LXXXV. BRADYCARDIA IN THE VITAMIN B_1-DEFICIENT RAT AND ITS USE IN VITAMIN B_1 DETERMINATIONS.

BY THOMAS WILLIAM BIRCH AND LESLIE JULIUS HARRIS.

From the Nutritional Laboratory, University of Cambridge, and Medical Research Council.

(*Received March 1st, 1934.*)

IN an earlier paper [Drury *et al.*, 1930] it was shown that rats suffering from vitamin B_1 deficiency had low heart rates. When vitamin B_1 was administered rapid cures resulted, the effect on the heart rate being proportional to the amount of vitamin allowed. It was suggested that this action on the heart might form the basis of a very convenient, rapid and accurate method for estimating the vitamin. Vitamin B_2 and various other food constituents were without similar action. During the past three and a half years this method has been in continuous use in this laboratory where it forms our usual routine method of test in vitamin B_1 assays. As a result of this further experience—during which several hundred tests in all have been carried out—we have had the opportunity of collecting further evidence as to the advantage of the method and have examined its degree of accuracy when carefully checked against other standard methods of vitamin B_1 assay. In the present paper we present a typical set of results, illustrating the use of the method in practice. The principal examples which we give relate to tests on the materials selected for investigation in 1930 by the sub-committee appointed in this country to examine methods and standards for vitamin B_1, preparatory to the adoption of the League of Nations International Standard. These materials were (1) the "activated acid clay" standard, and certain specimens of (2) yeast, (3) marmite and (4) wheat germ. Parallel determinations on each of these four materials were carried out by us by three other methods, *viz.* (1) cure of convulsions in rats, (2) growth rate in rats, and (3) cure of "polyneuritis" (head retractions) in pigeons, and are here compared with our results by the bradycardia method. These data were included in the memorandum on antineuritic vitamin B_1, placed before the International Conference on Vitamin Units and Standards of the Permanent Standards Commission of the Health Organisation of the League of Nations, London 1931.

PART I.

DETERMINATION OF VITAMIN B_1 BY THE BRADYCARDIA METHOD.

1. *Technique.*

The electrocardiographic records needed in this method are taken by a procedure already outlined [Drury *et al.*, 1930]. The unanaesthetised rat is held down firmly on its back on a board, its head being held by an adjustable metal clamp and its paws and hind-legs fixed by slip nooses attached to cleats at the

side of the board. The electrodes consist of two small needles, one being placed under the skin of the right fore-leg the other at the lower end of the thorax. These needles are connected to a Matthews's portable electrocardiograph on which permanent photographic records are then taken.

The method of carrying out an assay is as follows. Young rats weighing about 40 g. are placed on a basal diet of

Sugar	60
Arachis oil	15
"Light white casein"	20
Salt mixture	5
Autoclaved marmite (1 hr., 15 lbs., p_H 10) ...	6
Cod-liver oil	1 drop per rat per day.

After about 3 weeks, when the animals are beginning to decline in weight, electrocardiograms are taken. By this time the heart rate should have fallen from

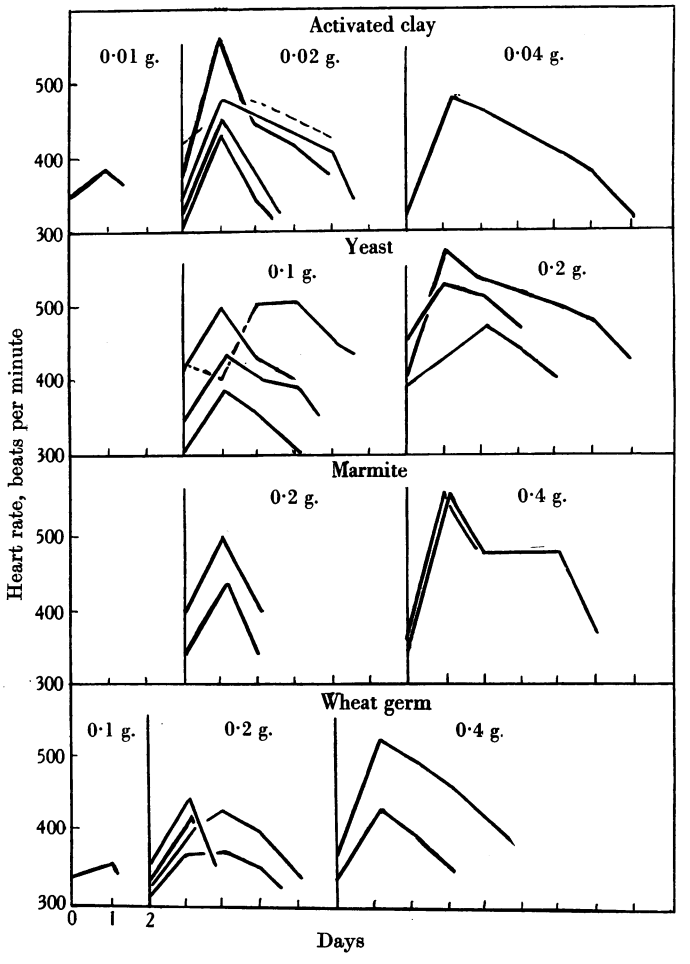

Fig. 1. Heart rate after administration of different sources of vitamin B_1.

Table I. *Duration of cures.*

Material	Dose fed g.	Individual duration of cure (days)	Average duration of cure (days)	Relative activity calculated from curve
Activated clay	0·01	1·5	1·5	
	0·02	2·5, 4·5, 2·5, 4, 4	3·5	100
	0·04	6	6	
Yeast	0·1	3, 3, 3·5, 4·5	3·5	21
	0·2	4, 5, 6	5	16·5
				18·8
Marmite	0·2	2, 2	2	(6)
	0·4	5, 5	5	8·3
				8·3
Wheat germ	0·1	1	1	5·5
	0·2	2, 2, 4, 3·5	3	9
	0·4	5, 3	4	6·5
				7

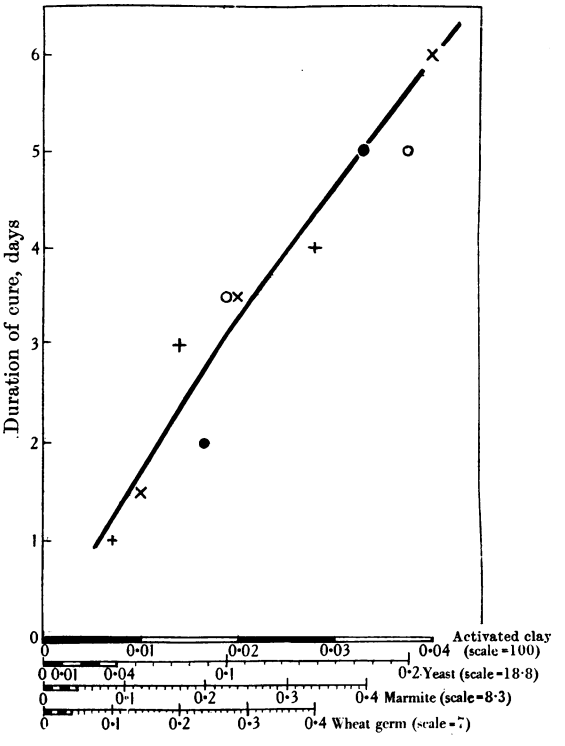

Fig. 2. Bradycardia method: dose-effect curve.

