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MEASUREMENT OF THYROID ACTIVITY BY THE MOUSE ANOXIA METHOD

BY

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Animals given substances with thyroid activity show a reduced resistance to anoxia (Duran, 1920), and Smith, Emmens, and Parkes (1947) have used this effect in a method of thyroid assay based on the survival times of mice in closed jars. We have used it for comparing the activities of thyroxine and some of its derivatives. We find it relatively simple to carry out; the results it gave have been confirmed in clinical trials, both with sodium thyroxine and with the N-formyl derivative (Hart and Maclagan, 1950).

We were particularly interested in two questions: has solubility an important effect on the activity of thyroxine and has thyroxine enhanced activity in the dried glandular extract? For this purpose a number of preparations of synthetic thyroxine and its derivatives were prepared (Clayton and Hems, 1949) and examined for thyroid activity. This paper records the results obtained biologically.

PROCEDURE

Throughout the whole of the experiment the mice were kept in a thermostatically controlled incubator room at a temperature of 25.5° ± 1°. For each experiment use was made of male fawn mice of the GFF strain, weighing between 18 and 24 grammes, distributed into groups of twenty animals to give uniform body weight representation. Usually six groups, at ascending dose levels, were employed, three for the standard and three for the test preparations; a further twenty mice were kept as controls.

The doses were administered on three alternate days, every animal in a group receiving the same dose irrespective of body weight and the volume of the solution injected being the same for all groups. All compounds for assay were either suspended in 0.9 per cent sodium chloride solution or dissolved in 0.01 M-sodium carbonate solution containing 0.9 per cent sodium chloride.

On the second day after the final injection the groups of ten mice were sealed in glass jars and the time of sealing was recorded. We used 32-oz. wide neck, screw-capped clear bottles, with soft paraffin smeared on the neck to ensure a complete seal. The volumes of the jars ranged from 980 to 1,020 ml., and they were randomly distributed. The time of survival was recorded for each individual mouse, the end-point being the last visible respiration, which is almost invariably preceded by marked terminal convulsions.

As standard throughout we have used the pure sodium salt of synthetic DL-thyroxine (Borrows, Clayton, and Hems, 1949). The dose levels used for the standard were generally 5, 10, and 20 μg. per mouse per injection. In the investigation of new derivatives a preliminary probing test was carried out on two groups of ten mice with dose levels of 20 and 100 μg. of test substance per mouse at each injection, the doses of test substance used in the subsequent full assay being based on the results.

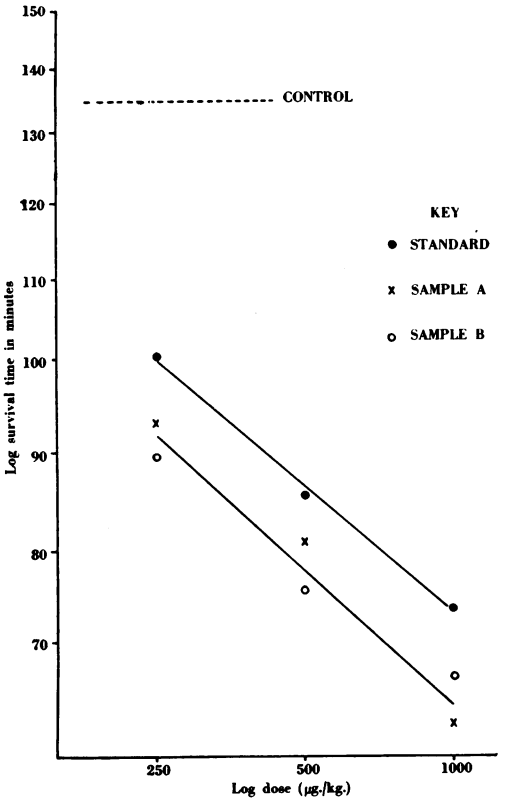

FIG. 1.—Dose response curves for two samples of sodium thyroxine and a standard preparation. Abscissae: dose in μg. per 20 g. mouse on a logarithmic scale. Ordinates: survival times on a logarithmic scale.

