The heats of formation calculated from the results of this work are given in Table 4, together with those for glucose and glycerol for comparison.

SUMMARY

1. The heats of combustion of dipotassium glucose 1-phosphate, disodium glycerol 1-phosphate and disodium glycerol 2-phosphate have been determined in a bomb calorimeter.

2. Possible errors in the determinations are discussed and the stoichiometry of the combustion reactions are established.

3. The heats of formation of the compounds examined have been calculated.

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Some Observations on the Measurement of 'Binding' of Cyanocobalamin by 'Intrinsic Factor' Preparations

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In certain body tissues and fluids, particularly gastric juice and serum, there are present substances having the property of combining with cyanocobalamin so that its activity for micro-organisms is altered and on dialysis it does not pass through a cellophan membrane. The ability of gastric juice to combine with added cyanocobalamin is closely associated with its 'intrinsic factor' activity, but there have been conflicting reports on the identity of 'binding' activity with intrinsic-factor activity. To some extent this confusion has come about because the different groups of workers have used different methods for measuring binding activities.

For example, Bird & Hoevet (1951) measured the binding activity of an intrinsic-factor concentrate by three methods and obtained three different results. The first method was that of Ternberg & Eakin (1949), in which graded doses of the concentrate were added aseptically to a series of autoclaved tubes, each containing assay medium plus 0.5 μ mg. of cyanocobalamin. The minimum amount of protein required to inhibit the growth of the test organism (*Lactobacillus leichmannii*) was taken as

the amount which bound 0.5 μ mg. of cyanocobalamin. In the second method, a constant amount of intrinsic-factor concentrate was added aseptically to increasing amounts of cyanocobalamin. The binding activity was calculated from the lowest level of cyanocobalamin at which growth occurred. This level was three times higher than that found by the first inhibition method. The apparent binding activity was increased a further tenfold if the method of measurement was based on the non-dialysability of the bound vitamin. Bird & Hoevet suggest that the last method is the more reliable and that in the growth-inhibition test *L. leichmannii* is able to utilize all but a small part of the bound vitamin.

Rosenthal & Sarett (1952) also used a dialysis method and *L. leichmannii* to measure the cyanocobalamin-binding activity of normal human serum. Their results were considerably higher than those obtained for normal serum by Pitney, Beard & Van Loon (1954), who measured the activity by the *Euglena gracilis* tube assay method of Ross (1952). This method depends on the assumption that bound cyanocobalamin is unavailable to *E. gracilis* unless

it is heated with the assay medium at 100° for 30 min. Pitney *et al.* (1954) added increasing amounts of cyanocobalamin (from 50 to 1000 $\mu\text{g.}$) to 1 ml. portions of normal serum, then assayed the mixture for free and bound cyanocobalamin. The binding capacity appeared to increase with increasing amounts of added vitamin. Raine (1955) used a similar method to measure the binding activity of an intrinsic factor preparation and found a similar non-stoichiometric relationship. She added an excess of cyanocobalamin to the intrinsic-factor preparation and measured the free vitamin by the *Escherichia coli* plate-assay technique. This method depends on the assumption that bound cyanocobalamin is inactive for *Esch. coli* in plate assays.

A wild type of *Esch. coli*, which can absorb large amounts of cyanocobalamin, was used by Hoff-Jorgensen (1952) for measuring the free and bound vitamin. The cyanocobalamin absorbed by the *Esch. coli* cells was measured by boiling the cells and determining the released vitamin with *L. leichmannii*. In the presence of intrinsic-factor preparations, the cells were unable to absorb the vitamin from the solution. Chow & Davis (1952) employed a similar method, except that they used resting *L. leichmannii* cells to absorb ^{60}Co -labelled cyanocobalamin. Thus they could estimate the bound vitamin directly by measuring the radioactivity of the cells, and no problem of releasing the cyanocobalamin for subsequent assay was involved. Using this technique they found that heparin and yeast nucleic acid prevented the uptake of vitamin B_{12} by the *L. leichmannii* cells, and they concluded that these substances combined with the vitamin.

