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Studies on the Vitamin B_{12}, Cyanocobalamin-Binding Capacity, Desoxyribosides, and Methionine in Some Commercial Milk Products and Cheese

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1. Part of the vitamin B_{12} in cow milk is destroyed during commercial pasteurization (74°C for 16 sec).

2. There is no "free" vitamin B_{12} (estimated as *E. coli* activity) in unpasteurized cow milk. On the other hand, this activity is considerable in pasteurized milk.

3. The cyanocobalamin-binding capacity of cow milk amounted on a average to 0.29 mμg/ml. Different results were obtained with different samples of milk, the range being 0.15–0.45.

4. Commercially pasteurized cow milk (74°C, 16 sec) has no cyanocobalamin-binding capacity.

5. The cyanocobalamin-binding capacity of cow milk, determined as shown in Table 4, is destroyed by heating to a temperature between 50–60°C.

6. The substance responsible for the cyanocobalamin-binding capacity of cow milk loses this property upon heating, at the same time as the cyanocobalamin is released from the complex.

7. Freezing has no influence on the cyanocobalamin-binding capacity of cow milk.

8. The "total" vitamin B_{12} activity of sour milk and yoghurt is lower than that of the milk from which they have been prepared. This indicates that the vitamin is used by the bacteria during the lactic fermentation of the milk. The total activity in yoghurt is due mainly to methionine and desoxyribosides, both of which are not at all present, or only in very small amounts, in the ultrafiltrate of cow milk.

9. Sour milk has no, or only a very low, cyanocobalamin-binding capacity. This property is on the other hand very high in yoghurt, being in the range 150–190 mμg/ml, *i. e.* about 500–600 times higher than in fresh unpasteurized milk.

10. Preliminary results with saline suspensions of *Lactobacillus bulgaricus* and of *L. thermophilus*, used in the preparation of yoghurt, indicate that these organisms are able to bind cyanocobalamin.

11. Cheddar and Roquefort had on average about the same B_{12} activity (28 and 27 mμg/g fresh basis, respectively), and so had Camembert and Svecia but on a lower level (11.5 and 11.3 mμg/g fresh basis).

The high contents of desoxyribosides made impossible the assay of the "free" vitamin B_{12} in Camembert and Roquefort by the micro-

biological method used here. The results obtained with Cheddar and Svecia show that the vitamin occurs in these cheeses mainly in a bound form.

13. Camembert contained about 400 μg methionine/g fresh basis (*Leuconostoc mesenteroides* activity) while the other varieties had amounts which were 3 to 7 times greater. The *L. delbrückii* activity of Cheddar and Svecia was of the same order of magnitude, 0.75—1.9 μg/g fresh basis, calculated as thymine desoxyriboside. The activity of Camembert and Roquefort was 20—35 times greater.

Numerous articles have been published in different countries on the determination of the content of cyanocobalamin in cow milk and the seasonal variations of this content as well as the influence of the animal diet composition etc. In the present work, determinations of the total vitamin B_{12} activity of cow milk were only made in order to see if the vitamin is destroyed during commercial pasteurization of the milk, and if so, to what extent. The total vitamin B_{12} activity in the whey was also determined as well as the "free vitamin B_{12}" activity in pasteurized and untreated cow milk. The results obtained are summarized in points 1—3 above.

In 1955 Gregory and Holdsworth [2] demonstrated that milk from different animal species possessed the property of binding added cyanocobalamin. The amount of cyanocobalamin which could be bound was different for different species, being greatest for sow milk (240 mμg Cy/ml) and smallest for cow milk (0.5 mμg Cy/ml). It has been found previously in this laboratory by Neujahr *et al.*[3] that dry skim milk powder has no cyanocobalamin-binding capacity and, even if not fully investigated, it was pointed out that also commercially pasteurized milk might have lost the ability to bind cyanocobalamin.

In the present work, the cyanocobalamin-binding capacity of pasteurized and untreated cow milk was further investigated. As it was found that the pasteurized product does not bind cyanocobalamin, an attempt was made to determine at which temperature this property is destroyed by heating. The results are summarized in points 3—7 above.