× = Activated acid clay standard.
o = Yeast.
• = Marmite.
+ = Wheat germ.

To determine the scales for the unknown, the points for the standard are first plotted; unknowns are next plotted roughly at approximately the correct scale; and then the value for each point of the unknown is calculated against the standard curve. The average of these values (shown in Table I) is used in re-plotting for final comparison, as above.

the normal rate of 500–550 per minute to a rate of about 350 per minute. As soon as the rate has fallen to this figure a single dose of the substance to be tested is fed. The animal is replaced in its cage and given basal diet and water *ad lib.* as before. After 24 hours its electrocardiogram is again taken. If the rhythm has not increased a larger dose must be tried. With a sufficiently large dose the rate is suitably increased and is measured every 24 hours, or better twice daily, until it has fallen again to about the same low value as when the dose was administered. The increase in rate and the time over which the increase lasts are roughly proportional to the dose given. Several levels of unknown are tested in this way, with about 4 to 6 animals in each group, and comparison is made simultaneously with the amount of standard reference material needed to produce the same effect. A dose-effect curve may be plotted from which relative activities may be determined for intermediate readings, whenever the "effects" are not quite identical (*cf.* Fig. 2). Cures of about 4 days' duration are found to be the most suitable basis for comparison. A quick method, when approximate results suffice, is to find the amount of supplement needed to give the smallest detectable effect on the heart rate lasting about 1–2 days. If doses lasting longer than about 4–5 days are given errors may be caused by apparently incomplete utilisation of the vitamin. This is especially the case with sources of moderate activity and less so with active concentrates.

2. *Illustrative results.*

In the tests taken for description here the "activated clay" standard was compared with yeast, marmite and wheat germ fed simultaneously at two or three different levels of activity. Fig. 1 gives the daily records of the heart rates after dosing. It is seen that 0·02 g. of activated clay is roughly equivalent to 0·1 g. of the yeast, or slightly more potent than 0·2 g. of marmite or of wheat germ, and other doses in proportion. The average durations of cure at each level of dosage are worked out in Table I, and the relative activities of the four substances can then be calculated more accurately from the combined dose-effect curve, Fig. 2. The activities so determined, taking activated clay as 100, are: yeast 19, marmite 8, wheat germ 7. These results will be found to agree excellently (Table VII) with those determined by the three other standard methods. We now turn to a discussion of the latter, giving various recommendations as to the technique which we have found most advantageous, and shall return later to a comparative discussion of the bradycardia method.

PART II.

CURE OF CONVULSIONS IN RATS.

1. *Method.*

Our technique is a modification of that described by Smith [1930].

Diet. The basal diet consists of

"Light white casein"	18
Salt mixture	4
Arachis oil	10
Sucrose	58
Autoclaved brewer's yeast	10
Dried brewer's yeast	0·4
Cod-liver oil	1 drop per rat per day.

The small addition of brewer's yeast is made because in our experience a larger percentage of the rats develop convulsions consistently when a trace of vitamin B_1 is provided in the diet, which is in conformity with the conclusions of Sherman and Sandels [1931] and Sebrell and Elvove [1931]. The 0·4 % level was chosen because after numerous trials it was found to give the best results. Sugar is used in place of starch in order to avoid risk of refection and also because we found that the substitution tended to increase the percentage incidence of convulsions. On this diet over 50 % of animals can be relied upon to develop convulsions at the regular time.

Preparation of animals. Young rats, either albino or piebald, weighing about 70–80 g. are placed on the above diet. After about 6–8 weeks, appetite having fallen considerably, "polyneuritic" symptoms begin to appear. The rat develops leg weakness and drags his limbs, the gait is "wobbly" and there is evidence of inco-ordination (swollen red paws said to be indicative of deficiency of Reader's "vitamin B_4" have not been seen). As a criterion for the presence or absence of definite "polyneuritis" the rat is picked up and suspended by the tail, and rotated between the thumb and finger for a second or two: if convulsions follow the rat is ready for use.

Method of test. A single dose of the substance to be tested is administered to each of a series of such rats. If, as in the work under description, the substance is a foodstuff, it is fed *per os*. If it is an active concentrate it is probably better to inject it into the peritoneum. Provided a sufficient dose has been given the animal is cured within 24 hours. If it is not cured in 2 days the test is regarded as negative, and a larger dose is then given. Animals which die within 24 hours of dosing are neglected, as when they are in the proper condition they should live for at least two days with the "polyneuritic" symptoms. If the dose has been sufficient and the animal is cured, the number of days' respite from convulsions is noted.

Normally, using the minimum dose of standard needed to effect a proper cure, the cure lasts about 5–6 days. With certain substances, however, such as marmite it tends to last slightly longer, *e.g.* about 8–12 days: this may be due to the slower rate of absorption.

Calculation of results. The agreement between duplicate animals as regards the duration of cure is very close. For example a 6-day dose will produce cures lasting somewhere between 4 and 9 days in the great majority of animals in any given group. Occasionally, however, an animal may be encountered which behaves abnormally, appearing to remain cured or partially cured over a protracted period: such animals are neglected in computing the results of the assay. In a few cases these same animals have also failed to respond normally during the depletion period, and coprophagy was suspected—it cannot always be obviated even with screened floors.

If an approximate idea only is desired of the potency of different preparations under test, it can be obtained with great ease and using very few rats by simply comparing the minimum doses of each needed to produce a definite cure—such cures usually lasting about 5 days. This method is especially useful in determining roughly the activity of solutions during a process of concentration.

At about this minimum level, however, results tend to be more variable and partial cures are more frequent. More precise results are obtained therefore by providing sufficient of the supplement to give cures lasting from about 6 to 12 days and feeding several dosage levels of both unknown and standard, using several duplicate rats at each level. Doses of standard and unknown are then compared which give approximately the same duration of response. To allow for slight

differences a dose-effect curve (Fig. 3) is plotted, but it is always advisable to restrict comparisons to doses of unknown and standard which give similar magnitudes of cure. At medium levels the duration of cure is found to be very nearly proportional to the weight of vitamin given, so that the method of calculating activity from "day-dose" (dose given/average duration of cure) is reasonably accurate. As higher levels are reached however the increase in duration of cure does not quite keep pace with the increase in dose.

