other mixtures, e.g. of $C_{22}H_{44}O_2$ and $C_{24}H_{48}O_2$, which have been examined [Chibnall, Piper & Williams, 1936], show too many irregularities to be used for direct comparison. Some estimate of the nature of the impurities present may, however, be made by correlating the X-ray measurements with the general observations made by Chibnall *et al.* [1936] on the behaviour of fatty acid mixtures. For this purpose measurements had to be made of both the B and C polymorphic modifications, the X-ray photographs usually being taken of samples pressed lightly on to glass slides.

A. The original preparation, when recrystallized from acetone, showed X-ray lines corresponding to the C modification alone. On recrystallization from benzene and from acetic acid, preparations were obtained, both of which showed lines due to B and C modifications on the same photograph. Such behaviour was noted by Piper and co-workers [Chibnall *et al.* 1936] in the binary mixture containing 2.5–10% and 80–90% of the lower constituent. Here the C spacing is slightly lower, the B spacing slightly higher than the best values for pure $C_{22}H_{44}O_2$. Both these types of spacing change may be due to mixture of a small quantity of $C_{26}H_{46}O_2$, say 2.5–10%, with $C_{22}H_{44}O_2$, but it seems very probable that the fraction still contains traces of other acids, particularly $C_{24}H_{48}O_2$.

IIIR 5. This fraction, when recrystallized from methanol-ethyl acetate, showed the B spacing alone. While the spacings, both of this, and of the methyl ester, are intermediate

between those of $C_{22}H_{44}O_2$ and $C_{24}H_{48}O_2$, the data do not fit very well with the observations that have been made on mixtures of synthetic $C_{22}H_{44}O_2$ and $C_{24}H_{48}O_2$, so that the mixture is presumably more complex.

Table 6. X-ray measurements

Acid fraction	M.P. ° C.	B spacing	C spacing	Methyl ester spacing
A	77.5	53.6	47.7	—
III R 5	81.9	56.5	—	61.0
Compare				
$C_{26}H_{46}O_2$	73.5	48.45	44.15	52.3
$C_{22}H_{44}O_2$	79.95	52.95	48.3	57.02

SUMMARY

1. The isolation of behenic acid from refined mona wax is described.

2. The results of X-ray crystallographic examination of the product, by C. H. Carlisle & D. Crowfoot, are reported. They confirm the conclusion that the isolated product consists largely of behenic acid.

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REFERENCES

- Buchner, E. & Meisenheimer, J. [1908]. *Ber. dtsch. chem. Ges.* **41**, 1414.
 Buxton, L. O. & Kapp, R. [1940]. *J. Amer. chem. Soc.* **62**, 986.
 Chibnall, A. C. [1934]. *Biochem. J.* **28**, 313, 2189.
 — Piper, S. H. & Williams, E. F. [1936]. *Biochem. J.* **30**, 106.
 Francis, F. & Piper, S. H. [1939]. *J. Amer. chem. Soc.* **61**, 577.
 — & Malkin, T. [1930]. *Proc. roy. Soc. A*, **128**, 214.
 Holde, D. [1924]. *Kohlenwasserstofföle u. Fette*, **5**, 763.
 Jantzen, E. & Tiedecke, T. [1930]. *J. prakt. Chem.* **127**, 279.
 Longenecker, H. E. [1937]. *J. Soc. chem. Ind., Lond.* **56**, 199T.
 Marcusson, J. & Lederer, P. [1931]. *Fettchem. Umsch.* **38**, 253.
 Reilly, J. & Wilson, J. P. [1940]. *Sci. Proc. R. Dublin Soc.* **22**, 321.
 Stadler, A. [1933]. *Mitt. Kohlenforsch. Inst. Prag.* **5**, 445.
 Stohmann, F. & Langbein, H. [1890]. *J. prakt. Chem.* **42**, 379.

A Study of the Excretion of Organic Antimonials Using a Polarographic Procedure

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Several attempts have been made to determine the rate of excretion of antimony compounds during the treatment of tropical diseases, and so to control dosage and avoid toxic symptoms. The early qualitative tests for the presence of the metal in urine [Di Cristina & Caronia, 1916; Mallardi, 1921] were replaced by quantitative methods based upon the colorimetric determination of antimony as sulphide

[Brahmachari, Das & Sen, 1923; Khalil, 1931] or by a modification of the Gutzeit test [Boyd & Roy, 1929]. A summary of this work is given in Table 1. All the quantitative procedures were tedious; the degree of accuracy was low, and the chemical treatment made it impossible to distinguish between trivalent and quinquevalent antimony in the original samples. The subjects were various human patients

and laboratory animals, and a number of different antimony compounds were used, with varying dosages and methods, so that no scale of comparison had as yet been provided. The present work provides such a scale for a number of widely used antimonials which have been injected in comparable doses into the same species of laboratory animal. It also introduces the rapid method of polarography for the direct determination of antimony in both trivalent and quinquevalent forms. A short summary of a few of our results has already been recorded [Goodwin & Page, 1942].

Roncato & Bassani [1934] and Kamegai [1939*a, b*]; for lead in blood by Teisinger [1938], in urine by Reed & Gant [1942], and in both blood and urine by Cholak & Bambach [1941]; for alkali metals in blood by Schweitzer [1936] and in urine by Kemula & Michalski [1939]. The determination of arsenic in biological materials has recently been reported by Bambach [1942]. The lengthy pretreatment is typical of the procedures usually required for the polarographic examination of such materials.

The polarographic determination of antimony was described by Page & Robinson [1942], who