In our work on the bound form of cyanocobalamin in milk and other tissues, we have used an ultrafiltration technique for measuring cyanocobalamin-binding activity (Gregory & Holdsworth, 1955). The purpose of this paper is to present some of our observations on the different methods of measuring binding activity, as they may be of some assistance to others in interpreting results obtained in different laboratories.

EXPERIMENTAL

Intrinsic-factor concentrate. This was a commercial preparation, Bendogen (G.E.A. Copenhagen), made from pig pyloric mucosa.

Sow's milk whey concentrate. The material, SF8, was that used in our previous experiments on binding activity (Gregory & Holdsworth, 1955).

Radioactive cyanocobalamin. Material with activity 100 $\mu\text{C}/\text{mg.}$ was kindly provided by Glaxo Laboratories Ltd., Greenford, Middlesex.

Tube assays. The *Lactobacillus leichmannii* ATCC 4797 tube-assay method was that of Skeggs, Nepple, Valentik, Huff & Wright (1950), modified as described by Gregory (1954).

Plate assays. For the *Escherichia coli* 113-3 plate assays we used the medium of Burkholder (1951) with 2% agar added, but substituting thiomalic acid for sodium thioglycollate. The *Esch. coli* 113-3 was obtained from Glaxo Laboratories, Ltd. Large plates (22 cm. $\times$ 37 cm.) were used. 2:3:5-Triphenyltetrazolium chloride (200 mg.) was added to the medium (300 ml.) before pouring, so that the growth zones would show up red on a colourless background (Ford & Holdsworth, 1953). Unless otherwise stated, 0.4 ml. of a 5 hr. culture of *Esch. coli* (approx. 15×10^9 organisms) was used to inoculate the plate. The test solutions were applied to the plate by the pad method of Jukes & Williams (1954), 0.01 ml. of cyanocobalamin solution or test preparation being applied to a 6 mm. disk of no. 4 Whatman filter paper. When Whatman A.A. disks (diam. 10 mm.) were used the volume added to the disk was increased to 0.1 ml. The plates were incubated for 18 hr. at 28° and the diameters of the red growth zones were measured.

Elution of the dyestuff from the growth zones. In addition to measurement of growth by the diameter of the zones, the amount of red formazan pigment produced by the micro-organism from the triphenyltetrazolium chloride was also estimated. The zones were cut out of the agar with a cork borer and the agar disk was placed in a stoppered tube together with sufficient tetrahydrofuran to make the volume to 10 ml. The eluted colour was measured in a Unicam SP. 600 spectrophotometer.

Measurement of binding activity of Bendogen by the plate-assay method. A solution of Bendogen in water was made up at a concentration of 100 mg./ml. Increasing amounts of cyanocobalamin were added to 1 ml. portions of the solution so that in some of the solutions excess of the binding factor and in others excess of cyanocobalamin was present. These solutions were diluted to concentrations of approx. 1.0, 0.1 and 0.01 $\mu\text{g.}$ of cyanocobalamin/ml. and assayed by the pad-plate method. In other experiments to measure binding activity we used the conditions and method of Raine (1955).

Effect of size of inoculum and of paper pads on the growth activity of bound cyanocobalamin in plate assays. A large plate was inoculated with 0.4 ml. of a 5 hr. culture of *Esch. coli* (approx. 15×10^9 organisms) and a second plate was inoculated with one-eighth of this amount. The cyanocobalamin standard solution at 1.0, 0.1, 0.01 $\mu\text{g.}/\text{ml.}$ and the test solutions were applied to both small (6 mm. diam.) and large (10 mm. diam.) pads, 0.01 ml. of solution being used for small pads and 0.1 ml. (of ten-times diluted solution) for the larger pads, so that the same amount of cyanocobalamin was present.

