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The Combination of some Vitamin B_{12}-like Compounds with Sow's Milk Whey and 'Intrinsic Factor' Concentrates

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In a previous communication from this laboratory, Gregory, Ford & Kon (1952) reported the presence in sow's milk of a substance that combined with vitamin B_{12} making it unavailable to assay micro-organisms. This 'binding factor' appeared to be associated with a particular fraction of whey proteins. Other naturally occurring materials have been shown to inactivate vitamin B_{12} in the same way. One of the first reports of this type of inactivation was that of Ternberg & Eakin (1949), who found that normal gastric juice and an aqueous extract of pig gastric mucosa contained a non-dialysable, heat-labile substance that combined with vitamin B_{12} and made it unavailable to assay micro-organisms. Prusoff, Meacham, Heinle & Welch (1950) fractionated extracts of desiccated pig stomach with ammonium sulphate and found that intrinsic factor activity was greatest in the fraction precipitated by 35–55 % saturation with ammonium sulphate. The ability of the various fractions to prevent the utilization of vitamin B_{12} by *Lactobacillus leichmannii* was investigated, but no figures were published in the communication.

The vitamin B_{12}-binding power of proteins such as egg albumin, globulins from blood and from soya beans, urease, lysozyme and an intrinsic factor concentrate has been investigated by Bird & Hoevet (1951). Only the intrinsic factor concentrate, however, showed any appreciable vitamin B_{12}-binding activity. Beerstecher & Altgelt (1951) observed a substance in saliva, similar to Castle's intrinsic factor from gastric juice in its ability to combine with and inactivate vitamin B_{12}. However, these two substances appear to differ in their heat stabilities (Beerstecher & Edmonds, 1951). Using dialysis techniques, in conjunction with microbiological assays, Rosenthal & Sarett (1952) have shown that vitamin B_{12} was present in a bound form in serum and that the serum was also capable of binding limited amounts of added vitamin B_{12}. Chow & Davis (1952) reported that yeast nucleic acid and heparin combined with vitamin B_{12}, but gastric juice was much more effective. Their measurements were made using radioactive vitamin B_{12} and no microbiological assays were carried out.

Compounds are known to exist, such as pseudovitamin B_{12}, first isolated by Pfiffner *et al.* (1951), and factors *A* and *B*, isolated from calf faeces by Ford & Porter (1952), that are not simple derivatives of vitamin B_{12}, but have 'vitamin B_{12} activity' for micro-organisms. Since the combination of vitamin B_{12} with intrinsic factor or other heat-labile substances is thought to be of importance in the metabolism of vitamin B_{12} (Ungley, 1951), it is of interest to find out whether the other vitamin B_{12}-like compounds also form a complex with intrinsic factor, particularly since pseudovitamin B_{12} has been shown to be clinically inactive in the treatment of pernicious anaemia (Pfiffner, Dion & Calkins, 1952). For this reason the microbiological inactivation of pseudovitamin B_{12} and of factors *A* and *B* by an 'intrinsic factor' concentrate and a concentrate from sow's milk has been investigated.

EXPERIMENTAL

Preparation of the sow's whey concentrate. Skimmed sow's milk was clotted with crystalline rennin at 40° and the curd removed. The whey was adjusted to pH 4·6 with glacial acetic acid and the proteins were fractionated by $(NH_4)_2SO_4$ saturation following the procedure used by Prusoff *et al.* (1950) for the concentration of intrinsic factor activity from powdered hog stomach. The proteins precipitated by

33–100% $(NH_4)_2SO_4$ saturation were suspended in a small volume of water, dialysed for 36 hr. against running water and freeze-dried. The freeze-dried proteins were dissolved in 0·1 M sodium acetate buffer at pH 5·5. Saturated $(NH_4)_2SO_4$ solution was added drop-wise with continuous stirring. Precipitates were collected at various concentrations of $(NH_4)_2SO_4$, dissolved in the minimum amount of water, dialysed for 7 hr. against running water and freeze-dried. The fraction precipitated by 66–100% $(NH_4)_2SO_4$ saturation contained the most vitamin B_{12}-binding activity. This was the concentrate used in these experiments. A 1% solution was made in sterile distilled water. From this, serial dilutions were made aseptically for addition to the assay tubes.