Table 1

Observer	Disease treated	Drug used	Method of assay
Di Cristina & Caronia [1916]	Mediterranean kala-azar	Tartar emetic	Qualitative test (sulphide)
Christopherson & Newlove [1919]	Schistosomiasis	Tartar emetic	Beam & Freak [1919] method presumably
Mallardi [1921]	Mediterranean kala-azar	Tartar emetic; stibacetin	Semi-quantitative modified Marsh test
Renault, Monier-Vinard & Gendron [1922]	Mediterranean kala-azar	Stibacetin	Iodine (?) titration
Brahmachari, Chaudhury, Das & Sen [1924]	Indian kala-azar	Na antimonyl tartrate; urea stibamine	Modified Beam & Freak method
Brahmachari & Sen [1924]	Indian kala-azar	Various antimonyl tartrates	Modified Beam & Freak method
Delius [1925]	Disseminated sclerosis	Antimosan	Marsh test
Boyd & Roy [1929]	Indian kala-azar	Na antimonyl tartrate; Neostibosan	Modified Gutzeit test
Peter [1929]	Schistosomiasis	Fouadin (stibophen)	Not stated
Khalil [1931]	Schistosomiasis	Fouadin	Modified Beam & Freak method
Piazza [1933]	Mediterranean kala-azar	Tartar emetic; Neostibosan	Semi-quantitative colorimetric test (sulphide)
Khalil [1936 <i>a, b</i>]	Schistosomiasis	Fouadin	Colorimetric test for pyrocatechol said to run parallel with the urine Sb content
Hassan [1937, 1938 <i>a</i>]	Schistosomiasis	Tartar emetic; Fouadin	Modified Gutzeit test on ashed urine
Weese [1937]	Normal rabbits	Neostibosan; Solustibosan	Not stated
Weese [1938 <i>a</i>]	Normal dogs	Fouadin	Not stated

The polarographic method of micro-chemical analysis was devised about 20 years ago by Heyrovsky, but it is only comparatively recently that self-contained instruments have become available for routine analytical procedures. Several comprehensive accounts [Kolthoff & Lingane, 1941; Müller, 1941] of the principles of analysis with the dropping mercury electrode have been published, and these should be consulted for practical details of the standard procedures. The general factors to be considered in quantitative work have been reviewed by Kolthoff [1942].

The polarograph has been widely used for the determination of the inorganic constituents of biological materials, but the procedures are frequently tedious, since many organic substances are reducible at the cathode and the materials have consequently to be prepared for analysis by special pretreatments. Such methods have been described for the determination of copper in liver ash by Shoji [1931] and Mandai [1931] and in blood by

demonstrated that in the presence of normal HCl the diffusion current for trivalent Sb is directly proportional to the concentration over the range 0.05–0.0001% Sb. Trivalent but not quinquevalent Sb in normal HCl has a half-wave potential versus the saturated calomel electrode of -0.15 V., consequently trivalent Sb can be determined directly in the presence of the quinquevalent form.

The determination of Sb in blood and urine is remarkably simple. Blood requires relatively little pretreatment, while for urine the determination is direct. The half-wave potential of trivalent Sb versus the saturated calomel electrode being -0.15 V., its characteristic step appears before those of other reducible constituents of urine. The latter would completely mask small quantities of a substance less readily reducible than trivalent Sb. A similar procedure can be adopted for the determination of bismuth [Page & Robinson, 1942].

METHODS

Technique of polarographic determination of antimony

In order to estimate quinquevalent Sb it must first be reduced by boiling with Na sulphite, and can then be determined in the usual way. For a sample containing both ter- and quinquevalent Sb, the tervalent Sb is first estimated, the sample reduced, and the total Sb determined, the quinquevalent Sb being obtained by difference. These points are illustrated in Fig. 1. Under favourable conditions, the error does not exceed $\pm 2\%$ over the concentration range 10^{-1} to 10^{-3} , $\pm 5\%$ over the range 10^{-3} and 10^{-4} , and $\pm 10\%$ over the range 10^{-4} and 10^{-5} g./100 ml.

Fig. 1. Antimony polarograms.

- (1) 0.0001 M sodium antimony^v gluconate.
- (2) 0.00005 M sodium antimony^v gluconate
- (3) 0.0001 M sodium antimony^v gluconate } reduced.
- (4) 0.00015 M sodium antimony^v gluconate

A Cambridge polarograph [Page & Robinson, 1942] was used for this investigation, the small cell being particularly useful, since it required only 5 ml. of solution. The supplementary 2000 μ F. electrolytic condenser ensured a sharp polarogram, which facilitated the measurement of small diffusion currents.

All solutions were made normal to HCl and the Sb content adjusted within the range 0.05–0.001%. The instrument was calibrated by recording on the same photographic paper as the unknown, the

polarogram of a standard Sb solution. So long as the same capillary was employed and the mercury column maintained at a constant height, there was very little difference in the standardization during the course of several months.

Polarographic estimation of antimony in urine

Collection of urine. It was found that metal metabolism cages were unsatisfactory in this work, as small quantities of metals derived from the cage interfered with the Sb determination (especially in polarographic estimations). An all-glass metabolism cage was therefore devised, in which the animals rested upon a grille of glass rods, and the urine came into contact with nothing but glass. Groups of ten 20 g. mice were used in the cages, and the bladder of each one emptied by gentle pressure on the abdomen at the end of every collection period. The cages were washed down with 50 ml. of distilled water, a sufficient quantity to ensure recovery of 80–90% of the Sb in the cages, without undue dilution of the sample.

Preparation of urine samples. (a) *For estimation of tervalent antimony.* The urine collected in the glass metabolism cage was made normal to HCl with strong acid and a sample examined immediately on the polarograph. The operation was performed as rapidly as possible after the addition of the acid as we have shown (*vide infra*) that many quinquevalent Sb preparations are hydrolysed to the tervalent state on standing with HCl.

(b) *For estimation of total antimony.* 5 ml. of the urine sample after treatment with HCl were boiled for 10 min. with about 0.05 g. Na sulphite, cooled, made up to 5 ml. with a few drops of strong HCl, and the total Sb determined. It is essential to boil off all the SO_2 , since Na_2SO_3 in *N* HCl yields a step of its own, which seriously interferes with the Sb step.

Polarographic estimation of antimony in blood

(a) *Estimation of tervalent antimony.* It is essential that the necessary treatment of the blood before polarography should not change the valency state of the Sb present, and that the reagents used should not interfere with the polarographic step for Sb. A preliminary investigation on the influence of protein precipitants was therefore necessary.

Whole blood, serum or plasma cannot be used directly, since on addition of HCl the protein is precipitated and carries down a considerable proportion of the Sb with it. Trichloroacetic acid, tungstic acid and zinc hydroxide interfere with the polarographic step. The only suitable protein precipitant is sulphosalicylic acid, which has been used by Brdicka [1938] and also by Waldschmidt-Leitz & Mayer [1939] for the removal of protein from

serum, in their investigations on the polarographic detection of cancer. However, some loss of Sb occurs with whole blood and sulphosalicylic acid, even if the blood is diluted to 1 in 20, but the loss with plasma is considerably less, due to the smaller amount of protein precipitated from it.