Both plates were divided into quarters and the positions of the large and small pads were arranged at random in opposite quarters, each standard or test solution being assayed in duplicate on both sizes of pad.

Measurement of binding activity by growth-inhibition method. The method was that described by Gregory & Holdsworth (1953).

Measurement of binding activity by ultrafiltration. The apparatus and methods were those previously described (Gregory & Holdsworth, 1955). All solutions contained NaCl at a final concentration of 0.9% (w/v). The first 0.3 ml. (approx.) of ultrafiltrate was rejected, the cellophane wiped with filter paper, then the next 1 ml. of ultrafiltrate was collected for assay by *L. leichmannii* or for estimation of radioactivity.

Use of ^{60}Co -labelled cyanocobalamin for measuring binding activity. An extension of the ultrafiltration method was the use of ^{60}Co -labelled cyanocobalamin so that the concentration of the vitamin in the ultrafiltrate could be determined by physical methods. The conditions of the experiment depended on the specific activity of the cyanocobalamin and the sensitivity of the counting equipment. The samples of [^{60}Co]cyanocobalamin used in our experiments had specific activities from 20 to 100 $\mu\text{C}/\text{mg}$. The 10 ml. of liquid used for ultrafiltration contained 2–4 μg . of cyanocobalamin and sufficient binding protein to combine with approx. half the vitamin (e.g. 10 mg. of Bendogen). When 1 ml. of ultrafiltrate had collected, it was diluted to 10 ml. and the radioactivity determined in a liquid counter type M6 (20th Century Electronics, New Addington, Surrey).

To test the effect of concentration on the determination of binding activity, experiments were made with cyanocobalamin of the lower specific activity (20 $\mu\text{C}/\text{mg}$.), 40 μg . being added to 100 mg. of Bendogen in 10 ml. of liquid for ultrafiltration.

Stability of the cyanocobalamin-protein complex to dialysis and to exchange of the cyanocobalamin. Bendogen (100 mg.) and [^{60}Co]cyanocobalamin (31 μg .), specific activity 92 $\mu\text{C}/\text{mg}$., were dissolved in 5 ml. of distilled water. The free vitamin was estimated by ultrafiltration, 0.5 ml. being collected. The material remaining in the cellophan tubing was dialysed against running tap water for 48 hr., then against four 500 ml. portions of distilled water. The final 500 ml. were freeze-dried and examined for radioactivity. A portion of the dialysed material was diluted and the amount of bound cyanocobalamin estimated by determining the radioactivity. The binding activity obtained by the dialysis method was compared with that given by the ultrafiltration technique.

The bound radioactive cyanocobalamin was diluted to 2 μg . of cyanocobalamin/ml. and 1 ml. portions were incubated for 4, 24 or 96 hr. at 30° with either 4 ml. of distilled water or 4 ml. of solutions containing 10 or 100 μg . of unlabelled cyanocobalamin. The amount of [^{60}Co]cyanocobalamin that had exchanged with unlabelled vitamin was estimated by ultrafiltering a portion of the liquid and measuring the radioactivity in the ultrafiltrate.

RESULTS

Plate-assay method for measuring binding activity

The results of an attempt to measure the binding activity of Bendogen by the plate-assay method are illustrated in Table 1. With 28 μg . of cyanocobalamin added to 100 mg. of Bendogen, the amount of the vitamin available to *Esch. coli* increased from 32 % of the total present when the test solution contained 1.4 μg . of bound cyanocobalamin/ml. to 100 % when the test solution was diluted a further hundredfold to contain 0.014 $\mu\text{g}/\text{ml}$. That this was not an effect of dissociation of the vitamin-protein complex was shown by ultrafiltering the most dilute solution and measuring any free vitamin in the ultrafiltrate. By neither radioactivity nor microbiological techniques could we detect in the ultrafiltrate more than 1 % of the total vitamin present in the original solution.

