COMPARISON OF BIOLOGICAL AND CHEMICAL ASSAY OF THYROID

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The biological and chemical examination of thyroid samples has demonstrated the inadequacy of the B.P. assay, which is based upon the determination of so-called "thyroxine iodine". It is suggested that, in the absence of specific methods for determining thyroxine and tri-iodothyronine in thyroid, the chemical assay should be replaced by a biological assay.

The mouse anoxia method is shown to be suitable for this purpose. The potencies of samples assayed thus over the past 3 years, together with chemical data and clinical comments are presented. The relative activities of the thyroid constituents 3,5,3′,5′-tetra-iodo-L-thyronine (L-thyroxine), 3,5,3′-tri-iodo-L-thyronine, 3,5-di-iodo-L-tyrosine, 3-mono-iodo-L-tyrosine, as determined by the mouse anoxia method, are in good agreement with their reported clinical effectiveness.

During 1958 it was found in these laboratories that the chemical assay for thyroid (B.P. 1958) gave results not always in agreement with the physiological activity in man. Most of the discrepancies were attributed to lactose used as a diluent which interfered with the assay, thus confirming the work of Doery (1945).

As a result, the chemical assay for thyroid was amended (B.P. 1958 Addendum 1960) to conform with that for thyroid tablets, in which the lactose is first removed by washing.

Some discrepancies, however, still remained, and were ascribed to differences in the relative proportions of biologically active constituents such as thyroxine and tri-iodothyronine. The greater activity of the latter is not taken into account in the B.P. assay.

This led us to examine several biological methods of assay for one suited to the routine standardisation of commercial samples and one which would give a better indication of clinical activity.

Since there may be species differences between thyroid derived from the three main commercial sources, ox, pig and sheep, which could lead to difficulties in their chemical or biological assay, samples from all three sources were examined.

Experimental Methods

Chemical

Determination of "Thyroxine Iodine" by the method described in B.P. 1958 and Addendum 1960.

Determination of Total Iodine, as for "thyroxine iodine", but ignoring the acid precipitation (B.P. 1958).

Biological

Oxygen Consumption Method in rats. Gaddum (1930).

Goitre Prevention Method in rats. Dempsey and Astwood (1943). Male albino rats, 100–140 g. in weight were used: the animals were killed by asphyxiation with carbon dioxide.

Mouse Anoxia Method. The method of Smith, Emmens and Parkes (1947) was modified. Male albino mice in a 2 g. weight range, within the limits of 15–20 g. were divided into 6 equal groups, each of 16–20 animals. A 3 + 3 assay design was used. Occasionally a further similar

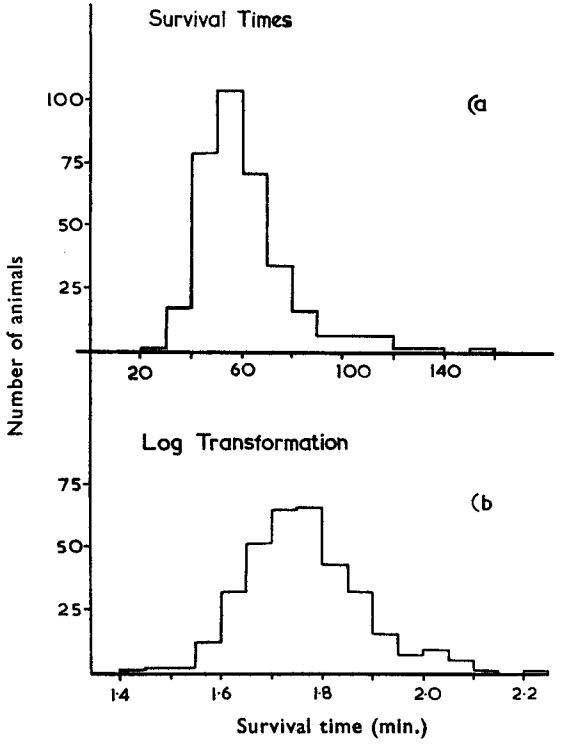

FIG 1. a. The distribution of survival times of 343 animals which received the same dose of thyroid.
b. Distribution of a log transformation of the data used in Figure 1a.