There seems to be no published data about the vitamin B_{12} activity and cyanocobalamin-binding capacity of yoghurt and sour milk. In the present work, the vitamin B_{12} content of both products was determined and compared with that of the milk used in their preparation. The ability to bind cyanocobalamin was also investigated. The results are summarized in points 8—10 above.

The vitamin B_{12} activity, total as well as "free", and the cyanocobalamin-binding capacity of four different kinds of cheese were determined. The cheese extracts were also analyzed for methionine and desoxyribosides. The results are summarized in points 11—13 above.

PART I. THE VITAMIN B_{12} ACTIVITY, TOTAL AND "FREE", IN PASTEURIZED AND UNPASTEURIZED COW MILK

Experimental

Determination of the vitamin B_{12} content. A 50 ml sample of milk, containing 100 μg/ml of KCN, was autoclaved for 10 min at 120°C. After cooling, the liquid was ultrafiltered and the ultrafiltrate analyzed for vitamin B_{12} using the tube method and *E. coli* 113—3 as test-organism.

The ultrafiltration was carried out using a piece of Visking cellulose tubing (The Visking Corp., Chicago), diameter 1/4 inch, as described by Gregory [1] but modified in the following way: Two small pieces of plastic or rubber tubing (c) were pushed on to the stem of a funnel (a) (Fig. 1) and a short piece of glass tubing (b). A 20—25 cm length of cellulose tubing was moistened with distilled water at both ends and then slipped over the sleeves (c). Cotton thread was tied tightly around the cellulose tubing in order to make both joints secure. Hence, in contrast to the original method, the cellulose tubing did not have a cotton-tied end with which the liquid to be ultrafiltered came into contact. To facilitate the fastening, the tube (b) can first be loosened from the rubber stopper and can then, after the joints have been made secure, be pushed back into its place. The cellulose tube must be carefully moistened in order to prevent it from cracking during the ultrafiltration. This was performed by keeping it overnight, after it had been fastened to the funnel and the glass tube, in a desiccator, the lower compartment of which was half-filled with distilled water. The desiccator was placed in a refrigerator in order to prevent any possible microbiological attack on the cellulose. To perform the ultrafiltration, the cellulose tubing *etc.* was put into a tube (d) which could be evacuated through (e). It was possible to connect several ultrafilters to the same suction device when there were numerous samples to be treated. During the present work, up to 30 simultaneous ultrafiltrations have been run successfully. After autoclaving, a 10 ml sample was poured

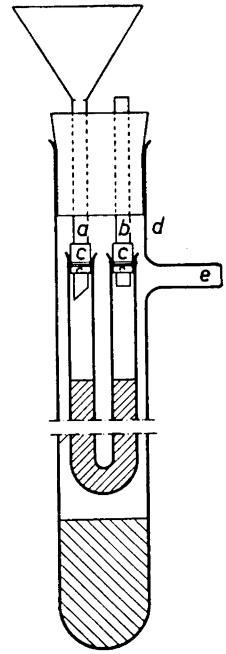

Fig. 1. Ultrafilter.

into the funnel. Suction was applied and then removed when about 1—1.5 ml ultrafiltrate had been collected. This took approximately one hour.

Working with this proportion between the sample and the ultrafiltrate, the amount of free vitamin B_{12} per ml in both of them should be the same [2]. The ultrafiltrate was diluted appropriately and its vitamin B_{12} content determined using the tube method and *E. coli* 113—3 as test organism. To get the standard curve, crystalline cyanocobalamin was added to six sets of tubes in quadruplicate in the following quantities: 0.025, 0.05, 0.1, 0.2, 0.4, and 0.8 mμg per tube. In several cases, the pH of the milk was adjusted to 5 before autoclacing. The whey, thus produced, was ultrafiltered and its vitamin B_{12} content determined. The values were fundamentally the same as those obtained by ultrafiltration of the whole milk, after the above treatment. This is in agreement, with the findings of Neujahr et al [3].