Colorimetric estimation of antimony in urine

A modification of Clarke's method [1928] was used. The urine was boiled with copper foil in the presence of HCl, the deposited Sb dissolved from the foil with Na_2O_2 and water, and any copper

Table 2. *Recovery of antimony after treatment with sulphosalicylic acid*

Sample	1 in 10 dilution			1 in 20 dilution		
	Sb ^{III} in solution g. $\times 10^{-4}$ /100 ml.		Recovery of Sb ^{III} %	Sb ^{III} in solution g. $\times 10^{-4}$ /100 ml.		Recovery of Sb ^{III} %
	Found	Added		Found	Added	
1. Citrated blood	40.4	59.0	70	24.8	32.2	80
2. Plasma	52.0	59.0	90	—	—	—
3. Water	59.2	59.0	100	32.4	32.2	100
4. Plasma	68.8	64.9	107	39.4	35.4	112
5. Corpuscles	2.5	51.3	5	—	—	—
6. Plasma	—	—	—	36.9	35.4	105
7. Corpuscles	—	—	—	4.5	29.0	15

Table 2 shows the percentage recovery of tervalent Sb from citrated blood and plasma to which known amounts of stibophen were added. The same quantity of stibophen was added to nos. 1, 2 and 3. Nos. 4 and 5 were obtained by centrifuging a sample of no. 1 immediately after the addition, while nos. 6 and 7 were obtained after the blood had been standing for 6 hr.

The technique finally adopted was as follows: 1 ml. of the citrated blood was mixed with 19 ml. of distilled water in a centrifuge tube. 2 ml. of 20% sulphosalicylic acid were added and the tube spun for 15 min. 5 ml. of the clear supernatant liquid were pipetted off, treated with 1 ml. of strong HCl, made up to 10 ml. with distilled water and examined immediately on the polarograph. The recovery of tervalent Sb was about 80%.

(b) *Estimation of total antimony.* 1 ml. of citrated blood was pipetted into 7.5 ml. of 10% HCl and refluxed in a 50 ml. flask on a sand-bath for 2 hr., during which time the contents of the flask became clear except for a few brownish specks. After cooling, filtering and diluting to 20 ml., the solution was ready for the polarograph.

In the course of this process, all the Sb was reduced to the tervalent form, whatever had been its state in the original blood. The recovery was good, amounting to over 90% of the theoretical value when tested upon blood containing known amounts of stibophen or Na antimony^v gluconate.

(c) *Distribution of antimony between corpuscles and plasma.* When the plasma was separated from a sample of blood to which Sb had just been added, the recovery was found to be more than 100%. The corpuscles, on the other hand, contained practically no Sb, indicating that little had been absorbed. After standing for 6 hr., the concentration in the corpuscles was still very much lower than that in the plasma.

precipitated from solution with H_2S . The filtered solution containing the Sb was acidified with H_2SO_4 , evaporated to small bulk, and then treated with KI, aqueous pyridine and SO_2 in the presence of gum acacia as a protective colloid. The Sb in the clear yellow colloidal solution of the complex iodide was estimated with a Hilger 'Spekker' absorptiometer, calibrated with standard tartar emetic solutions.

RESULTS

The rate of urinary excretion of organic antimonials by laboratory animals

(1) *Preliminary work using the colorimetric method.* Boyd & Roy [1929] studied the excretion of 'neostibosan' (diethylamine *p*-aminophenylstibonate) after intravenous and intramuscular injection into kala-azar patients, and showed that the initial rate of excretion of Sb in the urine was very high, about 20% of the dose being recovered in the first 2½ hr. Stibamine glucoside ('neostam') is a quinquevalent compound of the same type as neostibosan, and its rate of excretion by mice was determined to see how it compared with that substance. A few tests were made on rats and hamsters, and certain other Sb compounds were also used. The results in Table 3 are a useful check on the more accurate polarographic determinations recorded in the following section (b).

(2) *Results using the polarograph.* The excretion of the following tervalent and quinquevalent antimonials was investigated:

Tervalent. K antimonyl tartrate. Stibophen ('Fouadin'). Na antimony^{III} bis-pyrocatechol-3:5-disulphonate. 'Anthiomaline'. Li antimony^{III} thiomalate.

Quinquevalent. (a) ('emetic type'). Na Sb^v gluconate (= 'Solustibosan'?).

Table 3. *The excretion of antimony by laboratory animals (colorimetric method)*

Animals	Drug	Dose mg. Sb/kg.	Method of in- jection	% dose excreted in urine (hr.)								% dose excreted totally in	
				0-1	1-2	2-4	4-6	6-24	24-48	48-72	72-96	6 hr.	24 hr.
Mice (groups of 10)	Stibamine glucoside (Sb ^v)	62.5	Subcut.	11.4	6.9	6.6	6.1	13.7	—	—	—	31.0	44.7
				15.2	14.5	8.0	6.0	8.2	—	—	—	43.7	51.9
				8.3	5.1	5.3	8.1	2.8	—	—	—	26.8	29.6
			Intrap.	13.1	13.3	8.2	8.9	9.8	—	—	—	43.5	53.3
				16.6	4.8	6.9	4.0	7.7	—	—	—	32.3	40.0
				25.4	2.3	11.4	6.9	8.3	—	—	—	46.0	54.3
	Stibophen (Sb ^{III})	41.0	Intrav.	35.4	6.9	5.1	2.7	5.5	—	—	—	50.1	55.6
				32.7	10.2	3.2	2.3	5.1	—	—	—	48.4	53.5
				23.0	12.3	4.0	13.6	4.8	—	—	—	52.9	57.7
			Subcut.	32.4	3.8	13.0	3.2	5.4	—	—	—	52.4	57.8
				41.0	5.1	3.2	1.1	—	—	—	—	50.4	—
				Intrap.	40.4	9.6	2.2	4.1	5.7	—	—	—	52.2
14.0	12.8	17.6	6.6		7.8	—	—	—	51.0	58.8			
26.0	7.8	4.9	2.3		4.3	—	—	—	41.0	45.3			
Intrav.	66.0	6.5	2.5	1.7	5.4	—	—	—	76.7	82.1			
	Tartar emetic (Sb ^{III})	6.0	Intrap.	8.0	3.0	2.8	16.0	5.0	—	—	13.8	29.8	
Rats (groups of 6)	Stibamine glucoside (Sb ^v)	33.3	Intrap.	45.0					1.4	0.6	0.4	—	45.0
				41.0					1.7	0.6	0.3	—	41.0
				43.0					7.0	2.5	1.2	—	43.0
				41.0					5.0	1.6	1.2	—	41.0
Hamsters (mean values for 10 exps. with single animals)	Solusti- bosan	160.0	Subcut.	61.2					3.1	3.4	—	—	61.2
	Na Sb ^v gluconate	160.0	Subcut.	56.7					2.1	1.6	—	—	56.7

(b) (*derivatives of phenylstibonic acid*). Stibamine glucoside ('Neostam')-N-glucoside of Na *p*-aminophenylstibonate. 'Neostibosan'-diethylamine *p*-aminophenylstibonate. 'Urea stibamine'-*s*-diphenylcarbamide-4:4'-distibonic acid and other compounds [Gray, Trevan, Bainbridge & Attwood, 1931]. 'Stibacetin'-Na *p*-aminophenylstibonate.