group was kept as a control. Using a dose ratio of 2:1, doses of the standard and test preparations were given by subcutaneous injection on three alternate days, each animal in a group receiving the same dose in 0·5 ml. solvent irrespective of weight. Powdered thyroid was administered in suspension, and thyroglobulin in solution, in distilled water. The doses used were equivalent in effect to 20, 10 and 5 μg. of sodium-L-thyroxine per mouse per injection.

L-Thyroxine sodium salt (anhydrous), 3,5,3′-tri-iodo-L-thyronine sodium salt, 3,5-di-iodo-L-tyrosine and 3-monoiodo-L-tyrosine were dissolved in 0·1N sodium hydroxide solution and diluted to give a final alkali concentration of approximately 0·001N.

The experiment was conducted at 23° on the second day after the last injection. The mice were put into separate blood transfusion bottles (volume 585 ± 4 ml.) which were sealed with rubber stoppers. Each bottle contained 50 ml. of dry sawdust. The time of survival to the nearest half minute was recorded for each mouse, commencing at the closure of the bottle and terminating at the last visible respiration, which was usually preceded by marked convulsions. At the end of the experiment the mice were weighed to the nearest 0·5 g.

TABLE I

CHEMICAL AND BIOLOGICAL ASSAYS AND CLINICAL ASSESSMENTS OF COMMERCIAL SAMPLES OF THYROID

Sample No.	Total iodine	"Thyroxine iodine"		Biological determinations			Clinical effect
	As a percentage by weight of the stated amount of thyroid present			Potency expressed as per cent of thyroid tablets No. 13	Limits of error (P = 0·95)	Method	
		Washed	Unwashed				
Thyroid tablets							
1				37		Mouse anoxia	—
2				41		,,	—
3				72		,,	—
4				37		,,	—
				34		Rat goitre	
				43		Rat O_2 consumption	
5		0·050		39		Mouse anoxia	—
6				44		,,	—
7				109		,,	+
8				116	86–155	,,	
9		0·104		119	89–159	,,	—
10		0·102		128	95–171	,,	+
11		0·094		113	84–151	,,	+
12		0·074		73	48–102	,,	
13 (Standard)	0·328	0·102		100	—		
Powdered Thyroid							
14		0·055	0·140	19	14–26	,,	—
15		0·075	0·120	30	15–46	,,	
16		0·069	0·097	64	46–94	,,	
17		0·081	0·095	89	63–123	,,	
18	0·260	0·069	0·101	85	61–113	,,	
19	0·265	0·092		102	74–142	,,	

— = Ineffective. + = Satisfactory

Before the actual assay a preliminary test to determine the approximate activity of the material was usually performed on 6 groups of animals with 3 to 5 mice per group, at dose levels equivalent to 40, 10 and 2·5 μg. of sodium-L-thyroxine.

The standard used for all assays was a production batch of ox thyroid tablets, 2 gr. (Sample 13, Table I).

RESULTS

Mouse Anoxia Method: Distribution of Survival Times

Figs. 1a and 1b show the distribution of survival times and log survival times respectively of a group of 343 mice which had received the same dose of thyroid on 3 alternate days in a series of mouse anoxia tests.

Taking the χ^2 class intervals, mean $\pm \frac{1}{4}$, $\frac{1}{2}$, 1 and 1·5 times the standard deviation, the distribution of survival times in min. is significantly skew ($P < 0{\cdot}001$, $\chi^2 = 55{\cdot}6$ with 9 degrees of freedom). The distribution of the log survival times is not significantly skew ($0{\cdot}1 > P > 0{\cdot}05$ and $\chi^2 = 15{\cdot}10$ with 9 degrees of freedom) and this is in agreement with the findings of Basil, Somers and Woollett (1950). All the mouse anoxia experiments have therefore been calculated on the basis of log survival time. Although the weights of the mice were initially within a 2 g. weight range, at the end of the test the range had widened such that it was found necessary to correct all responses for body weight by covariance analysis.

