XCI. THE USE OF VARIOUS RAT ASSAY METHODS IN COMPARING CRYSTALLINE VITAMIN B₁ PREPARATIONS.

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IN continuation of the study of the parallelism between the ultraviolet absorption spectra of samples of vitamin B₁ preparations and their potencies [Heyroth and Loofbourow, 1932], three samples of crystalline material have been subjected to detailed assay. The diet of the rats and the symptoms produced have already been described in detail [Heyroth, 1932]. Considerable variation has been encountered in the time required before the symptoms become evident. No attempts have been made to determine the causes of this variation by altering the diet. When the symptoms became marked, the vitamin preparation in aqueous solution was injected subcutaneously and the subsequent developments noted. The weight changes, the number of days required to produce a cure and the duration of the cure were recorded. If the rat appeared normal 72 hours after the injection and was found paralysed 96 hours after injection, the cure was said to have persisted 3 days.

Three preparations were tested. (A) was preparation 64 : 19 of Prof. R. A. Peters, Oxford. (B) was oryzanin from yeast and (C) was oryzanin from rice polishings. (B) and (C) were prepared by S. Ohdake, Tokyo, and furnished through the courtesy of Prof. U. Suzuki and Dr Ohdake. To all these workers I wish to extend my thanks for making these preparations available.

(A) consisted of 2 ml. of a solution containing 1.19 mg. of crystals in 96 % alcohol at p_H 3 (approx.). Peters has reported [Peters and Philpot, 1933; Kinnorsley *et al.*, 1933] its pigeon day dose as 2.2 γ . (B) and (C) were furnished in crystalline form and were dissolved to make stock solutions subsequently diluted to strengths suitable for injections and for spectrographic work elsewhere reported [Heyroth and Loofbourow, 1936].

RESULTS.

Minimum curative dose.

Previous data of others with an early Windaus preparation [Windaus *et al.*, 1932; Seidell and Smith, 1930; Heyroth and Loofbourow, 1932, p. 37] suggested that the rat would require several times the pigeon day dose. For this reason the first doses of Peters's preparation employed were 12, 11 and 9 γ . The results showed that these were needlessly large; however, they furnished some information as to the effects of large doses. The following observations were made as a basis for a minimum curative dose of (A).

Three trials of 1.1 γ failed to cure. 1.95 γ failed in one trial. 2.25 γ failed in two trials, gave incomplete or very slow cures in two trials and produced delayed but complete cures in two trials. 3 γ cured in two trials. In a moribund rat, which may be neglected, death occurred shortly after injection. 4 γ produced

two cures. In a third rat traces of leg weakness persisted, but this rat was abnormal in that the paralysis was flaccid rather than spastic. All doses of 5γ or more produced cures.

It therefore appears that the minimum rat curative dose of this preparation lies between 2.25 and 3γ . This neglects the improvement noted in some rats which received doses of 2.25 or 1.5γ . Since Peters reported the pigeon day dose of this preparation as 2.2γ , the factor for converting the pigeon day dose into the rat minimum dose is 1.2 .

In the case of the two Japanese preparations no attempt was made to determine the minimum curative dose, since we wished to make every injection yield some quantitative value. As the smallest dose of the yeast preparation used (3.97γ) cured in several trials, the minimum curative dose must be less than this and so close to that of (A). Minimum curative doses have since been published by Ohdake and Yamagishi [1935], who give 3γ for each. This value possibly represents a very slightly lower potency than that of (A).

Mean day dose.

This has been determined for each of the preparations and affords direct comparison of the needs of the pigeon and the rat for identical crystalline specimens (sample A).

In determining the time of cure it is necessary to adopt a definite criterion as to the return of the deficiency symptoms. We have employed usually the return of severe symptoms in which the paralysed rat not only is unable to walk but is so incoordinated that on attempting to right itself after having been placed on its back it rolls over completely, usually suffering convulsions during the attempt. Such a state is commonly preceded by two or three days during which there are very slight symptoms, most frequently a spastic state of one hind leg. It is obvious that if the appearance of such slight symptoms be adopted as the criterion of the end of a cure, the day dose will be higher. In the case of the first preparation assayed, only the severe symptoms were employed with sufficient regularity to make the results of value. With the Japanese preparations, more attention was paid to this point, so that the results are equally trustworthy whether the appearance of slight or of severe symptoms be taken as marking the end of the cure. There has also been employed as a third criterion of the end of the effect of the injection the loss of weight gained shortly after injection.

