CLXXIX. THE ESTIMATION OF VITAMIN B₁ IN BLOOD BY A MODIFICATION OF SCHOPFER'S TEST

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SCHOPFER [1935, 1] has described a method for estimating small amounts of vitamin B₁ by means of its growth-promoting activity on a mould, *Phycomyces Blakesleanus*.

It has been found that this method, with some modifications, can be used as a comparatively simple test for vitamin B₁ in small samples of blood.

The method as modified for this purpose is here described. In the discussion that follows the quantitative validity of this test is considered.

METHOD

The medium. A series of 50 ml. conical flasks is prepared. Into each are measured 6 ml. of a medium composed of an aqueous solution of dextrose, asparagine and salt mixture, of such strength that when the final volume of medium is made up to 10 ml. the concentrations of reagents will be

	Conc.
Dextrose 10% (A.R.)	0.556 <i>M</i>
Asparagine 2 : 1000	0.015 <i>M</i>
MgSO ₄ 0.5 : 1000	0.002 <i>M</i>
KH ₂ PO ₄ 1.5 : 1000	0.011 <i>M</i>

Preparation of the blood. Blood is collected in the presence of potassium oxalate (3 mg. of oxalate for each ml. of blood taken). Samples of blood are then carefully measured out with a pipette, and each is added to a conical flask prepared as above and containing 6 ml. of medium. At least two samples of 2 ml. each, and preferably also one of 1 ml. and one of 3 ml. should be taken from each specimen of blood to be tested. (The 1 and 3 ml. samples act as a convenient check on the results given by the 2 ml. samples.) As an additional safeguard it is advisable to prepare also two flasks containing 2 ml. of blood to which 0.1γ of vitamin B₁ is added. This is necessary if it is desired to exclude possible errors due to the presence in the blood of substances modifying the action of the vitamin. (This point is considered in the Discussion, where it is shown that no such substances usually occur.) The test can thus be performed conveniently on 15 ml. of blood.

The flasks are placed in cold store (−2°) for 12 hr. or more, until a convenient time arises for the completion of the test. The corpuscles separate out in the presence of the strong dextrose, and settle in a layer at the bottom of the flasks. The pH of the surface layer is adjusted to 6.5–6.7 by the addition of 0.1–0.2 ml. *N*/10 NaOH. Water is added to bring the final volume of fluid in the flasks to 10 ml.

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Preparation of the standard. In each experiment a standard range is set up, against which the growth obtained with the blood can be compared. A very dilute aqueous solution of pure crystalline vitamin B₁ containing 1 γ per ml. is freshly prepared before each experiment from a standard concentrated alcoholic solution preserved at pH 4.5 in a refrigerator. (Traces of alcohol in the aqueous solution do not affect the subsequent growth of *Phycomyces*.) Carefully measured volumes of the dilute vitamin solution are made up in a series of 50 ml. flasks containing 10 ml. of medium. The medium contains the same concentrations of dextrose and salts as used in the flasks containing blood, asparagine in a concentration of 4 : 1000, and 0.3 ml. N/10 NaOH per 10 ml. to bring the pH to 6.7. The usual amounts of vitamin selected for the standard range are 0, 0.05, 0.10*, 0.15, 0.20*, 0.25 and 0.30 γ *. (Those concentrations marked with a star are set up in duplicate.)

Sterilization. Both sets of flasks, those containing the blood and the standard range containing known amounts of vitamin, are placed together in an autoclave. The temperature is raised to 107° and maintained at that point for 10 min.

Inoculation. A stock culture of *P. Blakesleeanus* (Sex) is required. This should be prepared about a fortnight previously, according to the following procedure. A medium is prepared consisting of

Malt extract (B.P.)	...	...	25 ml.
Agar	...	...	4 g.
Distilled water to	...	...	200 ml.

