109. THE DETERMINATION OF VITAMIN B₁ BY MEANS OF ITS INFLUENCE ON THE VAGINAL CONTENTS OF THE RAT

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THE influence of vitamin B₁ on the vaginal contents of the mature rat has been examined with a view to using it as a criterion for the assay of that factor. Evans & Bishop [1922] showed that in the absence of vitamin 'B' from the diet, the normal cycle of changes in the contents of the vagina was upset. No keratinized cells were found and leucocytes with some nucleated epithelial cells were found day after day up to the time of death of the animal. This was confirmed by Macy *et al.* [1927]. Up to this time vitamin 'B' had not been proved to consist of several factors and our first experiment showed that this upset of the cycle of changes in the vaginal contents could be brought about by a deficiency of vitamin B₁ only. A later experiment showed that it could also be brought about by a deficiency of riboflavin only. The regular cycle could in either case be restored by giving the missing factor. The main part of the work showed that after a deficiency of vitamin B₁, the response to vitamin B₁ was graded to the dose given, i.e. the larger the dose of vitamin B₁ given the more quickly was the normal cycle of changes restored. Thus it would be possible to use this property of vitamin B₁ for its assay, but the accuracy obtainable by its use proved to be less than that obtainable by the 'rat growth' method.

Experimental

Preparation of the rats. The rats used for this work had grown to about 140 g. on the ordinary stock diet of the colony, or they had been used in vitamin A determinations (therefore never suffered from a shortage of vitamin B₁) and had then been fed on the stock diet for several weeks. Vaginal contents were examined daily by the usual 'smear method', directly, without staining, to assure ourselves that cycles were occurring normally. No rat was found that did not have cycles regularly; none had ever been given a chance of mating. As soon as a rat was found to have had three normal cycles it was given a vitamin B₁-free diet which consisted of:

Caseinogen (physiological caseinate, Glaxo)	...	15 parts
Dextrinized rice starch		79 "
Agar-agar		2 "
Salt-mixture (Steenbock's 40)		4 "
Dried yeast, autoclaved at 120° for 6 hr.		25 "

In addition each rat was given 5 drops of a good sample of cod liver oil twice a week. Fresh tap water was supplied daily and the cages were provided with grids of $\frac{1}{2}$ in. mesh to prevent the rats from having access to their faeces.

The vaginal contents were examined daily. A rat was considered ready for experiment when no keratinized cells had been found in the vagina for 10 successive days. Most of the 107 rats used in this experiment had two or three cycles at normal intervals during the feeding of the vitamin B₁-free diet before the period of 10 days 'leucocytes only' began; only 4 rats ceased at once to have cycles. The average number of days that elapsed after the vitamin B₁-free diet was first given and the 10 days anoestrous period began was 14.25 with $\sigma=5.43$. 6 or 7 rats were kept together in each cage during the preparatory feeding but each rat was housed in a separate cage during the dosing period.

Dosing of the rats. As the rats became ready for the experiments, they were distributed into 6 groups of about 12 rats per group. The rats of the different groups were given daily doses of 0.01, 0.02, 0.03, 0.04, 0.05 and 0.1 g. respectively of a vitamin B₁ adsorbate that contained approximately 200 I.U. of vitamin B₁ per g. The powder was intimately mixed with finely ground dextrinized rice starch, partly to facilitate the daily measuring of doses and partly because the rats ate it more readily in this form. Each dose was given on a small dish, mixed with a little water. Vaginal smears were examined daily. When a rat had shown evidence of three cycles, it was given no further dose of vitamin B₁ until cycles had ceased and a further period of 10 days anoestrus had occurred. The whole procedure of dosing was then repeated, each rat being given the same dose as it had been given before. When its three cycles were completed for the second time it was again deprived of vitamin B₁ and the whole procedure repeated for a third time. This was done in order to determine whether the sensitivity of the rats changed with successive treatments or whether they could be used over and over again.

Results

The curve of response to graded doses of the adsorbate

It is evident (Table 1) that the responses of the different groups of rats were graded to the dose of vitamin B₁ given. Moreover, the curve of response obtained for the first series of three cycles was a steep one; but the curves obtained from the second and third series were less and less steep. Thus the rats became increasingly sensitive since they responded more readily to the dose of vitamin B₁ given. This makes it impossible to use the same rat over and over again in a determination of vitamin B₁ by this method, unless of course all the rats used in any one determination were exclusively first or second or third 'series' rats.

