CX. ON CATATORULIN.

A NEW METHOD OF COMPARING THE OXIDATIVE FACTOR IN VITAMIN B₁ CONCENTRATES.

BY REGINALD PASSMORE¹, RUDOLPH ALBERT PETERS
AND HUGH MACDONALD SINCLAIR².

From the Department of Biochemistry, Oxford.

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IN recent work Gavrilescu, Meiklejohn, Passmore and Peters [1932] have shown that potent vitamin B₁ concentrates in very small amount increase the oxygen uptake of avitaminous pigeon's brain in lactate solutions. The effect is proved to be catalytic, because the extra oxygen taken up is far in excess of the amount of vitamin B₁ added. To avoid begging the question as to the identity of the oxidase factor in the concentrates with vitamin B₁, and to economise words, we propose to call it "catatorulin." There is very strong evidence to support the view that catatorulin and vitamin B₁ are identical. In particular Meiklejohn, Passmore and Peters [1932, 1, 2] found that the catatorulin effect was directly related to the well-known symptom of head retraction in pigeons, and that it disappeared with cure of the birds by vitamin. The indirect evidence in favour of identity is strengthened by the fact, presented here for the first time, that the crystalline preparations of Kinnersley, O'Brien and Peters [1933] exhibit catatorulin action. Nevertheless these workers are not yet convinced that the crystals represent pure vitamin, and therefore any finding of parallelism between catatorulin and vitamin B₁ preparations is of importance.

With these objects in view, a method has been devised for comparing the effects upon oxygen uptake of two different vitamin B₁ preparations. Avitaminous brain tissue must be used because the normal shows no effect with added vitamin B₁. Attempts to obtain stable dry preparations so far have failed.

There has proved to be good correspondence between vitamin B₁ and catatorulin in the crystalline preparations; the method therefore promises to be of value as a new means of estimating the activity of potent vitamin B₁ preparations. It has the great advantage that bird variations are eliminated, and that a comparison is made upon an enzyme system rather than upon the whole animal.

EXPERIMENTAL.

The brains of polyneuritic birds showing well-marked head retraction must be used; the birds were fed by the usual method employed in this laboratory. It is an advantage to use animals which have already been under test, as the brains seem more generally deficient in the vitamin. After guillotining (best at the neck alone to avoid injury to the tissue), the blood is allowed to drain for

¹ Senior Scholar of Brasenose College.

² Senior Demy of Magdalen College.

1 minute from the neck; the brain is then uncovered, mopped lightly with filter-paper and removed; all parts except the cerebellum are finely minced with a bone spatula¹ and thoroughly mixed.

The cerebellum has been excluded owing to previous findings, which have not been further investigated. It would have been better if one part of the brain could have been selected, but this does not provide enough tissue for comparison.

Samples of approximately 100 mg. (up to 16 in number) are introduced into tared and corked bottles of Barcroft-Dixon apparatus, previously filled with Ringer-phosphate lactate solution (p_H 7.3)². The weight is determined by a second weighing.

The Ringer solution used has the composition NaCl 0.9 %, KCl 0.025 %, $CaCl_2$ 0.030 %, $NaHCO_3$ 0.015 %. To 786 cc. Ringer solution 200 cc. of KH_2PO_4 6.8 % are added, and then 13.9 cc. 20 % NaOH solution to bring to p_H 7.3. After standing for one day, the precipitate is removed by filtration. To each 49 cc. of the Ringer-phosphate is added 1 cc. of a neutralised Na lactate solution, containing sufficient lactic acid to make the final concentration 0.033 *M*. The lactate solution was prepared from recrystallised zinc lactate, as formerly described. It is convenient to neutralise, make up so that the required amount of lactate is present in 1 cc. solution, and then to introduce 1 cc. amounts into sterile tubes fitted with cotton-wool plugs. These can be sterilised on 3 successive days for 20 minutes in the steamer and kept in cold store. One tube serves for an experiment. Glass-distilled water is used throughout.