Na antimony^v gluconate, stibophen and tartar emetic were given by the subcutaneous, intraperitoneal and intravenous routes, the first two compounds in both large and small doses. The small dose level of about 3.5 mg. Sb/kg. of mouse was the equivalent of about twice the ordinary therapeutic human dose. Smaller doses were not used, as the limit of sensitivity of the method was reached in the case of the 6-24 and 24-48 hr. samples.

The large dose level of 35 mg. Sb/kg. was, of course, much larger than is ever given in clinical practice, and with some of the compounds given (tartar emetic, anthiomaline, urea-stibamine) was impracticable owing to its toxic effect. An intermediate dose was given in most of these cases. The full range of results is given in Table 4 and some examples are shown graphically in Figs. 2, 3 and 4.

A single experiment was made using a rabbit, to determine the rate of excretion of Na antimony^v

gluconate at shorter intervals than 1 hr. after the injection. The animal was anaesthetized with nembutal, and a cannula tied into the left ureter. A dose of the compound containing 10 mg. Sb was injected into the left jugular vein, and the urine collected at 15 min. intervals, the cannula being washed out at each collection. Quantities of 4.8, 5.1, 4.9 and 4.6 % of the Sb in the dose injected were recovered during the first hour—19.4 % in all.

For comparison with the excretion rates, the total blood Sb levels in mice given the largest doses (35 mg. Sb/kg.) of stibophen and Na antimony^v gluconate by the three routes of injection were determined at intervals. Mice weighing 20-25 g. were used, and groups of four killed from time to time. The heart blood from all members of one group was pooled, and 1 ml. taken for a determination (Table 5).

(3) *Discussion*. The excretion results show general agreement with earlier workers. The large doses of stibophen and Na antimony^v gluconate were excreted at a higher initial rate than the small doses, especially after intravenous injection. After the first hour, the rates of excretion at the two dose levels did not differ very greatly. By the end of 48 hr., almost the same total percentage of the dose

Table 4. The excretion of antimony by mice

Drug	Dose mg. Sb/kg.	Method of in- jection	% dose excreted in urine (hr.)								% dose excreted totally in	
			0-1	1-2	2-3	3-4	4-5	5-6	6-24	24-48	6 hr.	48 hr.
			Tervalent compounds									
Stibophen	3.9	Subcut.	10.5	9.5	4.0	5.5	2.5	4.0	16.0	11.5	36.0	63.5
	3.6	Intrap.	9.5	6.0	4.0	5.5	5.0	5.5	19.5	11.0	35.5	66.0
	3.6	Intrav.	18.0	8.0	9.5	7.5	6.0	7.0	14.0	12.0	56.0	82.0
	33.5	Subcut.	21.8	10.4	2.9	2.3	5.4	5.4	11.0	4.2	48.2	63.4
	33.5	Intrap.	25.4	7.5	6.7	7.6	6.3	4.9	9.4	4.4	58.4	72.2
	30.4	Intrav.	46.3	5.9	3.3	2.2	6.4	1.7	7.1	4.3	65.8	77.2
Tartar emetic	3.8	Subcut.	10.0	5.0	4.5	6.0	4.5	4.0	15.0	8.5	34.0	57.5
	3.8	Intrap.	5.5	6.0	6.5	4.5	4.5	4.0	14.0	12.0	31.0	57.0
	3.7	Intrav.	8.5	8.5	7.5	8.0	5.5	8.5	15.0	7.0	46.5	68.5
	10.9	Intrap.	5.0	2.0	9.5	8.0	8.0	4.0	12.0	7.5	36.5	56.0
*Anthiomaline	3.5	Intrav.	9.0	10.0	4.0	1.0	1.0	1.0	14.0	10.0	26.0	50.0
Quinquevalent compounds												
Na antimony gluconate	3.5	Subcut.	11.0	11.0	7.0	4.5	2.0	1.0	6.5	8.0	36.5	51.0
	3.5	Intrap.	11.5	11.5	5.5	3.0	1.5	2.5	10.0	8.0	35.5	53.5
	3.5	Intrav.	20.0	11.5	7.5	8.0	3.5	3.5	6.5	6.5	54.0	66.5
	32.5	Subcut.	38.0	12.0	4.0	3.5	2.5	2.0	4.0	3.5	62.0	69.5
	32.5	Intrap.	23.0	6.0	8.0	6.0	1.5	1.0	6.5	4.5	45.5	56.5
	32.5	Intrav.	40.5	10.5	2.5	1.5	5.5	1.5	4.0	2.5	62.0	68.5
*Stibamine glucoside	3.5	Intrav.	15.0	15.0	10.0	5.0	5.0	2.0	7.0	5.0	52.0	64.0
	33.3	Intrav.	32.0	12.0	5.0	3.0	2.0	1.0	4.0	2.0	55.0	61.0
*Neostibosan	3.6	Intrav.	14.0	11.0	7.0	0.0	4.0	3.0	7.0	4.0	39.0	50.0
	25.6	Intrav.	22.3	10.2	8.2	1.6	1.0	2.5	6.4	3.1	45.8	55.3
*Urea-stibamine	3.5	Intrav.	18.0	11.0	9.0	4.0	3.0	1.0	4.0	6.0	46.0	56.0
	11.2	Intrav.	13.7	16.5	8.0	6.2	1.8	5.3	11.6	3.1	51.3	66.0
*Stibacetin	4.2	Intrav.	14.0	11.0	13.0	7.0	5.0	2.0	10.0	5.0	52.0	67.0
	34.3	Intrav.	28.2	7.7	11.2	6.1	5.0	1.0	2.9	3.0	59.2	65.1

* Tests made on groups of 10 mice. All the other figures are means of two experiments, each using a group of 10 mice.