Preparation of the 'intrinsic factor' concentrate. The 'intrinsic factor' concentrate was prepared from 'Extomak', a commercial brand of desiccated hog stomach prepared by Benger's Ltd., Holmes Chapel, Cheshire. A saline extract was fractionated by the method described by Prusoff *et al.* (1950). The concentrate used in these experiments was the fraction precipitated by 35–100% $(NH_4)_2SO_4$ saturation, which was dialysed, freeze-dried, reconstituted in a 1% solution in sterile water and diluted aseptically in the same manner as the sow's whey concentrate.

Vitamin B_{12} and vitamin B_{12}-like compounds. The vitamin B_{12} used was the commercially available 'Cytamen' (Glaxo Laboratories Ltd.), which was ionophoretically pure. Factors *A* and *B* and pseudovitamin B_{12} were purified by repeated ionophoresis in 0·5 N acetic acid as previously described (Holdsworth, 1953). Each of the four compounds moved as a single spot on paper chromatograms or on ionophoresis on paper.

Optical density measurements. Since the ionophoretically pure factors were not available in amounts that could be weighed, standard solutions were prepared on a colorimetric basis. The absorption curve of a solution of vitamin B_{12} has a sharp peak at 361 mμ. and at this wavelength the $E^{1\%}_{1\,cm.}$ is 210. Pseudovitamin B_{12} and factor *A* show similar absorption curves to vitamin B_{12}. In the presence of excess of cyanide ion all three substances showed a shift of the absorption peak to 367 mμ. with only a slight change in optical density. Factor *B*, however, had an absorption peak in water at 355 mμ. which shifted to 367 mμ. in 0·01% KCN, and became more sharply defined; and the optical density increased approximately 20% (Fig. 1). Since the absorption peak at 367 mμ. is common to all these substances when tested in the presence of cyanide, colorimetric measurements were made at this wavelength in 0·01% KCN. The measurements were made with the Beckman spectrophotometer and the concentrations calculated on the assumption that the $E^{1\%}_{1\,cm.}$ for pseudovitamin B_{12} and factors *A* and *B* was the same as for vitamin B_{12}. On this basis the factors were diluted to a concentration of 1 μg./ml. with 20% (v/v) aqueous ethanol containing 0·01% (w/v) KCN, and stored at 1°.

Measurement of binding activities. The vitamin B_{12}-binding activity of the sow's whey or 'intrinsic factor' concentrates was measured by adding decreasing amounts of the concentrates to a fixed amount of vitamin B_{12}. As the amount of 'binding material' in the tubes decreased so the microbiological availability of the vitamin increased. Where the amount of 'binding material' in the tube was such that it completely 'bound' all the vitamin B_{12}, the assay organism was unable to grow. But where the quantity of 'binding material' present was only sufficient to inactivate a part of the vitamin B_{12} the assay organism responded to the residual 'unbound' vitamin. Quantitative measurement of this response by reference to a standard containing vitamin B_{12} alone allowed the calculation of the amount of the vitamin 'bound' by 1 g. of the freeze-dried concentrate (see Table 1).

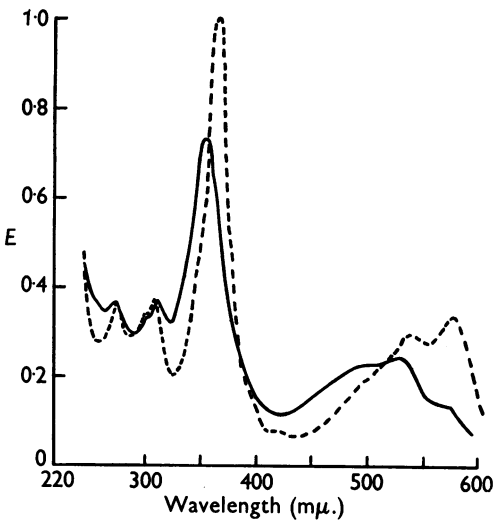

Fig. 1. Absorption spectra of factor *B* (solid line) and its cyano adduct (broken line).

Table 1. *Estimation of the vitamin B_{12}-binding activity of sow's whey concentrate*

(Assay organism: *Escherichia coli*. Vitamin B_{12} (0·4 μmg.) was added to each tube.)

Sow's whey concentrate/ tube (μg.)	Vitamin B_{12} available to *Esch. coli* (μmg.)	Vitamin B_{12} 'bound'/tube (μmg.)	Vitamin B_{12} 'bound'/g. concentrate (μg.)
5·000	0·00	>0·4	>80
2·500	0·15	0·25	100
1·250	0·28	0·12	96
0·625	0·34	0·06	96

The amounts of factors *A* and *B* and pseudovitamin B_{12} 'bound' were calculated in the same way by reference to a standard of the particular compound under test.

