CCLXXXIII. THE ESTIMATION OF VITAMIN B₁ IN BLOOD

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MEIKLEJOHN [1937] has described a method, based upon the work of Schopfer [1935], of estimating vitamin B₁ in "small samples" (15 ml.) of blood. From his evidence he concludes "that the method... provides a quantitative estimate of the true vitamin B₁ content of the blood". Since a clinical test for vitamin B₁ in blood is badly needed, and since Meiklejohn's method is being used by several workers in this country and in others, it seemed to be important to examine the justification of his claim and the value of the test. The basis of the method lies in the ability of vitamin B₁ to promote the growth of a fungus, *Phycomyces blakesleeanus*.

There is already sufficient evidence contained in Meiklejohn's paper and in Schopfer's numerous publications to show that the former's claim needs investigation. Schopfer & Jung [1937, 2] have published tentative conclusions: "Le sang donne une réaction positive. En exprimant en aneurine la totalité de l'action auxogène observée, on arriverait à des teneurs variant entre 0.2 et 0.4 γ par cm.³ de sérum." The animal used is not mentioned. In his other paper mentioning determinations on blood, Schopfer [1937] states: "Avec le sérum, nous avons trouvé une fois 39 mg. de récolte avec 1 ccm. (rat mâle) et une fois 69 mg. pour 1 ccm. (rat femelle); en comparant avec les données fournies par la vitamine pure, nous trouvons que 0.2 γ de cette dernière livre une récolte de 65 mg." He then warns us of the danger of expressing all growth-promoting activity in terms of vitamin B₁, since "nous savons que d'autres facteurs agissent également". Although results on two rats hardly merit discussion, it is instructive to examine the only experimental results that he has published (presumably obtained with the male rat):

Rat serum vol. in ml.	Dry wt. of fungus mg.	Approx. value for apparent vitamin B ₁ (γ /100 ml.) calculated from Schopfer's figures
0.5	34	17
1.0	39	10
2.0	47	6.5
Rat red blood corpuscles "about 2 ml."	77	15

It will be noticed that 0.5 ml. and 1 ml. serum give about the same actual growth; 0.5 ml. is nearly three times as effective as 2 ml. if the results are expressed per unit volume. These meagre results indicate that the method cannot be used for accurate quantitative estimations. They also indicate that rat serum contains an inhibitory factor.

Further, the method is not specific for vitamin B₁. Schopfer had great difficulty in identifying the growth-promoting factor owing to its great stability to heat, resistance to alkali and solubility in chloroform. Because of these

properties he at times discarded vitamin B₁ as the growth factor and supported bios [Schopfer, 1932], vitamin D, flavin [Schopfer, 1934, 1] and "factor M" [Schopfer, 1934, 2]. The only substances now known to promote the growth of the fungus are vitamin B₁ and its breakdown products; the pyrimidine and thiazole fragments (and certain closely related compounds [Robbins & Kavanagh, 1938]) are active when they are supplied together [Schopfer & Jung, 1937, 1; Sinclair, 1937; Robbins & Kavanagh, 1937]. It seems probable that "factor M" consists of these fragments; and since heating (e.g. in cooking) may destroy the vitamin, these substances may appear in blood and so invalidate the test [Schopfer & Müller, 1938].

Meiklejohn found that the blood of avitaminous pigeons had an adjuvant action on added vitamin. This fact strongly condemns the validity of the test, and it will be shown below that normal blood has a similar action.

Although these facts cast doubt upon Meiklejohn's claim, it seemed advisable to examine the test experimentally.

EXPERIMENTAL

Unless otherwise stated, the technique of these experiments has only differed from that described by Meiklejohn in a few particulars: 0.4% asparagine¹ has been used unless otherwise stated; MgSO₄ · 7H₂O in a concentration of 0.002 *M* was used instead of the anhydrous salt; the stock medium was brought to pH 6.5 with 10 *N* NaOH (the slight differences in pH produced by adding different amounts of blood did not affect the results), and was made up in stronger solution than used by Meiklejohn, so that only 4 ml. were added to each 50 ml. flask; 1 ml. spore suspension (containing about fifteen million spores) was used for inoculating. Since it has been found that the order in which the medium, blood, water and vitamin are added to the flask makes a difference to the results, unless otherwise stated the blood has been added to the medium and stood for at least half an hour; water (followed where necessary by vitamin) has then been added to the flasks and stood at -2° for at least 12 hr. before sterilizing by steaming for 20 min. on 3 successive days. The cultures have been grown for 10 days in the dark in a room kept at a constant temperature of 18°. The stock culture of *Phycomyces blakesleeanus* (sex -) has been grown on Sabouraud's medium to which malt extract (B.P.) was added to make a concentration of 2%. Most experiments have been set up at least in duplicate and results quoted are from typical experiments. Synthetic vitamin B₁ has been used throughout.

Composition of medium

In the method described by Meiklejohn, different concentrations of asparagine were used for the control flasks and for those containing blood. He found that in presence of 2 or 3 ml. blood, there might be some reduction of growth if the concentration of asparagine were increased above 0.2%. This effect was said not to be observed with 1 ml. or less; one result with 1 ml. and none with less were quoted. The results with 2 and 3 ml. show an inhibition in the higher concentration of about 14%. Most of Schopfer's experiments were done with 0.1% asparagine, which is well below the optimum concentration. I have found that results with different concentrations of asparagine vary, but that 0.4% with blood usually gives a greater growth than is given by 0.2 or 0.6%. Even 0.4% is suboptimal in the control flasks without blood (Table I).