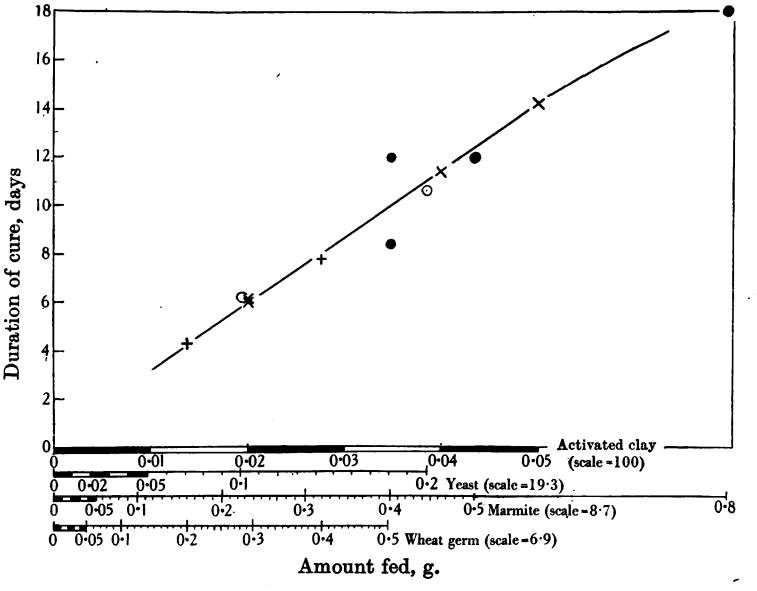

Fig. 3. Convulsion in rats: dose-effect curve.

× = Activated acid clay standard. ○ = Yeast.
● = Marmite. + = Wheat germ.

Scale divisions determined as for Fig. 2.

Degree of accuracy attainable. The relatively small degree of variation between individual animals on duplicate doses of a supplement is seen from reference to Table II. Thus at the level of 0·02 g. of activated clay, 13 out of 20 animals gave cures lasting 4 to 8 days, *i.e.* within 30 % of the median value of 6 days. Or again, with 0·4 g. of marmite 14 animals out of 22 gave a result within 20 % of the median value of 12 days, while 18 out of the 22 fell within 8–16 days, or 30 % of the median. Hence with 5 animals in a group, duplicate determinations based on the median value should certainly not differ by more than 30 %. From a practical point of view this may be taken as the "possible percentage error of the method," *i.e.* when comparing one single dose level of standard with the equivalent level of control and using 5 animals in each group. Actually, in any complete assay based on a series of such comparisons, the final error is naturally considerably less, as will be seen from Tables II and VII.

2. *Results.*

The determinations on yeast, marmite and wheat germ, taking activated clay as standard, are shown in Table II. They confirm the results given by our bradycardia method, the two sets of values being in excellent agreement.

Table II. *Cure of convulsions. Experimental results.*

Material	Dose g.	No. of tests	Days protected	Days protected, mean	Activity calculated from curve
Activated acid clay	0·01	6	(–), (–), (–), (–), (–), (–)	—	—
	0·02	13	10, 7, 6, 4, 4, 4, (20), 8, 7, 5, 7, (13), 5	6·1	—
		[7]	[5, 5, 9, 6, 11, (–), (–)]	[6]	100*
	0·04	4	13, 9, 11, 13	11·5	—
	0·05	3	17, 12, 14		
Yeast	0·05	6	(–), (–), (–), (–), (–), (–)	—	—
	0·10	9	5, 7, 5, 7, 10, 6, 6, 6, 4	6·2	20
	0·20	5	16, 8, 9, 12, 8	10·6	18·5
					19·3
Marmite	0·2	6	(–), (–), (–), (–), (–), (–)	—	—
	0·3	1	(–)	—	—
	0·4	7	10, 7, 7, 10, 6, 11, 8	8·4	7·3
		[15]	[13, 12, 14, 12, 11, 8, 12, 13, 17, 11, 12, 16, 11, 13, 22]	[12·5]	10·5
	0·5	2 [1]	[13], 12, 11	12·0	8·4
	0·8	[1]	[18]	—	—
					8·7
Wheat germ	0·1	3	(–)	—	—
	0·2	5	3, 3, 7, (–), (–)	4·3	7
	0·4	4	8, 6, 10, 7	7·8	6·8
					6·9

Figures in round brackets indicate partial cures, or abnormal response.
Figures in square brackets were determined at a separate time.
* = Standard of reference.

PART III.

Determinations by the growth rate method.

1. *Technique.*

Diet. We use a basal diet having the following composition:

Arachis oil	15
Sugar	60
"Light white casein"	20
Salt mixture	5
Alkaline autoclaved marmite after Guha and Drummond[1]	0·75 g. per rat per day
Cod-liver oil	1 drop per rat per day.

Experiments have also been carried out using rice starch in place of sucrose, and these gave identical results. In order to avoid risk of refection however we prefer to use sucrose, which has the additional advantage of being a more standard product. The "light white" variety of caseinogen is used in order to avoid any possibility of shortage of the "Coward factor".

Influence of variations in vitamin B_2 supplement. In one series of experiments 0·5 g. of autoclaved marmite was given per day in place of 0·75 g. Growth rate was consistently less. This experience emphasises the necessity, frequently overlooked, of always making comparison between animals receiving the same amount of vitamin B_2, in computing results.

[1] Made alkaline to litmus and autoclaved for 1 hour at 15 lbs.

Method of test. During the preliminary period albino rats of 50 to 70 g. weight are fed on the above diet. If smaller animals, around 40 g., are taken lower growth rates are obtained, and results are liable to be more variable. Groups should contain equal proportions of male and female, or preferably males only should be used. As soon as the animals begin to lose weight or are remaining constant, graded daily doses of unknown and standard are fed to groups of rats. The growth rate is then measured over a period of 2–3 weeks.

Calculation of results. Comparison between standard and unknown must be made over that range where small additions of the vitamin have the greatest effect on the growth rate, that is to say between 1 and 2·5 g. per day (Fig. 4). Over

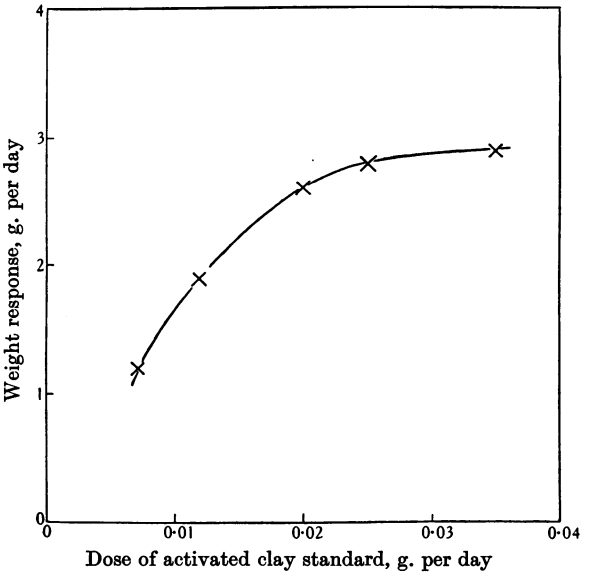

Fig. 4. Growth rate method. Dose-effect curve with international standard (0·75 g. autoclaved marmite per rat per day).

this range the dose is nearly proportional to the growth rate, so that it is legitimate to calculate activity by dividing average gain in weight by dose given. It is preferable however to plot a dose-effect curve for unknown and standard. This should include at least two points for the standard on the steep part of the curve and at least one intermediate or coincident value for the unknown (or *vice versa*).