After the second weighing the tissue is finely broken up with a flat-ended glass rod, and immediately following this is added the vitamin made up in Ringer-phosphate lactate; the volume of this should be at least 0.1 cc., and allowance must be made when filling the bottles, so that the solution with additions reaches 3.0 cc. If care is taken not to remove more fluid than absolutely necessary with the glass rod, then any error from this cause will be negligible. The roll of filter-paper is now inserted, and wetted with 2*N* KOH, after which the bottles are twice evacuated³ to 70 mm. and filled with oxygen, and the taps closed. Excessive evacuation seems to spoil the tissue. These operations take about 30 minutes from the time of guillotining. When evacuation is completed, the bottles are placed in the bath at 38°, the taps being opened temporarily to equalise pressure; they are shaken with several levellings for 9 minutes, and the taps finally closed. The first reading is taken 12 minutes after introducing into the bath, and thereafter at intervals of 30 minutes.

The temperature of 38° represents a compromise between the temperature of normal and avitaminous animals. Doubtless the experiments would be more efficient at 41°, but as the effect is well obtained at 38° we have adhered to this temperature, which makes all our observations comparable with those made earlier here.

Preparation of vitamin solutions.

The standard solutions (200 γ crystals of vitamin B_1 per 1.0 cc. acid alcohol *N*/1000), as made for the other work in this Laboratory, were used for most of the work. We are indebted to Mr H. W. Kinnersley for his help in their preparation. The necessary amount of vitamin, 0.05–0.1 cc., was measured into a clean small test-tube, one drop of water added, and the alcohol removed on the water-bath. Immediately before addition to the bottles, Ringer-lactate phosphate solution was added together with an appropriate volume of *N*/1000 Na_2CO_3 to neutralise the acid. A convenient volume for addition was found to be 0.5 cc. = 1 γ vitamin preparation. We tried to use dilutions in alcohol, but found that traces of alcohol were apt to increase irregularity of results.

We have assumed throughout that 1 curative pigeon dose = 1 vitamin B_1 International Unit [see Kinnersley, O'Brien and Peters, 1933].

¹ An envelope opener is ideal for this purpose.

² Other apparatus for the estimation of oxygen uptake will of course do equally well.

³ Erroneously described as 710 mm. in previous paper.

Note. Care should be taken to see that the tissue does not become permanently washed up and stuck upon the sides of the bottles. This happens especially with some avitaminous brains, for reasons which are not clear. It can usually be dislodged by careful manipulation.

Variation of catatorulin effect with concentration.

Fig. 1 shows a typical curve (*Exp. 316*) relating concentrations of crystalline vitamin B₁ to oxygen uptake for the 2nd hour. Within the hour period the general resemblance to an enzyme activity-substrate concentration curve is

Fig. 1. Influence of increasing concentrations of crystalline vitamin B₁ preparations upon oxygen uptake of avitaminous brain. Period, second hour. Circles=experimental points. Curve drawn from equation.

striking, if for concentration of substrate we substitute catatorulin concentration. For values from 0 to 0.1 vitamin B₁ unit, the uptake rises considerably; with higher concentrations, the increment is smaller. In the application to estimations, certain limitations appear. It is not practicable to obtain more than 16 samples from one brain, nor convenient to work with 2 brains. The experimental error is somewhat larger: though 2 samples give approximate values for rough tests, the highest accuracy can be reached only by an average of at least 4 samples. Therefore we require a means of reaching the probable shape of the curve from one or two points.

A reasonable explanation for the shape of the curve in Fig. 1, is to assume that among other limiting factors the oxygen uptake depends upon the adsorption of catatorulin at some active centre in the brain tissue. This adsorption will depend upon concentration of the factor in the same way as adsorption of substrate upon an enzyme depends upon concentration of substrate. It is reasonable therefore to apply the Michaelis equation, assuming that lactate concentration may be regarded as maximum [see Gavrilescu *et al.*, 1932], and that concentration of catatorulin is the limiting factor.

