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The Occurrence of a Cyanocobalamin-binding Protein in Milk and the Isolation of a Cyanocobalamin-Protein Complex from Sow's Milk

By MARGARET E. GREGORY AND E. S. HOLDSWORTH
National Institute for Research in Dairying, University of Reading

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A preliminary communication from this laboratory has described the occurrence of a vitamin B₁₂-binding substance in sow's milk whey (Gregory, Ford & Kon, 1952). More recently, we have shown that this binding substance, and an intrinsic-factor concentrate prepared from pig stomach, combined with other vitamin B₁₂-like substances in the same way as with cyanocobalamin (vitamin B₁₂) (Gregory & Holdsworth, 1953). The similarity between the effect on cyanocobalamin of the sow's milk whey and of the intrinsic-factor concentrates, led us to attempt the isolation of the binding substance from sow's milk, since a study of its properties may help to explain some aspects of the utilization and metabolism of cyanocobalamin.

The first part of this paper presents evidence that the milk of the cow, goat, pig, rat and woman possesses the property of combining with added cyanocobalamin. The second part describes the isolation from sow's milk of a cyanocobalamin-protein complex.

THE OCCURRENCE OF A CYANOCOBALAMIN-BINDING PROTEIN IN MILK

EXPERIMENTAL

Materials

The samples of milk from the cow, goat, pig, rat and woman and of colostrum from the cow and goat were the same as those described by Gregory (1954).

The sow's whey concentrate (SF 8) and the 'intrinsic factor' concentrate from pig stomach were prepared as described by Gregory & Holdsworth (1953).

Microbiological methods

Method of assay. The method of assay with *Lactobacillus leichmannii* ATCC 4797 was a modification of that of Skeggs, Nepple, Valentik, Huff & Wright (1950) and is fully described by Gregory (1954).

Measurement of cyanocobalamin in the cyanocobalamin-protein complex. Cyanocobalamin was quantitatively released from the protein complex by digestion with papain as described by Gregory (1954) and measured by *Lb. leichmannii*.

Measurement of cyanocobalamin-binding activity. Two methods were used. (a) The growth-inhibition method, described by Gregory & Holdsworth (1953). (b) The ultra-filtration method. A bag of 'Visking' cellulose tubing (The Visking Corporation, Chicago), diameter $\frac{1}{4}$ in., was suspended from the stem of a glass funnel held in the neck of a filtration tube by means of a rubber bung. The bag was made by knotting one end of the tubing tightly and tying the other, with cotton, over a piece of Polythene tubing fitted over the stem of the funnel. (A diagram of this apparatus will be found in the paper by Gregory, 1954.) A known amount of cyanocobalamin in 1 ml. of solution was added to a measured amount of the binding substance, and the mixture (total volume 10 ml.) poured into the cellophane bag. The filtration tube was evacuated, sealed off and left in the refrigerator for 1-2 hr. In this time, 1-2 ml. of ultra-filtrate could be collected.

The free cyanocobalamin, which was able to pass into the ultrafiltrate, was measured by *Lb. leichmannii*. The amount of cyanocobalamin bound by the binding substance was then calculated by subtracting the free cyanocobalamin from the total amount added.

This method was applied to the measurement of the binding of cyanocobalamin by the milks of different animal species. The approximate amount of cyanocobalamin bound by each of the milks was first estimated and then a more exact determination made. Three different levels of cyanocobalamin within a narrow range (e.g. 0.05, 0.10 and 0.20 μ g.) were added to 10 ml. milk, so that at least two of the levels gave an estimation of the binding activity.

Comparison of the bound form of cyanocobalamin in the milk of different species and in extracts of desiccated pig stomach

In order to establish whether the cyanocobalamin in the different milks was associated with one particular protein, the whey proteins were separated by electrophoresis on paper and the paper strips examined for proteins and

cyanocobalamin by the methods now to be described. An 'intrinsic factor' concentrate, prepared from desiccated pig stomach as described by Gregory & Holdsworth (1953), was treated similarly.

Preparation of the samples for electrophoresis

The milk was centrifuged and the cream removed and discarded. Glacial acetic acid was added to the skimmed milk to bring the pH to 4.6. The precipitated casein was removed by centrifuging and the precipitate washed once with 0.1 M sodium acetate buffer at pH 4.6. The combined whey and washings were dialysed against running tap water for 6 hr., then against 0.05 M veronal buffer at pH 8.6 for 16 hr. The whey was then concentrated by ultrafiltration (using the apparatus described above), and adjusted to a final concentration of 2% protein (estimated by the biuret reaction of Levin & Brauer, 1951). The 'intrinsic factor' concentrate and the sow's whey concentrate (SF 8) were each used as 2% solutions in 0.05 M sodium veronal buffer, pH 8.6.