Microbiological methods. The method of assay was based on the *Escherichia coli* tube assay described by Burkholder (1951), except that the sodium thioglycollate in the medium was replaced by thiomalic acid. The medium was made up at 5 times its single strength, and 1 ml. added to each tube making the final volume of liquid to 5 ml., as described below.

A standard of vitamin B_{12} was set up by preparing a solution containing 0·2 μmg./ml. and adding it to a series of assay tubes at levels of 0·2, 0·5, 1·0, 1·5, 2·0, 3·0 and 4·0 ml. per tube, i.e. over a range of 0·04–0·8 μmg. per tube. The volume of liquid in each tube was made up to 4 ml. with distilled water and 1 ml. of medium added.

For the measurement of 'binding activity' a series of tubes, containing the assay medium plus 0·4 μmg. vitamin B_{12} in a total volume of 4 ml. was sterilized by steaming for 30 min. After cooling, aseptic additions of 1 ml. portions of

the serial dilutions of the concentrates were made. A similar set of tubes was prepared in which the 'binding material' was added before sterilization to obtain a value for the heat-stable 'binding activity'. A preliminary trial assay was made to ascertain the approximate 'binding activity' of the concentrates. The dilutions were then arranged so that they fell within a narrow range, thus enabling two or three of the dilutions to give growth responses from which the 'binding activity' could be calculated (see Table 1).

The 'binding' of factor *A* was measured in the same manner, using as the standard a solution containing 0·2 μmg. of factor *A*/ml. (colorimeter potency). The method was the same for factor *B* and pseudovitamin B_{12} except that the standard solutions were made, respectively, 5 and 10 times stronger than the vitamin B_{12} solution. This was necessary because these factors were less active than vitamin B_{12} for *Esch. coli*, when their concentrations were calculated on optical density measurements. The tubes were inoculated with one drop of a saline suspension of *Esch. coli* cells, as described by Burkholder (1951) and incubated at 30° for 18 hr. with continuous shaking. After steaming for 10 min., the tubes were cooled, 5 ml. of water were added to each (making a total volume of 10 ml.) and the growth was measured turbidimetrically.

RESULTS

Factors *A* and *B*, and pseudovitamin B_{12}, were less active than vitamin B_{12} in promoting the growth of *Esch. coli*. For example, a solution of factor *A* containing 1 μg./ml. by colorimetric measurement had growth activity for *Esch. coli* equivalent to only 0·66 μg. vitamin B_{12}/ml. These activities have been called the 'vitamin B_{12} activities' of the compounds, since they are a measure of the growth-promoting activity of these compounds for *Esch. coli* in terms of vitamin B_{12}. In Table 2 they are compared with the colorimetric potencies. It follows that to obtain the same amount of growth as given by 1 μmg. vitamin B_{12}, 1·33 μmg. factor *A*, 4·5 μmg. factor *B* and 12 μmg. pseudovitamin B_{12} by colour potency are required.

The amounts of vitamin B_{12} and vitamin B_{12}-like compounds 'bound' by the 'intrinsic factor' and sow's whey concentrates are given in Table 3. These figures were calculated from the concentrations of each of the compounds measured colorimetrically as described above. Because of the different growth activities of the compounds for *Esch. coli*, their concentrations had to be chosen so that they gave approximately the same amount of growth as the standard vitamin B_{12} solution, which contained 0·2 μmg./ml. Consequently solutions at concentrations of 0·2 μmg./ml. for factor *A*, 1·0 μmg./ml. for factor *B* and 2 μmg./ml. for pseudovitamin B_{12} were used. This necessitated the use of stronger solutions of the 'binding' concentrates for factor *B* and pseudovitamin B_{12}. In spite of these differences in the way the 'binding activities' were measured, the amounts of the vitamin B_{12}-like compounds 'bound' by the concentrates were remarkably constant.

The 'vitamin B_{12} activities' were also used for calculating the amounts of the compounds 'bound'

Table 2. *The 'vitamin B_{12} activities' of factor* A, *factor* B *and pseudovitamin B_{12} measured by the* Escherichia coli *tube assay*

(The concentration of all compounds was 1 μg./ml. as estimated by colorimetric measurement. The number of determinations is given in parentheses.)