Variability of "standard reference curve." The suggestion has been made that trouble may be saved and less animals used by constructing a standard reference curve, showing the relation between dose and effect, which, it is suggested, may then be applied to any future experiment. We have found (as we reported in the memorandum to the Vitamin Conference in 1931) that this procedure is inaccurate, since the curve may vary appreciably from one experiment to another, owing to the considerable number of uncontrollable variables. Thus, in one experiment, summarised in Table III (and Fig. 4), in which five levels of standard were tested, using 4 or 5 rats, all males, in each group, the growth was found to be proportional to the dose given up to the level of about 2·5 g. per day and then fell off rapidly, whereas in the earlier experiment (Fig. 6, top), it remained proportional up to 3·5 or over. It follows that direct comparison between

Table III. *Growth responses with graded doses.*

Amount of standard fed mg. per day	Average gain in wt. g. per day	Mean gain in wt.
7	1·2, 1·7, 1·0, 1·1, 1·2	1·2
12	2·5, 2·0, 1·5, 1·9	1·9
20	2·5, 2·7, 2·4, 2·9, 2·6	2·6
25	3·1, 2·8, 2·7, 2·8, 3·1	2·8
35	3·0, 3·2, 2·6, 2·9	2·9

unknown and standard must be made in each individual experiment. This has since been accepted by Coward *et al.* [1933] in a recent paper.

Degree of accuracy attainable. Differences of ±20 % in activity can generally be readily detected by the method described. This is well illustrated by the experiment (Table IV) in which we fed supplements at 80, 100 and 120 mg. per day of marmite to groups of 4 to 5 animals only. Comparing groups of the same sex the differences in response are quite marked (*cf.* dose-response curve shown in

Table IV.

Amount of marmite fed per day mg.	Average daily gains in wt. (g.) ♂	Average daily gains in wt. (g.) ♀	Mean gains in wt. (g.) ♂	Mean gains in wt. (g.) ♀
80	0·44, 0·44	0·16, 0·12, 0·02	0·44	0·10
100	0·82, 0·82	0·24, 0·62	0·82	0·43
120	1·1, 1·3	0·45, 0·70	1·2	0·57

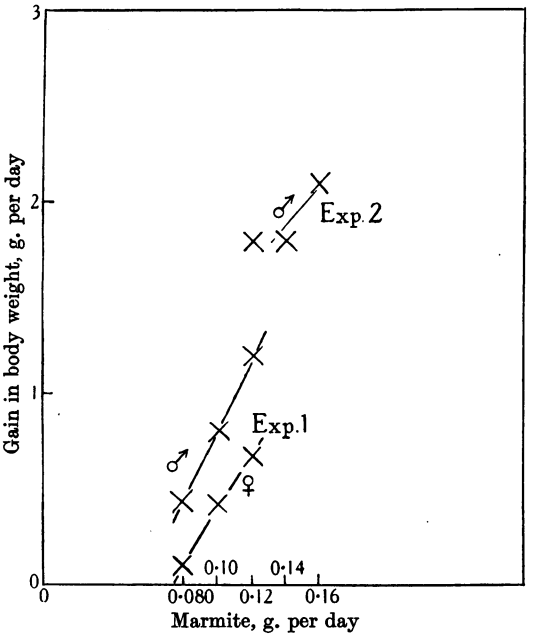

Fig. 5. Degree of accuracy of growth rate method.

Fig. 5). (In this experiment the animals grew at a somewhat slower rate than is usual for this level of marmite. This was attributed to their having started on experiment at the weight of 40 g. in place of the usual 50–70 g.)

To determine the largest individual variation in a larger group of animals and so obtain a more exact measure of the accuracy of the method, a further experiment was carried out. Doses of 160, 140 and 120 mg. of marmite respectively were fed to groups of 9, 5 and 5 animals, all males. The growth rates are given in Table V. It is seen that in the group of 9 the maximum variation is from 1·7 to

Table V. *Individual variability of animals in groups.*

Amount of marmite fed mg. per day	Average daily gains in wt. (g.)	Mean daily gain in wt. (g.)
120	0·9, 2·4, 1·8, 1·9, 1·9	1·8
140	1·8, 1·4, 1·9, 1·0, 1·8	1·8
160	2·4, 1·9, 2·3, 2·4, 2·6, 1·7, 2·0, 1·8, 1·9	2·1

2·6 g. per day, that is about 25 % either way of the median value of 2·1 g. per day. The remaining 7 animals are all within 14 % of the median. Four out of the 9 are within 10 %. In the two groups of 5, 3 or 4 animals agreed within 10 % of the median.

From the foregoing figures it may be expected that in a determination using 5 animals in a group (at a suitable level of dosage), 3 out of 5 animals should agree within 14 %; and an accuracy of this order is about the best that may reasonably be looked for. Actually in the experiment under discussion the difference between doses of 140 mg. and 120 mg. (= −14 %) was not detected when groups of 5 animals were used, but the difference between 140 and 160 mg. (= −12 %) was detected when groups of 5 and 9 animals respectively were used. On the

Table VI. *Growth method. Summary of results.*

Material	Dose (g.)	Average growth rate g. per day	Mean growth rate g. per day	Relative activities calculated from curve
1st series: 0·5 g. autoclaved marmite				
Activated clay	0·007	−1, −0·5	−0·75	
	0·014	2, 1·9, 1·7	1·87	100 (*a*)
	0·028	2·7, 2·2	2·45	
Yeast	0·04	0, 0	0	—
	0·08	1·7, 1·9, 2·6	2·07	20
	0·16	3, 2·4	2·70	21
				20·5
Marmite	0·1	−0·2, 0·5	0·15	—
2nd series: 0·75 g. autoclaved marmite				
Activated clay	0·03	4·2, 3·8	4	(103)
Yeast	0·05	1·8, 1·0	1·40	20
	0·10	3·3, 1·9	2·60	20
	0·20	3·9, 3·7, 3·3	3·63	—
				20 (*b*)
Marmite	0·07	0·75, 0·6	0·68	9·1
	0·14	2·4, 1·5, 1·6	1·83	9·6
	0·28	2·5, 3·4, 3·3	3·07	8·6
				9·1
Wheat germ	0·1	1·2, 0	0·60	6·5
	0·2	1·7, 2·3	2·00	7·5
	0·4	4·2, 3·6, 3·8	3·87	7·5
				7·2

(*a*) = Standard. (*b*) = Secondary standard.

other hand it should be pointed out that when an assay of an unknown is carried out by feeding several different levels of unknown and of standard, the final result