Asparagine was the best source of nitrogen that Schopfer [1934, 3] tried, although glycine was almost as good. I have tested various compounds, using amounts which supplied the same quantity of nitrogen as is present in 0.4% asparagine, and found that, as sources of nitrogen, guanidine sulphate, methyl

¹ B.D.H. asparagine and dextrose (A.R.) were used; the two inorganic salts were supplied by Kahlbaum ("puriss.").

Table I. *Effect of concentration of asparagine on the growth of Phycomyces*

In this and all the other tables, unless otherwise stated, the figures represent dry weight of fungus in mg.

Exp.		Concentration of asparagine (%)							
		0	0.1	0.2	0.3	0.4	0.6	0.8	1.0
1	0.1 γ vit. B ₁	0	—	30.8	—	41.0	38.4	40.2	—
	Ox blood 1 ml.	26.4	—	26.3	—	27.9	23.5	25.3	—
	„ 2 ml.	51.6	—	52.4	—	52.6	48.4	45.3	—
2	Ox blood 1 ml.	27.6	—	35.2	—	35.4	40.1	40.0	—
	„ 2 ml.	—	—	61.4	—	65.3	69.2	79.3	—
3	0.1 γ vit. B ₁	0	—	36.5	—	37.1	43.5	43.3	47.3
	0.5 γ vit. B ₁	0	—	73.8	—	101.4	115.6	120.7	132.3
4	0.5 γ vit. B ₁	—	—	—	—	101.0	113.8	—	—
	Ox blood 2 ml.	—	56.7	64.5	63.8	87.8	78.6	—	—
5	Ox blood 2 ml.	—	—	68.8	—	88.7	—	—	—
	+ 0.1 γ vit. B ₁	—	—	108.9	—	111.0	—	—	—
6	0.1 γ vit. B ₁	0	24.8	26.3	28.9	31.6	31.9	32.7	33.1
	0.5 γ vit. B ₁	0	38.1	60.0	66.8	93.7	100.9	101.2	98.4
	Ox blood 1 ml.	26.9	35.2	34.0	38.8	41.1	36.9	44.4	39.4
	+ 0.1 γ vit. B ₁	37.4	60.1	72.2	71.8	78.0	69.5	75.6	79.5
	+ 2.5 γ vit. B ₁	39.7	76.9	109.5	142.2	168.1	165.6	218.8	270.0
	Ox blood 3 ml.	43.9	66.4	86.3	92.4	100.2	93.4	97.5	97.2
	+ 0.1 γ vit. B ₁	52.6	81.4	104.2	110.7	126.9	115.8	124.0	128.2
	+ 2.5 γ vit. B ₁	59.5	88.5	135.6	169.7	195.5	219.3	233.5	309.3

guanidine HCl, dimethyl guanidine HCl, creatine and creatinine were useless; ammonium carbonate, urea and glycocyamine were very poor; alanine and arginine were poor; sodium aspartate and glycine were as good as asparagine, and "glutamine" much more effective (the glutamine was added direct to the medium, containing salts and glucose, before steaming; most of it will have been destroyed by this process).

Table II. *Comparison of the effect of different sources of nitrogen on the growth of Phycomyces*

Exp.	Vit. B ₁ γ	Source and % of nitrogen								
		Aspara- gine 0.4	Glycine			"Glutamine"				
			0.2	0.5	0.75	0.2	0.4	0.6	0.8	1.0 1.2
1	0.1	46.0	46.0	50.7	53.8	43.8	53.2	56.2	—	—
	0.3	87.4	81.1	86.2	90.5	66.4	110.0	115.7	—	—
2	0.5	101.0	—	—	—	—	—	128.2	136.6	175.0
3	0.5	101.4	—	—	—	—	—	130.6	136.8	— 160.0

Glutamine was not quite as effective as asparagine when added to blood (Table III).

Table III. *Comparison of the effect of different concentrations of asparagine and "glutamine" in the presence of 2 ml. ox blood*

	Source and % of nitrogen				
	0.1	0.2	0.3	0.4	0.6
Asparagine	56.7	64.5	63.8	87.8	78.6
"Glutamine"	—	—	61.2	75.7	75.7

The most satisfactory source of nitrogen, either with or without blood, has been found to be hydrolysed casein.

100 g. caseinogen (Glaxo) were heated with 300 ml. conc. HCl and 300 ml. water under a reflux condenser for $3\frac{1}{2}$ hr. After bringing to pH 6.5 with NaOH the total volume was 950 ml.

Table IV. *Comparison of asparagine and hydrolysed casein as sources of nitrogen for Phycomyces*

Exp.		Aspara- gine 0.4%	Hydrolysed casein (%)							
			1	3	5	6	7	8	10	12
1	0.1 γ vit. B ₁	37.1	—	—	—	58.7	58.7	60.3	58.0	45.3
2	0.1 γ vit. B ₁	41.0	26.8	49.6	51.5	—	—	62.9	59.0	—
	1 ml. ox blood	27.9	—	53.1	55.8	—	54.1	66.3	63.3	—
	2 ml. „	52.6	—	86.3	105.0	—	102.6	110.8	82.1	—
	3 ml. „	70.0	—	81.7	86.7	—	88.0	84.3	81.8	—

Table IV and four other experiments have shown that with or without blood about 8% is the optimum concentration of hydrolysed casein. The growth obtained with this concentration and 0.1 γ vitamin B₁ is about the same as is obtained with 0.4% asparagine and 0.2 γ vitamin B₁. Without added vitamin, hydrolysed casein causes no growth of the fungus.