Vitamin B_{12}-like factor	'Vitamin B_{12} activity' (±standard error) (μg./ml.)
Vitamin B_{12}	1·00
Factor *A*	0·58±0·09 (4)
Factor *B*	0·18±0·04 (5)
Pseudovitamin B_{12}	0·08±0·02 (3)

Table 3. *Amounts of vitamin B_{12}, factors* A *and* B *and pseudovitamin B_{12} 'bound' by 'intrinsic factor' and sow's whey concentrates*

(Calculated from the concentrations of each compound measured colorimetrically. Assay organism: *Esch. coli*. Individual experimental results are given with their mean. The result of each experiment was calculated from three determinations at different levels of the concentrate as shown in Table 1.)

		Vitamin B_{12} 'bound' (μg./g.)	Factor *A* 'bound' (μg./g.)	Factor *B* 'bound'* (μg./g.)	Pseudovitamin B_{12} 'bound' (μg./g.)
			'Intrinsic factor' concentrate		
Heated		13, 14, 10, 8	8, 12, 6, 7	11, 2, 2	14, 27, 13
	Mean	**11**	**8**	**5**	**18**
Unheated		190, 200, 210, 170	140, 150, 170, 130	120, 150, 160	150, 230, 220
	Mean	**190**	**150**	**140**	**200**
			Sow's whey concentrate		
Heated		30, 12, 15	12, 6, 9	4, 3	21, 13
	Mean	**19**	**9**	**3·5**	**17**
Unheated		110, 120, 100	100, 80, 100	90, 130	130, 110
	Mean	**110**	**93**	**110**	**120**

* Values were reduced by 20% if the colour was measured at 355 mμ. in the absence of cyanide.

by the concentrates. The results are not given in detail since it was found that, calculated in this way, the amounts of factor *A*, factor *B* and pseudovitamin B_{12} 'bound' were 66, 22 and 8 %, respectively, of the figures given in Table 3. That is, they merely reflected the growth-promoting activities of these compounds for *Esch. coli* (see Table 2).

DISCUSSION

The ideal way of calculating the amounts of the vitamin B_{12}-like compounds which combine with 'intrinsic factor' and sow's whey concentrates would be on an equimolecular basis. But, since the chemical constitution of these compounds is still unknown, this is not possible at present. By measuring the absorption of solutions of these compounds in the region 350–370 mμ. we have assumed that a functional part of the molecule, common to all of the vitamin B_{12}-like compounds tested, was being measured. The results based on these measurements (Table 3) seem to justify this assumption, since vitamin B_{12} and the other compounds are all 'bound' to the same extent.

As a temporary expedient, therefore, it may be useful to base microbiological assays of these vitamin B_{12}-like compounds on colorimetric concentrations, measured as described in this paper. We are well aware of the possible fallacy in our assumption that the $E_{max.}$ for a compound closely related to vitamin B_{12} is necessarily the same as for vitamin B_{12}. The presence or absence of a particular grouping could sharpen or depress the absorption peak. With pseudovitamin B_{12}, in which the dimethylbenziminazole nucleotide moiety of vitamin B_{12} is replaced by adenylic acid (Dion, Calkins & Pfiffner, 1952), the $E_{max.}$ is, however, the same as for vitamin B_{12}. This is also the case with factor *A*, although it is not yet known how this compound differs from vitamin B_{12}.

Factor *B* is a less complex molecule than vitamin B_{12}, but its constitution is not yet known; it has $E_{max.}$ at 355 mμ., but when a slight excess of cyanide is added the peak becomes sharper and shifts to 367 mμ. The concentrations of factor *B*, calculated from the absorption at 355 mμ. without cyanide and at 367 mμ. in the presence of cyanide, were both used for estimating the 'binding' of factor *B* by the concentrates. The results obtained in the absence of cyanide were 20 % less than those given in Table 3. Since the absorption peak of the vitamin B_{12}-like compounds at 367 mμ. in the presence of cyanide seemed to be common to all, the results in Table 3 were calculated using this value. It seems probable that absorption in the region 367 mμ. is due to a part of the molecule to which the protein concentrates become attached and which is common to all the factors.