It can also be seen from Table 1 that as the ratio of binding factor to added cyanocobalamin increased the microbiological availability of the vitamin decreased, but with each level of added vitamin the microbiological activity increased with increasing dilution of the test solution. The binding activity of the Bendogen calculated from the different assay levels is shown in the last column of the Table. The activity differed according to the dilution at which the Bendogen solution was assayed and also according to the ratio of binding factor to added cyanocobalamin.

Effect of increasing both the inoculum and diameter of the assay pads on the activity of bound cyanocobalamin in plate assays

The availability of the bound vitamin to *Esch. coli* on the different sizes of pad and with two levels of inoculum was calculated from the diameters of

Table 1. *Effect of varying the ratio of binding factor (Bendogen) to added vitamin B₁₂ on the measurement of 'binding activity' by an Escherichia coli plate assay*

	Assay level (μg . of bound vitamin B ₁₂ /ml.)	Vitamin B ₁₂ measured		Vitamin B ₁₂ - binding activity ($\mu\text{g}/\text{mg}$. of Bendogen)
		Amount detected ($\mu\text{g}/100$ mg. of Bendogen)	Percentage of that present	
100 mg. of Bendogen + 28 μg . of vitamin B ₁₂	1.4	9.0	32	0.19
	0.14	16.0	57	0.12
	0.014	28.0	100	0
100 mg. of Bendogen + 20 μg . of vitamin B ₁₂	1.0	0	0	>0.20
	0.125	1.3	6	0.19
	0.0125	10.2	51	0.10
100 mg. of Bendogen + 14 μg . of vitamin B ₁₂	0.7	0	0	>0.14
	0.07	4.4	31	0.10
	0.007	7.0	50	0.07
100 mg. of Bendogen + 10 μg . of vitamin B ₁₂	0.125	0	0	>0.10
	0.0125	0	0	>0.10
	0.00625	3.0	30	0.07

Table 2. *Effect of size of inoculum and size of the assay pad on the availability of bound cyanocobalamin to Escherichia coli in plate assays*

The availability of bound cyanocobalamin is expressed as a percentage of the total cyanocobalamin present.

		Availability of bound cyanocobalamin							
		Normal inoculum (15×10^8 organisms/plate)				Dilute inoculum (2×10^8 organisms/plate)			
		6 mm. Pad		10 mm. Pad		6 mm. Pad		10 mm. Pad	
Assay level ($\mu\text{g. of bound}$ cyanocobalamin/ ml.)		By zone diam. (%)	By colour (%)	By zone diam. (%)	By colour (%)	By zone diam. (%)	By colour (%)	By zone diam. (%)	By colour (%)
100 mg. of Bendogen + 28 $\mu\text{g. of cyanocobalamin}$	1.4	6	4	18	20	4	2	20	8
	0.14	34	21	52	48	28	27	57	53
	0.014	67	55	72	130	44	71	86	100
100 mg. of Bendogen + 20 $\mu\text{g. of cyanocobalamin}$	1.0	3	2	20	31	2	3	19	10
	0.1	29	24	46	55	30	21	58	50
	0.01	70	52	47	110	25	95	56	104
100 mg. of Bendogen + 14 $\mu\text{g. of cyanocobalamin}$	0.7	0	0	1	6	0	0	4	1
	0.07	22	24	22	43	19	15	43	13
	0.007	52	61	—	108	26	77	43	128

Table 3. *Cyanocobalamin-binding activity of Bendogen, measured by the ultrafiltration method*

A different sample of Bendogen from that quoted in Table 4 was used. Test organism, *Lactobacillus leichmannii*.

	$\mu\text{g. of cyanocobalamin}$ bound/mg. of Bendogen		
	Assay 1	Assay 2	Assay 3
2 mg. of Bendogen + 0.6 $\mu\text{g. of cyanocobalamin}$	0.292	0.283	0.292
2 mg. of Bendogen + 0.7 $\mu\text{g. of cyanocobalamin}$	0.299	0.289	0.297
2 mg. of Bendogen + 0.8 $\mu\text{g. of cyanocobalamin}$	0.306	0.301	—
Mean	0.299	0.291	0.295

diameter of the small pads. These were higher with the normal inoculum, but the activities measured by colour tended to be higher on the plate with the diluted inoculum.