TABLE II

CHEMICAL AND BIOLOGICAL ASSAYS OF THYROID SAMPLES FROM OX, PIG AND SHEEP

	Thyroid sample		
	Ox	Pig	Sheep
Chemical Assays (Values as percentage by weight)			
Total iodine	0·345	0·581	0·475
"Thyroxine iodine"	0·107	0·173	0·155
Thyroxine*	0·130	0·182	0·118
Tri-iodothyronine*	0·034	0·076	0·034
Biological Assays			
Mouse anoxia method (Potency in terms of "ox" sample)	100	255	113
Limits of error ($P = 0{\cdot}95$) as percentage of "ox" sample	—	162–399	77–167

* Results obtained by Mr. Devlin, Canadian Department of National Health and Welfare and published by permission of Dr. N. R. Stephenson.

Comparison of Biological, Chemical and Clinical Data on Thyroid Samples from Various Sources

Table I shows the results obtained in the chemical and biological assay of 13 samples of thyroid tablets, and 6 samples of powdered thyroid obtained from various sources. The table also includes clinical comments where these are known.

A marked variation occurs in the biological activity of the samples, and this is reflected in their clinical effects. There is also variation in the results of the chemical assay: such figures moreover are not always related to biological activity.

Studies on Thyroid from Ox, Pig and Sheep

Table II shows the results of biological and chemical assays obtained on samples of thyroid from ox, pig and sheep.

The method used by Mr. Devlin for the estimations of thyroxine and tri-iodothyronine is based upon the enzymatic hydrolysis of the thyroid followed by chromatographic separation, and spectrophotometric determination utilising the reaction with ceric sulphate-arsenious acid reagent.

In the chemical assays the results obtained for the sample of pig thyroid are higher than those for the other two samples. Further, the biological potency of the sample of pig thyroid is more than twice that of the other two materials.

The Relation Between Chemical and Biological Assays

Table III shows ratios of biological potency to "thyroxine iodine" (B.P. method) and to total iodine (U.S.P. method) calculated on the basis of taking that of the standard as unity. A significant deviation from unity

TABLE III

RELATIONSHIP BETWEEN CHEMICAL AND BIOLOGICAL ASSAYS

Sample No.	Biological potency "Thyroxine iodine" (B.P.)	Biological potency Total iodine (U.S.P.)
	Limits of error (P = 0·95)	
9	1·16 (0·87–1·56)	
10	1·28 (0·95–1·71)	
11	1·23 (0·92–1·64)	
12	1·01 (0·66–1·41)	
13 (Standard)	1·00 –	1·00
14	0·36 (0·27–0·48)	
15	0·41 (0·20–0·63)	
16	0·95 (0·68–1·39)	
17	1·11 (0·79–1·55)	
18	1·26 (0·90–1·67)	1·07 (0·76–1·42)
19	1·14 (0·83–1·58)	1·27 (0·92–1·75)
Ox	used as "standard" 1·00	1·00
Pig	1·58 (1·004–2·47)	1·51 (0·96–2·37)
Sheep	0·78 (0·53–1·15)	0·82 (0·56–1·21)

(samples 14, 15 and pig thyroid) indicates that the chemical method of assay (B.P.) gives results which are not in agreement with biological potencies determined by the mouse anoxia method. In no other instance was there a significant difference.