Protein patterns. A band of each protein solution 5 cm. long and 0.3 cm. wide was painted on to a wide strip of Whatman no. 3 MM paper and separated by electrophoresis in 0.05 M sodium veronal buffer at pH 8.6 under a potential gradient of 7 v/cm. Under these conditions a marker spot of 2% crystalline bovine albumin moved 16 cm. in 15 hr. In addition to the whey proteins a spot of 1% lactose solution was added to each strip to mark the extent of electro-osmosis.

The separated proteins on the paper strips were stained with bromophenol blue as recommended by Kunkel & Tiselius (1951) and the strips were scanned for the stained

protein at 2 mm. intervals in a densitometer (made by E. S. Holdsworth, unpublished).

Electrophoresis followed by microbiological assay. A piece of Whatman no. 3 MM paper was marked in 1 cm. strips at right angles to the electrical field, and 0.1 ml. of the protein solution was applied at the origin as a row of 0.01 ml. spots 1 cm. apart. When the electrophoresis in 0.05 M sodium veronal buffer had been completed, the wet paper was immediately cut into the 1 cm. strips. Each strip was rolled up and placed on a 3 cm. square of fine nylon stocking fabric, which was arranged in the mouth of a $\frac{1}{2}$ oz. screw-capped bottle so that its edges were overhanging. The cap was screwed on to the bottle tightly, making, in effect, a nylon bag in which the paper strip was suspended half-way down the bottle. The bottles were centrifuged at 2000 g for 15 min. and liquid from the paper strips collected in the bottom of the bottles. The papers were moistened with physiological saline and centrifuged again. In this way the protein was washed from the paper strips. These protein solutions were made to known volumes and portions removed for microbiological assay either for total cyanocobalamin or for binding activity measured by the growth-inhibition method.

Autoradiography. In some instances, e.g. with the sample of sow's milk and the 'intrinsic factor' and sow's whey (SF 8) concentrates, radioactive cyanocobalamin labelled with ^{60}Co was added before electrophoresis on paper. The position of material containing radioactive cyanocobalamin was shown by placing the dry electrophoresis strips in contact with Ilfex (Ilford Ltd.) X-ray film. After the required exposure, the film was developed according to the maker's instructions. This was a sensitive test for cyanocobalamin, e.g. 0.01 ml. of a solution containing 0.005 μg . cyanocobalamin/ml. (activity 420 $\mu\text{C}/\text{mg}$.) gave on the film,

Table 1. *Measurement of the 'binding' of cyanocobalamin by the milks of different species of animals, and of the cyanocobalamin naturally present in these milks*

Naturally occurring cyanocobalamin was measured after papain digestion by the method of Gregory (1954); cyanocobalamin-binding activity was measured by the ultrafiltration method described in the text; assay organism: *Lb. leichmannii*. Means in bold type.

Material	Amount of cyanocobalamin added/ml. milk (μg .)	Amount of cyanocobalamin found/ml. of ultrafiltrate (μg .)	Cyanocobalamin 'bound' ($\mu\text{g}/\text{ml. milk}$)	Natural cyanocobalamin present ($\mu\text{g}/\text{ml. milk}$)
Sow's milk	0.200	0.000	> 0.200	0.0017
	0.300	0.070	0.230	
	0.400	0.140	0.260	
Rat's milk (diluted 1:30)	0.0004	0.000		0.0120
	0.004	0.0006	0.102	
	0.050	0.0450	0.150	
Human milk	0.050	0.000	> 0.050	0.0003
	0.100	0.020	0.080	
	0.200	0.120	0.080	
Cow's colostrum	0.008	0.0000	> 0.008	0.0029
	0.010	0.0018	0.0082	
	0.0125	0.0042	0.0083	
Goat's colostrum	0.008	0.0023	0.0057	0.0050
	0.010	0.0044	0.0056	
	0.0125	0.0064	0.0061	
Goat's milk	0.004	0.0014	0.0026	0.0007
	0.006	0.0035	0.0025	
	0.008	0.0050	0.0030	
Cow's milk	0.002	0.0017	0.0003	0.0032
	0.004	0.0037	0.0003	
	0.006	0.0051	0.0009	

an easily discernible zone after an exposure of 7 days. An accurate measure of the darkening of the film due to the radioactivity was obtained by scanning the film at 2 mm. intervals in the densitometer used for examining protein patterns.