Note. In this particular instance it seems that we must be dealing with a case of heterogeneous equilibrium, in which the particles of brain tissue may be regarded as practically stationary when compared with the vitamin B₁. This does not invalidate the use of the equation, because it can be also derived from Langmuir's conceptions of heterogeneous catalysis.

Theoretically, if

k_c = equilibrium constant,
 v = velocity at concentration of added vitamin x (measured in $\text{mm.}^3/\text{g.}/\text{hr.}$),
 R = residual vitamin in tissue,
 E = amount of enzyme (here directly proportional to tissue),

$$\text{then } v = \frac{k_c \cdot E (x + R)}{k_c + (x + R)} \quad \dots\dots(1),$$

and if V = velocity when x is large, we have

$$k_c = (x + R) \left(\frac{V}{v} - 1 \right) \text{ for constant amount of enzyme system } E \quad \dots\dots(2).$$

The tissue is chosen to be approximately 100 mg. The catatorulin concentration is measured in terms of γ or $10^{-2}\gamma/3$ cc. solution.

Now the mixed brain provides 12–16 samples of about 100 mg. each, making 4 quadruplicate estimations possible as a maximum. Two estimations are made with a standard catatorulin preparation at concentrations of $100 \times 10^{-2}\gamma$ (1γ) and $3 \times 10^{-2}\gamma$ respectively and one or two at arbitrary concentration. From these an average curve is drawn by calculating V from the relation

$$V = \frac{v}{x + R} (k_c + x + R) \quad \dots\dots(3).$$

It is found that the curves are best satisfied when $k_c = 10 \times 10^{-2}\gamma$, i.e. $1/20$ vitamin B_1 unit gives $1/2$ possible velocity, and when $R = 20 \times 10^{-2}\gamma$ for 100 mg. tissue. The latter is quite reasonable; it assumes a figure of 1 vitamin B_1 unit (pigeon dose) per g. of brain tissue as the limiting concentration possible for life.

The curve is then drawn, using for the points

$$v = \frac{x + R}{k_c + x + R} \cdot V \quad \dots\dots(4).$$

That at $20 \times 10^{-2}\gamma$ will serve to test the equation. From the curve the concentration of the unknown can readily be found.

Correction for variation in the amount of the tissue. Variations are unavoidable in the amount of the tissue, though often they do not exceed ± 15 mg. Change in the amount alters E and also R , R changing in direct relation with E . From equation (1) the effect of changes in tissue amount may be calculated.

If E_1 and E_2 = amounts of enzyme system, in mg. tissue per apparatus, and $(x + R_1)$ and $(x + R_2)$ are the amounts of vitamin (in $10^{-2}\gamma$) present in each bottle, R_1 and R_2 being the amounts of tissue vitamin corresponding to E_1 and E_2 respectively, it can be shown that

$$\frac{v_1}{v_2} = \frac{E_1}{E_2} \cdot \frac{\left(\frac{k_c}{x + R_2} + 1 \right)}{\left(\frac{k_c}{x + R_1} + 1 \right)} \quad \dots\dots(5).$$

In calculating results, E has always been taken into account because the figures are reduced to $\text{mm.}^3/\text{g.}/\text{hr.}$; no account has been taken, however, of R in the past. Let us consider variations from the true value, introduced by this method of calculation in which R is considered as the amount in 100 mg. tissue. Table I gives results, calculated from equation (5) for $x = 0, 20, 100 \times 10^{-2}\gamma$; $k_c = 10 \times 10^{-2}\gamma$, $E_1 = 100$ mg. and E_2 varying.