Fig. 2. The excretion of stibophen by mice. (Dose approx. 35 mg. Sb/kg.)

Fig. 3. The excretion of tervalent antimony compounds after intravenous injection in mice. (Dose approx. 3.5 mg. Sb/kg.)

given had been excreted at both dose levels, the excretion rate during the 6-48 hr. interval being proportionately greater with the small doses. Weese [1938b] suggests that the large doses flood the whole body, and the 'overflow' is excreted very rapidly, the amount of the overflow depending upon the

Na antimony^v gluconate, but tartar emetic was much slower, and anthiomaline slower still. Intravenous injections were in every case the most rapidly excreted. Intraperitoneal and subcutaneous doses did not differ greatly in their rates of excretion.

Fig. 4. The excretion of quinquivalent antimony compounds after intravenous injection in mice. (Dose approx. 3.5 mg. Sb/kg.)

Table 5. Blood antimony in mice given injections containing 35 mg. Sb/kg.

Groups of four animals were killed at each time period, and the blood pooled

Drug	Method of injection	Blood antimony (mg./100 ml.) (min. after injection)										
		0	5	10	15	20	30	60	120	180	240	300
Stibophen (Sb ^{III})	Subcut.	0.6	1.7	2.0	—	2.0	1.7	1.4	1.1	0	0	0
	Intrap.	0.6	—	2.6	2.0	2.2	2.0	1.6	1.2	0.6	0	0
	Intrav.	4.8	0.7	1.7	—	1.7	1.7	1.1	0.3	0	0	0
Na antimony ^v gluconate (Sb ^v)	Subcut.	0.3	0.8	1.3	—	1.4	1.1	0.5	0	0.3	0	0
	Intrap.	0.2	1.0	1.2	—	1.0	1.2	0.8	0.4	0	0	0
	Intrav.	7.8	4.0	4.0	—	2.3	1.2	1.0	0.8	0	0	0

amount injected. Tartar emetic shows a fairly constant rate of excretion over the first 6 hr., but the rate is considerably greater with mice than has been reported in human clinical cases [Brahmachari *et al.* 1924; Boyd & Roy, 1929; Hassan, 1937]. The most rapidly excreted antimonial in the quinquivalent group was Na antimony^v gluconate, and then in order came stibacetin, stibamine glucoside, urea-stibamine and neostibosan—though there was little to choose between them. Stibophen in the trivalent group was not a great deal slower than

The rabbit experiment showed an almost constant rate of excretion during the first hour after a dose of Na antimony^v gluconate. If the other kidney is assumed to have excreted at the same rate, about 40 % of the dose was eliminated in the first hour, a result which agrees with the mouse experiments. From Table 5 it will be seen that in the course of about 3 hr., all measurable amounts of Sb had disappeared from the blood stream. It is interesting to see how efficient the absorption of the two

compounds investigated appears to be. In the time taken to kill a mouse and remove blood from the heart, appreciable quantities of Sb reached the blood stream after subcutaneous and intraperitoneal injection.

The excretion of antimony by human subjects

The polarographic method was applied to the determination of Sb excretion in human subjects. Doses were given intravenously or intramuscularly, and the urine and the capillary or venous blood collected at intervals. Polarographic measurements were much easier than with mice, owing to the larger amounts of Sb present. The results are shown in Tables 6 and 7 and Fig. 5, and though the number of experiments is small, it will be seen that in the case of Na antimony^v gluconate, the results correspond to the values for mice fairly closely. With stibophen, however, the rate of excretion is rather slower than in mice, though it should be noted that the small dose given to the mice was 5 times the equivalent of the largest human dose (subject 2). Individual variations were marked.

Fig. 5. The excretion of sodium antimony^v gluconate and stibophen by human subject (1).

Table 6. *The excretion of antimony by human subjects*

Drug	Dose mg./Sb	Method of injection	Subject no.	% dose excreted in urine (hr.)									% dose excreted totally in	
				0-1	1-2	2-3	3-4	4-5	5-6	6-12	12-24	24-48	6 hr.	24 hr.
Stibophen (Sb ^{III})	42.5	Intrav.	2	8.1	1.4	1.6	0.8	1.0	1.0	2.6	2.8	5.4	13.9	24.7
	8.5	"	2	6	3	2	1	2	2	2	1	1	16	20
			1	3	3	2	2	5		5	3	4	15	27
			4	3	1	2	1	2	2	4	2	1	11	18
	8.5	Intramusc.	5	11	5	3	2	2	2	2	4	4	25	35
Na antimony ^v gluconate (Sb ^v)	100	Intrav.	2	26.7	8.2	7.9	5.8	4.7	3.9	7.9	7.8	3.5	57.2	76.4
	50	"	3	17.5	15.1	8.9	6.1	7.0	2.4	20.4		—	57.0	77.4
			1	44.0	17.6	10.4	5.0	3.0	1.7	2.7	1.0	2.5	81.7	87.9
			6	43.6	17.4	8.6	7.2	4.7	2.2	4.0	2.3	2.4	83.7	92.4
			7	38.8	17.2	8.4	5.9	2.4	3.0	2.8	1.9	2.5	75.7	82.9
Stibamine glucoside (Sb ^v)	9.0	"	1	35.4	15.8	8.3	5.9	2.0	1.9	3.1	2.3	5.1	69.3	79.8
	50	"	3	21.0	9.0	6.0	4.0	3.0	3.0	—	—	—	46.0	—

Table 7. *Blood antimony in human subjects*

Drug	Dose mg./Sb	Method of injection	Subject no.	mg. antimony/100 ml. blood (hr.)									
				0.3	0.5	0.6	1.0	2.0	3.0	4.0	5.0	6.0	24.0
Na antimony ^v gluconate (Sb ^v)	100	Intrav.	2	1.0	—	0.6	—	0.5	0.3	0	0	0	0
			3	—	—	—	1.5	1.1	0.8	0.3	0.4	—	0
	50	"	1	1.3	—	0.4	0.3	0.3	—	0	—	0	0
			7	—	1.3	—	0.4	—	—	0.1	—	—	—
Stibamine glucoside	50	"	3	—	—	—	1.6	—	0.8	—	—	—	—

Subject (1) Normal male, 27.

(2) Normal male, 27.

(3) Male, 24, under treatment for Mediterranean kala-azar.

(4) Normal male, 19.

Subject (5) Normal male, 20.