About 70 ml. of this medium are placed in a 200 ml. conical flask and sterilized by heating in an autoclave for 15 min. at 110°. The flask is then inoculated with about 0.5 ml. of a suspension of *Phycomyces* spores. A good growth should be obtained in about 2 weeks. Several g. of spore-bearing mycelium are removed with sterile forceps and thoroughly washed in 10–20 ml. of sterile distilled water contained in a small beaker which is covered with a watch glass. The mycelium is then removed, leaving a suspension of spores in sterile water, which should contain at least 1,000,000 spores per ml. This can be checked, if necessary, with a blood-counting chamber. Each flask is then inoculated with 0.2 ml. of the spore suspension by means of a sterile pipette. The suspension should be frequently stirred during the inoculations, to ensure that the spores do not sediment.

Culture. The inoculated flasks are left for 10 days in the dark at room temperature. At the end of this time all growth has ceased. The mycelium in each flask is removed and carefully washed free from adherent medium under a running tap. This operation is facilitated by the fact that the mycelium forms a single adherent mat in the course of its growth, and small fragments of growth cannot be detached except with difficulty. The mycelium is placed on a watch-glass and dried to constant weight in an air oven at 110°. It is then weighed and the weight recorded correct to the nearest 0.5 mg.

Reading. The weights of mycelium obtained from the standard range are plotted on a graph; dry weight of mycelium obtained against amount of crystalline vitamin B₁ added. A smooth curve is drawn through the points on the graph (Fig. 1).¹ From this curve the amounts of vitamin corresponding to the weights

¹ The small growth (10 mg. or less) obtained in the absence of added vitamin is probably due to traces of vitamin in the constituents of the medium (probably the dextrose). This does not affect the validity of the test as the same amount of medium is present in all flasks.

of mycelium obtained from the blood samples are recorded. These figures are taken as representing the vitamin B₁ content of the blood.

Fig. 1. Curve showing weight of *Phycomyces* obtained with known amounts of vitamin B₁.

Discussion of method

Volume of medium. Schopfer sets up his tests in 25 ml. of medium, contained in 100 ml. flasks. It was felt that when the vitamin preparation to be tested can only be obtained in limited amounts, as in the case of blood samples from living animals, it would be more satisfactory to reduce the total bulk of medium, so that a smaller absolute amount of the preparation would be necessary to achieve the same concentration of vitamin in the medium. In this research the tests have therefore been made up in 10 ml. medium contained in 50 ml. conical flasks. This has the additional advantage of being economical in reagents, and the smaller flasks are more easy to handle in large numbers.

Composition of medium. In all experiments salt mixture and dextrose have been added in the same concentrations as used by Schopfer.

Asparagine. Schopfer [1935, 2] has shown that when *Phycomyces* is grown on a synthetic medium with asparagine as a source of nitrogen, the growth obtained with a given amount of vitamin B₁ is dependent on the concentration of asparagine present. The growth increases as the concentration of asparagine is increased up to an optimum concentration of 4:1000. (At concentrations higher than this he has found some evidence that the growth obtained is reduced in amount.) These results have been fully confirmed in these experiments. Fig. 2 shows the results of a typical experiment in which concentrations of asparagine between 1 and 6:1000 have been tested.

When the amount of vitamin present is sufficient, or more than sufficient, to produce maximum growth, it has been found that inconsistent results have been obtained with higher concentrations of asparagine; from a number of experiments it has not been possible to say whether an increase

from 4 to 6 : 1000 in the concentration of asparagine reduces or increases the growth obtained in the presence of excess of vitamin.

A similar and greater inconsistency has been found in the presence of blood to which sufficient vitamin has been added to produce a growth approaching the maximum (*v. infra*).

Fig. 2. Effect of varying concentrations of asparagine on growth of *Phycomyces*.

This observation is probably to be explained as follows. In the presence of excess of vitamin, the growth of the mycelium ultimately ceases as a result of a number of factors, of which the size of the vessel, and the accumulation of waste products of metabolism are probably the most important. The influence of these factors may be considerably affected by very slight changes in the conditions of the experiment.

This has no bearing on the main issue of this paper, since the volumes of blood chosen for test have given a growth of mycelium within the limits of the steep part of the growth/vitamin curve, where the limit to growth is determined solely by the amount of vitamin present. Over this part of the curve the results obtained have been always entirely consistent.