From Table I it is seen that no allowance for R need be made with amounts of vitamin $100 \times 10^{-2}\gamma$ ($1/2 B_1$ unit); failure to consider R will make a distinct difference at $x = 20 \times 10^{-2}\gamma$, and more so when $x = 0$. This accords with

Table I.

C_2 (mg.)	R ($10^{-2}\gamma$)	x_1 =0	x_1 = $20 \times 10^{-2}\gamma$	x_1 = $100 \times 10^{-2}\gamma$
150	30	112.5	104	100.5
120	24	106	102	100
100	20	100	100	100
90	18	96.5	99.0	100
80	16	92.5	97.5	100
70	14	87.5	97.0	100
50	10	76	96.0	99

experience. With maximum amounts of vitamin it has often been noticed that the amount of tissue could be varied within wide limits without influencing the results. With estimations of residual uptake, or with small amounts of vitamin, unaccountable variations have often appeared. Reference to the notes of previous work in this laboratory shows that these may be largely eliminated by taking into account variation in R in these cases. The use of equation (5) and Table I seems to be thoroughly justified. Where necessary, therefore, results are now corrected in this manner.

EXPERIMENTAL RESULTS.

Exp. 1 gives a protocol of a typical experiment, with a discussion of its probable accuracy.

No. 414. 29. iii. 33. Avitaminous pigeon's brain (except cerebellum) was finely minced and mixed. It was distributed in 12 tared bottles of Barcroft apparatus, which had been previously

Readings.

Bottles No.	1	2	3	4
Constant	3.01	2.78	2.94	3.05
Tissue wt. (g.)	0.1072	0.0979	0.1220	0.1171
	(a) (b)	(a) (b)	(a) (b)	(a) (b)
Time 4.16 p.m.	2.5 —	2 —	2 —	2.5 —
5.16 „	38.0 35.5	38.5 36.5	43.5 41.5	45.5 43.0
6.16 „	70.0 32.0	70.5 32.0	77.5 34.0	82.5 37.0
Bottles No.	5	6	7	8
Constant	2.78	2.76	2.73	2.98
Tissue wt. (g.)	0.1177	0.1008	0.1099	0.0996
	(a) (b)	(a) (b)	(a) (b)	(a) (b)
Time 4.16 p.m.	0.5 —	2 —	4 —	3.5 —
5.16 „	45.5 45.0	42.5 40.5	52.0 48.5	40.5 37.0
6.16 „	84.5 39.0	79.0 36.5	95.0 43.0	73.5 33.0
Bottles No.	9	10	11	12
Constant	3.04	2.81	3.25	3.48
Tissue wt. (g.)	0.1119	0.1195	0.1073	0.0912
	(a) (b)	(a) (b)	(a) (b)	(a) (b)
Time 4.16 p.m.	3 —	4 —	2.5 —	3.0 —
5.16 „	42.5 39.5	51.0 47.0	36.0 33.5	32.0 29.0
6.16 „	76.5 34.0	90.5 39.5	66.5 30.5	58.0 26.0

Bottles No. 1, 2, 3, 4 contained 0.34γ 64.19 (crystalline vitamin B_1 preparation)

„ 5, 6, 7, 8 „ 1.0γ 64.19

„ 9, 10, 11, 12 „ 0.5γ 78.27

(a) Difference in readings in scale divisions.

(b) Scale divisions per hour of differential apparatus.

corked after filling with Ringer-phosphate lactate solution, volume 3.0 cc. minus 0.1–0.5 cc. to allow for addition of vitamin. The bottles were reweighed; the brain was subdivided with a glass stirrer; the freshly diluted and neutralised vitamin was immediately added; the bottles were twice evacuated *etc.* and placed in the bath at $38 \pm 0.1^\circ$. Time from guillotining 40 minutes. Readings commenced at 52' = 4.16 p.m.

Summary.