In Table IV the growth obtained with 3 ml. blood and hydrolysed casein is in general lower than that obtained with 2 ml. In Tables IV, V and VI a comparison is made of the growths obtained with and without blood or vitamin and the two different sources of nitrogen. From these and other similar experiments it is concluded that (i) in absence of blood, hydrolysed casein is a better source of nitrogen than asparagine; (ii) this result is also usually found in the presence of 1 ml. blood, although the estimated vitamin tends to be lower when hydrolysed casein is used; usually with 3 ml. blood, the estimated vitamin per unit volume of blood is less than with 1 ml. when hydrolysed casein is used, more than with 1 ml. when asparagine is used; (iii) when 0.1 γ vitamin is added to blood, a greater growth than expected is usually produced in presence of asparagine, and a smaller growth than expected is produced in presence of hydrolysed casein; (iv) this adjuvant action of blood in presence of asparagine is very marked when excess vitamin is added; but with excess vitamin and hydrolysed casein, blood sometimes has an adjuvant action and sometimes an inhibitory effect. This inhibitory action is being further investigated. These experiments prove that part of the adjuvant action of blood in presence of asparagine is due to sources of nitrogen in the blood. In this connexion it is interesting to recall that Meiklejohn found that extracting the vitamin from blood by means of alcohol or removing protein by heating in acid solution resulted in a considerable loss of growth-promoting activity.

It must be stressed that Schopfer has made no attempt to choose, and has not claimed to have chosen, the best medium for this fungus. The one employed by him and by Meiklejohn is likely to be suboptimal since it is poor not only in sources of nitrogen but also in salts (MgSO₄ and KH₂PO₄ are the only salts added by Schopfer). The addition of small amounts of various salts to the medium increases the growth; Czapek-Dox salts produce a better growth, and addition of CaCl₂, FeSO₄, CuSO₄, MnSO₄ and pyrophosphate, all increase it to varying extents, as shown in Table VII. In all cases pure salts were used; they were brought to pH 6.5 and added before steaming.

Table V. *Comparison of the effects of asparagine, hydrolysed casein and blood as sources of nitrogen for Phycomyces*

Source of nitrogen: O = no added nitrogen; A = 0.4% asparagine; HC = 8% hydrolysed casein

Addition	Exp. 1			Exp. 2			Exp. 3			Exp. 4			Exp. 5			Exp. 6		
	Human blood			Ox blood			Ox blood			Human blood			Human blood			Ox blood		
	O	A	HC	O	A	HC	O	A	HC	O	A	HC	O	A	HC	O	A	HC
0.1γ vit. B ₁	0	15.3	24.2	0	27.1	35.2	0	31.6	38.4	0	24.0	36.8	0	26.3	35.0	37.1	60.3	—
2.5γ vit. B ₁	0	114.5	157.0	0	110.8	180.2	0	93.7	186.4	0	66.8	172.8	0	113.0	126.4	101.9	—	—
1 ml. blood	18.6	19.1	28.2	28.0	37.7	41.2	26.9	41.1	29.4	18.8	22.6	26.3	25.1	33.7	27.1	42.3	—	—
+ 0.1γ vit. B ₁	32.0	44.6	43.0	36.1	66.1	66.5	37.4	78.0	49.4	—	63.1	—	—	48.0	—	—	—	—
+ 2.5γ vit. B ₁	72.9	129.0	206.0	40.9	149.0	81.0	39.7	168.1	91.2	—	135.0	—	—	—	—	—	—	—
2 ml. blood	—	—	—	38.6	73.4	57.7	—	—	—	—	32.2	—	—	48.6	—	45.5	55.2	—
3 ml. blood	52.1	69.5	63.8	41.1	89.0	78.0	43.9	100.2	64.3	—	50.3	—	—	63.0	65.0	—	—	—
+ 0.1γ vit. B ₁	63.5	90.8	81.8	—	—	—	52.6	126.9	77.6	—	—	—	—	—	—	—	—	—
+ 2.5γ vit. B ₁	128.5	201.0	224.0	—	—	—	59.5	195.5	102.4	—	—	—	—	—	—	—	—	—

Table VI. *Comparison of the effects of asparagine and hydrolysed casein as sources of nitrogen for Phycomyces in presence of blood*

Vit. B ₁ γ	Ox blood (ml.)						
	0	1	2	3	4	5	6
	Hydrolysed casein (8%)						
0	0	29.4	63.6	64.3	113.5	118.2	124.5
0.1	38.4	49.4	75.4	77.6	116.3	123.4	137.1
2.5	186.4	91.2	99.6	102.4	136.5	163.7	169.9
Asparagine (0.4%)							
0	0	41.1	66.0	100.2	118.6	122.5	—
0.1	31.6	78.0	93.1	126.9	128.7	—	—
2.5	93.7	168.1	165.7	195.5	171.9	—	—

