CLXVII. A SURVEY OF THE RAT-BRADYCARDIA METHOD OF ESTIMATING VITAMIN B₁

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THE replacement of the first International Standard vitamin B₁ by the crystalline vitamin (Nov. 1938) makes the present time appropriate for a survey of the responses of the vitamin B₁-deficient rat heart to the acid-clay adsorbate and the use of such responses in routine assays.

Using the technique and basal diet recommended by Birch & Harris [1934], work on rat bradycardia was begun in these laboratories in July of that year. Early assays by this method were considered promising and the details of the technique were developed until we were able to maintain a group of 60–80 rats at a suitable degree of deficiency for 12–20 to be available daily for their curative dose. A colony of this number of rats has been studied over the last 3½ years and we have therefore an assembly of responses to the International Standard acid clay sufficient for an indication of the value of the duration of cure of bradycardia as a means of estimating vitamin B₁ activity.

It has been the custom to feed 5 or 6 doses of i.s. acid clay to groups of rats at each of 3 levels, at first once a fortnight, and after the first few months at weekly intervals. During 1935 and 1936 these weekly doses were fed at different groups of levels, e.g. 10, 20, 30 mg., 15, 30, 50 mg. etc., but for the last 2 years the regular doses have been 15, 25 and 40 mg. weekly (apart from short vacations at Christmas and Easter). In Table I will be found a record of the number of responses obtained with each of these latter doses.

Table I. *Number of responses to i.s. acid clay fed at different levels*

Dose level mg.	No. fed weekly	Total at each level
15	6	501
25	6	486
40	6	475

An examination of the 1462 responses (in Table I) yields the following information:

Table II

Dose in mg.	No. of doses fed	Mean response in days	Range in days	Standard deviation σ
15	501	3.10	0–7.0	1.26
25	486	4.49	0–12.0	1.58
40	475	5.97	2–11.0	1.80

Each group of observations is normally distributed about the mean value (Table III).

Table III. *Distribution of the values*

Mean no. of days	σ	$M \pm \sigma$	No. expected between these limits 68.27%	No. actually obtained	$M \pm \frac{2\sigma}{3}$	No. expected between these limits 50.5%	No. actually obtained
For 15 mg. 3.10	1.26	1.84-4.36	342	371	2.26-3.94	253	248
For 25 mg. 4.49	1.58	2.91-6.07	332	351	3.44-5.54	245	242
For 40 mg. 5.97	1.80	4.17-7.77	324	303	4.77-7.17	240	225

The agreement between actual and expected numbers is near enough for the distribution to be regarded as normal.

In common with biological responses used for other vitamin assays, the mean values plotted against the logarithm of the doses fall on a straight line (see Fig. 1), and in this respect our findings differ from those of Leong & Harris [1937]. As the graphs show, the responses given by other vitamin-containing substances fall on lines parallel with that obtained from the standard.

Fig. 1. $y = 6.98x + 1.7466$, $\odot - \odot$ Int. St. acid clay. $y = 7.42x + 1.522$, $\triangle - \triangle$ prepared germ. $y = 7.35x + 0.01$, $\square - \square$ yeast extract.

The variance and the error of the method are obtained as follows:

Table IV. *Variance of responses*

Dose mg.	No.	Mean response ($\bar{y}$)	$\Sigma (y - \bar{y})^2$	$n - 1$
15	501	3.10	792.60	500
25	486	4.49	1216.59	485
40	475	5.97	1542.03	474
			3551.22	1459

$$\text{Variance } (s) = \frac{3551.22}{1459} = 2.434.$$

The equation to the best straight line through these three points is represented by the expression

$$y = 6.980x + 1.7466,$$

where

$$x = \log \text{dose}.$$

It may be shown that the limits of error of a test interpreted with regard to this standard curve are as follows:

Table V. *Limits of error. Acid clay International Standard*

No. of rats	$P=0.95$ %	$P=0.99$ %
40	85-117	83-121
20	80-125	75-133
10	72-138	66-152
5	64-157	55-181

This degree of accuracy can be regarded as satisfactory when compared with that of other biological methods.

Specificity of the method

The method has recently been criticized by Williams & Spies [1938] on the grounds that the response may not be specific for vitamin B_1 . In this connexion we have grouped the responses obtained to two natural sources of the vitamin, prepared wheat germ and a yeast extract, samples of both of which we have frequently assayed. The results are given in Table VI.

Table VI. *Cure of bradycardia by natural sources of vitamin B_1*

Source of vitamin	Dose g.	No. of doses	Mean response	Variance	Equation to line relating response to log dose
Prepared wheat germ	0.2	232	3.77	3.73	$y = 7.42x + 1.522$
	0.3	223	5.04		
Yeast extract	0.2	66	2.36	2.91	$y = 7.35x - 0.01$
	0.3	69	3.21		
	0.5	10	6.01		

There is no significant difference between the slopes of these two lines and that of the equation to the i.s. acid clay. This strongly suggests that the active material being measured is the same in each case: any interfering material present in one, would, on this evidence, be equally present in all three.