To determine the amount of "free" vitamin B_{12} (as *E. coli* activity), samples of milk were directly ultrafiltered and the ultrafiltrate analyzed.

Bioautography. Spots of the samples were put on sheets of Whatman No. 1 paper and developed in two different solvent systems according to Neujahr [6], the compositions of which are given below, for 24 or 48 h. The spots were made by releasing drops from a capillary. After development, the paper sheets were dried and placed on agar plates seeded with the appropiate test microorganism.

Solvent system I		Solvent system II	
Sec. butanol	75 %	*Sec.* butanol	75 %
Acetic acid (conc.)	1 %	NH_3 (conc.)	1 %
H_2O	24 %	H_2O	24 %
KCN	0.01 %	KCN	0.01 %

Results and discussion

The samples were provided by Mjölkcentralen, Stockholm.

The values found for the total vitamin B_{12}, given as *E. coli* activity, were 3—6 mμg/ml for untreated milk and 2.5—5.3 mμg/ml for pasteurized milk. These results show that a part of the vitamin B_{12} is destroyed during the commercial pasteurization of the milk (16 sec at 74°C). In untreated milk, the "free" B_{12} content is zero, or very low, while in pasteurized milk it is 0.6—0.14 mμg/ml. During pasteurization the milk loses its cyanocobalamin-binding capacity and also a part of the naturally bound vitamin is released. (See Part II).

The results of two typical experiments are shown in Table 1.

Table 1. Total and "free" vitamin B_{12} in pasteurized and unpasteurized cow milk.

Unpasteurized cow milk		Pasteurized cow milk	
Total vitamin B_{12} activity mμg Cy/ml	"Free" vitamin B_{12} activity mμg Cy/ml	Total vitamin B_{12} activity mμg Cy/ml	"Free" vitamin B_{12} activity mμg Cy/ml
6	0.025	5.3	0.56
5.1	0	4.7	0.60

The samples were analyzed also by the bioautographic technique with solvent systems I and II [6] (see Experimental), using *E. coli* 113—3 and *L. leichmannii* 313. In this way, it was shown that the vitamin B_{12} activity was due only to cyanocobalamin.

PART II. CYANOCOBALAMIN-BINDING CAPACITY OF COW MILK. TEMPERATURE AT WHICH THIS PROPERTY IS DESTROYED. EFFECT OF LOW TEMPERATURES ON THE BINDING CAPACITY

Experimental

Determination of cyanocobalamin-binding capacity of pasteurized and unpasteurized cow milk. To four samples of milk from the same batch, 0.25, 1.0, 2.0, and 4.0 mμg cyanocobalamin/ml of milk was added. The samples were kept at 4—7°C for 2 h stirring for 5 min every 30 min. The samples were then ultrafiltered in the usual way, each sample being divided equally between two ultrafilters. In this way, two ultrafiltrates were obtained from each sample and their cyanocobalamin contents were determined separately, the average being taken as the cyanocobalamin content of the sample in question. The cyanocobalamin determination was made using the tube-method with *E. coli* 113—3 and/or *L. leichmannii* 313 A.T.C.C. 7830 as test-organisms. The quantity of cyanocobalamin bound additionally is obtained as the difference between the amount of cyanocobalamin added and that found in the ultrafiltrate. Samples of milk without any added cyanocobalamin were also ultrafiltered to determine the activity of the ultrafiltrate for the test-organism.

Influence of temperature on the cyanocobalamin-binding capacity of cow milk. A 2 l quantity of milk was heated under continuous stirring in a water bath. When the milk reached the desired temperature, a sample was taken. After having kept the milk at the same temperature for a certain time, another sample was taken (Table 4). The milk was then heated until the next temperature was reached and a fresh sample was taken, and so on. The samples were then kept at 4—7°C to permit them to reach a uniform temperature. The binding capacity of the different samples was then determined as above, after adding 2 mμg cyanocobalamin/ml. In the last four samples of assay No 3 taken at 70—85°C, some of the cyanocobalamin naturally present in the milk had been released (contents over the 2 mμg cyanocobalamin added). The remaining part of each sample was kept at —20°C for several days, and then ultrafiltered and analyzed. The results were the same.

