CCXXV. THE ESTIMATION OF VITAMIN B₁ IN CEREBRO-SPINAL FLUID

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THE most sensitive method for the estimation of vitamin B₁ is the one described by Schopfer; this consists of growing a fungus, *Phycomyces blakesleeanus*, upon a synthetic medium to which the substance to be assayed is added. The method has been applied to blood by Meiklejohn [1937], but the test is somewhat invalidated by the presence in blood of other substances, particularly protein, that affect the growth of the fungus [Sinclair, 1938]. For clinical purposes the estimation of the vitamin in plasma or cerebro-spinal fluid might be very useful; the former, however, cannot be done with sufficient accuracy because the amount of vitamin B₁ in plasma is very small (about 1 $\mu\text{g.}/100\text{ ml.}$) and is greatly affected by slight haemolysis of the blood [Goodhart & Sinclair, 1939]. Therefore estimations upon cerebro-spinal fluid were attempted, and the results on 110 cases have already been published [Sinclair, 1939, 1].

Karrer [1937] had previously reported that he found no vitamin B₁ in normal c.s.f.; but he used the thiochrome method which was not sensitive enough to detect it. Kasahara *et al.* [1938], who tested the c.s.f. of rabbits, to which 1 to 4 mg. vitamin B₁ had been given intravenously, by the crude method of injecting 1 ml. of the fluid into polyneuritic pigeons, found none in the c.s.f. of normal rabbits but found it in the c.s.f. of meningitic rabbits. Villela [1939] has reported results obtained by adsorbing the vitamin from 10 to 15 ml. of c.s.f. on frankonite and then testing the adsorbate either by the thiochrome method or by Schopfer's method; the latter method gave higher values than the former. No details of the method have yet appeared and it seems doubtful if the fungus would elute all the vitamin from the frankonite. The values found in 30 cases of mental disease averaged 2.5 $\mu\text{g.}/100\text{ ml.}$; the range is not given. Villela states that "In all cases in which the chemical test was negative, it was possible to detect the vitamin with the *Phycomyces* test." Later this is contradicted by the statement that in some cases "no trace of aneurin could be found by either test".

EXPERIMENTAL

The method has been similar to that already described for blood [Sinclair, 1938]. Amounts of c.s.f. between 1 and 4 ml. are added to flasks containing the medium (with 0.4% asparagine), the flasks are sterilized by tyndallization and inoculated with a suspension of *Phycomyces blakesleeanus*. The weight of the fungus produced after 10 days is proportional to the amount of vitamin present.

The only substances that are known to promote growth of the fungus are vitamin B₁, its phosphate esters, its degradation products and certain substances very closely related to these. That the slight growth obtained with c.s.f. is due to vitamin B₁ or its phosphate esters is indicated by the observation that no growth is obtained if the vitamin is converted into thiochrome by alkaline ferricyanide (Table I).

Table I. *Effects of alkali and ferricyanide upon the growth factor in c.s.f.*

C.s.f. ml.	Vitamin B ₁ μg.	K ₃ FeC ₆ N ₆ (1%) drops	NaOH (5N) drops	Wt. of fungus mg.	Vitamin equivalent μg.
0	—	—	—	0	0
0	0.1	—	—	23.5	0.10
0	2.5	—	—	106.5	Excess
1	—	—	—	3.2	0.003
1	0.1	—	—	24.2	0.105
1	2.5	—	—	110.2	Excess
2	—	—	—	6.6	0.007
2	0.1	—	—	25.8	0.11
2	2.5	—	—	116.5	Excess
2	—	—	1	6.2	0.006
2	0.1	—	1	22.2	0.095
2	0.1*	—	1	23.1	0.10
2	—	2	1	0	0
2	0.1	2	1	0	0
2	0.1*	2	1	23.9	0.105

* Vitamin added after alkali.

In experiments such as the one summarized in Table I, c.s.f. and then 2 drops of 1% potassium ferricyanide followed by 1 drop of 5N NaOH were added to the flasks, which were then placed in boiling water for 1 min. After cooling, the pH was adjusted to 6.5 with HCl; the medium was then added and the flasks sterilized by tyndallization. In control flasks vitamin was added either before or after the ferricyanide to show that the treatment was sufficient to destroy the growth-promoting properties of the vitamin without the reagents themselves altering the growth of the fungus in presence of vitamin subsequently added. It will be seen from Table I that heating with alkali does not abolish the growth produced by c.s.f., no doubt because this treatment breaks vitamin B₁ into its constituent parts which are together active [Sinclair, 1937]. Heating with alkaline ferricyanide, however, abolishes the growth produced by c.s.f.; this treatment oxidizes the vitamin to thiochrome, which does not act as a growth factor for the fungus [Meiklejohn, 1937].