The curves of response were asymptotic to both axes and indicated that 0.02 g. of this adsorbate (i.e. 4 I.U.) was about the lowest dose that would bring about a series of three cycles. A dose of 0.01 g. (i.e. 2 I.U.) failed entirely to do this in any of the 7 rats to which this dose was given. In only 2 of the 7 was even one cycle produced. During an average period of 60 days of receiving this dose, the average loss of weight of the rats was 12 g.; this loss of weight was almost entirely confined to those rats which had been longest on the experiment. Thus, apparently, a minimum of 0.01 g. of this adsorbate (or 2 I.U.) is required to maintain female rats at about 140 g. weight, but it is not sufficient to maintain the normal functioning of the ovaries (as evidenced by the vaginal contents).

To test the usefulness of the curve of response, 10 rats prepared as above were given doses of 0.25 g. of a sample of dried yeast which we had previously assayed for vitamin B₁ potency by the 'rat growth' method. 9 rats were simultaneously given doses of 0.075 g. of the International Standard (adsorbate from rice polishings) for vitamin B₁. There was not enough material left to carry on the dried yeast group to the third series of cycles, but it is evident (Table 1) that

Table 1. *The length of time required to restore normal cycles in the vaginal contents of rats which had been deprived of vitamin B₁ until the anoestrous period had lasted 10 days and which had then been given graded doses of that factor, groups of about 10 rats each being used for testing each dose*

Dose, g.	1st series of three cycles			2nd series of three cycles			3rd series of three cycles		
	Aver. time days	St. dev. σ	Coeff. of var.	Aver. time days	St. dev. σ	Coeff. of var.	Aver. time days	St. dev. σ	Coeff. of var.
0.02 adsorbate product	75.6	31.1	0.412	38.6	11.7	0.304	34.0	7.1	0.208
0.03 „	43.7	21.0	0.480	26.8	6.0	0.223	22.8	4.7	0.205
0.04 „	31.4	16.2	0.516	24.2	13.6	0.560	20.9	4.7	0.223
0.05 „	27.0	11.1	0.410	25.1	9.4	0.373	20.6	6.4	0.312
0.1 „	28.3*	19.7	0.696	22.3	11.3	0.507	16.9	4.0	0.234
0.25 dried yeast	23.4	9.0	0.383	15.5	4.5	0.291	—	—	—
0.075 Int. Stand.	27.6	8.2	0.300	20.1	5.3	0.261	18.2	5.7	0.311
Average			0.457			0.360			0.249

* The time for one rat in the group was 87 days; the rest ranged from 15 to 36, average 23.0 days, $\sigma=7.21$, coeff. of var. = 0.31. In the 2nd and 3rd series, its times were 20 and 23 days respectively.

Table 2. *Total amount of adsorbate taken per rat before three cycles were completed in each series*

Daily dose of adsorbate g.	1st series		2nd series		3rd series	
	No. of days	Total dose g.	No. of days	Total dose g.	No. of days	Total dose g.
0.02	75.6	1.512	38.6	0.772	34.0	0.680
0.03	43.7	1.311	26.6	0.798	22.8	0.684
0.04	31.4	1.256	24.2	0.968	20.9	0.836
0.05	27.0	1.350	25.1	1.255	20.6	1.030
0.1	28.3	2.830	22.3	2.230	16.9	1.690

0.25 g. dried yeast was rather more potent than 0.075 g. of the International Standard, i.e. it contained more than 30 I.U. of vitamin B₁ per g. A smooth curve was drawn through the 'first series' results, extrapolating beyond the 0.05 point, since the 0.1 point was obviously 'out'. The abscissa corresponding to the dose of 0.25 g. dried yeast was about 0.08. The abscissa corresponding to the dose of 0.075 g. International Standard was 0.045. Thus the dried yeast contained about 50 I.U. of vitamin B₁ per g. The potency obtained by the 'rat growth' method was 40 I.U. per g. In Table 1 is recorded the standard deviation (σ) for each period taken to produce three cycles. It may be seen that the values of σ decrease with decreasing times taken, so the coefficient of variation was determined for each time taken. These coefficients of variation also decrease in the successive periods, hence the responses of the rats were becoming more regular as the treatments were repeated, whether measured absolutely or relatively to the lengths of the periods (Table 1).

The time taken to produce the three cycles is inversely proportional to the dose of vitamin B₁ given, but not exactly so, which indicates that vitamin B₁ is used for other purposes than the direct restoration of normal cycles, and also, in our experiment, that 0.1 g. of the adsorbate was much in excess of the rats' needs. In Table 2 may be seen the total amounts of adsorbate needed to restore the cycles according to the different daily doses given. For the first series of cycles, apparently the most economical dose was 0.04 g. daily; for the second and third series the lowest of the doses given, 0.02 g., was the most economical

of those tried. Apparently a certain threshold value of concentration must be reached and beyond that the animal body can utilize only a proportion of the extra amount given.