Results and discussion

The cyanocobalamin-binding capacity of pasteurized and unpasteurized cow milk was investigated using the ultrafiltration technique (see Experimental). The results show that the pasteurized milk (Table 3) has no binding capacity while the unpasteurized milk (Table 2) does bind cyanocobalamin.

Table 2. Cyanocobalamin-binding capacity of unpasteurized cow milk. The results are given as "coli activity".

Added Cy mμg/ml	Cy found in the ultrafiltrate mμg/ml	Bound Cy mμg/ml	Average
1	0.76	0.24	
2	1.61	0.39	0.29
4	3.77	0.23	

Table 3. Cyanocobalamin-binding capacity of pasteurized cow milk. The results are given as "coli-activity".

Assay No.	Activity of the milk before adding Cy mμg/ml	Cy added mμg/ml	Activity of the ultrafiltrate mμg/ml
1	0.20	0.25	0.5
		1.0	1.11
		2.0	2.10
		4.0	4.10
2	0.28	1.0	1.1
		2.0	2.3
		4.0	4.3
3	0.14	0.25	0.45
		1.0	1.08
		2.0	2.0
		4.0	4.2

This is in agreement with the results of previous investigations in this laboratory performed by Neujahr *et al.*[3] The table shows the average of a large number of determinations using *E. coli* and *L. leichmannii* as test-organisms in the tube method.

The cyanocobalamin-binding capacities of different samples of unpasteurized milk were different, the range being 0.15—0.45 mμg cyanocobalamin/ml. Since the amount of vitamin is small, this variation can be due to the method employed *.

Since pasteurized milk has no binding capacity, the temperature at which this property is destroyed was determined. It was found (Table 4) that, for the conditions under which this work was done (see Experimental), the critical temperature is between 50 and 60°C. Above this temperature the "free" vitamin B_{12} content increases considerably with increasing temperature. This indicates that part of the bound vitamin B_{12} is released by heating over 50—60°C and is thereafter unable to rebind. Hence, this seems to indicate that the substance responsible for the binding of cyanocobalamin in milk is altered by the heating at the moment when the vitamin is released from the complex. To make this conclusion more certain, samples of milk, heated to different temperatures above 50—60°C, were ultrafiltered after they had reached room temperature and also after they had been kept for several days at either 4 or 7°C. It was found that the quantities of vitamin B_{12} in the ultrafiltrates were the same. No recombination of the vitamin took place.

* It it also possible that milk samples from different cows, and even from the same cow on different occasions, have not the same cyanocobalamin-binding capacity. Such possibilities were not especially studied it the present work.

In several assays, the values of which were discarded, a considerable increase in the binding capacity was observed when the amount of cyanocobalamin added was increased. This observation has been made by many authors before, and has been attributed by Gregory [1] to the method of determination of the vitamin.

Table 4. Influence of heating on the cyanocobalamin-binding capacity of cow milk. The results are given as "coli-activity".

Assay No.	Temp °C	Time used to pass from a given temp. to the next. Min	Time kept at given temp. Min	Cy added mμg/ml	Cy found in the ultra- mμg/ml (a)	(b)
	15			2	1.8	
	30	8	5	2	1.8	1.85
	40	6	5	2	(1.35)	(1.32)
1	50	6	5	2	*1.92*	*2.15*
	60	6	5	2	2.32	2.7
	70	7	5	2	2.32	2.52
	80	6	5	2	2.50	2.8
	90	6	5	2	3.2	3.5
	17					
	30	7	5			
	40	6	5	2	1.15	(1.92)
2	50	6	5	2	1.20	1.27
	60	6	5	2	*2.45*	*2.45*
	70	7 1/2	5	2	2.20	2.07
	80	6	5	2	2.37	2.80
	90	7		2	3.90	
	18					
	50	11	0	2	1.77	
	55	1	0	2	*2.15*	
	60	1	0	2	broken	
3	65	1	0	2	2.10	
	70	1	0	2	2.73	
	75	1	0	2	2.45	
	80	1	0	2	(1.97)	
	85	1	0	2	3.90	

(a) Samples were taken at the moment at which the milk reached the given temperature.
(b) Samples were taken after keeping for 5 min at the given temperature.