*Binding activity determined by
ultrafiltration and dialysis*

Table 3 shows the binding activity of a sample of Bendogen assayed on three separate occasions. Each ultrafiltrate was assayed by *L. leichmannii* and the estimate of binding capacity calculated from three assay levels with duplicate tubes (cf. Gregory & Holdsworth, 1955). Also three different amounts of cyanocobalamin were added to a fixed amount of Bendogen and the same binding capacity was obtained.

When 100 mg. of Bendogen and 31.0 $\mu\text{g. of } [^{60}\text{Co}]$ cyanocobalamin reacted in 5 ml. of solution, ultrafiltration showed that 8.5 $\mu\text{g. of cyanocobalamin}$ was present in free form, i.e. 22.5 $\mu\text{g.}$ was bound by 100 mg. of Bendogen. After the solution remaining in the cellophan sac had been dialysed it contained a total of 21.0 $\mu\text{g. of cyanocobalamin}$, presumably in bound form since the free vitamin is readily diffusible. No radioactivity was detected in the last 500 ml. of dialysate. Thus ultrafiltration and dialysis methods gave 0.23 and 0.21 $\mu\text{g./mg.}$ as the cyanocobalamin binding capacity of Bendogen.

A comparison of the binding capacity of a particular sample of Bendogen, determined by different methods, is shown in Table 4. The ultrafiltration method (with either microbiological or radiometric assays) gave similar results over a wide range of conditions. The plate assays, however, gave variable results as described above.

the zones of growth and from the elution and measurement of the red colour present in the growth zone. It is shown in Table 2, expressed as a percentage of the total cyanocobalamin present.

Decreasing the inoculum made the growth zones larger and less densely coloured. With both sizes of pad and with both normal and diluted inocula, the growth activity of the Bendogen-cyanocobalamin solutions (measured by the zone diameter, or colour) increased with increasing dilution of the solution. However, although both showed the same trends, the activities measured by the zone diameters were not always in agreement with those measured by the colour of the zone, particularly with the more dilute solutions.

Further, different growth activities were obtained with different sizes of pad, the larger pads generally giving the higher activity. The amount of inoculum used in the plate also had an effect on the growth activity measured by the growth-zone

Table 4. Comparison of different methods used for measuring the cyanocobalamin-binding activity of extracts of pig pyloric mucosa (Bendogen) and sow's whey (SF 8)