RESULTS

Responses to thyroxine.—The results of a typical comparison of two samples of thyroxine sodium with the standard preparation are shown in Fig. 1 and their analysis in Table I. Estimates of the activities and their respective fiducial limits ($P=0.95$) are shown in Table II. Examination of the results from a series of experiments indicates that distribution of survival times within a group is skew ; of the various conversions tried, log-time gave the least skew and sharpest peak (Fig. 2). Hence the metameter log-time (y) was adopted for calculation of results. Relative activities, together with their fiducial ranges, were calculated by the method of Bliss and Marks (1939) and the errors of the slopes according to Irwin (1937). The dose response lines tended to be shallow, the mean slope, b, relating units

TABLE I

ANALYSIS OF VARIANCE OF THE RESULTS PLOTTED IN FIG. 1

Source	Degrees of freedom	Mean square	F	P
Between controls and treated groups ..	1	22,107	402	<0.001
Between samples	2	893	16	<0.001
Common linear regression	1	12,779	232	<0.001
Departure from parallelism	2	99	1.8	N.S.
Curvature	3	51	0.93	N.S.
Other orthogonal effects	4	183	3.33	>0.001 <0.01
Residual error	488	55	—	—

TABLE II

RELATIVE ACTIVITIES OF SAMPLES OF THYROXINE SODIUM BY THE SUBCUTANEOUS ROUTE

Sample	Activity	Fiducial limits (P = 0.95)
Standard	Taken as 1.0	—
Sample A	1.52	1.27 to 1.85
Sample B	1.61	1.34 to 1.97

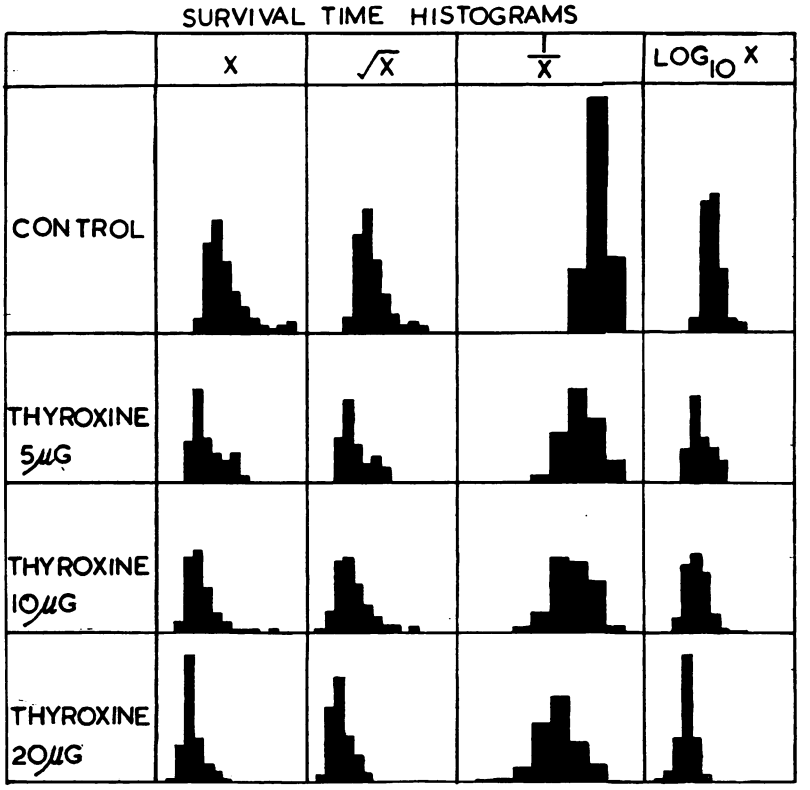

FIG. 2.—Distribution curves for different transformations of survival time from data for a series of experiments. Log-time gives the least skew and sharpest peak.

of increase in y per tenfold increase in dose, from five separate assays of thyroxine sodium by the subcutaneous route being -20.8 with a standard error (P=0.95) of ± 3.1. The mean value for s/b was 0.35, from which it was calculated that to achieve an accuracy of ± 20 per cent (P=0.95) it would be necessary to employ 240 animals on the standard (i.e., 80 at each of three dose levels) and the same number on the test preparation. Results accurate enough for our particular purpose could, however, be obtained on a quarter of this number. By the oral route the

slopes were even shallower, b being -14.8 and s/b -0.48. Owing to the highly significant departure from parallelism it was not possible to make valid comparisons of activities by the oral and parenteral routes. There is some indication that DL-thyroxine sodium in the mouse is about half as active orally as parenterally. This would suggest a wide species difference in the oral effectiveness of thyroxine, since Bailey, Bartlett, and Folley (1949) calculated that for the cow the oral parenteral activities of L-thyroxine sodium are approximately 16 : 1.