Table VII. *The effect of salts on the growth of Phycomyces*

Exp. 1	0.3 γ vitamin B ₁	96.0
	0.3 γ vitamin B ₁ + CaCl ₂ (52 mg.)	131.5
	0.3 γ vitamin B ₁ + sodium pyrophosphate (12 mg.)	116.2
Exp. 2	0.1 γ vitamin B ₁	46.0
	0.1 γ vitamin B ₁ + CaCl ₂ (6 mg.)	53.4
	0.1 γ vitamin B ₁ + CaCl ₂ (50 mg.)	65.4
	0.1 γ vitamin B ₁ + sodium pyrophosphate (1.7 mg.)	45.9
	0.1 γ vitamin B ₁ + sodium pyrophosphate (8.0 mg.)	53.4
	0.3 γ vitamin B ₁	87.4
	0.3 γ vitamin B ₁ + CaCl ₂ (6 mg.)	104.1
	0.3 γ vitamin B ₁ + CaCl ₂ (50 mg.)	126.2
Exp. 3	0.1 γ vitamin B ₁	37.1
	0.1 γ vitamin B ₁ + FeSO ₄ (2 mg.)	41.1
	0.1 γ vitamin B ₁ + CuSO ₄ (2 mg.)	50.0
Exp. 4	0.1 γ vitamin B ₁	39.5
	0.1 γ vitamin B ₁ + MnSO ₄ (1 mg.)	40.0
	0.4 γ vitamin B ₁	83.0
	0.4 γ vitamin B ₁ + MnSO ₄ (1 mg.)	95.6

These salts were supplied in larger amounts than would be present in the blood added. But since the medium selected is not a good one for the growth of the fungus, it would be expected that there might be substances present in blood that would have an adjuvant action upon it. Experiments now to be described show that such is the case.

Other substances in blood affecting growth

Meiklejohn concluded that the presence of blood does not usually alter the activity of the vitamin. The three lines of evidence that he advances are not convincing.

(1) He states that adding known amounts of vitamin to blood that has been autoclaved at pH 9 gives the expected growth, and the four figures he quotes support this. Yet this treatment, as he points out, is so drastic that it destroys other substances; for instance the reaction shifts during the process to pH 5. It is shown below that the adjuvant factor is thermolabile.

(2) He states that "the addition of a small amount of vitamin B₁ usually increases the growth of mycelium to the same extent in the presence of added blood as in its absence". Results on three samples of 2 ml. blood with and without 0.1 γ vitamin are quoted in support of this. As already mentioned, he found that the blood from avitaminous pigeons had an adjuvant action on added vitamin and this I have confirmed. He stated that it was important to be aware of the possibility of blood containing an adjuvant factor, "and if necessary to control it" by including in the test a 2 ml. sample of blood to which 0.1 γ vitamin had been added. He says that this gives a growth "considerably less" than the maximum, but his own figures show that it may be equal to the growth obtained with excess vitamin B₁.

As this point is important, I have examined it in some detail, and find that blood always contains an adjuvant factor. In experiments with ox blood summarized in Table VIII, known amounts of vitamin B₁ were added to the flasks and the total vitamin content was estimated. In some cases it was possible to estimate the apparent vitamin B₁ in the blood by subtracting the amount of vitamin B₁ added from the estimated total vitamin content; in such cases the apparent vitamin B₁ content, expressed in γ /100 ml. blood, has been printed in

Table VII in ordinary type. In other cases the amount of growth was greater than that obtained even with excess of added vitamin in the absence of blood, so that it was not possible to translate the weights of growth into apparent amounts of vitamin B₁; when this was the case the actual weights of growth have been given and have been printed in black type, as have also the maximum growths obtained with excess of vitamin alone.

The three experiments illustrated in Table VIII and many others show that adding small amounts of vitamin to ox blood produces in about 75 % of cases a

Table VIII. *Effect of added vitamin B₁ on the estimated vitamin in blood*

(For explanation of figures, see text.)

Vit. B ₁ γ	Ox blood (ml.)																
	0	0.5	1	2	3	4	5	0	1	2	3	4	0	1	2	3	4
0	0	11.0	8.0	9.0	10.5	132	139	0	10.0	11.0	110	132	0	7.5	8.0	13.5	11.5
0.025	—	—	—	—	—	—	—	—	11.0	13.0	114	131	—	—	—	—	—
0.05	—	9.0	10.0	10.5	9.5	137	166	—	11.5	19.0	117	138	—	9.0	11.0	—	—
0.1	—	6.0	8.5	9.0	9.5	140	—	—	16.0	108	134	139	—	11.5	16.0	—	—
0.15	—	8.5	13.0	8.5	—	—	—	—	31.0	—	—	—	—	—	—	—	—
0.2	—	15.0	18.0	7.0	129	—	—	—	109	129	135	—	—	—	—	—	—
Excess (2.5-5.0)	118	136	132	147	157	175	205	105	132	143	—	—	104	166	204	266	278
	Exp. 1							Exp. 2					Exp. 3				

greater growth than would be expected from the amount of vitamin added. The blood itself therefore usually has an adjuvant effect upon the growth of the fungus apart from the vitamin it contains.

Experiments with human blood have given similar results. In 150 l ml. samples of blood from different cases the addition of 0.1γ vitamin B₁ has produced an increase over the expected of 50 % or more in 63 % of cases, an increase of less than 50 % or no difference in 30 %, and a diminution in 7 %. From this it appears that with small amounts of vitamin there is usually an adjuvant factor in blood, but occasionally an inhibitory factor.