Whichever criterion was employed, certain of the values were regarded as questionable. These represented either resistant rats which remained cured for extremely long periods, rats which died from undetermined causes before the return of symptoms, or rats in which the symptoms were of an unusual type, *e.g.* flaccid rather than spastic paralysis. The mean values of all injections, and the means with the questionable cases eliminated (marked corrected) are given separately in Table I.

Table I. *Summary of the rat day doses in γ of three preparations.*

Preparation	Mild symptoms		Severe symptoms				Weight maintenance			
	All results	Corrected data	All results	σ as %	Corrected data	σ as %	All results	σ as %	Corrected data	σ as %
(A) Peters, 64 : 19	—	—	0.452 ± 0.14	64	0.411 ± 0.086	36	0.539 ± 0.144	46	0.551 ± 0.144	37
(B) Ohdake, yeast	0.717	0.600	0.531 ± 0.119	53	0.509 ± 0.085	35	0.774 ± 0.24	66	0.713 ± 0.232	63
(C) Ohdake, rice	0.675	0.508	0.604 ± 0.284	91	0.450 ± 0.114	44	0.843 ± 0.506	108	0.588 ± 0.192	51

Table II. *Comparative potencies of three preparations, that of Peters taken as 100.*

Preparation	Severe symptoms		Weight maintenance	
	All results	Corrected data	All results	Corrected data
(A) Peters, 64 : 19	100.0	100.0	100.0	100.0
(B) Ohdake, yeast	85.1	80.7	69.6	77.3
(C) Ohdake, rice	74.8	91.3	63.9	93.7

From the results of 14–22 injections for each preparation, the standard mean deviations (σ) have been computed for the severe symptoms and weight maintenance doses.¹ In Table I these have been presented as % of the mean value in order to afford a more ready comparison of the deviations found when employing the various criteria for the end of the cure. To each mean there has been appended 2ϵ , where ϵ is the standard error of the mean, $\epsilon = \frac{\sigma}{\sqrt{n}}$.

The weight criterion has the advantage of requiring less experience of the symptoms, but it demands a somewhat larger number of injections, since in some cases the symptoms recur before the weight has returned to its value at the time of injection. No attempt has been made to employ this criterion in rats declining in weight but not yet showing paralysis. It is open to the serious objection that the presence of other members of the vitamin B complex might lead to error, although in the present series there is no indication that such has been the case.

DISCUSSION.

There has been much discussion as to the validity of the results of the day dose method. Peters [Kinnersley *et al.*, 1935], who has employed it extensively, regards it as not very satisfactory for the crystalline vitamin in the pigeon, whilst Birch and Harris [1934] think that, properly employed, it is satisfactory. Two points require consideration, the question as to whether the size of the dose influences the value of the day dose and the great deviations of the results.

Previous workers [Kinnersley *et al.*, 1935; Van Veen, 1932; Coward *et al.*, 1933; Birch and Harris, 1934] have found that the duration of cure does not increase in direct proportion to the size of the dose, so that the day dose method can be safely employed only over a very limited range of duration of cure. The potency of a given preparation may appear less if large doses are employed than if smaller ones are used.

For the present series, these considerations are of relatively little importance, since the preparations were so nearly alike in potency that it would have been futile to attempt to select different dose levels for each to make the lengths of cure the same. Most of the doses injected were 4–5 γ , and the average time for the recurrence of severe symptoms was about 12 days. Only in the case of Peters's preparation were a few doses of 9 γ or more employed. If the higher doses should give higher day doses they would make this preparation appear less active in comparison with the other two. Therefore the inclusion of these few higher doses in the case of this preparation tends to reinforce the evidence that this preparation is slightly more potent than the others.

To test the validity of the view that the duration of cure influences the results, the data were divided into two groups in which the cures were (a) over and (b) under 10 days. For each preparation, using all values, there were computed the standard deviations from the means, as well as σ in % of the means.

¹ $\sigma = \sqrt{\frac{\sum d^2}{n-1}}$. The number of observations varied from 12 to 19.

