SUMMARY

1. A table of the change of weight of various foods during domestic cooking is presented. Loss of weight varied between 13 and 37% for puff pastry, 7 and 33% for grilled fish and 13 and 39% for roast meats. Green leafy vegetables generally gained weight on boiling.

2. A list of the wastage of fruits, vegetables and fish during preparation for the table is given and average values are compared with those in tables of food composition. Unavoidable waste is so great for many commodities that persons handling foods should avoid wastage due to careless preparation.

REFERENCES

Andross, M. (1940). *Chem. & Ind.* **59**, 449.
Andross, M. (1941). *Chem. & Ind.* **60**, 176.
Andross, M. (1946). *Proc. Nutr. Soc.* **4**, 155.
Lowe, B. (1944). *Experimental Cookery*, 3rd ed., p. 251. London: Chapman and Hall.
McCance, R. A. & Shipp, H. L. (1933). *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 187.
McCance, R. A. & Widdowson, E. M. (1942). *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 235.
McCance, R. A., Widdowson, E. M. & Shackleton, L. R. B. (1936). *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 213.
Medical Research Council: Accessory Food Factors Committee (1945). *M.R.C. (War) Memor.* no. 14.
Simpson, J. I. & Halliday, E. G. (1941). *Food Res.* **6**, 189.
World Health Organization (1951). *Tech. Rep. Wld Hlth Org.* no. 45, p. 15.

The Microbiological Assay of 'Vitamin B_{12}' in the Milk of Different Animal Species*

BY MARGARET E. GREGORY

National Institute for Research in Dairying, University of Reading

(*Received 2 April* 1954)

Values for the 'vitamin B_{12}' content of the milk of different species of animal have been published by Collins, Harper, Schreiber & Elvehjem (1951), who used *Lactobacillus leichmannii* ATCC 4797 as the assay organism, and by Sreenivasamurthy, Nambudripad & Iya (1950), who used *Lactobacillus lactis* Dorner. Both groups of workers made their determinations on diluted whole milk. Results obtained at this Institute have shown that for some milks a preliminary treatment was necessary before the 'vitamin B_{12}' was fully available to the assay organism (Gregory, Ford & Kon, 1952).

The assay organisms used by Collins *et al.* (1951) and Sreenivasamurthy *et al.* (1950) are not specific for cyanocobalamin (vitamin B_{12}). Thus *Lb. leichmannii* responds to factor A, pseudovitamin B_{12} and deoxyribosides besides cyanocobalamin (Ford, 1953*a*), and deoxyribosides can replace cyanocobalamin as a growth factor for *Lb. lactis* (Shive, Ravel & Harding, 1948). For this reason, the term 'vitamin B_{12}' is

* This investigation forms part of a thesis for the degree of Ph.D. in the University of Reading.

used throughout this paper. It refers to the total vitamin B_{12} activity measured by the test organisms and may include responses due to other factors besides cyanocobalamin. Recently, Ford (1953*b*) has published a method for assaying cyanocobalamin with the protozoan, *Ochromonas malhamensis*, according to him specific for cyanocobalamin. The results presented in this paper were obtained with this organism, with *Lb. leichmannii* ATCC4797 and with a mutant of *Bacterium coli*. The effect of different extraction methods in the microbiological assay was also investigated.

EXPERIMENTAL

Collection of the milk samples

All the milks used in this study were deep frozen as quickly as possible after collection. The cow's milk was a sample of milk from the Institute's herd of Shorthorn, Friesian and Guernsey cows. Cow's milk was also collected in an aseptic manner straight into sterile glass bottles for the experiments with unheated cow's milk. The goat's milk was a sample of bulk milk from the Institute's herd of British Saanen goats and the sow's milk was a bulked sample from four Large White sows in the 3rd–8th week of lactation, collected as described by Braude, Coates, Henry, Kon, Rowland, Thompson & Walker (1947). Samples of colostrum were taken from one goat 6 h after kidding. The cow's colostrum was collected from Shorthorn and Friesian cows on the 1st and 2nd days after calving.

Rat's milk was collected from stock-colony hooded Norwegian rats at the end of the 3rd week of lactation, using the apparatus of Temple & Kon (1937). The rats were maintained on the stock diet of Folley, Ikin, Kon & Watson (1938) modified by Henry & Kon (1952). The ewe's milk was a single sample from an experimental sheep in mid-lactation and the human milk was a bulk sample of early milk (up to about the 10th day of lactation) collected at a maternity hospital.