One simple method of testing the validity of the method has not been used by Schopfer and has been disparaged by Meiklejohn. If there is no substance in blood other than vitamin B₁ which affects the growth of the fungus, then the growths obtained with and without blood when excess vitamin B₁ is added should be the same. They are not. In 50 experiments at least 2.5γ vitamin B₁ have been added to the flasks with and without the addition of different samples of human blood (1 ml.); this amount of vitamin is excessive because under the conditions of the experiments a maximum effect is obtained with about 0.5γ. The flasks containing the blood have always produced a growth that is greater than the control; the increase varies from 14 to 160 %, with an average for the fifty of 78 %. Further figures will be found in Tables I, V, VI, VIII and XI. Meiklejohn stated that "when large amounts (e.g. 0.4γ) of vitamin have been added [to blood] the effect has been less satisfactory. As noted previously, however, anomalous results are frequently obtained when the amount of vitamin is sufficient to produce nearly maximum growth." The size of the vessel and the accumulation of waste products of metabolism, amongst other factors, are suggested by him as probably explaining his inconsistent results. However, the results that I have quoted are consistent and significant and show that vitamin B₁ is not the only substance in blood that affects the growth of the fungus, since the addition of blood always produces a greater growth in presence of excess vitamin. An unusually large adjuvant action was shown by blood taken shortly after death

from a patient with aplastic anaemia; the following weights of fungus were produced:

	mg.
0.1 γ vitamin B ₁	25.8
2.5 γ vitamin B ₁	65.8
1 ml. blood	61.5
1 ml. blood + 0.1 γ vitamin B ₁	90.7
1 ml. blood + 2.5 γ vitamin B ₁	193.6

Meiklejohn's inconsistencies with nearly maximal growth are possibly partly due to insufficient drying of the mycelium before weighing; the mycelium takes a long time at 110° to reach a constant weight. Further, the adjuvant factor is thermolabile, and slight differences in the temperature at which the flasks are sterilized make considerable differences to the growth. With these precautions in mind, I have obtained consistent results.

Another fact that proves that blood has an adjuvant action is that large samples (more than 3 ml.) of blood (to which no vitamin has been added) usually give a growth that is greater than that obtained with excess vitamin B₁ (see Tables VI and VIII).

(3) The third line of evidence advanced by Meiklejohn to show that blood does not usually contain an adjuvant factor is that with separate samples of 1, 2 and 3 ml. blood the values obtained are in the ratio 1:2:3. He quoted six selected cases of human blood in support of this and states that he discarded the figures when this result was not obtained. I have found that there is usually an adjuvant factor present in blood so that the 3 ml. sample gives a value of more than three times the 1 ml. sample. Very rarely the blood has a depressant effect. Figures for ox blood have already been quoted. Some selected figures for human blood are as follows:

Table IX. *The effect of different volumes of blood upon the estimated vitamin in blood*

		Apparent vitamin B ₁ in blood (γ /100 ml.)						
ML. blood	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8
1	13	8	6	7.5	8	6	10	13
2	18	18	11	15	12	12	9	6.5
3	113	107	17	17	15	17	6	0
	(5 γ gives 106)	(2.5 γ gives 78)						

The figures in black type represent actual weights of fungus obtained (cf. Table VIII). Determinations upon samples of human blood from 235 different cases have given the following results: in 155 samples, the 3 ml. value (expressed in γ /100 ml. blood) has been 1 γ , or more, higher than the 1 ml. value, and in 38 samples 1 γ , or more, lower; in 213 samples the average 3 ml. value (expressed per unit volume) was 23 % higher than the average 1 ml. value, and in 22 samples the growth with 3 ml. blood added was greater than that given by excess vitamin. It must be admitted therefore that blood usually has an adjuvant action on the growth of the fungus; rarely there is an inhibitory action. This proves that Meiklejohn's method does not provide a quantitative estimate of the true vitamin B₁ content of blood.

The nature of this adjuvant factor is not fully known. It has already been shown that one cause of it is the additional sources of nitrogen, and possibly the salts, of blood. A further cause of it is to be found in the buffering power of blood. The fungus, in presence of excess vitamin, stops growing mainly because of the accumulation of products of metabolism. Although the medium is buffered

Exp.		ml. blood				
		1	2	3	4	
1	Blood alone	35.0	64.5	93.0	—	
	+CaCO ₃	49.3	83.1	81.3	—	
2	Blood alone	40.9	57.8	88.4	—	
	+CaCO ₃	55.3	72.2	122.7	—	
3	Blood alone	44.1	79.6	96.8	105.0	
	+CaCO ₃	38.4	64.5	112.4	124.6	
4	Blood alone	22.8	59.3	93.2	—	
	+CaCO ₃	24.8	43.8	84.7	—	
5	Blood alone	28.3	39.0	54.5	—	
	+CaCO ₃	29.4	44.9	—	—	
6	Blood alone	26.8	43.6	70.0	—	
	+CaCO ₃	43.3	64.2	—	—	
	+CaCO ₃ (added after steaming)	29.4	49.0	—	—	
7	No vitamin B ₁	{ Blood alone	30.9	59.8	96.0	—
		{ +CaCO ₃	36.3	57.5	88.9	—
	0.1 γ vitamin B ₁	{ Blood alone	59.3	82.8	110.4	—
		{ +CaCO ₃	66.6	94.2	98.9	—
	2.5 γ vitamin B ₁	{ Blood alone	132.3	146.8	147.0	—
		{ +CaCO ₃	122.7	136.3	39.8	—

B. Human blood (1 ml.)		
Exp.		
1	Blood alone	20.5
	+CaCO ₃	31.0
2	Blood alone	19.2
	+CaCO ₃	22.8
3	Blood alone	29.8
	+CaCO ₃	40.0
4	Blood alone	26.1
	+CaCO ₃	40.2
5	Blood alone	27.6
	+CaCO ₃	21.8