For (A) they were 68 % for the low and 49 % for the high group. For (B) they were 36 and 40 %, and for (C) 84 and 47 %, respectively. The view of Birch and Harris [1934] that the deviations are somewhat greater with lower than with higher duration of cure is borne out.

To determine whether the order of activities of the three preparations is influenced by the duration of cure the means, with questionable values omitted, were compared in each of these two groups. The results are for the group with longer cures: (A), 0.384 γ ; (B), 0.398 γ ; (C), 0.391 γ . For those with shorter cures they are: (A), 0.484 γ ; (B), 0.710 γ ; (C), 0.634 γ . The order is the same in both groups, and it is shown in Table I that it is also the same when the results are not so grouped. The differences in the case of the group with longer cures, where the uniformity is also greater, are not statistically significant, which tends to lessen somewhat the significance of the differences found in the ungrouped results. No attempt was made to group the results by sizes of doses injected, since only a very limited range of doses was employed.

The magnitude of the deviations is of interest. Table I indicates that for all values this varies from 64 to 91 % of the means. With the omission of abnormal cases, this is lessened to about 35–44 %. In the pigeon, Peters usually found the deviation to be 44 %, while the results of Ohdake and Yamagishi [1935] appear somewhat more uniform as they calculate it to be 20–30 %. In the case of the weight criterion the results in (C) were worse. Such deviations greatly limit the value of curative methods in the intact animal for the comparison of crystalline preparations of a high degree of purity.

Histological studies [Hofmeister, 1922; Prickett, 1934] of the brains of rats showing the deficiency symptoms have shown disseminated foci of haemorrhage or intense congestion in the pons and medulla involving important cranial nerve nuclei. Peters and Thompson [1934] have found an impaired ability of the deficient pigeon's brain to oxidise pyruvic acid. The cure of the metabolic disturbance by the vitamin may be obscured by the presence of the complicating anatomical lesions. The large deviations encountered in the durations of the cures may have their origin in individual anatomical variations. The measurement of the oxygen uptake of minced brain, as in the catatorulin test of Peters, would be expected to approximate to the metabolic effects of the vitamin more closely.

Some speculation as to the reason for the dependence of appetite on the vitamin may perhaps be permitted from the histological work of Prickett [1934]. Lesions occurring in the regions of the nuclei of the fifth and ninth nerves might well destroy or lessen the sense of taste. Disturbances of other cranial nerves, *e.g.* exophthalmos, and more rarely nystagmus, have been encountered in the series.

To determine whether the differences in activity of the preparations indicated by the means in Table I are statistically significant, for each pair of means the sums of their variances, $\frac{\sigma^2}{n}$, have been divided into the differences of the means. If these quotients are large numbers, the differences may be judged significant. These values, from symptom criteria, are 27 for the (A)-(B) pair, 8 for the (A)-(C), and 12 for the (B)-(C). By the weight test they are respectively 9, 0.5 and 7. While these results are not large numbers, it is believed that they are sufficient, except in the case where weight was the criterion in the pair (A)-(C), to indicate that (A) has a significantly greater potency than (B); the deviations in the case of (C) were greater and it is not possible to be certain as to its comparative rating beyond the fact that it is not more potent than (A). In

Table I comparative values are given for the three preparations on the basis of 100 % for (A). Although, as has just been indicated, its significance is limited, it is included for comparison with spectrographic data in the following paper.

The variations in the weights of the rats employed at the times of injection were not great enough to permit a conclusion as to the influence of the weight of the individual upon the dose required. In the few instances in which heavier rats were used, the day doses obtained were among the lowest, and any weight influence may have been masked by the great deviations encountered in the entire series.