I have already shown that blood contains substances other than vitamin B₁ that affect the growth of the fungus [Sinclair, 1938]. But since protein is probably the most important of these, c.s.f. might be expected to give more satisfactory results than blood. Experiments show that this is the case. Unlike blood, c.s.f. is unable to promote growth of the fungus in absence of added medium; and if in presence of c.s.f. the asparagine is omitted from the medium so that it contains no source of nitrogen, lower weights of fungus are obtained and there is no effect of added vitamin B₁. Whereas relatively higher values for vitamin B₁ in blood are obtained when hydrolysed casein (0.8%) is substituted for asparagine in the medium, identical values are obtained with c.s.f. Unlike blood, samples of c.s.f. up to 4 ml. give the same values per unit volume (Table II), and the growth obtained by adding 0.1 μg. vitamin B₁ to samples of c.s.f. is the same as (or very slightly more than) would be expected from the result obtained without added vitamin (see Table I).

However, it is easy to show that there is usually an adjuvant factor in c.s.f., although very much less than in blood. If excess vitamin B₁ (2.5 μg.) be added to samples of 1–4 ml. c.s.f., the growths obtained are usually larger than those in the control flasks without added c.s.f.; results with 1 and 2 ml. c.s.f. are summarized in Table III and compared with similar results for blood [Sinclair, 1938].

Table II. *Effect of size of sample of c.s.f. upon estimated content of vitamin B₁ ($\mu\text{g.}/100\text{ ml.}$)*

Sample	ml. c.s.f.			
	1	2	3	4
	$\mu\text{g.}$	$\mu\text{g.}$	$\mu\text{g.}$	$\mu\text{g.}$
1	0.2	0.2	0.2	—
2	0.5	0.7	0.7	—
3	0.5	0.5	0.7	—
4	0.6	0.7	0.7	0.8
5	0.7	0.7	0.7	—
6	1.3	1.5	1.8	—
7	1.5	1.6	1.7	—
8	2.0	2.5	2.3	2.4
9	3.0	3.5	3.3	—
10	5.0	4.0	3.0	—
11	5.5	5.5	4.3	—
Mean	1.89	1.95	1.76	

Table III. *Effect of c.s.f. upon the growth of Phycomyces in presence of excess vitamin B₁*

Vol. of c.s.f. ml.	No. of observations	Increase in presence of c.s.f. (%)	
		Range	Mean
1	110	0-78	22
2	105	0-116	37
Blood 1 ml.	50	14-160	78

That part of this adjuvant effect of added c.s.f. is due to the fluid buffering the medium is indicated by Table IV. In this table a comparison is made of the pH of the medium after the fungus has stopped growing in presence of c.s.f. or in presence of approximately the same amount of vitamin. It will be seen that the change in pH from the original 6.5 is less in the flasks containing c.s.f. than in the controls.

Table IV. *Effect of c.s.f. upon the final pH of cultures of Phycomyces*

C.s.f. ml.	Added vitamin B ₁ $\mu\text{g.}$	Approx. conc. of vitamin present $\mu\text{g.}$	Final pH	Added vitamin B ₁ $\mu\text{g.}$ (no c.s.f.)	Final pH
1	—	0.035	4.8	0.025	4.0
1	0.1	0.15	3.8	0.15	3.3
2	—	0.07	4.6	0.10	3.5
2	0.1	0.165	3.8	0.20	3.3
1	2.5	Excess	4.6	2.5	3.7
2	2.5	Excess	4.8		