In setting up the standard range, with known amounts of crystalline vitamin, a concentration of 4 : 1000 of asparagine in the medium has therefore been used throughout.

In the presence of blood, however, there may be some reduction of growth if the concentration of asparagine is increased above 2 : 1000. This effect is not shown when the volume of blood is 1 ml. or less, but becomes apparent when 2 or 3 ml. samples of blood are taken. At the same time in the presence of 2 or 3 ml. blood a fall has been observed in the weight of mycelium produced if the amount of added asparagine is reduced to 1 : 1000 or less. This is shown in the following experimental results. Samples of sheep's blood were tested with different concentrations of asparagine. The dry weights of mycelium (in mg.) obtained are recorded in Table I.

The concentration of asparagine in the medium has therefore been reduced to 2 : 1000 when samples of blood are to be added.

Table I

Volume of sheep's blood ml.	Concentration of asparagine				
	0	1 : 1000	2 : 1000	4 : 1000	6 : 1000
1	—	30	29.5	29	—
2	—	44	51	—	—
2	—	60	78	—	—
2	43.5	—	50.0	45.5	—
2	—	—	51.5	42.5	39.0
3	—	71	78	—	—
3	—	77	90	79	—
3	—	—	73.5	60.5	61.0

Collecting the blood. It is most convenient to collect the blood in the presence of an anticoagulant, and afterwards to measure out samples accurately with a pipette. For this purpose potassium oxalate is used; 3 mg. for each ml. of blood. Potassium oxalate even in greater concentrations than this was found to be without effect on the growth of *Phycomyces*.

	Dry weight of mycelium (mg.)	
	Without oxalate	With 30 mg. potassium oxalate
With 0.4 crystalline vitamin B ₁	89	86
With 2 ml. sheep's blood	40	39.5

Most suitable volume of blood. It has been found that 1–3 ml. is a convenient volume for human, sheep's and pigeon's blood. A growth of mycelium is obtained which can be correctly weighed to within 1%, and is yet considerably less than the maximum growth that can be obtained under these experimental conditions.

Preparation of the blood. From the point of view of technical convenience it is most satisfactory if the test can be performed directly on whole blood, with a minimum of preliminary manipulations. This, however, has the following disadvantages.

Several ml. of whole blood, made up in Schopfer's medium, coagulate when the medium is sterilized by heat, and when the mycelium of *Phycomyces* is subsequently removed for weighing, it is found impossible completely to separate it from the coagulum.

It is necessary to ensure that the total vitamin content of both plasma and corpuscles is available for the growth of *Phycomyces*.

Tests on whole blood are not valid unless it can be shown that no interfering substances are present. This last difficulty is discussed later in the paper.

I. *Separation of mycelium.* It has been found that the difficulty of separating the mycelium from the coagulated protein can be overcome by allowing the corpuscles to separate out and then adjusting the pH of the supernatant fluid layer to 6.5–6.7. At this pH, treatment in the autoclave at 107° while effectively sterilizing the medium, produces only a partial coagulation of the plasma proteins, from which the mycelium is easily separated.

Attempts at extracting the vitamin from blood by means of alcohol, and removing the interfering protein by heating in acid solution, have both been found to result in a considerable loss of growth-promoting activity.

II. *Availability of vitamin in plasma and corpuscles.* This requires further discussion.

It has been found that whole blood has a greater growth-promoting activity for *Phycomyces* than has separated plasma.

In a number of cases the vitamin B₁ contents of whole blood and of plasma obtained from the same source have been determined separately (*v.* Table II).

Table II. *Apparent vitamin B₁ contents of samples (in γ)*

	2 ml. blood	1 ml. blood	Vitamin per ml. of blood	2 ml. plasma	1 ml. plasma	Vitamin per 0.5 ml. plasma	Approximate % of total blood vitamin B ₁ carried in plasma*
Human	0.25	—	0.125	0.094	—	0.023	19
Human	0.185	—	0.092	—	0.03	0.015	16
Human	0.14	—	0.070	—	0.025	0.012	17
Sheep	0.115	—	0.058	0.075	—	0.019	33
Pigeon	—	0.40	0.40	0.44	—	0.11	27
Pigeon	—	0.21	0.21	—	0.13	0.07	33

* Assuming that plasma accounts for approximately 50 % of the total blood volume.