C. Without blood						
Vitamin B ₁ (γ)						
	0.0125	0.05	0.1	0.5	2.5	4.0
Control	—	—	22.9	—	—	103.9
+CaCO ₃	—	—	43.9	—	—	164.2
Control	—	17.7	32.1	73.5	107.1	—
+CaCO ₃	—	22.4	38.9	80.8	120.8	—

Vitamin B ₁ (γ)					
	0.1	0.2	0.3	0.5	2.5
Control	45.1	70	87.5	113	—
+CaCO ₃	48.6	78	103.5	123.5	—
Control	33.7	61.5	84.5	118	140
+CaCO ₃	51.4	75	133	162	285
Control	34.2	—	—	—	115
+CaCO ₃	47.0	—	—	—	161
+CaCO ₃	42.5	—	—	—	135.5

(added after steaming)

with phosphate, the solution has a pH of about 2 at this time. When blood is present the final pH is between 4 and 5. Blood therefore buffers the medium and allows the fungus to continue to grow for longer than it would otherwise do. This is undoubtedly one factor that contributes to the adjuvant effect. It can be tested by adding excess $CaCO_3$ (about 200 mg.). This does not alter the original pH of the medium; free acids produced by the fungus are neutralized by the $CaCO_3$ and the pH of the medium when the fungus has ceased to grow is still 6.5. When this is done it is found that a greatly increased weight of mycelium is produced (Table X c). The technique can be applied to blood, although the original pH must be carefully controlled, particularly if excess oxalate was used as an anticoagulant. As a result of a number of experiments it has been found that the addition of $CaCO_3$ usually, but not always, produces an increase (averaging 16 %) in the growth produced by blood; but it invariably produces an increase (36 % on the average) without blood, showing that the buffering of blood is responsible for part of the adjuvant action (see Table X). This is very important, because Meiklejohn believed that over the steep part of the growth/vitamin curve (i.e. with concentrations of vitamin below about $0.3\gamma/10$ ml.) "the limit to growth is determined solely by the amount of vitamin present"; the results summarized above show that his conclusion is not justified. It is interesting that pyruvic acid (isolated as the 2:4 dinitrophenylhydrazone) accumulates in large amounts in the flasks containing small amounts of vitamin (e.g. 0.1γ), but there is practically none in the flasks containing excess vitamin. This indicates that the vitamin may be concerned with the removal of pyruvate as much in the fungus as it is in the animal body.

Fig. 1.

The growth produced in presence of blood differs in two further respects from the controls. First, the fungus in the flasks containing blood starts to grow more quickly than that in the control flasks containing approximately the same concentration of vitamin; the difference is particularly marked if vitamin be added to the blood (Fig. 1). Secondly, the ratio wt. of aerial hyphae/total wt. of fungus is much less in the flasks containing blood than in the control flasks containing approximately the same concentration of vitamin; and when excess vitamin is added to blood a very pale growth is produced which contrasts strongly

with the green hyphae in the control flasks with excess vitamin. These facts show that blood contains substances other than vitamin B₁ that affect the growth of the fungus.

There is one further piece of evidence that proves the existence of an adjuvant factor in blood. Meiklejohn stated that "some loss of growth-promoting activity has been found if the temperature has been raised above 110° or the time, at 107°, increased to more than 15 min." This presumably is meant to apply only in cases in which blood has been added, since the flasks without blood can stand autoclaving at higher temperatures for longer times without loss of activity. Vitamin B₁ is fairly stable at pH 6.5, and even if autoclaved for 10 min. at pH 8—which will undoubtedly cause some destruction—there is no loss in activity as Table III in Meiklejohn's paper shows. Schopfer & Jung [1937, 1] and Sinclair [1937] have indicated the reason for this, namely, that the vitamin is probably broken down to its constituent parts, and these can be utilized by the fungus. Schopfer & Müller [1938] have in consequence stated that it is necessary, when assaying vitamin B₁ with *Phycomyces*, first to adsorb the vitamin on fuller's earth and then to determine the vitamin in eluates made from this adsorbate. The loss of activity in the presence of blood which occurs on autoclaving at 107° (pH 6.5) for longer than 15 min. may be due to destruction of the vitamin but is more probably due to destruction of an adjuvant factor present in blood.

The results recorded in Table XI were obtained with human blood; the flasks were filled with medium and vitamin (where required), sterilized by steaming for 20 min. on three successive days and then filled under sterile conditions with the

Table XI. *The effect of heat upon the ability of blood to promote the growth of Phycomyces*

Addition	Temperature (°)							
	50	60	80	100	104.5	111.5	115.5	130.5
0.1 γ vit. B ₁	—	—	—	15.3	15.0	15.5	15.2	12.3
2.5 γ vit. B ₁	—	—	—	114.5	113.5	114.2	114.2	99.6
1 ml. blood	10.2	29.8	27.4	18.6	16.4	16.6	15.3	13.0
+ 0.1 γ vit. B ₁	77.6	65.0	52.5	44.6	46.4	35.3	30.9	19.7
+ 2.5 γ vit. B ₁	236.0	235.5	165.1	129.1	127.2	123.4	121.8	114.8
3 ml. blood	23.4	55.5	85.9	69.5	68.2	58.7	54.0	26.5
+ 0.1 γ vit. B ₁	77.8	103.0	109.8	90.8	85.6	81.7	78.2	47.1
+ 2.5 γ vit. B ₁	272.1	284.3	204.1	201.0	190.6	188.5	188.3	184.0

blood. The flasks were then heated at the required temperature for 10 min. I have already stated [Sinclair, 1938] that there is but little growth if the flasks containing blood are not heated because the vitamin in blood is bound; maximal growth is obtained after heating to about 60°; at higher temperatures there is a fall in growth due to destruction of adjuvant factor (shown by the fall in the flasks containing excess vitamin B₁). Since no change is produced by heating to 115° in the control flasks without blood, it is very important carefully to control the exact temperature in estimations; sterilizing at 110° for 10 min. will give a value about 15% lower than that obtained by sterilizing by steaming and 5% lower than after heating to 105°. This effect is also illustrated by Table XII.