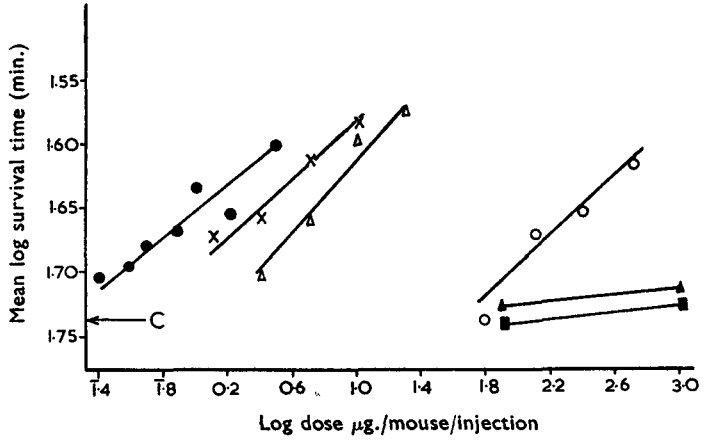

FIG. 2. Dose response curves of thyroid and thyroid constituents using the mouse anoxia method.

△ L-Thyroxine sodium salt. ● 3,5,3′-Tri-iodo-L-thyronine. (Na salt). ■3,5-Di-iodo-L-tyrosine. ▲ 3-Mono-iodo-L-tyrosine. X Thyroid tablets sample No. 13 (Standard). ○ Thyroglobulin. C Control (untreated mice).

The responses for thyroxine tablets sample No. 13 are plotted on the basis of sodium thyroxine content calculated from B.P. 'thyroxine iodine' values.

Relative Potencies of Thyroid and Thyroid Constituents

Using the mouse anoxia method the relative activities of several thyroid constituents were obtained. Fig. 2 shows the mean log survival times of groups of 10–20 mice plotted against log dose in μg./mouse for each substance. The dose response curves of active materials show no significant deviation from parallelism. 3-Mono-iodo-L-tyrosine and 3,5-di-iodo-L-tyrosine are inactive. 3,5,3′-Tri-iodo-L-thyronine sodium salt is 4·5 times as potent as L-thyroxine sodium salt (w/w).

Data obtained for sample No. 13 are plotted in terms of the expected sodium thyroxine content, calculated from the "thyroxine iodine" (B.P. 1958). The activity of the sample is 206 per cent of that expected (limits of error, P = 0·95 from 135–318 per cent). This was confirmed using the rat goitre prevention method (potency 246 per cent, limits of error, P = 0·95, from 116–409 per cent).

The potency of the thyroglobulin sample in terms of L-thyroxine is 2 per cent.

Discussion

The basis for the standardisation of commercial thyroid preparations as described in the British and United States pharmacopoeias is a chemical assay and occasionally marked differences have been reported between the results of the chemical and biological assays (Gaddum and Hethering-ton, 1931).

The method of assay in the British Pharmacopoeia 1958, Addendum 1960, takes the acid insoluble iodine content (so called "thyroxine iodine") as the measure of activity, whilst that of the United States Pharmacopeia XVI uses the total iodine in organic combination.

The present method of assay, first described in the British Pharmacopoeia 1932, Addendum 1936, is based upon the work of Harington and Randall (1929). It involves the separation of clinically active, acid insoluble thyroxine from clinically inactive, acid soluble di-iodotyrosine. This takes no account of the very potent thyroid hormone, 3,5,3′-tri-iodo-L-thyronine, discovered by Gross and Pitt-Rivers (1952).

The method described in the U.S.P. XVI is based upon a relation, empirically determined, between the total iodine content of a sample and its physiological activity, as shown by its effect on myxoedema in humans by Means, Lerman and Salter (1933). It is assumed that in samples from different species there is the same proportion of thyroxine, tri-iodothyronine and inactive iodinated organic compounds such as 3-mono-iodo-L-tyrosine and 3,5-di-iodo-L-tyrosine. 3,3′-di-iodothyronine and 3,3′,5′-tri-iodothyronine also have been found in the thyroid (Roche, Michel, Wolf and Nunez, 1956) and are inactive (Gemmill, 1956, and Stasilli, Kroc and Meltzer, 1959). The results obtained in Dr. Stephenson's laboratory for the thyroxine and tri-iodothyronine contents of 3 samples of thyroid from ox, pig and sheep (Table II) do not indicate that this is so.