It will be seen that less than 20 % of the total vitamin B₁ content of human blood is present in the plasma. The remainder must either be contained in the corpuscles or adsorbed on their surfaces.

In estimating the vitamin B₁ content of whole blood it is essential to ensure that vitamin contained in the corpuscles is available for the growth of the *Phycomyces*.

In the method here described the corpuscles have been allowed to separate out slowly in the presence of 6 ml. of medium containing 17 % dextrose. (In this way, as previously explained, the difficulty of separating the mycelium from the coagulated corpuscles is avoided.) It is believed that this procedure results in an adequate extraction of the vitamin from the corpuscles, so that in practice all the vitamin in the blood is available for the growth of the *Phycomyces*. The evidence for this is as follows.

I. If any significant quantity of vitamin were retained in the corpuscles it would be expected that this retention would be most marked when the number of corpuscles present is large, and the efficiency of extraction therefore reduced. In fact, however, it has been found that approximately the same result is obtained for the vitamin content per ml. of blood, whether the estimation is performed on 1 or 3 ml. of blood. (For results *v. infra*.) Under the conditions employed, therefore, the vitamin in the corpuscles must be freely available whether 3 or 1 ml. of blood are employed.

II. In one experiment a culture flask containing 2 ml. of sheep's blood was prepared for inoculation. The fluid surface layer was then separated from the coagulated corpuscles, and the growth-promoting activity of each was tested separately. The corpuscular layer was thoroughly ground up in the usual volume of fresh medium before inoculation:

Growth given by surface layer	...	...	...	27.5 mg. of mycelium.
Growth given by corpuscles	...	...	...	5.0 mg. ,,
Growth given by a separate sample of 2 ml. of whole blood prepared without additional manipulations				33.0 mg. ,,

This demonstrates that the corpuscular layer retains little of the vitamin, and the little it does retain evidently diffuses out into the upper fluid layer during the course of the experiment and becomes available for the growth of the *Phycomyces*.

III. The corpuscular layer, at the end of an experiment, was found to possess no growth-promoting activity, when ground up in fresh medium and re-inoculated.

It is concluded, therefore, that by this method the total vitamin B₁ content of both plasma and corpuscles is measured, without any significant loss of the vitamin contained in the corpuscles.

Storage. Oxalated blood will keep for some hours at room temperature without loss of vitamin content. 1–3 ml. measured out into 6 ml. synthetic medium in conical flasks will keep in cold store (-2°) for days without significant loss of vitamin.

	Time kept in cold store days	Dry wt. of mycelium mg.
1 ml. sheep's blood	5	30
	15	29
3 ml. sheep's blood	5	78
	15	75

pH. The growth of *Phycomyces* is slightly influenced by the initial *pH* of the medium, as shown by the following results:

Table III

Initial <i>pH</i> of medium	Wt. of mycelium in mg.
(a) With 0.2γ vitamin:	
5.0	72
6.0	74.5
6.5	79
7.0	79.5
7.5	79
8.0	80
(b) With 1 ml. pigeon's blood:	
6.5	81
6.8	86.5
7.0	82.5

In this series of experiments the initial *pH* of the culture flasks has been adjusted in all cases to 6.5–6.7. A *pH* in this region has been found to be most satisfactory for the following reasons:

(a) It is sufficiently close to the optimum to give a growth that will not differ appreciably from the maximum.

(b) It is not sufficiently alkaline to give rise to a risk of any destruction of vitamin in the autoclave.

(c) It corresponds to the most acid reaction at which, when blood is present, a sufficiently fluid medium is obtained to ensure that the mycelium can be freely separated from the medium (see above).

Phycomyces produces acids in the course of its growth. In the presence of 0.2γ of pure vitamin, the final *pH* of the medium is about 3.5. When blood is present the final *pH* is 4–5.