The effect of a low temperature on the cyanocobalamin-binding capacity of cow milk was also studied. Three assays were made with the same sample: before freezing and after keeping at —20°C for 3 days and 10 days. No differences were found.

PART III. VITAMIN B_{12} ACTIVITY AND CYANOCOBALAMIN-BINDING CAPACITY OF SOUR MILK AND YOGHURT. PRELIMINARY STUDY ON BINDING CAPACITY OF *LACTOBACILLUS BULGARICUS* AND *L. TERMOPHILUS*.

Experimental

1. *Determination of "total" vitamin B_{12} activity.* The samples of both sour milk (pH 4.2—4.4) and yoghurt (pH 4.0—4.05) were diluted with an equal amount of distilled water. The pH was adjusted to 5.5 and 100 mμg KCN/ml of diluted sample was added

before autoclaving for 10 min at 120°C. After cooling to room temperature, the solutions were ultrafiltered as usual and the ultrafiltrates analyzed for vitamin B_{12} by the tube method using *E. coli* 113—3 and/or *L. leichmannii* 313 A.T.C.C. 7830 as test-organisms.

2. *Determination of "free" vitamin B_{12} activity.* The samples were diluted with an equal amount of distilled water, ultrafiltered and the ultrafiltrates were assayed as above.

3. *Determination of cyanocobalamin-binding capacity.* This assay was performed as described for milk (see Part II), the appropriate amount of cyanocobalamin being added to the samples after they had been diluted with an equal amount of distilled water.

4. *Chromatography in order to identify methionine* was done on sheets of Whatman No. 1 paper, using a solvent system composed of methylethylketone (55 %), acetone (25 %) and NH_3 conc. (10 %), for 5—8 h. The papers were, after drying, developed by spraying with 0.5 % ninhydrine in acetone [5].

Results and discussion

Yoghurt and sour milk are obtained by the lactic fermentation of milk. Industrially, the milk is first pasteurized and then inoculated with the appropriate microorganism. The vitamin B_{12} activity of these products and also that of the pasteurized milk from which they were prepared was investigated in the present work. As shown above (see Part II), pasteurized milk does not bind cyanocobalamin. The cow milk used for the production of yoghurt and sour milk is treated more rigorously (85°C for 16 sec) than the commercially pasteurized milk (74°C for 16 sec). Any cyanocobalamin-binding capacity in the soured products must therefore be due to changes occurring during the fermentation. The results are shown in Table 5 and can be summarized as follows:

Sour milk. The total B_{12} activity was 30—40 % lower than that of the milk from which the sour milk was prepared. Thus a large part of the vitamin is used by the *Lactobacillus* during the fermentation. Bioautographs were performed using solvent systems I and II (see Part I) and *E. coli*. Only one spot corresponding to cyanocobalamin was obtained. Furthermore, the agreement between the results obtained in assay No 4 with two different test-organisms also indicates that the activity is due to only one factor.

In all assays, the "free" vitamin B_{12} content was found to be greater than that in the milk from which the sour milk was prepared. Bioautographs were, in addition, performed with the ultrafiltrates in which the "free" vitamin B_{12} had been determined. Also here, only one spot, corresponding to cyanocobalamin, was obtained. As before, the results with two different test-organisms (assay No 4) were the same.

The presence of "free" B_{12} would indicate that sour milk has no binding capacity for this vitamin. This seems to be confirmed, in general, by the assays made to study this property. As shown in Table 5, there seems to exist, in some cases and especially in assay No 2, a certain cyanocobalamin-binding capacity since it should be remembered that the solution already contained a certain amount of "free" vitamin B_{12}. In conclusion, it can be said that sour milk has only a very small, or no, cyanocobalamin-binding capacity.