Method	Test conditions	Binding activity ($\mu\text{g./mg.}$)
	Bendogen	
1. Ultrafiltration: <i>L. leichmannii</i>	0.24–0.28 $\mu\text{g.}$ of cyanocobalamin/mg. of Bendogen in 10 ml. of saline	0.22
2a. $\left\{ \begin{array}{l} \text{Ultrafiltration:} \\ [^{60}\text{Co}] \text{cyanocobalamin} \end{array} \right.$	$\left\{ \begin{array}{l} 2\text{--}4 \mu\text{g. of cyanocobalamin/10 mg. of} \\ \text{Bendogen in 10 ml. of saline} \\ 40 \mu\text{g. of cyanocobalamin/100 mg. of} \\ \text{Bendogen in 10 ml. of saline} \end{array} \right.$	0.22
2b. $\left\{ \begin{array}{l} \text{Ultrafiltration:} \\ [^{60}\text{Co}] \text{cyanocobalamin} \end{array} \right.$	$\left\{ \begin{array}{l} 40 \mu\text{g. of cyanocobalamin/100 mg. of} \\ \text{Bendogen in 10 ml. of saline} \\ 31 \mu\text{g. of cyanocobalamin/100 mg. of} \\ \text{Bendogen in 5 ml. of water} \end{array} \right.$	0.21
2c. $\left\{ \begin{array}{l} \text{Ultrafiltration:} \\ [^{60}\text{Co}] \text{cyanocobalamin} \end{array} \right.$	$\left\{ \begin{array}{l} 31 \mu\text{g. of cyanocobalamin/100 mg. of} \\ \text{Bendogen in 5 ml. of water} \end{array} \right.$	0.23
3. Dialysis: [^{60}Co]cyanocobalamin	31 $\mu\text{g.}$ of cyanocobalamin/100 mg. of Bendogen in 5 ml. of water	0.21
4. <i>Esch. coli</i> plate assay	10–28 $\mu\text{g.}$ of cyanocobalamin/100 mg. of Bendogen at different dilutions in water*	0 to > 0.20
	Sow's whey concentrate (SF 8)	
1. Ultrafiltration: <i>L. leichmannii</i>	0.10–0.20 $\mu\text{g.}$ of cyanocobalamin/mg. of SF 8 in 10 ml. of saline	0.12
2. Ultrafiltration: [^{60}Co]cyanocobalamin	2.0 $\mu\text{g.}$ of cyanocobalamin/10 mg. of SF 8 in 10 ml. of saline	0.12
3. Growth inhibition: <i>Esch. coli</i> tube assay	0.4 $\mu\text{mg.}$ of cyanocobalamin/assay tube. 0.625–5.0 $\mu\text{g.}$ of SF 8 added aseptically	0.11

* See Table 1.

Table 4 also shows the binding capacity of a concentrate from sow milk whey (SF 8) determined by the growth-inhibition and ultrafiltration methods. Good agreement was found between the two methods.

Stability of the complex

When the radioactive cyanocobalamin-protein complex was incubated with water for 4, 24 or 96 hr., no radioactivity could be detected in the ultrafiltrate. When amounts of 10 or 100 $\mu\text{g.}$ of unlabelled cyanocobalamin were added to the complex no exchange was detectable in 4 hr. After 24 hr. at the 100 $\mu\text{g./4 ml.}$ level 2.5 % of the bound radioactive cyanocobalamin had been exchanged. After 4 days at the 100 $\mu\text{g.}$ level 7.5 % of the original 2 $\mu\text{g.}$ of radioactive cyanocobalamin was released.

DISCUSSION

The results of our experiments with cyanocobalamin combined with an extract of pig pyloric mucosa (Bendogen) show that the bound vitamin has growth activity for *Escherichia coli* in plate assay. The amount of growth obtained from a certain amount of bound cyanocobalamin depended on the dilution at which it was assayed, on the amount of inoculum used on the plate, on the size of the disk on which the test solution was spread and finally on the ratio of vitamin to binding factor. The higher growth

activity obtained with the more dilute solutions of the vitamin complex appears to be connected with the amount of complex presented to a given number of micro-organisms. In *Esch. coli* plate assays the diameter of the growth zone varies linearly with the log of the concentration of the vitamin and it is difficult to obtain accurate results. We have found that an error of 0.05 mm. in measuring the zone diameters could alter the estimate of binding activity by as much as 66 %. It is not surprising that Raine (1955), using *Esch. coli* plate assays, was unable to find a stoichiometric relationship between added cyanocobalamin and the binding substance.