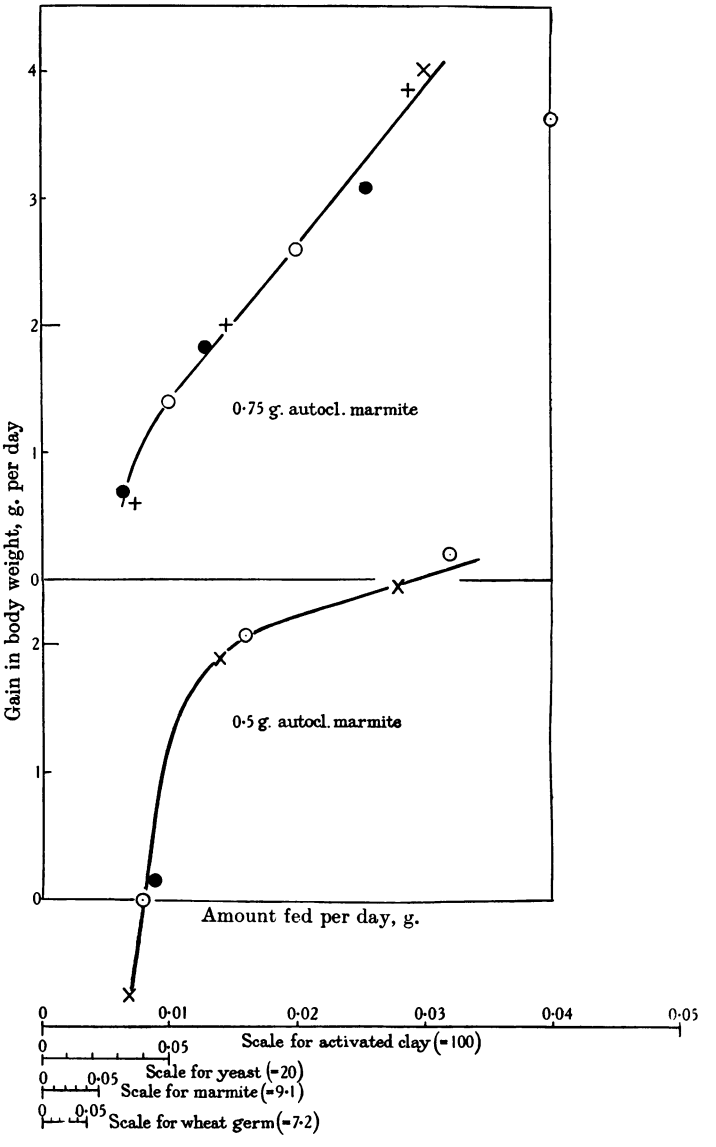

Fig. 6. Vitamin B_1 determination by growth rate method.

× = Activated clay standard. ○ = Yeast.
● = Marmite. + = Wheat germ.

Scale for abscissae determined as in Fig. 2. Note difference in response with 0·5 g. autoclaved marmite per rat per day (below) and 0·75 g. (above).

is then more accurate than just indicated, for although there is a possible error of 14–20 % in each single group, these tend to cancel each other when the final averages are determined (see Table VII).

2. *Results.*

Determinations on activated clay, yeast, marmite and wheat germ gave results virtually identical with those determined by the bradycardia method. Two determinations were carried out: in the first the yeast was compared directly with the acid clay as standard; in the second yeast was used as secondary or derived standard for determining the activity of the marmite and the wheat germ (Table VI and Fig. 6).

PART IV.

COMPARISON OF THE "BRADYCARDIA" AND OTHER METHODS OF ASSAY.

Specificity of test. The results described in Sections I, II and III show that the "bradycardia method" gave identical values with the two other methods of assay, when checked against four materials of diverse origin having activities ranging from 7 to 100. The same figures were given in a few pigeon tests which we also carried out (cure of head retractions)[1]. The comparison of these results, together with further values obtained independently in other laboratories, is given in Table VII. The values obtained by the three rat methods appear to agree within an experimental error of $\pm 5\,\%$.

Table VII. *Agreement between bradycardia and other methods.*

	Relative activities			
Method	Activated clay standard	Dried yeast	Marmite	Wheat germ
Bradycardia method	100	18·8	8·3	7
Cure of convulsions in rats	100	19·3	8·7	6·9
Curative growth tests on rats	100	20	9·1	7·2
Pigeon method	100	18	8·5	—
"Institute B" (rat growth tests)	100	(15)	9	7
"Institute C" (pigeon tests)	100	20	(11)	(14)

Values in brackets are somewhat divergent from five other concordant readings on the same material and are considered less reliable.

This very close agreement (which is confirmed by tests on vitamin B_1 concentrates and other materials) indicates that the factor measured by the bradycardia method runs parallel in its distribution with that measured by the other

[1] The basal diet used was polished rice and water. Only such pigeons as developed head retraction within 30 days were used. These amounted to about 30 % only of the total. Minimum doses were compared giving approximately the same duration of cure and corrected for the "day-dose." Our experience confirms that of other workers that the pigeon gives more erratic results than the rat owing to the greater variation in individual responses. Among the disadvantages of the pigeon method are: (1) a frequent failure to absorb the vitamin properly, especially with materials of only moderate activity, and the occurrence of a "crop bound" condition; (2) an inability to consume many types of foodstuffs of relatively low potency, but of great practical dietetic importance (which of course can be readily tested by the rat method); (3) false cures (*e.g.* as with glucose, change in temperature, *etc.*); (4) lack of economy in use—*e.g.* (*a*) the space needed to house 1 pigeon will accommodate about 10–20 rats, and (*b*) each pigeon is available for test only once in every 6 weeks, whereas with "bradycardia" or "convulsions" tests on a rat several tests per fortnight may be carried out per rat.

better known methods of assay, and it seems very probable that the same single factor is responsible for the loss in weight, for the production of the polyneuritis and for the bradycardia. Physiological arguments in favour of this view are presented in a later section of the paper (Part VI). We have found also that highly purified specimens of vitamin B_1, including the crystalline preparations of Jansen and of Windaus, retain the characteristic effect on the heart beat. At the same time we recognise that in view of the present complicated position of the vitamin B problem it would be premature to make any dogmatic claim on this point.

Advantage of the bradycardia method. The bradycardia method has many advantages over the other methods. All animals are ready for test about 4 weeks after having been placed on the deficient diet, and all consistently develop the symptom. This may be contrasted with the delay and irregularity in the production of head retractions in pigeons or convulsions in rats. Again, results are obtained very rapidly and with little trouble: only one dose has to be fed to each rat and the test is concluded in a few days, one measurement only per day being needed and taking but a few moments to carry out. Also much less material is needed than for a growth test. An additional economy is that the same animal can generally be used two or three times over. The sensitivity of the method is similar to that of the others, although it is probably slightly inferior in this respect to the growth rate method, which however is more laborious and depends on a less specific symptom. A further advantage, and a very important one from the practical standpoint, is that the heart rate method can be used for testing foodstuffs containing only slight traces of the vitamin. Neither the curative pigeon test nor the rat convulsion test is applicable to this important class of estimation, as it is impossible to get the severely ill animal to eat sufficiently large quantities of the food, as for example when the diet must consist entirely of the food in question. (The bradycardia develops at an earlier stage than the polyneuritic symptoms and while the animal is still able to eat normally.) Indeed in certain estimations of this kind, such as in comparisons of white and brown breads and of certain wheat products we have been driven back to the heart rate method as the only one available, since in growth tests on rats we were defeated by the further complication of refection so that no comparison was possible. In the heart rate method an assay can be concluded in 3 or 4 days before refection has had time to develop. It is in this type of problem, *i.e.* comparing the vitamin B activity of naturally occurring and especially farinaceous materials, that the heart rate method seems particularly useful, and we have accordingly added experimental details (Part V) of its application in certain such cases.