The results of the chemical assay of three thyroid powders (Table II), are not in good agreement with the biological potencies, and with the pig thyroid sample the difference is statistically significant.

In most instances in Table I the biological potencies relative to the working standard are of the same relative order as the "thyroxine iodine" values, although in two thyroid samples, numbers 14 and 15, the differences are significant, $P < 0.05$ (Table III). In the few instances where total iodine values are quoted they are in reasonable agreement with the biological potencies. It is possible therefore that in some thyroid samples the total organically bound iodine is related to biological potency. This cannot obtain where decomposition of active constituents or adulteration with iodinated casein has taken place; the ratio of organically bound iodine to physiological activity in iodinated casein is dependent upon the conditions under which the iodination is carried out.

Since the biological potency of the standard (sample No. 13) is 206–246 per cent of the potency expected from analysis of "thyroxine iodine" (B.P. 1958) it follows that for most of the samples examined "thyroxine iodine" does not provide a valid indication of potency in terms of L-thyroxine. This confirms the observations of Frieden and Winzler (1948).

Therefore, in our opinion, neither of the methods at present described in the British Pharmacopoeia or the United States Pharmacopeia for the assay of thyroid is valid, as both are capable of providing misleading indications of physiological activity. In theory, the quantitative estimation of the two important thyroid hormones, 3,5,3′-tri-iodo-L-thyronine and 3,5,3′,5′-tetra-iodo-L-thyronine (L-thyroxine) should provide a better chemical assay for thyroid.

The results in Fig. 2, obtained by the mouse anoxia method, show that the activities of several iodine-containing compounds found in the thyroid are in good agreement with their reported clinical effectiveness.

3,5,3′-Tri-iodo-L-thyronine, which was approximately 4·5 times as effective as L-thyroxine in mice, has a potency variously reported as 3–10 times more effective than thyroxine in man, in increasing oxygen consumption (Gross, Pitt-Rivers and Trotter, 1952, Asper, Selenkow and Plamondon, 1953) and 5–10 times more potent than L-thyroxine in man in its effect on plasma cholesterol levels (Boyd and Oliver, 1960). The clinical inactivity of 3,5-di-iodo-L-tyrosine (Strouse and Voegtlin, 1909) is also reflected in the mouse anoxia test.

The present studies show therefore that the mouse anoxia method gives results which are in good agreement with clinical activity. The assay is easy to perform, the apparatus required is cheap and simple, and results are obtained within 7 days. Over a period of 3 years they have been reasonably consistent and the statistical weight per mouse has remained at about 1·5. We believe that the mouse anoxia method is suitable for the routine standardisation of thyroid.

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The papers were presented by MR. K. L. SMITH and MR. WEBB. The following points were made in the discussion.

Not all strains of mice were suitable for the mouse anoxia method. The mice needed to be kept at constant temperature throughout the assay. When compared with a reference thyroid sample in mice, oral administration of pig thyroid had given a potency within 5 per cent of that obtained by the subcutaneous route. Lactose should be replaced as a diluent by calcium phosphate which does not interfere with the chemical assay. There are formidable manipulative difficulties, caused largely by the three filtrations, in the B.P. assay, and inconsistent results have been encountered although there had been rigid adherence to B.P. conditions. A method of determining the physiologically active constituents of thyroid, viz., thyroxine and 3,5,3-tri-iodo-thyronine by paper chromatography was being investigated. Two difficulties had to be overcome; 1, the thyroid protein must be hydrolysed quantitatively to amino-acid and 2, there must be a minimum of di-iodination in the assay.