It is possible that under certain experimental conditions other assay micro-organisms, e.g. *Euglena gracilis*, can utilize the bound cyanocobalamin in the presence of the free vitamin, and this would explain the discrepancies between the various methods of measuring binding activity. For this reason we have separated the measurement into two parts: (a) the separation of a specimen of free cyanocobalamin by ultrafiltration from a mixture of binding substance with excess of vitamin and (b) the estimation of the concentration of the free cyanocobalamin in the ultrafiltrate by microbiological or radiometric techniques. If only a small proportion of the liquid is ultrafiltered, the concentration of free vitamin in the ultrafiltrate will be the same as that in the mixture and the amount

of cyanocobalamin bound can be calculated. Over a wide range of concentration of Bendogen the combining capacity was the same, 0.21–0.23 $\mu\text{g.}/\text{mg.}$ Further, when excess of [^{60}Co]cyanocobalamin was added to Bendogen, the excess dialysed away and the residual vitamin measured, it was again found that the sample of Bendogen bound 0.21 $\mu\text{g.}/\text{mg.}$ Thus the dialysis and ultrafiltration methods used as described here give results in good agreement. However, for routine estimations the ultrafiltration method is the quicker. It takes only about 1 hr. to collect 1 ml. of filtrate, whereas a period of about 48 hr. is required to dialyse away all traces of free vitamin B_{12} from the mixture. In their studies on the binding of cyanocobalamin by serum Rosenthal & Sarett (1952) dialysed 5 ml. of serum plus 0, 10 or 30 $\mu\text{mg.}$ of cyanocobalamin against 25 ml. of saline for 48 hr. or against frequent changes of Clark & Lubs buffer for 65 hr. Since their experiments were designed to demonstrate that the cyanocobalamin of normal serum was firmly bound and that the serum could bind limited amounts of the added vitamin, they did not report precise figures for the binding activity of different samples of serum. Similarly we found that equilibrium dialysis of this type did not give reproducible results in experiments of up to 24 hr. duration.

An additional advantage of the ultrafiltration method is that the ultrafiltrate contains only free cyanocobalamin, which can be measured microbiologically simply by diluting the filtrate to the appropriate vitamin concentration. With the dialysis method, the dialysate is usually too dilute for accurate measurement of the free vitamin and therefore the bound vitamin remaining in the cellophan bag has to be estimated. This requires some treatment, such as digestion with papain, to make the cyanocobalamin fully available to the test organism. This difficulty can be overcome by the use of [^{60}Co]cyanocobalamin, the bound vitamin being estimated from the radioactivity of the liquid remaining inside the dialysis sac. The Bendogen has been tested by the method depending on the inhibition of uptake of cyanocobalamin by *Esch. coli* cells (Hoff-Jorgensen, 1952), and it was found that 1 mg. of Bendogen prevented the uptake of 0.22 $\mu\text{g.}$ of cyanocobalamin (E. Hoff-Jorgensen, private communication). There is a danger, however, in using bacterial cells to differentiate between free and bound cyanocobalamin, that the test substance may alter the surface of the cell so that it can no longer absorb the free vitamin. Chow & Davis (1952), using a method in which the excess of free vitamin was absorbed by resting suspensions of *L. leichmannii*, reported strong cyanocobalamin-binding activity by heparin, chondroitin sulphate and nucleic acid preparations. None of these substances bound the vitamin when tested by our ultra-

filtration procedure and it is conceivable that these materials interfered in some non-specific manner with the uptake of the free vitamin by *L. leichmannii*. Similarly Bird & Hoever (1951) found that by the growth-inhibition methods lysozyme appeared to bind cyanocobalamin, whereas the dialysis method indicated there was no true 'binding' by lysozyme.

The reaction between free cyanocobalamin and the specific protein from pig pylorus is rapid and a stable complex results. The amount of free vitamin present in an aqueous solution of the complex could not be measured accurately but was much less than 1 % of the total vitamin. Further, dialysis for 48 hr. against running water did not remove measurable amounts of cyanocobalamin. Attempts to replace the radioactive vitamin in the complex by incubation with a large excess of the unlabelled vitamin showed that the exchange was very slow.

Our work shows that the well-defined complex of cyanocobalamin and binding protein can, under certain conditions, support the growth of micro-organisms. We therefore believe that the most suitable method for routine measurements of binding capacity is the ultrafiltration method, which avoids growing the test organism in the presence of the vitamin-protein complex and is more rapid and easier to manipulate than the dialysis method.