Yoghurt. As can be seen in Table 5, practically all the vitamin B_{12} of the milk was used during the lactic fermentation. Bioautographs were made, in solvent systems I and II (see Experimental Part I) and using *E. coli* 113—3

Table 5. Cyanocobalamin-binding capacity of sour milk and yoghurt, vitamin B_{12} activity of pasteurized cow milk, sour milk, and yoghurt.

Assay No.	Pasteurized cow milk				Sour milk								Yoghurt									
	"Total vitamin B_{12}" mμg/ml		"Free vitamin B_{12}" mμg/ml		"Total vitamin B_{12}" mμg/ml		"Free vitamin B_{12}" mμg/ml		Added Cy mμg/ml	Cy found in the ultrafiltrate mμg/ml		Bound Cy mμg/ml	"Total vitamin B_{12}" mμg/ml		"Free vitamin B_{12}" mμg/ml		Added Cy mμg/ml	Cy found in the ultrafiltrate mμg/ml			Bound Cy mμg/ml	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)		(a)	(b)	*	(a)	(b)	(a)	(b)		(a)	(b)	Average		Average
1	6.5		0.95		4.7		1.23		2 4 8	3.1 6.7 11.0			1.6		0.52		2 4 8	0.56 0.52 0.52			>2 >4 >8	
2	5.5		0.6		3.25		0.92		2 4 8	2.6 4.0 6.8												
3	4.7		0.56		3.5		1.14		2 4 8	2.46 6.30 9.00			1.25		0.42		160 200 300 400	16 56 140 206			144 144 160 194	160
4	3.6	3.41	0.025	0.067	2.44	2.22	0.6	0.51	2 4 8	2.34 4.16 7.40	2.50 4.54 7.20		0.49	0.10	0.55	0	80 160 200 300	0 3.4 4.9 64.0	0 2.8 2.7 62.0	0 3.1 3.8 63.0	>80 157 196 237	197

(a) The results are given as *E. coli-activity.*
(b) » » » » » *L. leichmanii-activity.*
* See page 744.

as test-organism, with the ultrafiltrates corresponding to the assays of "total" and "free" vitamin B_{12}. Several diffuse spots were obtained in this way, none of them being cyanocobalamin. The different results obtained in the determination of vitamin B_{12} using *E. coli* and *L. leichmannii* as test-organisms in the tube method (assay No 4) also indicate that the activity is due to more than one factor. Hence, a qualitative assay of the ultrafiltrates was made by putting them simultaneously on four agar plates seeded with *E. coli* 113—3, *L. leichmannii* 313 A.T.C.C. 7830, *L. delbrückii* A.T.C.C. 9649, and *Leuconostoc mesenteroides* P. 60 A.T.C.C. 8042 (the first microorganism is sensitive to cyanocobalamin and methionine, the second to cyanocobalamin and desoxyribosides, the third to desoxyribosides and the last one to methionine). This assay showed that the active factors in the ultrafiltrates were mainly methionine and desoxyribosides. Methionine was later also identified by chromatography (see Experimental).

The cyanocobalamin-binding capacity of yoghurt is surprisingly high (Table 5), *viz.* 150—190 mμg/ml.

As mentioned before, yoghurt is prepared from pasteurized cow milk by inoculating with *Lactobacillus bulgaricus* and *L. thermophilus* and fermenting at 45°C. Obviously, the cyanocobalamin-binding capacity of yoghurt appears during this fermentative process and can be due to transformations of the natural constituents of milk and/or can be caused by the substances belonging to the metabolism of the above-named lactobacilli. Hence, preliminary assays were made with suspensions of *L. bulgarius* and of *L. thermophilus* in saline solution. It was found that they are able to bind cyanocobalamin. A further investigation will be carried out on this subject.