Practically the only drawback to the heart rate method is the original expense of purchasing the electrocardiograph. Accurate determinations are not possible with simpler types of apparatus.

PART V.

Application of bradycardia method to wheat products.

To illustrate the practical uses of the bradycardia method, its application to the determination of the vitamin B_1 content of two classes of wheat products will be described. These were (*a*) white compared with brown bread, (*b*) whole wheat grain as influenced by different soil treatments.

(a) White compared with brown bread.

We originally became interested in this problem because it had been claimed that white bread contains adequate amounts of vitamin B [Hartwell, 1924] and that the substitution of brown for white bread in a dietary experiment on rats resulted in little improvement in growth [Mottram and Hartwell, 1929].

Our attempts to compare the vitamin contents of white and brown bread by the rat growth method failed, as the rats became refected. Efforts were made to apply the rat convulsions method, but with unsatisfactory results. Determinations by the bradycardia method were then carried out, and these proved the striking difference between the two kinds of bread.

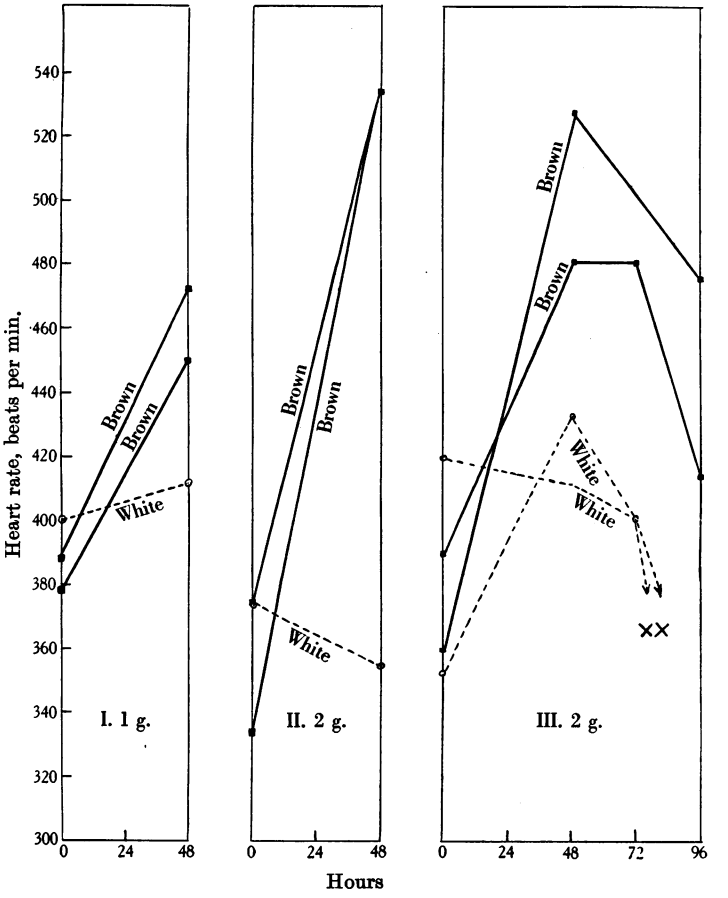

Fig. 7. Comparison of white and brown bread by the heart rate method.

Three tests were carried out. In each case the specimens of brown and white bread were purchased from a local branch of the Co-operative Society. The first test (Fig. 7, I) showed that 1 g. of brown bread was sufficient to cause a marked rise in heart rate whereas 1 g. of white bread had a very slight effect only. The difference was brought out more sharply when the brown bread was fed at a

higher level (Fig. 7, II) and a further experiment (Fig. 7, III) furnishes the basis for a rough quantitative comparison.

Conclusion. The white bread contained only insignificant amounts of vitamin B_1 compared with the brown bread. Detailed results on different types of wholemeal, brown and white and germ breads will be presented in a later paper.

(*b*) *Effect of soil treatment on vitamin value of wheat.*

This work was undertaken to test the claim that the vitamin content of seeds and grain is influenced by the nature of the manure or artificial fertiliser [McCarrison and Viswanath, 1926; Rowlands and Wilkinson, 1930].

Through the co-operation of Sir John Russell of the Rothamsted Experimental Station we were provided with specimens of whole wheat from plots treated as follows:

Plot No. 3. No manure.
2B. 14 tons of dung per acre.
5. Complete mineral manure.
7. Complete mineral manure +412 lbs. of sulphate of ammonia per acre.
10. 412 lbs. of sulphate of ammonia only.

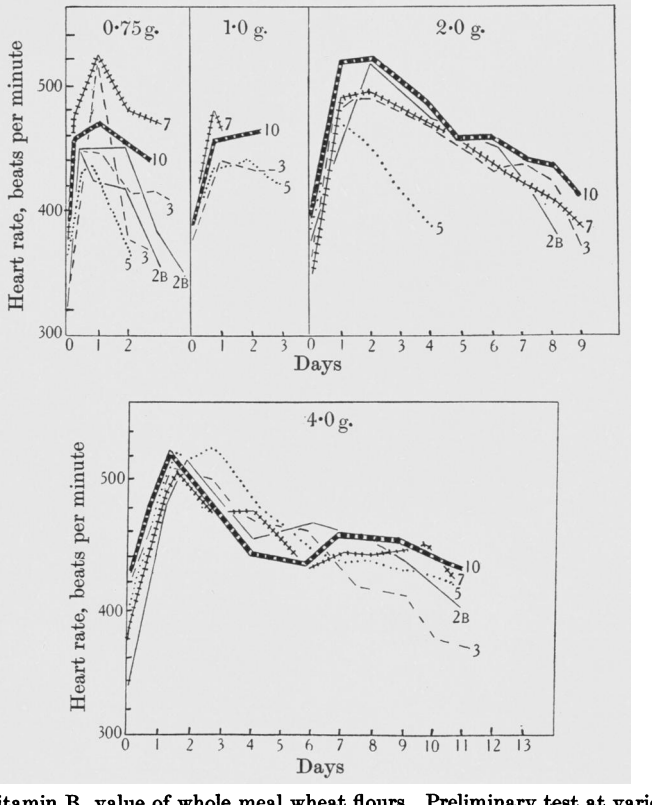

Fig. 8*a*. Vitamin B_1 value of whole meal wheat flours. Preliminary test at various levels (illustrating extent of variation of individual animals).

———— = Plot 2B. — — — = Plot 3. ····· = Plot 5.
+++++++ = Plot 7. ▬▬▬ = Plot 10.