Sterilization. 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.

Number of spores. In all cases not less than 200,000 spores have been added to each flask in the inoculum. On several occasions the sufficiency of the inoculum has been tested by inoculating some flasks with half the usual amount. This did not result in any diminution in the amount of mycelium obtained.

Conditions of growth. As Schopfer [1935, 2] has shown, the growth in the presence of very small amounts of vitamin ceases in a few days, but when the amount of vitamin present is sufficient to produce maximum growth, the growth continues for about 10 days.

In this series of experiments 10 days have been allowed for the growth of the organism, to ensure that in all cases the mycelium is not weighed until all growth has ceased. It is thought probable that this time may be unnecessarily long. The growth on 2 ml. of blood is very slow in the last 3 days, and it is probable that a sufficiently accurate estimation might be obtained by stopping the experiment in 7 days or less. In this way a more speedy result might be obtained, which would be an advantage in clinical cases. This point still requires investigation.

Accuracy. In preparing the standard range with known amounts of vitamin, the use of ten flasks in each experiment makes it possible to draw a curve relating weight of mycelium to vitamin present with considerable accuracy. The differences between duplicate figures for the growth obtained with a given dose of vitamin have in most cases been less than 3%. The error in reading off the "vitamin equivalent" of a given weight of mycelium from these experimental curves is not greater than $\pm 0.005\gamma$ (between the limits of 0 and 0.3γ) in the majority of cases.

The difference between duplicate estimations of the weight of mycelium produced by a given sample of blood is in most cases less than 5%, and the average may therefore be taken as being correct to within $\pm 3\%$, which in the case of 2 ml. samples of blood, is equivalent to a maximum error of $\pm 0.01\gamma$ in terms of "vitamin equivalent". In a typical experiment the maximum possible error in reading the vitamin content of a 2 ml. sample of blood is therefore $\pm 0.015\gamma$, or less than $\pm 10\%$. In many experiments the maximum possible error, as judged by the difference between duplicate estimations with standard and blood samples, has been considerably less, and it might be still further reduced if all estimations were performed in triplicate.

With one assumption, therefore, that is that the growth of mycelium obtained with a given sample of blood represents the true maximum growth that the vitamin present in the blood can give, it is possible to state that the vitamin B₁ content of a given 15 ml. sample of blood can be correctly estimated to within 10% or less by this method. The justification for provisionally accepting this assumption is given in the final section of this paper.

Specificity of the test. It is believed that the growth-promoting activity for *Phycomyces* possessed by blood is due to its vitamin B₁ content. The evidence for this is as follows:

I. The growth-promoting activity is reduced in the blood of pigeons fed on a vitamin B₁-deficient diet. This has been shown in a series of experiments, in

Table IV. *Apparent vitamin B₁ per ml. blood (in γ)*

Normal birds	Vitamin-deficient birds
0.27	0.055
0.21	0.065
0.375	0.055
0.225	0.065
0.28	0.055
0.39	0.050
0.31	0.065
—	0.040
—	0.050
Averages 0.295	0.055

which the full technique described above has been used to compare the apparent vitamin B₁ contents of blood from normal pigeons and from pigeons suffering from opisthotonus—resulting from a dietary deficiency of vitamin B₁.

It will be seen that in pigeons suffering from a dietary deficiency of vitamin B₁, the growth-promoting activity of the blood is reduced to 20% of the normal value.

II. It has been found that the growth-promoting factor of blood is completely destroyed by heat at pH 9.

2 ml. blood in 6 ml. medium were brought to pH 9 with *N* NaOH and autoclaved for 1 h. at 125°. The pH fell to 5 in the process. The sample was then set up for culture in the usual manner. No growth of *Phycomyces* was obtained.

The growth-promoting factor therefore resembles the vitamin in being thermolabile in alkaline solution.

III. Substances with growth-promoting activity for other organisms have been tested during the course of this research, and found to be without influence on *Phycomyces*.