PART IV. VITAMIN B_{12} ACTIVITY AND CYANOCOBALAMIN-BINDING CAPACITY OF FOUR DIFFERENT KINDS OF CHEESE. DETERMINATION OF METHIONINE AND DEOXYRIBOSIDES IN THE EXTRACTS USED TO TEST VITAMIN B_{12} CONTENT

Experimental

1. *Determination of total vitamin B_{12} activity.* 200—300 g of cheese was cut into small pieces and mixed, in order to obtain a random sample. Three different methods for the extraction of the vitamin B_{12} were compared:

a) 20 g of cheese was emulsified in a blender for 5 min with 70—75 ml of fresh aqueous emulsifying solution (see below) at 50°C. The volume was adjusted to 100 ml with the same solution. The emulsion was ultrafiltered as usual (see Part I) and the ultrafiltrates diluted and assayed for vitamin B_{12} activity with *Lactobacillus leichmannii* 313 A.T.C.C. 7830 in the tube method. The emulsifying solution had the following composition [4]:

KH_2PO_4	1.36 g
$Na_4P_2O_7$	0.75 g
KCN	0.005 g
H_2O	to 100 ml

b) 20 g of cheese was emulsified as in (a). Before adjusting the volume to 100 ml, KCN was added in order to get a final concentration of 100 μg KCN/ml of emulsion. The emulsion was then autoclaved for 10 min at 120°C, the volume readjusted to 100 ml and the solution ultrafiltered and assayed as before.

c) 20 g of cheese was emulsified as in (a) using, however, glassdistilled water containing 100 μg KCN/ml instead of the emulsifying solution. The volume was adjusted to 100 ml and the pH to 5.5. The emulsion was then autoclaved for 10 min at 120°C, the volume readjusted before ultrafiltering and the ultrafiltrates assayed.

As mentioned before, no appreciable differences in the vitamin B_{12} activity were found when using the methods described. The aqueous extraction (c) was thus adopted.

2. *Determination of "free" vitamin B_{12}.* 20 g of cheese was emulsified in a blender for 5 min with 70—75 ml glass-distilled water at 40—45°C. The emulsion was made up to 100 ml, ultrafiltered and the ultrafiltrate assayed.

3. *Determination of cyanocobalamin-binding capacity.* Emulsions of the cheeses, obtained as described above, were assayed for cyanocobalamin-binding capacity using the ultrafiltration method (see Part II). The activity of the emulsions without addition of cyanocobalamin was also determined after ultrafiltering.

Results and discussion

Several methods for the extraction of vitamin B_{12} from cheese were tested (see Experimental) but no significant difference was found. Consequently, the aqueous extraction method was adopted because of its simplicity.

The determination of the vitamin B_{12} activity was performed by taking the whole of the cheese. The variations in vitamin B_{12} content which may exist in different parts of the same cheese were not tested.

The samples of cheese were extracted (see Experimental) and ultrafiltered. The ultrafiltrates were assayed, using first of all the cup method and *E. coli* 113—3 as test-organism in order to get an approximate idea of the activity. All the four cheeses tested (see below) gave double zones of growth, the inner ring being sharper. This indicates that there was present in the extracts more than one substance which stimulated the growth of *E. coli*. It was thought that the smaller and sharper ring could be due to vitamin B_{12}, and the larger and more diffuse to methionine.

A simultaneous qualitative test was then made using four agar plates, each seeded with a different microorganism (see Part III). The results were as follows:

	Vitamin B_{12}	Methionine	Desoxyribosides
Camembert	+	++	+++
Roquefort	+	++++	+++
Svecia	+	++++	+
Cheddar	+	++++	+

The presence of methionine was confirmed by chromatography and bioautography (see Part III).