The accuracy obtainable in a determination of vitamin B₁ by this method

In order to form an estimate of the accuracy obtainable by this method for determining the vitamin B₁ content of a substance, the reciprocal of the time taken by each rat to produce three cycles was used. The average for each group was calculated together with the standard deviation, σ (Table 3). The average

Table 3. *The reciprocals of times taken to restore normal cycles in the vaginal contents of rats which had been deprived of vitamin B₁ until the anoestrous period had lasted 10 days and which had then been given graded doses of that factor, groups of about 10 rats each being used for testing each dose*

Dose, g.	1st series of three cycles			2nd series of three cycles			3rd series of three cycles		
	Aver. of re-ciprocals of times taken	σ	Coeff. of re-ciprocals of times taken var. %	Aver. of re-ciprocals of times taken	σ	Coeff. of re-ciprocals of times taken var. %	Aver. of re-ciprocals of times taken	σ	Coeff. of re-ciprocals of times taken var. %
0.02 adsorbate	0.0187	0.01424	76.2	0.0279	0.00815	29.2	0.0301	0.00629	20.9
0.03 "	0.0290	0.01473	50.8	0.0396	0.01209	30.5	0.0457	0.01008	22.1
0.04 "	0.0379	0.01406	37.1	0.0497	0.01909	38.4	0.0504	0.01320	26.1
0.05 "	0.0427	0.01440	33.7	0.0439	0.01265	28.8	0.0527	0.01430	27.1
0.1 "	0.0441	0.01636	37.1	0.0529	0.01849	34.9	0.0622	0.01462	23.5
0.25 dried yeast	0.0477	0.01476	30.9	0.0681	0.01654	24.2	—	—	—
0.075 Int. Stand.	0.0401	0.01488	37.1	0.0530	0.01431	27.0	0.0584	0.01323	22.7
Average		0.01506	43.3		0.01562	30.4		0.01311	23.7

Table 4. *Limits of error obtainable in an assay in which it is supposed that 10 rats have been used for the International Standard and 10 rats for the substance under examination*

The results from the highest dose have been omitted for it was obviously excessive.

	1st series	2nd series	3rd series
	$b=0.06142$	$b=0.04236$	$b=0.04478$
	$\sigma=0.01481$	$\sigma=0.01417$	$\sigma=0.01278$
	$\epsilon=0.006623$	$\epsilon=0.006337$	$\epsilon=0.005716$
$P=0.67$	78–128 %	71–141 %	75–134 %
$P=0.95$	61–164 %	50–199 %	56–180 %
$P=0.99$	52–192 %	41–243 %	47–213 %

values of σ for the groups producing the first, second and third series of cycles respectively were determined ($\sqrt{\{\sum d^2/(N-M)\}} = \sigma$ in which d = the difference from the mean of a group, N = the total number of animals used and M = the number of groups). The curves of response were approximately logarithmic and more nearly so when the result from the highest dose, which was obviously excessive, was ignored. The equations of the curves through the four points of each series were determined. From these and the average variances for the four points in each series was calculated the accuracy of a test in which 10 rats were supposed to be used for the Standard and 10 for the substance under examination, according as the first, second or third series of cycles was used (Table 4). The three series gave an average range of limits of error for $P=0.99$ of 47–216 %. This compares

unfavourably with the limits of error of the 'rat growth' method given in the British Pharmacopoeia Commission's Report of the Subcommittee on the Accuracy of Biological Assays [1936], which are 74–135 % for $P=0.99$.

As the values for σ increased almost regularly with increasing averages of times taken to produce three cycles, the coefficients of variation were determined. These also decreased in successive series for each dose of adsorbate tested.

SUMMARY

Rats were deprived of vitamin B₁ until no keratinized cells had been found in the vagina for 10 successive days. They were then distributed in order, as they became ready, into 5 groups, and the groups were given a graded series of daily doses of 0.01, 0.02, 0.03, 0.04, 0.05 and 0.1 g. respectively of an adsorbate containing approximately 200 I.U. of vitamin B₁ per g., each rat in any group receiving the same dose.

The response was graded to the dose of vitamin B₁ given; the larger the dose the shorter was the time required to produce a series of three cycles in the vaginal contents of the rat.

When a rat had completed three cycles, vitamin B₁ was withheld until 10 successive days had passed without keratinized cells in the vagina. The rat was then given the same daily dose as before until a second series of three cycles had occurred. Then the whole process was repeated again.

The rats became increasingly sensitive to their respective doses, so that in any assay of vitamin B₁ by this method all rats used would have to be first, second or third 'series' rats, but not a mixture of the three.

One assay by this method (50 I.U./g.) of the vitamin B₁ content of a sample of dried yeast in comparison with the International Standard tested simultaneously agreed fairly well with an assay (40 I.U./g.) carried out by the 'rat growth' method.

The 'limits of error' of this method have been estimated as 47–216 % for $P=0.99$, a result which is not as good as the 74–135 % 'limits of error' of the rat growth method given in the B.P.

REFERENCES

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