When, however, the rat and the pigeon are compared, the interesting result is obtained that the day dose is proportional to the mean weights of the two species. In an earlier paper Peters *et al.* [1928] gave the weights of 17 pigeons at the onset of symptoms as between 205 and 310 g., the mean being 254 g. This agrees with the mean of 255 g. reported by Windaus *et al.* [1932] for 16 pigeons. The mean weight of our rats in the assay of Peters's preparation was 44 g. Multiplication of the dose for this preparation found by Peters for the pigeon by the ratio of the weights of the animals ($2.2\gamma \times 44/254$) gives 0.39γ . The rat day dose was found to be 0.41γ (Table I). The mean weight of Ohdake's pigeons [Ohdake and Yamagishi, 1935] at the time of injection was 207 g., and of our rats used in assaying his preparations, 44 g. Ohdake's pigeon day dose for either the yeast or the rice preparation was 2.4γ . (The identity of these preparations with the specimens sent to us is uncertain.) The calculated rat day dose is 0.51γ . There were found 0.509γ (yeast) and 0.450γ (rice). This fails to confirm the observations of Kinnersley *et al.* [1930] that the day doses are the same for the pigeon and rat, irrespective of the difference in weights of the two species. Nor does it confirm the view of Cowgill [1934] that relative to body weight the rat requires more vitamin B₁ than the pigeon. (Cowgill, however, appears to have used as his criterion of activity growth for the rat and maintenance of a weight level for the pigeon.) Should man also have the same requirement, the curative day dose would be 0.6 mg. On the basis of Cowgill's expressions, a man of 154 lb. requires 122 times as much of a given vitamin preparation as does a pigeon of 254 g. Since the pigeon day dose for the prevention of the recurrence of severe symptoms is 2.2γ , the man would require about 0.27 mg. per day, or about half the amount calculated upon the assumption that weight for weight the demands of the pigeon and of man are equal.

From Ohdake's data [Ohdake and Yamagishi, 1935], the pigeon appears to differ in one respect from the rat. He reports the minimum pigeon curative dose as 3γ and the pigeon day dose as 2.4γ . In previous work in this laboratory [Heyroth and Looffbouro, 1932], the minimum curative dose for the rat was found to be 6.6 times the curative day dose. This is confirmed in the case of Peters's preparation, since 6.6 times the day dose of 0.41γ would give 2.7γ as the minimum curative dose. Direct determination gave its value as between 2.25γ and 3γ . If this same ratio applied in the case of the pigeon, the minimum curative dose for that species would be about 16γ . The minimum curative doses seem about the same for the two species whilst the day doses stand in the ratio of their mean weights.

Since the rat day dose is 0.185 times that for the pigeon and the minimum curative dose for the rat is 6.6 times as great as the day dose, it follows that the minimum dose for the rat is 6.6×0.185 , or 1.2 times the pigeon day dose. This confirms the statement of Williams and Eddy [1931-32] that the minimum curative dose for the rat is certainly not less than the pigeon day dose, a statement based on the observation that prophylactic feeding tests on pigeons showed that

0.25 g. of rice polishings daily protects the pigeon indefinitely; 1 g. of the polishings yielded on thorough extraction with alcohol 3–5 curative doses.

It may also be noted that Ohdake found 2.3 γ as the curative day dose of his preparations when given orally and about the same value or 2.4 γ when given by injection. Kinnersley *et al.* [1935] believed a given sample to be 30 % more active by injection than when given orally. Van Veen [1932] found that preparations which were ineffective for the rat when given orally cured when injected. All doses in the present series have been injected.

SUMMARY.

1. Three samples of crystalline vitamin B₁ have been assayed by the rat method. Their potencies are, in the order of decreasing activity: Peters (64 : 19), Ohdake (rice), Ohdake (yeast). The differences between them appear to be only slightly beyond the limits of accuracy of the assay. The variations indicated should not be taken as an implication that the vitamin has not been isolated, but only as suggesting a contamination with slight but different amounts of impurities. Peters has recorded other samples of somewhat greater activity.

2. The minimum curative dose for the rat is 1.2 times the pigeon day dose, and the rat minimum curative dose is 6.6 times the day dose required for preventing the recurrence of severe symptoms.

3. In determining the curative rat day dose a definite criterion must be adopted as marking the return of symptoms. Different values are found when this is taken as the first spasticity of the hind leg, or as convulsions and inco-ordination. The order of activity of different preparations is the same in either case.

4. As a third criterion, giving still different numerical values, there may be employed the number of days which elapse before the rise of weight following the injection has been lost. This assumes the preparations to be uncontaminated by other vitamins of the B group.

5. Histological reasons are advanced to account for the rather large standard deviations encountered in these assays.

6. In the cases of rats and pigeons, the day doses required to prevent the recurrence of severe symptoms are proportional to the mean weights of the animals of the two species at the times of injection.

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