Because of this adjuvant effect, the values for vitamin B₁ that are given by the test are higher than the true values. But it will be shown in a paper now in preparation that similar apparent values for blood can be corrected to give approximately true values by multiplying the weights of fungus produced in the presence of, say, 1 ml. blood by the ratio of the weight obtained with excess vitamin to the weight obtained with 1 ml. blood in presence of excess vitamin; the corrected weight thus obtained is then used to obtain the true value from the growth-vitamin curve. For instance, taking the figures given in Table I, we obtain the corrected weight for 1 ml. c.s.f. by multiplying the observed

weight (3.2 mg.) by $\frac{106.5}{110.2}$, and the corrected weight for 2 ml. by multiplying 6.6 mg. by $\frac{106.5}{116.5}$. The corrected weights are 3.1 and 6.0 mg. respectively, equivalent to 0.003 and 0.006 $\mu\text{g.}$ vitamin B₁. The weights obtained in the presence of small amounts of added vitamin (0.1 $\mu\text{g.}$ in Table I) can be corrected in the same way. It is believed that this method gives approximately true values for the estimation of vitamin B₁ in 1–4 ml. samples of c.s.f.

The method described above estimates both free vitamin B₁ and cocarboxylase; normal c.s.f. contains none of the latter [Goodhart & Sinclair, 1939]. I have previously shown that estimations on blood are much affected by the temperature to which the blood is heated; very low values are obtained with unheated blood because most of the vitamin is combined with protein. This is not true of c.s.f. If sterile c.s.f. be added to flasks that have previously been filled with medium and sterilized and then the flasks be inoculated without further heating, the same values are obtained as when the flasks are tyndallized before inoculation. This shows that the vitamin in c.s.f., unlike that in blood, is uncombined. This conclusion is confirmed by the fact that all the vitamin in c.s.f. is diffusible [Sinclair, 1939, 2].

RESULTS

The results already quoted, with the exception of those in Tables II and III which include all relevant estimations, have been obtained with samples of human c.s.f. that have shown no marked pathological features. The method has been applied to 272 different samples of c.s.f. obtained from hospital patients. Excluding all the samples that either showed marked pathological features (increased protein or cells, or non-sterility) or came from patients who might be deficient in vitamin B₁, the values (in $\mu\text{g./100 ml.}$) were as follows:

	No. of observations	Range	Mean ($\pm \sigma$)
Apparent	177	0.0–2.0	0.7 $\pm$ 0.5
True	150	0.0–1.3	0.5 $\pm$ 0.4

Taking the limits of $\pm 2\sigma$, it may be said that the amount of vitamin B₁ in the c.s.f. of “normal” individuals is 0.0–1.3 $\mu\text{g./100 ml.}$, mean 0.5 $\mu\text{g.}$ It happens that this statistical variation is the exact range within which all the 150 true values have fallen.

The corresponding figures for the sterile pathological samples were as follows:

	No. of observations	Range	Mean ($\pm \sigma$)
Apparent	95	0.1–12.0	3.5 $\pm$ 2.0
True	83	0.1– 6.5	2.2 $\pm$ 1.3

It will be seen that these values tend to be much higher, and it is instructive to correlate the values with the different abnormalities in the samples. A mere increase in protein in the fluid does not usually cause abnormally high true values for vitamin B₁: for instance, samples of c.s.f. withdrawn on different days from a patient with an intracerebral tumour contained 0.2, 0.8, 0.2 and 0.1 $\mu\text{g.}$ vitamin B₁ per 100 ml., and the corresponding protein values were 320, 360, 300 and 340 mg./100 ml. (the second sample contained 2240 red blood corpuscles per $\mu\text{l.}$); it is interesting to mention here that ascitic fluid containing 1600 mg. protein per 100 ml., from a patient with mitral stenosis, contained no vitamin B₁. But increase in the cells is accompanied by abnormally high values for the vitamin. Therefore high values are found in cases of meningitis, as shown in Table V, which includes all the sterile samples that were obtained from such cases.

Table V. *Variation of vitamin B₁ with cell count in c.s.f. from cases of meningitis*

Case no.	Type	White cells per μ l.	Differential count (%)		Red cells per μ l.	Vitamin B ₁ μ g./100 ml.
			Neutrophils	Lymphocytes		
1	Meningococcal	2850	88	12	0	4.6
2	Meningococcal	548	67	28	0	3.5
		287	100	—	0	3.5
		278	59	30	0	3.4
		61	31	68	0	1.5
3	Tuberculous	154	—	100	127	3.0
4	Meningococcal	130	46	54	1606	4.0
		127	74	26	86	3.0
5	Pneumococcal	38	72	38	0	0.0
		27	40	95	0	0.4
6	Tuberculous	17	—	100	1200	1.5
		9	—	100	580	0.1
		6	—	100	780	0.6
		3	—	100	260	0.5
		1	—	100	180	0.7
7	Pneumococcal	6	—	100	0	0.5
8	Tuberculous	1	—	100	0	0.5