Effect of storing blood

In any elaborate quantitative test, such as this, which is to be used for clinical purposes, it is often necessary to send samples of blood by post, or to keep samples until an experiment can be set up. (It may be mentioned that the

time consumed in the estimation on each sample of blood is about 2 hr. The test takes about a fortnight to complete.) Meiklejohn stated that oxalated blood will keep for "some hours" at room temperature without loss of vitamin; in presence of medium at -2° it would keep "for days".

A few experiments have been done to test the effect of keeping the blood. The vitamin content was found to remain the same for about 6 days at -2° . If blood is kept for more than one week at this temperature the growth-promoting power usually increases owing to increase in adjuvant factor (see Table XII); this is probably due to autolysis producing compounds with adjuvant action. In seven experiments in which the blood was kept for between 8 and 24 days at -2° , the average increase in growth compared with the same sample tested at once was 32% (16–50%). In the experiment shown in Table XII human blood was drawn under sterile conditions. Some was added at once to the flasks containing medium etc. and sterilized next day (column I). Some was kept for 12 days at -2° and then treated like the first sample (column II); it was also tested for sterility.

Table XII. *The effect of keeping blood upon its ability to promote the growth of Phycomyces*

		I	II
1 ml. blood	Unheated	—	8.5
"	Steamed	31.0	40.6
"	Autoclaved (110° for 10 min.)	27.4	36.5
1 ml. blood + 0.1 γ vitamin B ₁	Steamed	60.0	58.7
1 ml. blood + 2.5 γ vitamin B ₁	"	106.0	160.0
2 ml. blood	"	53.4	62.4
3 ml. blood	"	63.4	78.3

Specificity of the test

It has already been stated that the method is now known not to be specific for vitamin B₁. In his paper Meiklejohn reported tests for four substances possessing growth-promoting activity for other organisms with negative results. Two of his observations I have been unable to confirm.

(1) Meiklejohn stated that a specimen of bios supplied by Mr J. R. O'Brien was without effect. I obtained specimens of bios from the same source and found that they had a remarkable effect (see Table XIII).

Table XIII

	mg.
Bios (0.1 ml.)	170
Bios (0.1 ml.) + 0.1 γ vitamin B ₁	164
4.0 γ vitamin B ₁	104

It will be seen that bios (1 ml. = 21 mg. solids = 500 "doses") had a very much greater effect than excess vitamin B₁.

(2) Mannitol was stated by Meiklejohn to have no effect on the growth of the fungus. This confirmed the previous report by Schopfer [1934, 3].

(3) Knight's staphylococcal factor was stated to have only negligible activity in amounts of 100 γ . Since Knight [1937] has shown that his factor consists partly of the breakdown products of vitamin B₁, and since three groups of workers [Schopfer & Jung, 1937, 1; Sinclair, 1937; Robbins & Kavanagh, 1937] have all found that these products are active in very small amounts, Meiklejohn's results are difficult to interpret. The remainder of Knight's factor is nicotinic acid or its amide; neither of these had any action on the growth of the

fungus. Coenzymes I and II, pure riboflavin and β -alanine were also inactive. A highly purified preparation of vitamin B₆ had no activity as a growth factor in the absence of vitamin B₁, but when added with the latter it doubled the growth.

In addition to bios, autoclaved "marmite", autolysed yeast and "Peters's eluate" all produced growths much greater than those obtained with excess vitamin B₁, even after they had been autoclaved at pH 9 to destroy this vitamin. I have already stated [Sinclair, 1937] that cocarboxylase is about as active as vitamin B₁, and under the conditions of the test the same is true of vitamin B₁ monophosphate. These results will be reported in detail later. It may be mentioned that several substances have been found that inhibit the growth of the fungus in small concentrations. For instance, 1 mg. indole in 10 ml. produces complete inhibition of growth.

Availability of vitamin in blood

In Meiklejohn's method, the corpuscles are allowed to settle into a thick mass at the bottom of the flask. Since he states that over 80% of the vitamin in blood is associated with the corpuscles it is important to ensure that it all becomes available for the fungus. Meiklejohn advances three lines of evidence in support of this. The first is not satisfactory because, as shown above, the value obtained with 3 ml. blood is usually more than three times that obtained with 1 ml. and therefore the smaller relative growth which would be expected in the larger sample if some of the vitamin were not available would be masked by the adjuvant action. The second is not fully convincing: in one experiment only, the corpuscular layer was apparently autoclaved twice, and this, Meiklejohn states, caused some loss of activity; in any case, the difference in the growths given by the surface layer (27.5 mg.) and whole blood (33.0 mg.) was not large. The third statement—that the corpuscular layer at the end of an experiment possesses no growth-promoting activity—is not surprising, since the fungus is known to produce an inhibitory substance during growth which would presumably be present in the corpuscular layer.