In our microbiological assays we have always been able to measure some form of 'binding activity' that was stable to steaming for 30 min. The original report of sow's milk 'binding activity' (Gregory *et al.* 1952) referred to this heat-stable binding component. Table 3 shows that the unheated concentrates 'bind' many times more vitamin B_{12} (or vitamin B_{12}-like compounds) than the heated concentrates. Since the completion of the above experiments, however, we have found that the heat stability of the binding substances depended on the particular reducing agent used in the medium. Substitution of ascorbic acid for thiomalic acid increased stability considerably, so that the binding activity, measured after heating at 100° for 30 min. in medium containing ascorbic acid, was about half the unheated activity.

In a review of the work done on the 'intrinsic factor', Welch & Nichol (1952) stress the fact that the term 'intrinsic factor' should not be used synonymously with 'vitamin B_{12}-binding substances'. The 'intrinsic factor' concentrate used in these experiments was not tested for its clinical activity, nor was the sow's whey concentrate. However, Latner, Ungley, Cox, McEvoy-Bowe & Raine (1953) have fractionated human gastric juice by electrophoresis and found two peaks, one moving to the anode and the other to the cathode, which possessed both intrinsic factor and vitamin B_{12}-binding activities. Examination of our concentrates by electrophoresis showed only one zone with vitamin B_{12}-binding activity moving towards the anode with similar mobility to Latner's peak. Only a small proportion of the total solids of our concentrates was present in the zone of binding activity. In the case of the sow's whey concentrate it was 5 % of the total protein present. Further work on the isolation and purification of the binding substance from sow's whey will be published elsewhere.

If combination with intrinsic factor is important for the absorption of vitamin B_{12} or serves to protect it from assimilation by the intestinal flora, then the same process would apply to the other vitamin B_{12}-like compounds, since they are 'bound' to the same extent as vitamin B_{12}. It follows then that the clinical inactivity of the compounds other than vitamin B_{12}, e.g. pseudovitamin B_{12} (Pfiffner *et al.* 1952), is not due to their inability to combine with intrinsic factor.

SUMMARY

1. Optical density measurements have been used to determine the concentrations of solutions of ionophoretically pure vitamin B_{12}, factors *A* and *B* and pseudovitamin B_{12}.

2. From these measurements the microbiological activities for *Bact. coli*, in a tube assay, have been established. Factor *A* had 66 %, factor *B* 22 % and

pseudovitamin B_{12} 8% of the growth activity of vitamin B_{12}.

3. The amounts of these factors which combined with an 'intrinsic factor' concentrate and a sow's whey concentrate were identical with the amount of vitamin B_{12} 'bound'.

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Note. Since this paper was written, J. B. Armitage *et al.* (*J. chem. Soc.* in the Press) have identified factor *B* as vitamin B_{12} less the dimethylbenzaminazole nucleotide portion.

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The Pigments in Colour Phases of the Larvae of *Plusia gamma* L. (the Silver-Y Moth)

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Uvarov first proposed his theory of phases in 1921 (see Uvarov, 1928), one of the characteristics of insects in the 'gregarious' phase being that they are much darker in colour than those in the 'solitary phase'. The biochemical changes accompanying the colour variations in gregarious and solitary phases of *Locusta migratoria* and *Schistocerca gregaria* (order Orthoptera) have recently been investigated (see Goodwin, 1952*a*). Phases in the larvae of Lepidoptera were first observed by Faure in South Africa (1943*a*, *b*), namely, *Laphygma exigua* (the lesser army worm) and *L. exempta* (the army worm); they also probably exist in *Spodoptera abyssinia*.

Colour variations have recently been noted in this country between crowded and solitary larvae of the lepidopteran *Plusia gamma* both reared from the same batch of eggs. The solitary larvae remained pale green with thin white longitudinal stripes, whilst the crowded larvae showed forms ranging from a similar colour to a much darker green with yellow stripes. This variation was also observed in the field where unusually dark larvae were associated with mass outbreaks (Williams & Long, 1950).

This paper describes an investigation into the pigments responsible for the colour changes in *Plusia gamma*.

EXPERIMENTAL

Rearing of larvae. The larvae were reared at Rothamsted Experimental Station on sprigs of agricultural mustard stood in water in 1000 ml. short beakers fitted with fine muslin covers. A pad of filter paper in the bottom of each beaker prevented excessive dampness and provided a suitable surface for the larvae.

A few hours after hatching the young larvae from each batch of eggs were distributed at random over the breeding jars to provide both crowded cultures of eighty larvae and solitary cultures. Under these conditions the solitary larvae become a light green, whilst the vast majority of those in crowded cultures become a dark olive green.