Vitamin	mm. ³ /g./hr.		Average	
	First hour	Second hour	First	Second
0.34γ 64.19	995	905	1040	900
	1040	915		
	995	815		
	1120	960		
1γ 64.19	1060	920	1120	990
	1110	995		
	1205	1060		
	1110	995		
0.5γ 76.27	1070	930	1070	940
	1105	935		
	1010	920		
	1105	990		

The mean standard deviation for the 24 samples is 47.5 (48), and the standard error of mean $\frac{\sigma}{\sqrt{n}}$ for 4 observations is 24. Hence 2/3 of such tests should fall within ± 24 , and 21/22 within $\pm 2\epsilon = 48$. For 64.19, we have

	First hour	Second hour
0.34γ $V =$	1230	1070
1.0γ $V =$	1215	1070
Average	1220	1070

The standard error of mean (ϵ) for V will be $\frac{\sigma}{\sqrt{8}}$, i.e. $\frac{48}{2.83} \times \frac{123}{110} = 19$ for first hour and $\frac{48}{2.83} = 17$ for second hour.

Hence V for first hour = 1220 ± 38 and for second hour 1070 ± 34 , if we accept $\pm 2\epsilon$ as the limits between which 21/22 such tests must fall. We may remember that 2/3 of such tests would fall between the limits of 19 and 17 respectively, or approximately 1.6 %.

Fig. 2 shows the curves drawn through $V = 1220$ and 1070 , with dotted curves for ± 1.6 %.

From the curve, we learn that for 58.27, $0.5\gamma = 0.48\gamma$ and 0.51γ respectively of 64.19. Average 0.50γ , i.e. the preparations are identical in catatorulin activity within experimental limits. On curve A (first hour), we have plotted the area representing $\pm \epsilon$. This is theoretically the area within which 2/3 of the tests should fall. Of course by increasing the number of observations to 2 in an experiment, and taking the mean average of 4 observations the standard error of an estimation is again reduced by half. The method should therefore reach with a few birds a much higher accuracy than does the curative or growth test for vitamin B₁.

Table II shows additional evidence of the concordance obtained between observed results and the points calculated from a theoretical curve with constant $k_c = 0.1\gamma$. Considering the sources of error, including the difficulty of thoroughly mixing tissue, the observed results give a reasonable agreement with theory. Table III gives some of the comparisons which have been made already by the final methods upon crystalline specimens. Though preliminary, they show a satisfactory parallelism between catatorulin and vitamin B₁ effects in the preparations studied. If supported by further work, the method should

prove an accurate means of assaying vitamin B₁. It is especially interesting to note that the catatorulin activity of the German preparation is approximately half that of our own, which is in striking agreement with the conclusions for vitamin B₁ activity by curative test.

Fig. 2. Oxygen uptake and vitamin B₁ concentration. Curves A and B theoretical curves for first and second hour, drawn from the two experimental points (circles). Dotted curves represent $\pm\epsilon$ for curve A.

Table II. *Comparison of observed with calculated oxygen uptake in various experiments.*

Experiment	Amount of vitamin in $10^{-2}\gamma/3$ cc.	O ₂ uptake in first hour		O ₂ uptake in second hour	
		Observed	Theoretical	Observed	Theoretical
403	20	1315	1315	960	965
	10	1240	1235	910	905
404	10	1080	1090	700	685
	5	1050	1040	640	655
407	100	[1320]*	1530	940	945
	30	1380	1380	855	860
	15	1300	1290	800	800
408	100	1345	1400	960	970
	30	1250	1265	915	915
	15	1230	1180	810	815
410	100	1450	1450	1080	1090
	30	1380	1310	990	985
	15	1230	1225	860	915
411	100	• 1290	1295	985	970
	40	1215	1205	900	900
	20	1120	1120	840	840
414	100	1120	1125	990	990
	34	1040	1030	900	900
415	100	1280	1250	950	925
	34	1120	1140	810	840
417	100	1270	1290	940	965
	30	1195	1170	875	865

* This figure is a marked exception, and is possibly due to experimental error.

