits for measuring T_4 by CPBA are available: Resomat T_4 (Mallinckrodt Chemical Works, St Louis, Missouri 63160, USA); Tetralute (Ames Company, Elkhart, Indiana 46514, USA); Tetrasorb (Abbott Laboratories, North Chicago, Illinois 60064, USA); Thyopac-4 (Radiochemical Centre, Amersham, Bucks) etc. Alternatively the binding proteins may be induced (antibodies) and T_4 measured by radioimmunoassay (Chopra, 1972; Mitsuma, Colucci, Shenkman, and Hollander, 1972).

Commercial kits adapted from the chromatographic method of Pileggi *et al* (1961) are also available from Biorad, Curtis, or Oxford (BioRad Laboratories, 32nd and Griffin Avenue, Richmond,

California 94804, USA, Curtis Chemical Division, Curtis Nuclear Corporation, 1948 East Fortysixth Street, Los Angeles, California 90058, USA, and Oxford Laboratories, 107 North Bayshore Boulevard, San Mateo, California 94401, USA.) Reproducibility appears to be quite satisfactory with a coefficient of variation within batch of up to 3% but they do have the problem of contamination already mentioned. Figure 3 (Lee *et al*, 1972) shows the relationship between protein-bound iodine and thyroxine iodine as determined by column chromatography and demonstrates that much of the contaminating iodine is removed using this method. Figure 4 (Lee *et al*, 1972) makes a comparison of the column chromatographic method with that using competitive protein-binding assay and shows good correlation between the two. Solvent partition (West *et al*, 1966) appears rather complex technically and again the problem of iodine contamination is not entirely eliminated.

The most commonly used method in the radioisotopic group is that of involving competitive protein-binding assay (table IV). Figure 5 (Ekins *et al*, 1969) illustrates the principle of the procedure diagrammatically. As a first step thyroxine is

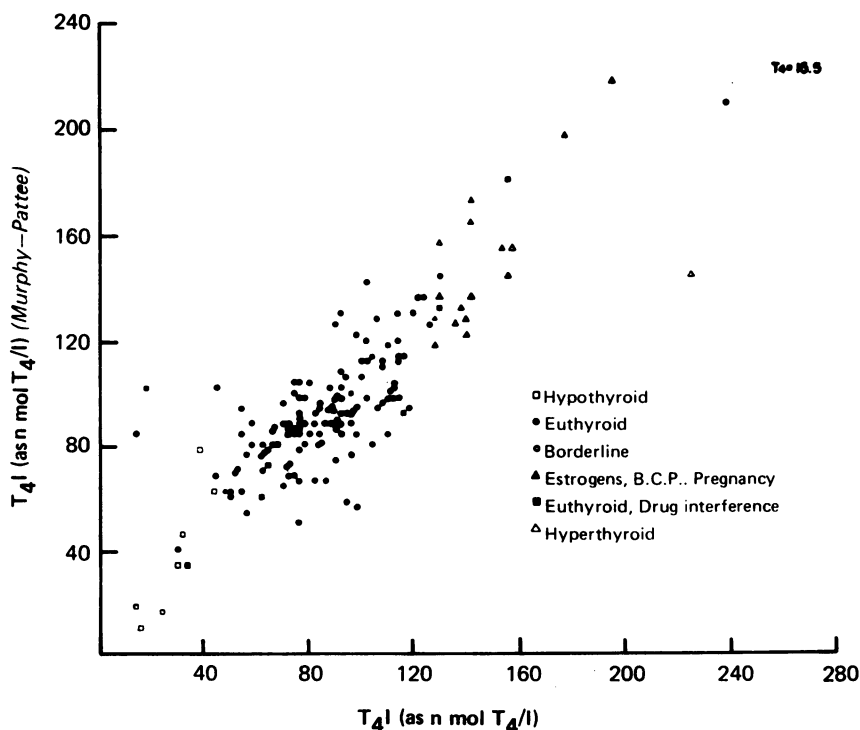
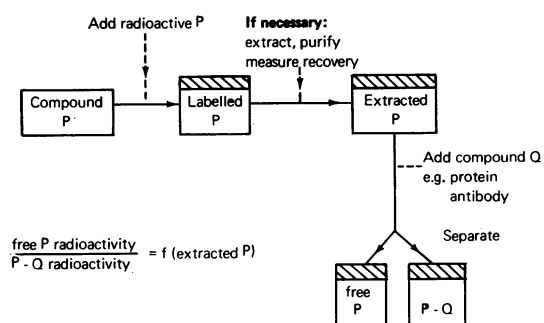


Fig 4 Relationship between values obtained for T_4 by competitive protein-binding assay and by a modified T_4 -by-column.