The availability of the vitamin in the corpuscular layer can be simply tested by comparing laked blood with blood in which the corpuscles have been allowed to settle. The results of 24 experiments show that there is no significant difference between the two methods and therefore Meiklejohn's method of allowing the corpuscles to settle out is fully justified.

Since in the control flasks the medium is liquid and in the flasks to which the larger samples of blood have been added it is partly solid, the effect of the fluidity

Table XIV. *The effect of the fluidity of the medium on the growth of Phycomyces*

A. Ox blood				
Vitamin B ₁ (γ)				1 ml. blood
	0.1	2.5		
Control	30.7	117.5		20.7
With agar	43.2	142.5		31.3
B. Human blood				
	1	2	3	4 ml. blood
Control	27.4	63.7	96.7	110.0
With agar	39.4	67.0	87.5	114.1

of the medium was tested by adding agar (50 mg.) to some flasks. The addition caused a slight increase in growth (Table XIV) although agar without added vitamin or blood produced no growth. It appears therefore that the fungus grows rather better on a solid medium, and part of the adjuvant action of the blood may be due to this fact.

DISCUSSION

Williams, who originally [1919] suggested the use of yeast for the assay of vitamin B, has recently [1937] stated that "the use of fungi in quantitative testing for vitamin B₁ in extracts appears hazardous in the extreme". Orr-Ewing & Reader [1928] elaborated a quantitative test for vitamin B₁, using its growth-promoting activity with *Streptothrix corallinus*; the test was useful with fairly pure solutions of the vitamin, but interfering substances were present in cruder preparations. Heating in alkaline solution destroyed the vitamin, but some growth-promoting activity with the *Streptothrix* still remained [Peters *et al.* 1928]. Peters [1930] made the excellent suggestion that the micro-organism required the vitamin and could reactivate "alkalized" torulin. In these respects this micro-organism behaves like *Phycomyces*. In his earlier papers Schopfer realized that the fungus could only be used for the assay of fairly pure solutions of the vitamin. In his recent work he has become bolder and used the method with plant extracts, animal tissues, milk and blood; it has already been shown that his results on blood are not satisfactory. The fungus gives an appreciable growth with only 10⁻⁷% vitamin B₁, and there is no doubt that Schopfer's method is the most sensitive one for assaying pure solutions of the vitamin. For this purpose it has proved very successful [Faguet, 1937; Villela, 1938].

But blood contains other substances that influence the growth of the fungus; this is not surprising when it is remembered that it contains "phytotoxins" [Macht, 1936]. The method is not specific; according to Schopfer & Jung [1937, 2], *Phycomyces* "nécessité pour sa culture en milieu synthétique, la présence d'une constellation de facteurs de croissance de nature vitaminique parmi lesquels se trouve la vitamine B₁". Therefore Schopfer & Müller [1938] state that the vitamin must be adsorbed on fuller's earth before it can be assayed by this method. Moreover, it has been shown above that blood has an adjuvant action upon the growth of the fungus under the experimental conditions of the test. It was shown that addition of small amounts (about 0.1γ) of vitamin to blood usually produced a growth greater than would be expected from the amount of vitamin added, that adding excess vitamin to blood invariably produced a growth greater than the control without blood, that large samples of blood (greater than 3 ml.) usually gave a growth greater than that obtained with excess vitamin and that the adjuvant action was partly destroyed by heat and usually increased when sterile blood was kept at -2° for more than a week. The adjuvant action is probably due to more than one factor; sources of nitrogen and salts in the blood, the buffering power of the blood and the more solid medium produced when blood is added, particularly in large amounts, probably all contribute to it. Under certain conditions, blood may be shown to have an inhibitory action on the growth of the fungus. Probably the inhibitory factor is normally masked by the adjuvant factor and is only apparent when the adjuvant action of blood is diminished by such devices as improving the sources of nitrogen in the medium.

These facts prove that blood contains substances other than vitamin B₁ that influence the growth of *Phycomyces* under the conditions of the test. Meiklejohn's conclusion cannot therefore be substantiated. I agree with Van Veen [1937] that in testing impure extracts (including blood and urine) by this method "the

results were far from reliable; in fact, that they were at times entirely unserviceable". Yet this is the only method available at the present time for attempting to assay vitamin B₁ in blood. If the sources of error mentioned in this paper are borne in mind and are controlled as far as possible, then the method is valuable for comparing the apparent vitamin B₁ in different samples of blood.

SUMMARY

1. Following the work of Schopfer with the fungus *Phycomyces*, Meiklejohn described a method of estimating vitamin B₁ in blood. His conclusion that it provides a quantitative estimate of the true vitamin B₁ content of the blood has not been confirmed.

2. It is shown that under given conditions blood always contains substances that affect the growth of the fungus.

3. Hydrolysed casein is the most satisfactory source of nitrogen that has been found. By means of this it can be shown that sources of nitrogen in the blood affect the growth of the fungus even in presence of optimal concentrations of asparagine.

4. Under the conditions of the test the addition to blood of small amounts of vitamin B₁ usually produces a greater growth than expected, and addition of excess vitamin invariably does so.

5. The method is known not to be specific for vitamin B₁.

6. The importance is emphasized of carefully controlling factors such as the temperature of autoclaving and the length of time the blood is stored.

7. If the possible sources of error are borne in mind and controlled as far as possible, the method is valuable for comparing the apparent vitamin B₁ in different samples of blood.

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