Although these various treatments greatly influence the chemical composition of the grain and the amount of grain produced our results (Figs. 8*a* and 8*b*) show

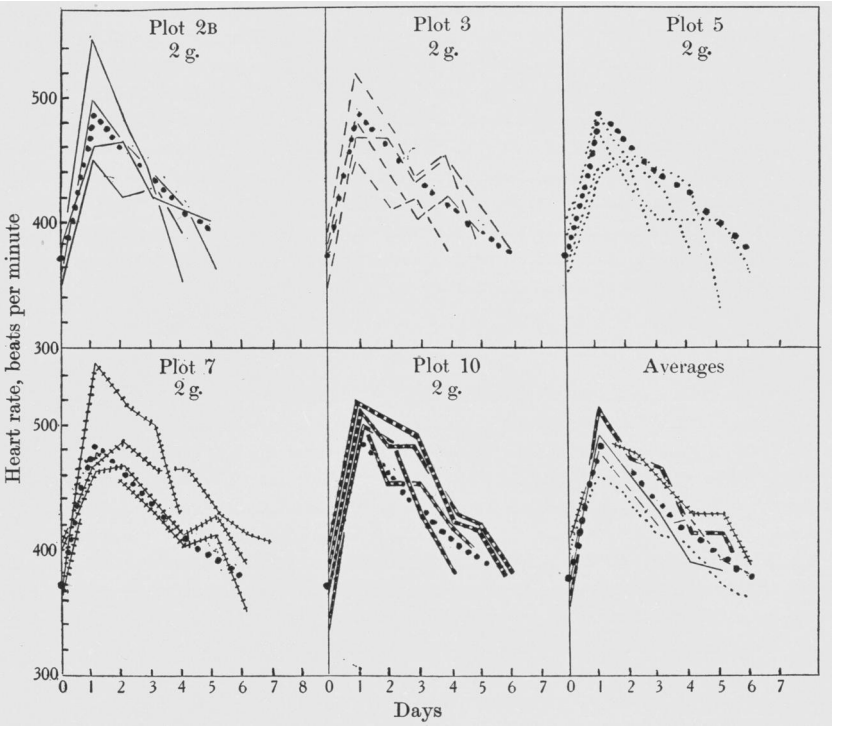

Fig. 8*b*. Comparison of vitamin B_1 activity of whole meal wheat flours, using 4 animals per group. The indication obtained in the preliminary test is confirmed, *viz.* showing the slight superiority of plots 7 and 10 and slight inferiority of plot 5, compared with 2B or 3. *Days protected:* Plots 2B and 3 = 5 days; plot 5 = 4 days; plots 7 and 10 = 6 days. *Relative activities:* Plots 2B and 3 = 100; plot 5 = 80; plots 7 and 10 = 120.

•–•–• = Comparison curve, mean of all specimens. ——— = Plot 2B.
– – – = Plot 3. ····· = Plot 5. +++++++ = Plot 7. ▬▬▬ = Plot 10.

that the vitamin B_1 content when expressed per g. of whole grain is not affected to any important extent (the fuller data will be presented elsewhere). In conformity with this, growth tests on the "sharps" prepared from the grain from these same plots showed no appreciable variation in content of vitamin B complex.

PART VI.

Physiological significance of the bradycardia in vitamin B_1 deficiency.

1. *Relations between bradycardia and "polyneuritic" symptoms.*

As mentioned in Part I, the incidence of convulsions in our vitamin B_1-deficient rats was greatly increased when they were given small traces of vitamin B_1 in their diet. This symptom seems to be associated, that is, with hypovitaminosis rather than complete avitaminosis or with chronic rather than acute

vitamin B_1 deficiency. This finding confirms earlier conclusions of Sherman and Sandels [1931] and Sebrell and Elvove [1931]. (A similar relation appears to apply also to certain symptoms of vitamin B_1 deficiency in the pigeon.) Measurements of the heart rate which we have carried out suggest that this small amount of vitamin B_1 probably has the function of keeping the heart rate at a high enough value to enable the animal to survive, thus allowing the chronic symptoms of polyneuritis to develop. We find that the heart rate of a "hypovitaminotic" rat with symptoms of polyneuritic convulsions is generally around 400 beats per minute, while a rat rapidly run out on a diet completely devoid of vitamin B_1 has a much lower heart rate, about 300 beats per minute or lower, and no symptoms of "polyneuritis." In other words the biochemical lesion caused by vitamin B_1 deficiency seems to affect the heart almost immediately and may so cause sudden death from heart failure, while its poisoning action on the central nervous system proceeds more slowly.

2. *Relation to the biochemistry of vitamin B_1 action.*

In the earlier paper on this question [Drury *et al.*, 1930] the suggestion was made that the bradycardia might be correlated with the accumulation of lactic acid which is known to occur in vitamin B deficiency [Bickel, 1924; 1925; Collazo, 1923; Collazo and Morelli, 1926]. This error in lactic acid metabolism seems to be a consistent feature of vitamin B_1 deficiency, is not restricted to any one organ or type of tissue and is common to various species. Work on beri-beri patients in Japan, for example, has shown how the lactic acid tends to reach abnormally high levels, especially after exercise. Hayasaka [1930, see also Hayasaka and Inawashiro, 1928; 1930; Inawashiro and Hayasaka, 1928] has found that the recovery process after muscular exercise is more prolonged in beri-beri than in normal individuals, and, in the case of the dog, he has pointed out that in vitamin B deficiency even quite light exercise sufficed to keep the blood-lactic acid above its normal value for prolonged periods. He therefore concludes that lack of vitamin B leads to a disturbance in the resynthesis of glycogen from lactic acid. In keeping with this view he has found that when sodium lactate was injected into beri-beri patients the resynthesis to glycogen was much lower than in normal controls or cured beri-beri subjects. The accumulation of lactic acid in vitamin B_1 deficiency has been confirmed further by Collazo and Bayo [1931] and others, and Kinnersley and Peters [1930] and their co-workers have recently found a correlation between the imminence of convulsions in pigeons and the excess of lactic acid accumulating in certain parts of the brain. From what is known of the influence of lactic acid on heart rhythm [Clark *et al.*, 1932] it is to be expected that an excess will diminish the rate of beat *in vivo*, as it does in isolated preparations. Measurements of blood-lactic acid (Fig. 9) in vitamin B-deficient rats have confirmed our belief in a correlation between the lactic acid excess and the low heart rate. On the other hand it must be recognised that this does not

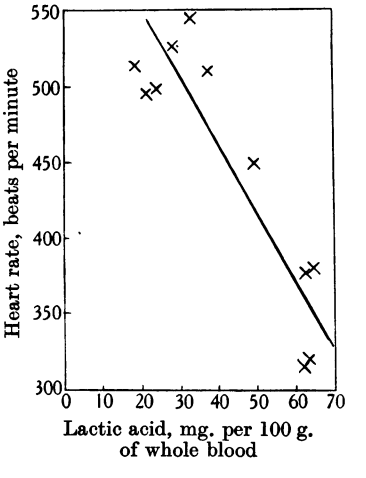

Fig. 9. Correlation between fall in heart rate and rise in lactic acid content of blood in vitamin B_1 deficiency.

prove that the correlation is a direct causative one. Attempts to influence the rate by the administration of large doses of sodium lactate have been unsuccessful.