(Ekins, Williams & Ellis. *Clin. Biochem* 1969, 2, 253-288)

Fig 5 Fundamental principle of saturation assay.

extracted from serum (tracer amounts of labelled thyroxine may be added before this so that a measure of the efficiency of extraction can be made). Extracted thyroxine is then added to some binding protein (TBG) together with labelled thyroxine and the bound/free ratio of isotope is determined after separation of the two fractions. If constant amounts of binding protein and labelled T_4 are used in the system the ratio of bound to free isotope will depend on the original amount of thyroxine in the sample. The unknown can then be compared with the results from known standards determined simultaneously and plotted graphically as a standard curve. Extraction of thyroxine can be carried out by various agents (butanol, ethanol¹, dimethoxypropane, ethanol-water-ammonia, or by alkali-stripping at high pH). Extraction efficiency using ethanol is of the order of 77% ($\pm 4\%$) (own observations) but as has been pointed out (Ekins *et al*, 1969) it can vary between sera and for this reason a check on each occasion may be desirable. The ethanol-water-ammonia system is more efficient (98% extraction) but the residue is poorly soluble. If the extract is not evaporated to dryness a flatter slope is obtained for the standard curve with a resultant decrease in sensitivity. Alcoholic extraction should be carried out above pH 9 or esters of thyroxine are formed which may invalidate results (Bellabarba and Sterling, 1969). An analysis of solvent extraction efficiency has been made by Watson and Lees (1973).

Radioimmunoassay of thyroxine in unextracted serum has been reported by Chopra (1972) and by Mitsuma *et al* (1972). Thyroxine binding to TBG is blocked by anilino-naphthylsulphonic acid and

binding to thyroxine-binding pre-albumin (TBPA) by barbital. Chopra uses a rabbit antithyroglobulin technique with goat antirabbit serum as the second antibody and Mitsuma a rabbit antithyroxine-albumin conjugate with subsequent separation by charcoal/dextran (an immunoassay for triiodothyronine being performed concurrently).

The specificity of methods using saturation analysis is much greater than that of PBI estimation (Ekins *et al*, 1969). However, certain drugs do interfere with these methods, for example, the thyroxine analogues dextrothyroxine (D- T_4), triiodothyronine and diiodothyronine, as well as phenylbutazone and diphenylhydantoin. The competition by D- T_4 is reported as 100% and by T_3 as 30%, although Ekins *et al* (1969) found an eight-fold difference of reactivity between T_4 and T_3 . Diphenylhydantoin 20 $\mu\text{g/ml}$ is equivalent to a T_4 concentration of 1 nmol/l (0.75 ng/ml). In the radioimmunoassay reported by Mitsuma *et al* (1972) there is very little cross reaction with triiodothyronine. As with PBI, total thyroxine will vary with a change in TBG capacity and in this situation will have the same limitations.

Some information on the reliability or precision of the various methods available for measuring total serum thyroxine, together with their normal ranges and correlation with other techniques, is shown in table V. It can be seen that precision varies con-

Method	Precision (CV)		Normal Range (n mol T_4 /l)	Correlation	
	Within Batch	Between Batch		PBI	CPBA
Chemical Chromatographic Column	2.8	16.7	50-120	0.30	0.75
		62-148			
		86-174			
	7.1	62-148			
Thin layer Solvent partition	14.7	62-146			
Radioisotopic CPBA					
	Murphy	32.3 11.2 12.1	52-144	0.82	
Ekins	4.3	12.0	58-150		
	3.8	7.8			
	6.0	5.8			
MacLagan	7.0	10.0	52-142	0.83	
Nakajima Braverman	27.3	5.1	72-156	0.95	
	4.4		78-138		0.95
	2.4				
Seligson RIA	4.3	3.4	60-130		0.94
		7.1	58-172		

Table V Comparison of various methods for measuring serum total T_4 in terms of precision, normal range, and correlation with other methods