(6) Normal male, 26.

(7) Male, 25, although suffering from renal stones, is at present in good health.

A dose of Na antimony^v gluconate was much more rapidly excreted than one of stibophen containing an equivalent amount of Sb. Hassan [1937] published some results about stibophen excretion, but subsequently corrected these [1938*a*], as it was found that a considerable proportion of the Sb in the drug used in the earlier experiments had become oxidized to the quinquevalent state. Our results agree with those given in Hassan's first paper, though the drug we used was completely free from quinquevalent Sb. It is perhaps worth noting that the 'rise' in excretion rate reported by Hassan after 24 hr. is due to the fact that the period of collection of urine was longer than for the earlier samples. If the excretion *per hour* is calculated, the results lie upon a steadily falling curve.

The influence of the valency of injected antimony on its metabolic fate

The low toxicity of the quinquevalent compounds must be due, in part at least, to their high rates of excretion. Their therapeutic advantages over the tervalent compounds in the treatment of kala-azar have never been satisfactorily explained. A direct leishmanicidal action is not generally favoured, owing to the low toxicity of the compounds to mammals, and it is unlikely that mere stimulation of the host's reticulo-endothelial system could account entirely for their activity.

It has therefore been assumed by analogy with the quinquevalent arsenic compounds that reduction takes place in the body to the tervalent state, the stibinoxide grouping ($R-Sb=O$) being parasiticidal. Since it has been shown above that the polarographic method will distinguish between tervalent and quinquevalent Sb, this method seemed to offer a new approach to the problem.

Brahmachari *et al.* [1924] showed that although the initial excretion of urea-stibamine was rapid, the rest of the dose came away slowly at about the same rate as a dose of tartar emetic, and advanced this slender evidence as proof that the slowly excreted fraction of the quinquevalent compound had been reduced. Bock [1927] thought to show from the results of toxicity tests that the Sb of stibophen was oxidized to the quinquevalent state and so detoxicated. Before the application of the polarograph to the problem, one of us had shown that it was very unlikely that any change in valency had occurred in the initially excreted Sb. The 24 hr. urine collected from groups of 10 mice injected with stibophen or Na antimony^v gluconate was passed through a Seitz filter, and the Sb content determined colorimetrically. Doses of the excreted Sb were then injected into mice infected with *Trypanosoma equiperdum*, using animals dosed with fresh solutions as controls. No trypanocidal activity at all was observed with Na antimony^v gluconate or

with the corresponding urine, but with stibophen the activity at three dose levels was almost identical with that of urine containing the equivalent amounts of Sb. As Na antimony^{III} gluconate has a marked trypanocidal action, and the quinquevalent analogue of stibophen no action at all, it appears that no valency change could have taken place.

By means of the polarograph, it could easily be demonstrated that up to the sixth hour no measurable amounts of Sb of changed valency were excreted either in animals or in man. Also, by precipitating the protein in mouse blood with sulphosalicylic acid, it was shown that the Sb in the clear solution was unchanged in valency after subcutaneous or intravenous injection of Na antimony^v gluconate. It was, however, more difficult to be sure of the fate of the Sb remaining in the tissues after the first few hours of excretion. The amounts of substance in every case were very small, and it was difficult to be sure that any valency change observed had occurred in the body, and not during subsequent treatment.

The presence of HCl is essential for these polarographic estimations, and this introduces certain limitations, e.g. it was found that when quinquevalent compounds were treated with normal HCl, hydrolysis took place with the formation of tervalent Sb. Thus with 0.02 % solutions of stibamine glucoside, neostibosan, urea-stibamine and stibacetin, about 30–50 % of the Sb was recorded as tervalent on the polarogram. The hydrolysis took place immediately upon addition of the acid, and no appreciable change was observed on standing for 1 hr. In the case of Na antimony^v gluconate however, the reduction was only very slight—about 1–2 % with a 0.02 % solution, and very much less with more concentrated solutions. This is presumably because, unlike other quinquevalent antimonials, the Sb is here joined to the carbon through the oxygen atom, and not directly.

We are grateful to Mr W. H. Gray of the Wellcome Chemical Research Laboratories for the suggestion that the hydrolysis of the *p*-aminophenylstibonic acid derivatives probably takes place as follows:

We have shown that treatment with HCl, followed by neutralization with NaOH, doubles the toxicity of stibamine glucoside as compared with the untreated substance to which an equivalent amount of NaCl had been added. This is further

evidence that the toxic tervalent compound had been produced.

Our choice of a quinquevalent compound for the investigation of valency changes was therefore limited to Na antimony^v gluconate, which had to be free from any tervalent Sb, since the presence of as little as 0.1 % of tervalent Sb has been found to be associated with increased toxicity.

Another source of difficulty when dealing with the valency state of Sb excreted in 24 hr. samples of urine, was the change which occurred on standing.

Table 8. *The effect of bacterial growth in urine containing stibophen equivalent to 0.0014 % Sbⁱⁱⁱ*

Time in hr. ...	26	52	71	95
% of Sb ⁱⁱⁱ in test sample (no HCl)	90	80	73	63
% of Sb ⁱⁱⁱ in control (+HCl)	99	100	—	—
Time in hr. ...	124	148	169	
% of Sb ⁱⁱⁱ in test sample (no HCl)	56	45	34	
% of Sb ⁱⁱⁱ in control (+HCl)	—	—	103	

The Sb in stibophen, for example, becomes progressively oxidized to the quinquevalent form if bacterial growth is not prevented. Table 8 shows the effect of unrestrained bacterial activity on urine containing a known amount of stibophen. Urine

cultures were of the type used for the routine preparation of yellow-fever virus, and were kindly provided by Dr F. O. MacCallum. Control cultures which had been killed by heating were incubated at the same time, and although the differences shown in Table 10 are small, there was a distinctly larger amount of tervalent Sb in the living than in the dead tissue cultures. A similar small effect was observed upon incubating with liver slices in Tyrode solution.

When a known amount of Na antimony^v gluconate (≈ 2 mg. Sb^v) was incubated with an emulsion of mouse liver (2 g. in 20 ml. saline) 8 % of the Sb was reduced in 3 hr., and 9 % in 24 hr. With an emulsion of kidney (0.5 g. in 20 ml. saline) about 4 % was reduced in 24 hr. No *in vivo* trypanocidal activity could be detected in the incubated solutions, and it appears therefore that the reduction of Sb by living tissues is less rapid than the reduction of quinquevalent As [Levaditi, 1909 *a*, *b*; Lourie, Murgatroyd & Yorke, 1935].