Liquid counting. Accurate measurement of radioactivity was unnecessary for the protein fractionation procedures. A McMichael radiation monitor was used as a rate meter by immersing the graphite-electrode counter to a standard

depth in a shielded measuring cylinder containing the solution to be assayed. The activity of each fraction was expressed as a percentage of the activity present before the beginning of the fractionation.

RESULTS

Cyanocobalamin-binding activity of the milks of different animal species. The addition of cyanocobalamin to the different milks resulted in some of the vitamin being bound to protein and therefore no longer able to pass through a cellophan membrane. It can be seen from Table 1 that each of the milks bound a definite amount of cyanocobalamin, and that cyanocobalamin added over and above this amount was recovered in the ultrafiltrate. A solution of the pure vitamin could be ultrafiltered quantitatively.

The bound form of cyanocobalamin in the different milks. The whey proteins from each of the milks were separated by electrophoresis on paper as described in the experimental section. Figs. 1 and 2

Fig. 1. Electrophoretic patterns of milk wheys. Electrophoresis in sodium veronal buffer, pH 8.6. The blocked-in curves represent cyanocobalamin measured microbiologically, the unblocked curves represent protein distribution. The ordinates are arbitrary units (optical density) of protein or cyanocobalamin concentrations: (a) cow's whey proteins, (b) cow's whey natural cyanocobalamin, (c) cow's whey with added cyanocobalamin, (d) goat's whey proteins, (e) goat's whey with added cyanocobalamin, (f) human whey proteins, (g) human whey with added cyanocobalamin.

Fig. 2. Electrophoretic patterns of sow's milk whey and an 'intrinsic factor' concentrate. Electrophoresis in sodium veronal buffer, at pH 8.6. The blocked-in curves represent added cyanocobalamin (^{60}Co) measured as the optical density of the autoradiographs: (a) sow's whey proteins, (b) sow's whey with added cyanocobalamin, (c) 'intrinsic factor' concentrate with added cyanocobalamin, (d) cyanocobalamin control.

illustrate the results of analysing the paper strips, after electrophoresis, for the position of the proteins (stained by bromophenol blue) and of the cyanocobalamin (measured by *Lb. leichmannii* or by autoradiography).

Owing to the low natural content of cyanocobalamin in goat's and human milks, the wheys from these milks were saturated with cyanocobalamin before electrophoresis (using the figures shown in Table 1), so that the position of the bound vitamin could be measured with more accuracy. Cyanocobalamin added to cow's whey moved to the same position as that naturally present in the whey (Fig. 1) and furthermore the bound cyanocobalamin had the same mobility in cow's, goat's, human and sow's wheys, although the protein patterns were dissimilar. In addition, Fig. 2 shows that cyanocobalamin bound by extracts of desiccated pig stomach (the 'intrinsic factor' concentrate) had the same mobility as the bound form in milk. Under the experimental conditions, cyanocobalamin itself is uncharged and has zero mobility.

Fig. 3. Electrophoretic patterns of the sow's whey protein fraction SF 8. Electrophoresis in veronal buffer pH 8.6. The ordinates are arbitrary units of protein or cyanocobalamin concentration or of cyanocobalamin-binding activity: (a) SF 8 protein pattern, (b) cyanocobalamin-binding activity of SF 8, (c) cyanocobalamin naturally present in SF 8, (d) SF 8 with added cyanocobalamin (^{60}Co).

The bound form of cyanocobalamin in sow's milk. Examination of the protein patterns of the sow's whey protein concentrate (SF 8), which contained the bulk of the cyanocobalamin-binding activity of the whey, revealed only one major protein constituent, lactalbumin (see Fig. 3a). The binding substance had a mobility slightly less than the lactalbumin (Fig. 3b). Fig. 3b-d shows that the binding substance, the cyanocobalamin-protein complex occurring naturally in the whey, and the complex formed by saturating the whey with added cyanocobalamin, all had the same electrophoretic mobility. The similarity between the bound form of cyanocobalamin occurring naturally and that produced by adding cyanocobalamin to the milk led us to adopt the procedure of fully saturating the whey with cyanocobalamin in order to obtain larger yields of the cyanocobalamin-protein complex as described in the next part of this paper.