Thyroxine derivatives.—In Table III the relative thyroid activities of a number of thyroxine derivatives by the oral and subcutaneous routes are given. To relate solubility with activity we have when possible included solubilities in the Table. Of all compounds examined, the most active was the mono-sodium salt of thyroxine. No correlation was evident between the solubilities of the derivatives and their activities, with the possible exception of free thyroxine and its mono-sodium salt. The higher activity of the mono-sodium salt compared with that of free

TABLE III

ACTIVITIES AND SOLUBILITIES OF SOME DERIVATIVES OF DL-THYROXINE BY THE SUBCUTANEOUS AND ORAL ROUTES

Activities in terms of thyroxine sodium (standard) taken as unity. The figures in parentheses represent the maximum daily dose (in micrograms), given three times, that produced no significant effect.

Derivative	Activity		Solubility in water (25°) g./100 g.
	Subcutaneous route	Oral route	
Thyroxine sodium (standard)	1.0	1.0	0.0003
Thyroxine (free acid)	0.2	0.25	<0.0001
Methyl ester	0.66	0.53	0.017
Ethyl ester	0.51	0.58	0.002
N-carbamido thyroxine	Inactive (80)	Inactive (80)	0.6
N-Formyl	0.33	0.23	0.16
N-Acetyl	0.33	0.27	0.25
N-Carboxy-formyl	0.33	0.49	0.14
N-β-Carboxy-propionyl	Inactive (80)	Inactive (160)	0.95
N-Phthalyl	Inactive (40)	—	<0.02
O-Methyl	0.49	0.50	—
O-Carboxy-methyl	0.12	—	—
Thyroxamine	Inactive (1,000)	—	—
3 : 5-Diiodo-4-(3′ : 5′-diiodo-4′-hydroxy-phenoxy) hydrocinnamic acid	Inactive (320)	—	—

thyroxine is in accord with the clinical results of Thompson, Thompson, Dickie, and Alper (1933) for patients with myxoedema and with the more recent studies of Monroe and Turner (1949) on the domestic fowl. We did not, however, find the disodium salt more active than the mono-sodium salt, which is in agreement with Monroe and Turner but not with Thompson *et al.*

Solutions and suspensions of thyroxine.—The relative activity of free thyroxine and its mono-sodium salt might be due to differences in solubility. We have compared the activity of a solution of sodium thyroxine in 0.01 M-sodium carbonate solution with a suspension of it in 0.9 per cent sodium chloride solution. There was no significant difference (Table IV).

TABLE IV

COMPARISON OF A SUSPENSION AND A SOLUTION OF THYROXINE SODIUM BY THE SUBCUTANEOUS AND ORAL ROUTES

Activity of suspension (standard) taken as unity for both routes

Route	Activity of suspension in 0.9% NaCl (standard)	Activity of solution in 0.01 M-Na_2CO_3	
		Estimate	Fiducial limits (P = 0.95)
Subcutaneous	1.0	1.035	0.69–1.49
Oral	1.0	1.165	0.78–1.75

Free thyroxine could only be given as a suspension. We found that the activity of free thyroxine was increased with decrease in particle size, but this was not true for suspensions of the sodium salt (Table V). This suggests that the lower activity of free thyroxine is due to its insolubility and that decrease in particle size enhances the activity by aiding absorption.

The relative potency of L- *and* DL-*thyroxine.*—We next turned to the relative activity of L- and DL-thyroxine; this matter has been reviewed by Pitt Rivers and Lerman (1948) and by Griesbach, Kennedy, and Purves (1949). We compared the activity of L-thyroxine sodium, synthesized by the method of Chalmers, Dickson, Elks, and Hems (1949) [$(\alpha)_D^{20°} = -5.7°$ in ethanol and N-sodium hydroxide (2 : 1)] with standard DL-thyroxine sodium (Borrows, Clayton, and Hems, 1949). By the

TABLE V

INFLUENCE OF PARTICLE SIZE ON THE ORAL ACTIVITY OF SUSPENSIONS OF FREE THYROXINE AND ITS MONO-SODIUM SALT

Suspension	Mean particle size in μ	Activity
DL-Thyroxine sodium	74	Taken as 1.0
	29	0.87
DL-Thyroxine (free acid) ..	158	Taken as 1.0
	39	4.47

TABLE VI

COMPARISON OF L- AND DL-THYROXINE BY THE SUBCUTANEOUS AND ORAL ROUTES

Activity of DL-thyroxine sodium taken as unity for both routes.