(b) *Excretion experiments.* It is in the later fractions of urine collected after a dose of Sb that any reduced substance must be sought. Since, when mice are used, the absolute amounts present in these fractions are very small, we turned our attention

Table 9. *The effect of incubating at 37° rabbit blood containing Na antimony^v gluconate equivalent to 0.001 % Sb^v*

		% antimony recovered (hr. after incubation)											
		0	0.5	1.0	2.0	3.0	4.0	5.0	6.0	24.0	72.0	96.0	
Experimental (blood)	Sb ^{III}	6	7	6	7	—	7	—	5	9	10	15	
	Total Sb ^{III} and Sb ^v	95	—	—	—	—	98	—	—	—	105	100	
Control (water)	Sb ^{III}	6	—	6	—	4	—	4	—	4	6	6	
	Total Sb ^{III} and Sb ^v	100	—	—	—	93	—	—	—	—	108	95	

was, therefore, always collected from the metabolism cage in vessels containing HCl, since this favours reduction, as well as preventing bacterial growth.

By a series of observations, we have satisfied ourselves that a small proportion of injected Sb is reduced in the body. The evidence for this is as follows:

(a) *In vitro experiments.* Table 9 shows the effect of incubation at 37° of normal rabbit blood containing Na antimony^v gluconate equivalent to 0.001 g./100 ml. of Sb. Samples were withdrawn from time to time, treated with sulphosalicylic acid to remove the protein, and the Sb content determined.

It will be seen that there is a small but significant increase in the proportion of tervalent Sb present after 72 hr. The tervalent Sb recorded in the fresh solution is due to the slight reduction by HCl.

Table 10 shows the effect of incubating small quantities of Na antimony^v gluconate with living chick-embryo tissue in serum Tyrode. The tissue

to human experiments. The urines collected from subjects 2 and 3 showed a considerable amount of tervalent Sb, but as they had been allowed to stand

Table 10. *The effect of incubation of Na antimony^v gluconate with living chick embryo tissue*

Culture	Conc. of total Sb in culture mg./100 ml.	% Sb recovered as Sb ⁱⁱⁱ
Experimental (living tissue culture)	0.48	4.2
	0.80	6.3
	1.26	11.1
	1.40	10.7
Control (culture killed by heat before addition of Sb)	0.68	0
	0.80	0
	1.09	5.5
	1.40	7.1

a considerable time with HCl, this evidence was worthless. The urine samples from subject 1, however, were acidified and polarographed immediately

on collection, so that any tervalent Sb present must have been produced in the body.

The proportions of tervalent to total Sb present are shown in Table 11. The fractions collected at all periods except the 12-24, 28-33, and 33-45 hr. collections were from a single emptying of the bladder, and they show a considerable, and increasing, proportion of tervalent Sb.

Table 11. *Antimony excretion by subject 1 after a dose of Na antimony^v gluconate equivalent to 50 mg. Sb^v*

Time of collection hr.	Vol. of urine ml.	Total Sb excreted mg.	Sb ^{III} excreted mg.	Sb ^{III} %
0-1	700	22.0	0.49	2.2
1-2	193	8.8	0.10	1.1
2-3	212	5.2	0.16	3.1
3-4	68	2.5	0.19	7.6
4-5	62	1.5	0.04	2.7
5-6	42	0.88	0.01	1.1
6-9	172	1.16	0.18	15.5
9-12	312	0.19	0.06	32
*12-24	714	0.50	0.50	100
24-28	360	0.25	0.14	56
*28-33	504	0.55	0.30	55
*33-45	408	0.41	0.28	68
45-48	84	0.06	0.03	50

* Collected in several fractions, and allowed to stand until end of collection period.

(c) *Antimony in the body organs.* We have confirmed the findings of other workers [Boyd, Napier & Roy, 1931; Hassan, 1938b] that Sb is concentrated in the liver, and is present in only very small quantities in the other organs if the animals are not killed immediately after injection. The livers of treated rabbits and mice contained easily measurable amounts of Sb determined by the method for total Sb in blood as is shown in Table 12. In order to

Table 12. *The recovery of antimony from the livers of animals injected with Na antimony^v gluconate*

Animal		Sb present mg./g. liver	Re-covered Sb ^{III} % of total
Rabbit given 11 doses of Na antimony ^v gluconate (170 mg. Sb in all)	Test	0.01	100
	Control (normal liver + Sb ^v)	0.02	1
Nine mice given 10 doses of Na antimony ^v gluconate (180 mg. Sb in all)	Test (9 livers)	0.03	25
	Control (10 normal livers + Sb ^v)	0.023	4

determine the valency of the liver Sb, weighed amounts of liver from animals which had received a series of doses of Na antimony^v gluconate were ground with sand and ice-cold saline as soon as possible after removal from the body. We did not

hope by this method to extract all the Sb present, but merely to determine the valency state. A control experiment was made in each case by grinding up normal liver with a known amount of Na antimony^v gluconate and saline. The protein was precipitated with sulphosalicylic acid, the solution was centrifuged, and HCl added to the supernatant solution which was polarographed at once. It will be seen from Table 12 that far more tervalent Sb was recovered from the livers of the injected animals than from the control experiments. We have no information as to the fate of the rest of the Na antimony^v gluconate molecule.

SUMMARY

1. The rapid polarographic method of analysis has been used for the determination of antimony in biological materials.

2. The rates of urinary excretion of a series of organic antimonials (tartar emetic, 'anthiomaline', stibophen, stibacetin, stibamine glucoside, 'neostibosan', 'urea-stibamine', sodium antimony^v gluconate) have been measured after injection into laboratory animals. Three compounds (stibophen, stibamine glucoside and sodium antimony^v gluconate) have been tested in a small number of human subjects.

3. It has been shown that about 30-40% of the antimony content of an injection of stibophen or of one of the quinquevalent compounds is excreted by mice in the urine during the first 1 or 2 hr. The subsequent excretion rate is less rapid. Small doses are excreted more slowly than large ones. Tartar emetic and 'anthiomaline' do not show such a high initial outflow. In the human subjects, a dose of Na antimony^v gluconate was excreted more rapidly than one of stibophen containing the same amount of antimony.

4. The blood antimony was determined at intervals after injection. No measurable amount could be detected after about 3 hr.