Table III.

A. *Catatorulin tests on vitamin B₁ preparations.*

Exp. No.	Unknown vitamin preparation	Standard used for comparison		Vitamin B ₁ mm. ³		% diff.	Remarks
		53	64·19	First hour	Second hour		
380	63. A. 9	—	—	1790	1455	81·2	Triplicate
383	56·20 64·19	0·3 (56) = 0·37 γ (1) = 0·37 γ (2) 0·3 (64) = 0·27 γ (1) = 0·2 γ (2)	—	1720	1280	74·5	„
403	Windaus <i>et al.</i>	—	0·2 γ W = 0·15 γ (1) = 0·10 γ (2) 0·4 γ W = 0·31 γ (1) = 0·30 γ (2)	1645	1205	73·5	„
410	Windaus <i>et al.</i>	—	1·2 γ W = 0·75 γ (1) = 0·6 γ (2)	1570	1170	74·5	„
414	78·27	—	0·5 γ 78 = 0·51 γ (1) = 0·48 γ (2)	1220	1070	88	Quadruplicate
415	78·27	—	0·4 γ 78 = 0·55 γ (1) = 0·55 γ (2)	1350	1000	74	Quintuplicate

(1) First hour; (2) Second hour.

B. *Amounts having similar activity.*

Preparation	Vitamin B ₁ units	Catatorulin	B ₁ /C
64·19	10 γ	10 γ	1·0
German	17·8 γ	16 γ	1·1
78·27	11·1 γ	8·5 γ	1·3
56	11·2 γ	6·0 γ	1·85
53	10 γ	6·3 γ	1·6

DISCUSSION.

It is clear that the complicated nature of the systems studied precludes more than provisional conclusions. With the proviso that the catatorulin in vitamin B₁ crystals is not an actual substrate, it is interesting to compare k_c with k_m for the enzymes proper. Assuming a molecular weight of not less than 300 for factor, k_c is of the order of $10^{-8}M$. This is even a higher affinity than that found for the oxidising enzymes; it will be interesting if similar facts hold for k_c of other co-enzymes. So far as we know, this is the first measurement. It is reasonable and consistent with the facts to think that a co-factor would be more highly adsorbed than the substrate. In this case, of course, we are still uncertain of the nature of the substrate.

Variations in R (the residual vitamin) should theoretically influence the curve as shown in Fig. 3. If therefore R increases to $40 \times 10^{-2}\gamma$ per 100 mg. (2 pigeon doses/g.), we should expect to see only very small catatorulin effects with changes in added vitamin. This has been shown to be the case for the normal brain, and suggests that normal tissue contains at least 2 units vitamin B₁ per g. It is likely that with further experience estimations of R in small amounts of tissue will be possible by this method.

Theoretically there is no reason why these principles should not be used generally for the estimation of substrate with tissue preparations.

Exp. 1 shows that k_c does not change significantly during the 1st and 2nd hours, when working with crystalline vitamin preparations. This is found even though the total oxygen uptake decreases. It indicates that this decrease is due to a steady inactivation of the enzyme system, in which E progressively decreases.

Fig. 3. Variation in position of theoretical curves for increasing concentrations of residual (tissue) vitamin (R); $V=1500$.

SUMMARY.

1. A method is described for comparing the co-ferment effect upon the oxygen uptake of avitaminous birds' brain of two vitamin B_1 concentrates. The specific factor responsible is called "catatorulin."

2. Crystalline specimens of vitamin B_1 are active sources of catatorulin; distinct effects are shown with 0.1γ in 3.0 cc. solution.

3. There is a wide parallelism between vitamin B_1 and catatorulin activity, which suggests strongly that they are identical and that the method estimates vitamin B_1 .

4. The affinity constant (k_c) for catatorulin is of the order of $10^{-8} M$, which is greater than that of the oxidases for their substrates.

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