Experimental. It was found necessary to use about 3 rats in order to obtain enough blood for each lactic acid estimation. The blood samples were obtained by decapitating the rats with scissors over a weighed basin of 20 % trichloroacetic acid. The blood was allowed to drain from the animals for about half a minute, the basin was then again weighed and the amount of blood obtained calculated from the difference in the weighings. The amount of blood used was usually between 6–10 g. this giving a reading of approximately 1–3 ml. of $N/100$ I in the final titration. When sufficient blood had been obtained the trichloroacetic acid mixture was made up to a definite volume so as to give a final concentration of about 5 % trichloroacetic acid. This solution was then filtered and precipitated with the copper-lime reagent to remove carbohydrates and the lactic acid estimation carried out according to the method of Friedemann *et al.* [1927].

As will be seen from Fig. 1 the heart rate of a normal rat is between 500 and 600 beats per minute, and the lactic acid content is about 30 mg. per 100 g. of blood. When the rats become deficient in vitamin B_1 the heart rate drops to 300–350 beats per minute and the lactic acid content rises to about 70 mg. per 100 g.

3. *Vitamin B_1 as oxidative coenzyme.*

The existing evidence warrants the suggestion that the physiological rôle of vitamin B_1 is to intervene at some stage in that complex cycle of reactions in carbohydrate metabolism involving the formation and oxidation of lactic acid, and in a capacity corresponding with that of coenzyme. Banga and Szent-Györgyi [1932] announced the isolation of a coenzyme for lactic acid dehydrogenase. One of us has drawn attention elsewhere [Harris, 1934] to the fact that a number of salient properties of the coenzyme bear a resemblance, superficially at least, to those of vitamin B_1—*e.g.* it is needed for the normal functioning of the heart muscle (it was from this source that it was isolated), and it is concerned in the removal and oxidation of lactic acid. In this connection also its suggested relationship to adenine, or adenylic acid, which is known to be of significance for the physiology of the heart beat, is of further interest.

It seems certain of course that vitamin B_1 itself cannot be identical with Banga and Szent-Györgyi's coenzyme (*cf.* Boyland, 1933); nevertheless there appears to be a close interconnection between the two classes of substances and their physiological rôles in the related and interdependent series of reactions which they control. The nature of the lactic acid dehydrogenase system is discussed further in the following paper [Birch and Mann, 1934].

Summary.

Part I.

The technique of estimating vitamin B_1 by the heart rate method which has been in use in this laboratory for several years as a standard method is described. Single doses of the unknown are fed to rats suffering from vitamin B_1 deficiency and the heart rate measured at daily intervals for a few days afterwards. The magnitude and duration of the effect are proportional to the amount of vitamin so given. The accuracy of the method was checked by tests on four substances chosen for investigation by the Sub-committee on Vitamin B_1 Standardisation, *viz.* activated acid clay standard, dried yeast, marmite and wheat germ. These were assayed by separate control tests by the independent methods described in Parts II and III.

Part II.

A method of carrying out vitamin B_1 determinations by curative tests on polyneuritic convulsions in rats is described. A feature of this method is the close agreement in response between animals receiving duplicate doses.

Part III.

Results with a growth rate method are also given and the conditions defined for the most accurate results. The number of variables differs too much to permit the use of a standard dose-effect reference curve—as for example with the amount of autoclaved marmite given as the source of vitamin B_2. Under the conditions described differences of ± 20 % in activity are readily detected, and 3 out of 5 animals at least in a group fall within ± 15 % of the median value while the values reached in the assays by any of the three rat methods appeared to vary within an experimental error of about ± 5 %.

Part IV.

Results by the heart rate method correspond with those given by the other methods, the comparative values found, taking activated clay standard as 100, being yeast 18·8, 19·3, 20, 18; marmite 8·3, 8·7, 9·1, 8·5; wheat germ 7, 6·9, 7·2, by the bradycardia, convulsions, growth rate and pigeon (head retraction) methods respectively. Among the advantages of the heart rate method are its convenience, rapidity, simplicity and economy, and the fact that it can be readily used for determining foodstuffs containing only small amounts of vitamin B_1 which are beyond the scope of the curative pigeon or rat (convulsions) method, and—when they contain starch and give rise to refection—the rat growth test.

Part V.

As an example of the use of the heart rate method tests are described on white and brown breads and other wheat products. These could not be readily estimated by the pigeon or rat curative methods, and growth tests on rats were vitiated by the occurrence of refection.

Part VI.

A rat given no vitamin B_1 and dying from acute avitaminosis has a very low heart rate (*e.g.* 300 per min.) and no polyneuritic convulsions. A rat given slight traces of vitamin B_1 and developing chronic hypovitaminosis has a less severe bradycardia (*e.g.* heart rate 400) but convulsions. It is suggested that the traces of vitamin B_1 act by keeping the heart rate at a high enough level to enable the animal to survive and so permit the more slowly developing chronic symptom to develop.

The earlier suggestion is confirmed that the bradycardia is correlated with the accumulation of lactic acid in the vitamin B_1-deficient animal. Vitamin B_1 appears to have the property of a coenzyme-like substance intervening in the chain of carbohydrate oxidation reactions.

REFERENCES.

Banga and Szent-Györgyi (1932). *Biochem. Z.* **246**, 203.
Bickel (1924). *Biochem. Z.* **146**, 493.
—— (1925). *Biochem. Z.* **166**, 251.
Birch and Mann (1934). *Biochem. J.* **28**, 622.
Boyland (1933). *Biochem. J.* **27**, 786.
Clark, Gaddie and Stewart (1932). *J. Physiol.* **75**, 331.
Collazo (1923). *Biochem. Z.* **136**, 278.
—— and Bayo (1931). *Biochem. Z.* **238**, 335.
—— and Morelli (1926). *J. Physiol. Path. Gén.* **24**, 76.
Coward, Burn, Ling and Morgan (1933). *Biochem. J.* **27**, 1719.
Drury, Harris and Maudsley (1930). *Biochem. J.* **24**, 1632.
Friedemann, Cotonio and Schaffer (1927). *J. Biol. Chem.* **73**, 335.
Harris (1934). *Ann. Review Biochem.* **3**.
Hartwell (1924). *Biochem. J.* **18**, 120, 1323.
Hayasaka (1930). *Tôhoku J. Exp. Med.* **14**, 72, 85, 283, 487.
—— and Inawashiro (1928). *Tôhoku J. Exp. Med.* **12**, 29.
—— —— (1930). *Tôhoku J. Exp. Med.* **14**, 53.
Inawashiro and Hayasaka (1928). *Tôhoku J. Exp. Med.* **12**, 1.
Kinnersley and Peters (1930). *Biochem. J.* **24**, 711.
McCarrison and Viswanath (1926). *Ind. J. Med. Res.* **14**, 351.
Mottram and Hartwell (1929). *Lancet*, ii, 892.
Rowlands and Wilkinson (1930). *Biochem. J.* **24**, 199.
Sebrell and Elvove (1931). *U.S. Pub. Health Reps.* **46**, 917.
Sherman and Sandels (1931). *J. Nutrition*, **3**, 395.
Smith (1930). *U.S. Pub. Health Reps.* **24**, 116.