SUMMARY

1. Cyanocobalamin bound to extracts of pig pyloric mucosa (intrinsic-factor concentrates) is active for *Escherichia coli* in plate-assay techniques; therefore the use of plate assays, and possibly other assays, with micro-organisms for measuring binding activity is unsound.

2. The ultrafiltration method, previously described by us, for measuring binding capacity has been extended by the use of radioactive cyanocobalamin. Further evidence of the reliability of this method is presented and its advantages over the dialysis method are discussed. The technique is not subject to interference by such substances as lysozyme, heparin, chondroitin sulphate and nucleic acids, as are methods depending on uptake of free vitamin by bacteria.

3. The cyanocobalamin-protein complex is stable to dialysis and only slowly exchanges its bound vitamin *in vitro*, even in the presence of a large excess of free cyanocobalamin.

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Sucrose Formation by Taka-diastase: Action of the Enzyme on Methyl β -Fructofuranoside and Raffinose

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Invertase preparations from several sources (see Bacon, 1954, for summary; Allen & Bacon, 1956) have been shown to possess transfructosylase activity. Fischer, Kohtès & Fellig (1951) first suggested that this could be explained by a reaction mechanism involving a fructosyl-enzyme intermediate:

equation (2) representing 'transfer' and equation (3) 'hydrolysis'. Similar mechanisms have been proposed for other transglycosylases (see review by Edelman, 1956).

The fructosyl radical is usually transferred to a primary alcoholic group in the acceptor, the one notable exception being the transfer to the reducing group of glucose, which accounts for a large part of the total transferring activity of mould invertase in sucrose solution; by this action sucrose is re-synthesized, as may be demonstrated by the use of radioactive glucose (Edelman & Bealing, 1953; Edelman, 1954). The failure of yeast invertase to bring about resynthesis of sucrose (Edelman, 1954) could be due to differences in the stereochemical specificity of the enzyme, or perhaps to the failure of the hypothetical fructosyl-enzyme compound to preserve the 'high-energy' fructoside bond of sucrose (cf. Hestrin, Feingold & Avigad, 1956).

Edelman (1954) mentioned, without giving details, that when sucrose was replaced by methyl β -fructoside as substrate for mould invertase, no

sucrose or other oligosaccharides were formed in the presence of glucose. Since this observation raised doubts about the validity of the fructosyl-enzyme hypothesis (cf. Edelman, 1956), we thought it worth while to investigate it more fully, and also to see whether the enzyme would synthesize sucrose by fructose transfer to glucose when raffinose was the substrate. With methyl β -fructoside as substrate no sucrose formation was detected. With raffinose, sucrose was formed, and it was shown by the use of radioactive glucose that this arose by transfructosylation, not by the removal of the galactose residue from the trisaccharide.

In the following account we include details of the preparation of methyl β -fructoside by the action of yeast invertase on solutions of sucrose and methanol, a preliminary account of which has been published (Bacon, 1952).

EXPERIMENTAL

Methods

The analytical methods used were essentially those described by Bacon (1954). The handling and counting of radioactive glucose followed that described by Edelman (1954); all the counts given are corrected for background. Uniformly labelled [^{14}C]glucose, from the Radiochemical Centre, Amersham, Bucks (1.3 mg. containing 0.1 mc), was dissolved in 0.5 ml. of 2% (w/v) glucose solution and was purified as described by Allen & Bacon (1956) before use.

In addition, the test of Raybin (1933) for sucrose and some of its derivatives was adapted for use on paper chromatograms: 15 mg. of diazouracil was dissolved in 10 ml. of 0.1N-NaOH. The solution, which changed in colour from red to yellow-green within 30–40 sec., was sprayed at once on the chromatogram. This was then partly dried by heating in an oven at 60–80° for 3 min., dipped through 2% aqueous MgCl_2 and blotted between filter papers. Sucrose, raffinose,

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