Precautions taken in the use of the method

(1) During the early months of work on the method, the accuracy obtained was less than it has been more recently. Certain anomalous results, for example, were found to be associated with the use of rats on their first test-dose of a vitamin-containing substance, and it was soon clear that many rats gave a longer response to the first dose than they did to the same size of dose of the same sample given subsequently. When the heart rate first shows the effect of the vitamin deficiency the fact that it falls below 500 beats per minute does not mean that still lower rates can be definitely expected, for, without any dose being given, the rate may be increased again for the next day or two. Some control must thus be exercised in choosing the time for dosage if unreliable results are to be eliminated. Yet if rats with such a fluctuating heart rate are left without any dose until the rate is decreasing steadily, a considerable number die. The practice was therefore adopted of giving each rat an initial dose when the rate was about 450 beats per minute. Cardiographic readings are begun after 28-32 days of vitamin B_1 depletion. The first test dose is deferred until the rate shows a steady regular decline from the maximum recorded after the initial dose.

In most cases in which an abnormally long or a fluctuating response is recorded to the initial dose, the subsequent behaviour of the rat is regular as regards both length and type of response (Table VII).

Table VII. *Typical responses to initial and subsequent doses; beats per minute*

		Rat no. 147	
Initial response		433, 500, 460, 480, 455, 480, 400, 488, 450, 420	
Response to 1st test dose		420, 450, 460, 412	
"	2nd	"	412, 420, 433, 430, 420, 400
"	3rd	"	400, 480, 450, 450, 412, 412, 375
"	4th	"	375, 450, 400, 400, 375
"	5th	"	375, 450, 350
"	6th	"	350, 463, 400, 400, 380, 350 (convulsions. Killed by CHCl ₃)

(2) After a rat has been used for several tests and the rate is under 350 it may die at any time with or without convulsions even soon after eating a dose containing vitamin B₁. For this reason no rat is used if it shows convulsive signs with such a slow rate, and test doses are not given when the rate is below about 330. This practice is regarded as important on account of the behaviour of the control rats, of which there are always two or three.

A rat is allocated as a control when its rate is dropping after its initial dose, the only one it receives. Its life after a reading of about 430 is 6–9 days. It is not unusual for such control rats after showing a lowered heart rate in the expected manner to show an accelerated rate on the day before or the actual day of death. Should such an increase follow a test-dose given to a rat in a severely depleted state, it might be regarded as an indication of vitamin activity and give rise to confused results.

Control rats, 24-hourly readings:

- (1) 433, 410, 400, 386, 360, **375**, 233, death.
- (2) 450, 420, 350, **380**, 300, death.
- (3) 430, 412, 412, 320, **340**, death.

(3) In counting the rate by electrocardiograph tracings, certain abnormalities are occasionally, though not commonly, found in the rhythm. Extra-systoles arise in a few animals; their positions are avoided in the selection of the ends of the length of strip to be counted and they do not affect the record. Rarely a rat shows a form of heart block in which a 24-hourly reading is approximately halved. When this happens it is usually during the early part of its time on test and if the animal is left alone the rate is up again by the following day, e.g.

433 (dose), **290**, 500, 450, 420.
 420 (dose), 525, 470, 440, **200**, 440, 150.
 450 (dose), 488, 500, 480, 470, 450, 460, **300**, 445, 436.

Tracings are also rarely obtained which show dropping of one beat in every three. In 2:1 block the rhythm is counted, not the actual number of beats. These and other changes in the heart of the vitamin B₁-deficient rat have been described by Weiss *et al.* [1938].

(4) Since the rat heart rate responds to such small quantities of vitamin B₁, the absence of all traces of the vitamin from the basal diet is essential. The usual precautions must be taken in preparing the diet, but the behaviour of the control animals remains as a constant check. The presence of traces of the vitamin may prolong the readings sufficiently to produce confusing results.

The bradycardia method has been used for a wide variety of different substances and has been found useful for materials possessing widely different degrees of activity, from a rice containing as little as 0.3 I.U./g. to concentrates

containing 200 or more I.U./g. When the substance to be tested is such that it can be diluted accurately, the method is applicable to materials of high activity such as crystalline aneurin.

Table VIII gives the doses employed for substances of differing activities and the actual limits of error determined directly for each assay.

Table VIII

Sample	Dose g.	No. of doses*		I.U./g.	Limits of error		Limits of potency $P=0.95$
		Sample	Standard		$P=0.95$	$P=0.99$	
Rice A	2.0	20	16	0.9	79-126	74-135	0.7-1.1
	3.0						
Rice B	3.0	20	16	0.3	71-141	64-157	0.2-0.4
	5.0						
Malt food	1.0	20	18	1.0	72-138	67-150	0.7-1.5
	1.5						
Cereal germ A	0.2	19	16	11.4	74-135	68-148	8.4-15.4
	0.3						
Cereal germ B	0.2	20	16	11.5	87-115	82-122	10-13
	0.3						
Cereal germ C	0.2	19	15	12.2	78-128	73-137	9.5-15.6
	0.3						
Yeast extract A	0.2	18	18	11.0	71-141	64-157	7.7-15.4
	0.3						
Yeast extract B	0.3	20	18	8.2	72-139	65-154	5.9-11.4
	0.5						
Yeast concentrate	0.075	12	18	33.1	68-148	60-168	22.4-48.8
	0.150						
Rice concentrate	0.010	10	18	183.2	77-130	71-141	141-236
	0.015						
Germ bread	2.0	10	15	1.2	71-141	64-157	0.85-1.70
	3.0						
Whole-meal bread	3.0	9	18	0.5	60-166	52-194	0.3-0.8
	4.0						

* In each case the dose levels of standard were 1.5, 2.5, and 4.0 I.U.

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