THE ISOLATION OF A CYANOCOBALAMIN-PROTEIN COMPLEX FROM SOW'S MILK

EXPERIMENTAL AND RESULTS

The binding substance present in 2700 ml. of skimmed milk was fully saturated by adding 540 μg . radioactive cyanocobalamin, labelled with ^{60}Co . The activity of this material was 0.24 $\mu\text{C}/\text{mg}$. The pH of the milk was adjusted to 4.6 with glacial acetic acid and the precipitated casein removed by centrifuging. The casein was washed twice with 0.1M sodium acetate buffer at pH 4.6, and the washings were added to the whey. The combined washings and whey were saturated to 33% with $(\text{NH}_4)_2\text{SO}_4$. The precipitated proteins were removed by centrifuging and washed with a 33% saturated $(\text{NH}_4)_2\text{SO}_4$ solution. Since the precipitate contained no radioactivity, measured in the liquid counter, it was discarded.

The whey and washings were fully saturated with $(\text{NH}_4)_2\text{SO}_4$. The precipitate was removed and suspended in 300 ml. 0.1M sodium acetate buffer at pH 5.5 containing a few mg. of cysteine. The insoluble material was removed by centrifuging and discarded, since it had no radioactivity. The supernatant fluid was treated with saturated ammonium sulphate solution and the following fractions were collected.

Fraction	Saturation with $(\text{NH}_4)_2\text{SO}_4$ (%)	Radioactivity (%)
A	0-33	0
B	33-50	0
C	50-60	15
D	60-66	15
E	66-100	70

Each of the fractions C, D and E was dissolved in 30 ml. water and dialysed for 6 hr. against dilute sodium acetate buffer containing cysteine. Fraction E was freeze-dried and 1 g. of a pink powder containing 520 μg . cyanocobalamin obtained. The powder was dissolved in 20 ml. 0.025M sodium veronal buffer at pH 8.6 and separated by continuous electrophoresis on paper (Holdsworth, 1953). Whatman no. 54 paper, with sodium veronal buffer at

pH 8.6 (0.025M) and a potential of 900v were used; Fig. 4 shows the paper at the end of the experiment after staining with bromophenol blue. Tubes 9 and 10 contained the bulk of the cyanocobalamin, measured both microbiologically and by the radioactivity, and thus good separation from the bulk of the proteins present was obtained.

The contents of the tubes with the bound cyanocobalamin were combined, and concentrated by ultrafiltration to a volume of 5 ml. This concentrated solution at pH 8.6 was fractionated with isopropanol at -5° .

Precipitate	isoPropanol (%)	Radioactivity (%)
1	0-28	5
2	28-45	90
3	45-55	5
4	55-75	0

Precipitate 2 was suspended in 2 ml. 0.1M sodium acetate buffer at pH 4.8. The insoluble material was removed, washed with buffer and discarded, as it had no radioactivity. The supernatant fluid and the washings were refractionated at -5° with isopropanol. The precipitate obtained at 45-55% isopropanol was red. This red precipitate was purified by further fractionations with isopropanol at pH 4.8 until the ratio of the extinction coefficients at 278 (due to protein) and 362 m μ . (due to cyanocobalamin) remained at a steady value.

In the final purification procedure, where the quantity of material handled was very small, the

Fig. 4. Photograph showing the separation of the sow's whey proteins after continuous electrophoresis on paper. The arrows mark the position of the cyanocobalamin-protein complex (tubes 9 and 10).

main object was to obtain a pure material and the overall yield was of secondary importance. A 45-fold concentration of the cyanocobalamin-protein complex, present in 1 g. of crude material (fraction E) was achieved. The pink powder, finally obtained, weighed 3.3 mg. and contained 78 μ g. cyanocobalamin (calculated from the absorption at 362 m μ .), that is 23.6 μ g./mg. of the complex. It was readily soluble in water and soluble in 50% (v/v) ethanol.

The amount of material obtained was insufficient to apply the usual criteria for protein purity. Therefore the assessment of purity was based on the fact that the substance was homogenous when examined by electrophoresis on paper at both pH 6.5 (phosphate buffer) and pH 8.6 (veronal buffer), and that the ratio of the concentration of cyanocobalamin to protein was unaltered by further precipitation with isopropanol.

DISCUSSION

Gregory *et al.* (1952) found that the cyanocobalamin of sow's milk could not be measured by a procedure which gave good results with cow's milk (see also Gregory, 1954). They found further that cyanocobalamin, added to the milk to a concentration of 0.06 μ g./ml. was unavailable to the assay organism, which responded, however, to additions over and above that amount. In other words, sow's milk could inactivate 0.06 μ g. cyanocobalamin/ml. This figure was obtained after heating the diluted whole milk with the assay medium and cyanocobalamin. In a previous paper (Gregory & Holdsworth, 1953) we have shown that an unheated protein concentrate from sow's milk bound more cyanocobalamin than the heated concentrate. It was necessary, therefore, to devise some method for measuring the amount of cyanocobalamin bound by unheated whole milk. Natural inhibitors present in raw milk complicate the interpretation of the microbiological responses when unheated milk was added aseptically to the assay tubes (Gregory, 1954).