5. The antimony excreted during the first 24 hr. after injection of ter- or quinquevalent compounds was unchanged in valency. After an injection of Na antimony^v gluconate, part of the antimony remaining in the body was reduced to the tervalent state, but the quantity was small. Tervalent antimony was recovered from the livers of injected animals.

6. *In vitro* experiments have demonstrated the reduction of quinquevalent antimony by living tissues.

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REFERENCES

- Bambach, K. [1942]. *Industr. Engng Chem. (Anal. ed.)*, **14**, 265.
- Beam, W. & Freak, G. [1919]. *Analyst*, **44**, 196.
- Bock, G. [1927]. *Z. Hyg. InfektKr.* **107**, 396.
- Boyd, T. C. & Roy, A. C. [1929]. *Indian J. med. Res.* **17**, 94.
- Napier, L. E. & Roy, A. C. [1931]. *Indian J. med. Res.* **19**, 285.
- Brahmachari, U. N., Das, J. & Sen, P. B. [1923]. *Indian J. med. Res.* **11**, 417.
- Chaudhury, S. C., Das, J. & Sen, P. B. [1924]. *Indian J. med. Res.* **11**, 829.
- & Sen, P. B. [1924]. *Indian J. med. Res.* **12**, 113.
- Brdicka, R. [1938]. *Acta intern. Verein. Krebsbekämpf.* **3**, 13.
- Cholak, J. & Bambach, K. [1941]. *Industr. Engng Chem. (Anal. ed.)*, **13**, 583.
- Christopherson, J. B. & Newlove, J. R. [1919]. *J. trop. Med. (Hyg.)*, **22**, 129.
- Clarke, S. [1928]. *Analyst*, **53**, 373.
- Delius, K. [1925]. *Med. Klinik*, **21**, 1198.
- Di Cristina, G. & Caronia, G. [1916]. *Malar. Mal. Paesi caldi*, **7**, 245.
- Goodwin, L. G. & Page, J. E. [1942]. *Biochem. J.* **36**, Proc. xi.
- Gray, W. H., Trevan, J. W., Bainbridge, H. W. & Attwood, A. P. [1931]. *Proc. roy. Soc. B*, **108**, 54.
- Hassan, A. [1937]. *J. trop. Med. (Hyg.)*, **40**, 161.
- [1938a]. *J. Egypt. med. Ass.* **21**, 126.
- [1938b]. *J. Egypt. med. Ass.* **21**, 123.
- Kamegai, S. [1939a]. *J. Biochem., Tokyo*, **29**, 439, 453.
- [1939b]. *J. Biochem., Tokyo*, **30**, 33, 45.
- Kemula, W. & Michalski, M. [1939]. *Atti X Congr. int. Chim.* **3**, 419.
- Khalil, M. [1931]. *Arch. Schiffs- u. Tropenhyg.* **35**, Suppl. 2, 100.
- [1936a]. *J. Egypt. med. Ass.* **19**, 285.
- Khalil, M. [1936b]. *Lancet*, **2**, 132.
- Kolthoff, I. M. [1942]. *Industr. Engng Chem. (Anal. ed.)*, **14**, 195.
- & Lingane, J. J. [1941]. *Polarography*. New York: Interscience Publishers.
- Levaditi, C. [1909a]. *Bull. Soc. Path. exot.* **2**, 45.
- [1909b]. *Ann. Inst. Pasteur*, **23**, 604.
- Lourie, E. M., Murgatroyd, F. & Yorke, W. [1935]. *Ann. trop. Med. Parasit.* **29**, 265.
- Mallardi, M. [1921]. *Pediatrics*, **29**, 878.
- Mandai, H. [1931]. *Acta Sch. med. Univ. Kioto*, **14**, 167.
- Müller, O. H. [1941]. *J. chem. Educ.* **18**, 65.
- Page, J. E. & Robinson, F. A. [1942]. *J. Soc. chem. Ind., Lond.*, **61**, 93.
- Peter, F. M. [1929]. *Arch. Schiffs- u. Tropenhyg.* **33**, Suppl. 3, 153.
- Piazza, G. [1933]. *Ricerche e studi sulla leishmaniosi viscerale del Mediterraneo*, p. 189. Catania.
- Reed, G. & Gant, V. A. [1942]. *Industr. Med.* **11**, 107.
- Renault, J., Monier-Vinard & Gendron, G. [1922]. *Bull. Soc. méd. Hôp. Paris*, **46**, 1624.
- Roncato, A. & Bassani, B. [1934]. *Arch. Sci. biol., Napoli*, **19**, 51.
- Schweitzer, H. [1936]. *Acta brev. neerl. Physiol.* **6**, 718.
- Shoji, K. [1931]. *Bull. Inst. phys. chem. Res. Japan*, **10**, 162.
- Teisinger, J. [1938]. *Pr. méd.* **46**, 676.
- Waldschmidt-Leitz, E. & Mayer, K. [1939]. *Hoppe-Seyl. Z.* **261**, 1.
- Weese, H. [1937]. *Chin. med. (Miss.) J.* **52**, 421.
- [1938a]. In Schmidt, H. & Peter, F. M. *Advances in the Therapeutics of Antimony*, p. 224. Thieme: Leipzig.
- [1938b]. In Schmidt, H. & Peter, F. M. *Advances in the Therapeutics of Antimony*, p. 232. Thieme: Leipzig.

The Biological Assay of Vitamin D₃

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Two methods are generally used for the biological assay of vitamin D₃ on chicks, viz. the A.O.A.C. method which uses the bone ash of the tibia as a criterion of response, and the method of Olsson [1936], in which the tarso-metatarsal distance (t.m.t.) measured on an X-ray film is so used. Both methods use criteria which are measured in fundamental units (mass or length, respectively). This gives them an advantage over those methods used in rat assays where a visual estimate is made by comparison with an arbitrary set of standards. The Olsson radiographic method also involves less time, expense and labour, since the chicks are not killed.

The technique has been described elsewhere [Olsson, 1936; 1939; Baker & Wright, 1940] and the method has been adopted (1940) as standard by the British Standards Institute.

The present paper deals with some investigations on the nature of the test diet and its effect on the t.m.t. distance, and shows that on a suitable diet, gain in weight runs parallel with decrease in t.m.t. distance and affords a criterion for assay of vitamin D₃ of the same order of accuracy as the t.m.t. method. Experience with diet no. 1 of the British Standards Institute Specification 911/1940 indicated that differences in t.m.t. distances between