¹Ethanol at room temperature has recently been shown to extract from serum a thyroxine-binding factor, probably TBG, which produces false low results by CPBA. See Goldie *et al* (*J. clin. Path.*, 1974, 27, 74) and Irvine (*J. clin. Endocr. Metab.*, 1974, 38, 468). ED.

siderably. In the chemical methods, the within-batch CV is only 3% while the between-batch CV is as high as 17%. With CPBA methods values for CV varied from 32% overall at low values of serum thyroxine to 12% at high values (Murphy and Pattee, 1964). Ekens *et al* (1969) found a within-batch CV of 4.3% at low thyroxine levels whereas Braverman *et al* (1971) obtained one of 27.3%, although at higher levels a much lower variability was noted (2.4%). Between-batch variability was usually not much greater than that within a batch. In a useful analysis of four commercial kits based on CBPA (Watson and Lees, 1973) a CV of the order of 5% was obtained, which was superior to that calculated for their PBI analysis (CV 10%). With Thyopac-4, Badman and Platten (1973) found within-batch CV ranging from 22% at a T₄ level of 26 nmol/l (2.0 µg/100 ml) and 4% at 208 nmol/l (16.0 µg/100 ml); lower values for CV resulted if autodilution was carried out, between batch CV being 9% for the manual and 5% for the autodiluter method. With the same kit in our own laboratory overall CV for T₄ values was 17% between 0 and 65 nmol/l (0 and 5 µg/100 ml) and was 5% between 65 and 130 nmol/l (5 and 10 µg/100 ml) and between 130 and 260 nmol/l (10 and 20 µg/100 ml).

The diagnostic efficiency of the various methods for measuring T₄ is of the order of 90% (mean of several published figures) and good correlation is found with the PBI if contaminated samples are excluded (correlation coefficient 0.82-0.95) (table V).

The sensitivity of thyroxine assays is rarely reported but Ekens *et al* (1969) were able to estimate as little as 65 to 190 pg/ml (85 to 247 pmol/l) of incubation mixture.

The relative economics (time taken to perform test, cost of material) of the T₄ and PBI assays are difficult to establish. Our own experience and that of Horn (personal communication) and Boss *et al* (1973) favours the PBI but Watson and Lees (1973) consider the latter obsolete, and certainly with the advent of increasing contamination of serum samples with iodine from various sources the PBI is becoming less attractive as a routine screening test for the evaluation of thyroid status. Toft, Seth, Kirkham, Marshall, and Irvine (1973) have recently made an assessment of several *in-vitro* thyroid function tests on 100 consecutive patients referred to a thyroid clinic. Included in the tests were the PBI and serum T₄. They found that the T₄ had a much higher diagnostic accuracy than the PBI as the latter gave spuriously elevated results in 48% of euthyroid patients, presumably due to an excess intake of iodine-containing compounds. These results suggest that if the PBI were to be replaced by the more specific serum T₄ as the first-line *in-vitro* thyroid

function test, a further investigation of many euthyroid patients would prove unnecessary.

The recently introduced radioimmunoassay methods for determining thyroid hormones are still being evaluated but it would seem that they offer advantages over established methods in terms of diagnostic accuracy and specificity; they too can be adapted to a mass production system.

Conclusions

The serum protein-bound iodine (PBI) has been used for a number of years as a generally satisfactory indicator of the level of thyroid function. Its major drawback is lack of specificity. However, the method is precise, sensitive and fairly inexpensive and can be automated.

Techniques for separating thyroxine by butanol extraction or column chromatography have improved specificity but may still be in error if organic iodinated compounds are present in serum.

With the advent of a specific assay of serum thyroxine by saturation analysis (competitive protein-binding assay and radioimmunoassay) there has been a swing away from the earlier methods. These new procedures have their own problems in that thyroxine usually has to be extracted from serum first and this may entail loss of accuracy. In addition, their precision is generally less satisfactory than that of PBI, they are technically more complex, and all require equipment for counting radioactivity.

On balance it would appear that for general clinical use the PBI remains the single most useful test of thyroid status at the present time. If invalid because of contamination, thyroxine can be determined after a separation procedure.

Radioimmunoassay of thyroid hormones seems to be the method of choice in the near future.

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