In the ultrafiltration method finally adopted, the actual binding process was separated from the microbiological assay. The technique consisted of mixing the milk or binding protein with a slight excess of cyanocobalamin, and ultrafiltering the mixture. By removing only a small proportion of the fluid by ultrafiltration, the equilibrium of the system was not appreciably disturbed and the concentration of cyanocobalamin measured in the ultrafiltrate represented the concentration of the free vitamin in the whole system. (The Donnan effect for neutral cyanocobalamin is negligible.) When the amount of cyanocobalamin bound by a sow's whey concentrate was measured by this ultrafiltration method, the result was identical with that obtained

previously by a growth-inhibition method (Gregory & Holdsworth, 1953). The use of ultrafiltration seems more justifiable than the techniques depending on dialysing the protein-cyanocobalamin mixture for long periods against large volumes of buffer or saline as used by Bird & Hoewet (1951) and Rosenthal & Sarett (1952). This improved method has shown that sow's milk can combine with $0.24 \mu\text{g}$. cyanocobalamin/ml. (see Table 1), a figure 4 times that obtained when the milk was heated with the basal medium for microbiological assay (Gregory *et al.* 1952). It has also shown that the milk and colostrum of other animal species have the capacity of binding additional cyanocobalamin, a property not detected when the milks were heated with the basal assay medium.

The wheys from the various milks showed different protein patterns when examined by electrophoresis on paper (Figs. 1 and 2), in good agreement with results obtained by Deutsch (1947) for electrophoresis in free solution using the conventional Tiselius apparatus. Microbiological assays and autoradiographs of milk extracts, after separation by electrophoresis on paper showed that the bound form of the vitamin in the milks, and the complexes formed when the vitamin was added to the milks, were very similar if not identical. Moreover, the bound form of cyanocobalamin from milk had the same mobility as the cyanocobalamin-protein complex isolated from desiccated pig stomach. The different heat stability of the complex in these milks makes it impossible to state that the milks all contain the same bound form of cyanocobalamin. For example, the cyanocobalamin in cow's milk is readily available to *Lb. leichmannii* after heating with the basal assay medium, whereas the cyanocobalamin in sow's milk is not released by this treatment (Gregory, 1954). We have found (Gregory & Holdsworth, 1953) that the heat-stability of the complex from sow's milk depended on the composition of the assay medium and therefore the composition of the different milks may have some influence on the stability of the complex.

Earlier experiments showed that the substance responsible for inactivating cyanocobalamin was not one of the major components of sow's whey (Fig. 3). Attempts to isolate the binding factor from sow's whey were unsuccessful because of considerable losses during fractionation and a more satisfactory procedure was to saturate the binding factor with radioactive cyanocobalamin and isolate the more stable complex by following the radioactivity.

We have shown that cyanocobalamin occurs in a bound form in milk from different animal species. The addition of cyanocobalamin to desiccated pig stomach or to human gastric juice produces a

similar bound form of the vitamin. Whatever the mechanism by which Castle's intrinsic factor causes the absorption of cyanocobalamin, it seems certain that the protein-bound vitamin is implicated.

The chemical nature of the complex, and studies on the mode of linkage between its two components, are described in the following paper.

SUMMARY

1. An ultrafiltration method has been used to demonstrate that the milks of different animal species have the property of combining with added cyanocobalamin. The amount of the vitamin bound per ml. of milk was: pig, $0.24 \mu\text{g}$.; rat, $0.13 \mu\text{g}$.; woman, $0.08 \mu\text{g}$.; goat, $0.0027 \mu\text{g}$.; and cow, $0.0005 \mu\text{g}$.

2. The whey proteins of each of the milks were separated by electrophoresis on paper and the position of the proteins on the paper compared with the position in which the cyanocobalamin occurred. In this way it was shown that the major proteins of the wheys did not combine with the vitamin and that the cyanocobalamin-protein complex had the same mobility in each of the milks tested.

3. The isolation of the pure cyanocobalamin-protein complex from sow's milk whey was achieved by fractional precipitation with ammonium sulphate, separation from the bulk of the proteins by continuous electrophoresis on paper, and finally fractional precipitation with isopropanol.

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