Substance	Subcutaneous route		Oral	
	Activity	Fiducial range (P = 0.95)	Activity	Fiducial limits (P = 0.95)
DL-Thyroxine sodium ..	1.00	—	1.00	—
L-Thyroxine sodium ..	2.00	1.24–3.22	2.34	1.83–3.02

subcutaneous route we found the L-isomer to be twice as active as the DL-mixture (Table VI) and closely similar results were obtained from oral administration, in accord with the findings of Foster, Palmer, and Leland (1936) and of Reineke and Turner (1943 and 1945). The results suggest that the D-isomer is inactive, but it would be unwise, in view of the large experimental errors involved, to draw final conclusions from these results, especially as a number of workers have found D-thyroxine active in man (Salter, Lerman, and Means, 1935; Pitt Rivers and Lerman, 1948). We have not yet been able to examine the pure D-isomer by this method.

Activity of thyroxine in dried glandular extracts.—It has been supposed that thyroxine is less active by the oral route alone than when combined with thyroid protein, owing to the insolubility and poor absorption of the free hormone (cf. reviews by Harington, 1933; Means, 1937; Elmer, 1938; and Salter, 1940). However, the results obtained are conflicting, probably because of the varied biological methods employed and their large inherent errors, and also owing to the possible unspecificity of chemical tests for thyroxine in materials containing protein. Few workers have found good agreement between physiological results and chemical assays based on either total iodine or acid-insoluble iodine determinations (Gaddum and Hetherington, 1931; Wokes, 1938).

We have determined the activity of one commercial sample of Thyroid B.P. by the mouse anoxia method. The sample, assayed by the British Pharmacopoeia (1948) acid insoluble iodine method, contained 0.11 per cent of iodine in combination as thyroxine. The results from three separate assays are shown in Table VII.

TABLE VII

COMPARISON OF SYNTHETIC DL-THYROXINE SODIUM AND THYROID B.P. BY THE ORAL ROUTE

Substance	Activity	Fiducial limits (P = 0.95)
DL-Thyroxine sodium	1.00	—
Thyroid B.P. 1st assay	2.05	1.56–2.64
Thyroid B.P. 2nd assay	1.61	0.89–2.56
Thyroid B.P. 3rd assay	2.73	1.82–4.11
Mean	2.22	1.80–2.68

The thyroxine present in the glandular extract appears twice as active as the DL-thyroxine sodium standard, a result to be accounted for on the assumptions that thyroxine in the gland is entirely in the L-form and that L-thyroxine is twice as active as the DL-mixture. The activity of this particular thyroid powder is thus in agreement with its chemical thyroxine content determined by the acid-insoluble iodine method.

Discussion

The mouse anoxia method of Smith, Emmens, and Parkes appears to provide a reasonably simple and rapid method for the routine assay of thyroid preparations. Its precision leaves something to be desired, but the results obtained appear to be related to clinical findings in man. Mice are, moreover, less removed from man in the evolutionary scale than are amphibia and would therefore seem more specific as test animals in this method. We find that the mono-sodium salt is the most active of a number of derivatives and preparations of thyroxine, free thyroxine itself being considerably less active. The difference is probably a question of absorption, free thyroxine, owing to its insolubility, being poorly absorbed (cf. Thompson *et al.*, 1933 ; Monroe and Turner, 1949). We have shown in a previous communication (Clayton, Free, Page, Somers, and Woollett, 1949), using thyroxine sodium labelled with ^{131}I, that absorption of this salt from the intestine does occur, but these studies have not been extended to free thyroxine itself.

We have obtained no evidence that thyroxine combined with protein in the natural gland is more active orally than thyroxine sodium, if it is assumed that thyroxine in the natural gland is present as the L-compound with twice the potency of the racemic mixture used as the standard.

The availability of pure synthetic L-thyroxine may make the biological assay of thyroid activity of less immediate therapeutic importance than hitherto. Nevertheless it will remain essential for studying the relationship between chemical constitution and thyroid or anti-thyroid activity, and also indirectly for fundamental investigations of thyroid function. The method described here seems the best at present to hand for reasonably quantitative estimations.

Summary

1. The mouse anoxia method of Smith, Emmens, and Parkes has been used for evaluating the thyroid activity of a number of derivatives and preparations of thyroxine.

2. The most active preparation was the mono-sodium salt of L-thyroxine.

3. By both the oral and subcutaneous routes the mono-sodium salt of L-thyroxine was twice as active as the corresponding DL-compound.

4. No evidence has been found to suggest that thyroxine combined with the natural protein of the gland is more active than the mono-sodium salt of L-thyroxine, on a weight for weight basis.

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