A statistical analysis of the figures in Table V shows that there is a positive correlation between the white cell count and the vitamin content: the correlation coefficient is 0.59 ± 0.16 . An analysis by the method of partial correlation fails to demonstrate any significant effect of the red cell count on the vitamin content in these particular figures: the correlation coefficient is 0.06. The regression of amount of vitamin B₁ on white cell count calculated from the figures in Table V indicates that a single white cell contains 1.4×10^{-8} μ g. vitamin B₁. These results agree well with our earlier conclusions. Mammalian leucocytes were shown to contain much more cocarboxylase than erythrocytes: as a very rough approximation it was stated that one leucocyte, in a sample of ox blood, contained as much cocarboxylase as 1600 erythrocytes [Goodhart & Sinclair, 1939]. In estimations of the true vitamin B₁ in the blood of 45 healthy adults, I have obtained a mean ($\pm \sigma$) of 7.4 ± 1.4 μ g./100 ml.; and plasma contains about 1 μ g./100 ml. Therefore, assuming a blood count of 5×10^6 r.b.c. and 7×10^3 w.b.c. per μ l. and that plasma represents 50% by volume of whole blood, we have: $7.4 = 0.5 + \left(\frac{5 \times 10^6}{1600} + 7 \times 10^3 \right) \times x \times 10^5$, where x is the mean amount of vitamin B₁ (in μ g.) in one leucocyte. From this equation $x = 0.7 \times 10^{-8}$, which is of the same order as 1.4×10^{-8} deduced above. In fact a higher figure should be obtained with the figures for meningitic c.s.f. than with figures for normal blood, since the former fluid is likely to contain disintegrated leucocytes. From these results it appears very probable that the increased values for vitamin B₁ obtained with pathological samples of cerebro-spinal fluid are mainly due to the increased cell count, particularly the white cell count. These results obtained with c.s.f. therefore agree very well both with the estimations of cocarboxylase in blood made by a chemical method and with the estimations of total vitamin B₁ made by a biological method.

DISCUSSION

It is known that vitamin B₁ acts in the body as a catalyst, necessary for the degradation of pyruvate, only after it has been converted into its diphosphate ester, cocarboxylase. The vitamin circulates in plasma in the free unphosphory-

lated form; in this form it diffuses readily and phosphorylation of it with formation of cocarboxylase occurs inside the cells. It has been shown that the vitamin B₁ in normal c.s.f. is free and unphosphorylated; its concentration, 0.0–1.3 $\mu\text{g.}/100\text{ ml.}$, is of the same order as the amount of free unphosphorylated vitamin in human plasma or blood [Sinclair, 1939, 2]. The cells of human blood contain about seven times as much vitamin as plasma, most of the vitamin in them being phosphorylated. Therefore it is not surprising that in pathological samples of c.s.f., with an increased number of cells, the amount of vitamin tends to be increased (as discussed above) and cocarboxylase may be found [Goodhart & Sinclair, 1939].

Unfortunately, estimations of the vitamin in c.s.f. seem to be useless for clinical purposes; the amount is extremely small, and zero values have been obtained in 22 patients who were almost certainly not deficient in the vitamin (such as patients with intracerebral tumours on normal diets and with no vomiting). It may be mentioned, however, that positive values have been found in untreated cases of diabetic neuritis, subacute combined degeneration of the cord and sciatica. The highest values in sterile c.s.f. have been obtained in cases of meningitis, cerebral abscess, intracranial tumour and hydrocephalus.

SUMMARY

1. A method is described, based upon the work of Schopfer, of estimating vitamin B₁ in 1–4 ml. samples of cerebro-spinal fluid. The method gives approximately true values.

2. In normal c.s.f. the vitamin is in the free unphosphorylated form.

3. The method has been applied to 272 different samples of c.s.f. obtained from hospital patients. The normal range is 0.0–1.3 $\mu\text{g.}/100\text{ ml.}$, with a mean ($\pm\sigma$) of 0.5 ± 0.4 .

4. Pathological samples of fluid tend to give higher values; the values show a positive correlation with the white cell count.

5. These results are discussed in relation to other work on the metabolism of vitamin B₁.

6. The method seems to be useless for clinical purposes.

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