The presence of considerable amounts of methionine and desoxyribosides in the extracts made uncertain the use of *E. coli* or *L. leichmannii* for the determination of vitamin B_{12}. It was first necessary to determine these substances quantitatively in order to calculate the appropriate degree of dilution of the extracts required to prevent their interference in the vitamin B_{12} assay. Methionine and desoxyribosides were determined by the tube method using *Leuconostoc mesenteroides* and *Lactobacillus delbrückii*, respectively, as test-organisms. As can be seen in Table 6, all four cheeses have a considerable methionine content, high enough to make the determination of vitamin B_{12}

impossible using *E. coli* as test-organism in the tube method without any special treatment of the extracts.

The amount of desoxyribosides in Cheddar and Svecia is 0.6 — 1.5 μg/g fresh basis. In Camembert and Roquefort the content is 20—40 times higher (*L. delbrückii* activity, calculated as thymine desoxyriboside). Because of the ratio between the quantities of cheese used and the final volume of the extract (see Experimental), the amount of desoxyribosides per ml of extract enabled a direct determination of vitamin B_{12}, using the tube method and *L. leichmannii* as test-organism, provided that the vitamin B_{12} content was not too low, in which case the necessary dilution of the extracts would be impossible.

Preliminary assays were made to determine the approximate vitamin B_{12} activity of the extracts. It was found that, at the dilution necessary in order to prevent the influence of deoxyribosides on the *L. leichmannii* growth, there was sufficient of the vitamin to permit an accurate assay. The results obtained using this microorganism in the tube method are shown in Table 6.

Table 6. Cyanocobalamin, methionine, and desoxyribosides contents of aqueous extracts from four kinds of cheese. The results are given as *L. leichmanii, Leuconostoc mesenteroides* and *L. delbrückii activity*, respectively, each being the average of three different assays.

	Extract from "total vitamin B_{12}" determination			Extract from "free vitamin B_{12}" determination		
	Methionine μg/g fresh basis	Desoxyribosides μg/g fresh basis	Cy mμg/g fresh basis	Methionine μg/g fresh basis	Desoxyribosides μg/g fresh basis	Cy mμg/g fresh basis
Cheddar	1 478	0.75	28	1 635	1.2	1.7
Camembert	390	70	11.5	565	67	*
Roquefort	2 700	42	27	2 515	40	*
Svecia	1 848	1.35	11.3	1 710	1.9	0.8

* The high amount of desoxyribosides present in the extract made impossible the determination of cyanocobalamin by the method used.

An attempt was made to determine whether the vitamin B_{12} is bound or free in the cheese. The samples of cheese were emulsified in a blender with glass-distilled water at 40—45°C, ultrafiltered and assayed for vitamin B_{12}, methionine, and deoxyribosides. As can be seen in Table 6, these last two substances are present more or less in the same amounts as in the ultrafiltrates corresponding to the determination of the total vitamin B_{12} activity. In contrast to this, the "free" vitamin B_{12} content in Cheddar and Svecia is much lower than the values obtained for the total vitamin B_{12}. This indicates that the vitamin is mainly bound in these cheeses. The determination of "free" vitamin B_{12} in Camembert and Roquefort was not possible with the method employed because of the high desoxyriboside concentration.

Table 7. Cyanocobalamin-binding capacity of four kinds of cheese. The results are given as *Lactobacillus leichmannii* activity.

	Natural activity of the ultrafiltrate mμg Cy/ml	Amount of Cy (mμg) added/ml cheese emulsion	Amount of Cy (mμg) found/ml ultra-filtrate
Cheddar	0.3	10	13.7
		20	24.0
Camembert	1.67	10	14.4
		20	23.0
Roquefort	2.5	10	13.0
		20	20.0
Svecia	0.2	10	9.6
		20	23.0

Desoxyribosides were also identified by bioautography in solvent system I and II (see Part I) with *L. delbrückii* as test-organism and thymine desoxyriboside as standard. Further investigations will be made to determine whether the activity detected with L. delbrückii is due only to desoxyribosides.

The cyanocobalamin-binding capacity of the cheeses was also investigated using the ultrafiltration technique (see Experimental).

The mean results obtained are shown in Table 7.

Hence, it can be concluded that none of the cheeses investigated has any cyanocobalamin-binding capacity.

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