(a) A specimen of bios possessing powerful growth-promoting activity for yeast was kindly supplied by Mr J. R. O'Brien. 100γ of this preparation had no effect, either by itself or on the growth obtained with a small dose of vitamin B₁.

(b) 5% mannitol, which acts as an adjuvant to the activity of vitamin B₁ on *Streptothrix* [Reader, 1929], has no action on *Phycomyces*, either by itself or in the presence of crystalline vitamin B₁.

(c) A preparation kindly supplied by Dr B. C. J. G. Knight with growth-promoting properties for staphylococci, had only negligible activity for *Phycomyces*. 100γ gave a growth corresponding to 0.16γ vitamin B₁.

(d) Two specimens of thiochrome gave no growth in doses of 0.2γ.

Possible modification of the activity of the vitamin by other substances present in blood. It is thought that no such modification usually occurs. The evidence for this view is as follows.

I. The addition of known amounts of the vitamin to blood which has been freed of vitamin by heating at pH 9, gives a growth/vitamin curve which follows closely the curve obtained in the absence of added blood. This is illustrated by the following experimental results:

Vitamin B ₁ added in γ	Weight of mycelium produced in mg.	
	Without other additions	With 2 ml. autoclaved sheep's blood
0.1	42	44
0.2	65	69
0.3	80	87
0.4	—	93
0.5	111	112

It is concluded that if any substance exists in blood which can modify the action of the vitamin, it is also destroyed by autoclaving at pH 9 (125°).

II. 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.

2 ml. samples of blood have been tested, with and without the addition of 0.1 γ of vitamin:

Sample	Vitamin content of 2 ml. of sheep's blood as estimated	Vitamin content of 2 ml. of sheep's blood with 0.1 γ vitamin B ₁ added, as estimated
I	0.185	0.280
II	0.187	0.277
III	0.130	0.230

When large amounts (e.g. 0.4 γ) of vitamin have been added the effect has been less satisfactory. As previously noted, however, anomalous results are frequently obtained when the amount of vitamin present is sufficient to produce nearly maximum growth.

In one group of cases the effect of added vitamin has produced a greater growth than the same quantity of vitamin in the absence of blood. This has occurred when 0.1 γ of vitamin has been added to 1 ml. of blood from pigeons fed on a vitamin B₁-deficient diet. The vitamin equivalent of the growth of mycelium obtained is increased by the addition of 0.1 γ from 0.05 to 0.25 γ . The blood from avitaminous pigeons has therefore an adjuvant action on added vitamin. The significance of this is not yet clear. It shows at least that the reduction of growth on vitamin B₁-deficient blood is not due to any inhibitory substance in the blood.

III. With separate samples of 1, 2 and 3 ml. of blood, the growths of *Phycomyces* obtained correspond with the growth given by $x\gamma$, $2x\gamma$ and $3x\gamma$ of vitamin. This is shown in the following results:

Volume of human blood tested ml.	Vitamin B ₁ content as calculated per ml. blood					
	Case					
	A	B	C	D	E	F
1	0.085	0.065	0.105	—	0.088	—
2	0.082	0.070	0.107	0.128	0.085	0.098
3	0.088	0.073	0.103	0.140	—	0.105

(When this result has not been obtained, in the course of routine tests, the figures obtained have been discarded.)

If any factor were present in blood which possessed any adjuvant or depressant action on the vitamin, it would be unlikely that it would cause the same percentage increase or decrease at three different concentrations of blood. It is concluded that the presence of blood does not usually alter the activity of the vitamin. But since it has been found to do so in the case of blood from vitamin B₁-deficient pigeons, it is important to be aware of this possibility, and if necessary to control it by including in the test a sample of blood to which a small amount of the vitamin has been added.

It is concluded from the evidence presented that the apparent vitamin B₁ content of blood samples as estimated by this method is satisfactory for comparative purposes and that the method also provides a quantitative estimate of the true vitamin B₁ content of the blood.

SUMMARY

1. A method is described for the estimation of vitamin B₁ in small samples of blood.

2. The technique, and the quantitative validity of the method are discussed.

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