Extraction methods

Method 1. *Extraction with cyanide.* The milk sample was diluted with an equal volume of water and 0·1 N-HCl added until the pH was 4·6. One drop of 1% (w/v) NaCN solution was added and the mixture autoclaved for 10 min at 10 lb. pressure. After cooling, the extract at pH 4·6 was filtered and the pH of the filtrate adjusted to 5·5. For assays with *Ochromonas malhamensis* the extract was diluted to contain about 0·2 mμg 'vitamin B_{12}'/ml. This same extract was used for the assay with *Bact. coli* but with the pH adjusted to 6·4. For assay with *Lb. leichmannii* the milk extract was diluted to contain about 0·04 mμg 'vitamin B_{12}'/ml. and the pH was 5·5.

Method 2. *Extraction with cyanide.* The second method consisted of adding 1 ml. of 0·1 M-sodium-acetate buffer at pH 4·6 and one drop of a 1% (w/v) NaCN solution to a 1 ml. sample of the milk under test. The mixture was heated in steam for 30 min, cooled, diluted, made to pH 4·6 and filtered. The pH of the filtrate was adjusted to 5·5 with 0·1 N-NaOH. If necessary a further dilution of the filtrate was made so that the final concentration of 'vitamin B_{12}' in the extract was about 0·04 mμg/ml.

Method 3. *Digestion with papain.* For digestion with papain, 1 ml. milk was mixed with 1 ml. 0·1 M-sodium-acetate buffer (pH 4·6), warmed to 60° in a water-bath and 50 mg papain (British Drug Houses Ltd.) in 1 ml. water were added. The papain was

activated by adding to it one drop of a 1% (w/v) NaCN solution. The mixture was incubated for 1 h at 60°, then steamed for 10 min to inactivate the enzyme, diluted, made to pH 4·6 and filtered. The filtrate, adjusted to pH 5·5, was diluted to contain about 0·04 mμg 'vitamin B_{12}'/ml.

Alkali treatment of the milk extracts

A portion of an ultrafiltrate, or of a clear extract obtained by any of the treatments just described, was brought to pH 11 with 1N-NaOH, and autoclaved at 15 lb. pressure for 15 min. After cooling, the pH was readjusted to 5·5, with 1N-HCl and the extract added to the assay tubes.

Ultrafiltration

A bag of 'Visking' cellulose tubing (The Visking Corporation, Chicago), diameter ¼ in., was suspended from the stem of a glass funnel held in the neck of a filtration tube by means of a rubber bung as shown in Fig. 1. 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. The milk, either whole or diluted with water, was poured into the cellulose bag and the outer tube evacuated and sealed. In about 2 h 2–3 ml. of ultrafiltrate could be collected. It was a clear fluid from which no proteins could be precipitated by trichloroacetic acid.

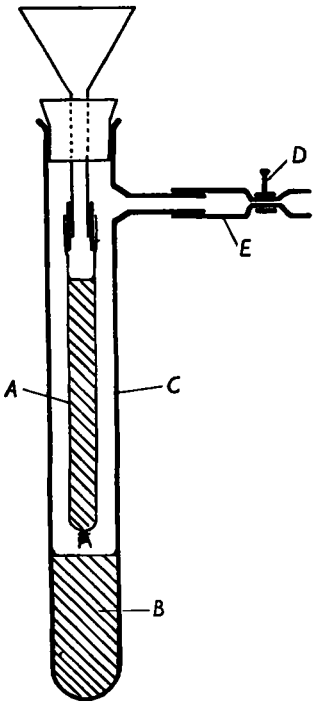

Fig. 1. Apparatus for preparing milk ultrafiltrates. The cellulose bag (*A*), suspended from the funnel, contains the whole milk. The ultrafiltrate (*B*) collects in the outer tube (*C*) when the apparatus is evacuated and sealed by means of a clip (*D*) on a piece of rubber tubing (*E*).

Each of the whole milks was ultrafiltered undiluted (except rat's milk which was diluted 1:10 with water), and the ultrafiltrates were assayed by *Lb. leichmannii* to ascertain whether the 'vitamin B_{12}' was present in the milks in an ultrafiltrable form.

Experiments with unheated cow's milk

The milk, which was collected aseptically from a cow free from mastitis, was diluted 1:100 with sterile distilled water and added unheated aseptically to the sterile assay tubes just before inoculation.

Microbiological methods

1. *Assays with* Lb. leichmannii *ATCC*4797. The method of assay was based on that described by Skeggs, Nepple, Valentik, Huff & Wright (1950). The ribonucleic acids or nucleotides were omitted from their medium and the initial pH of the medium was reduced to 5·5. The modification resulted in better growth under the conditions of assay described here.

The test preparations were added to duplicate optically matched Pyrex test tubes,

19 × 150 mm, at levels of 0·5, 1·0, 2·0 and 4·0 ml. A standard solution of pure cyanocobalamin, containing 0·04 mμg/ml., was added to a duplicate series of tubes at levels of 0·2, 0·5, 1·0, 2·0, 3·0 and 4·0 ml. The volume in all the tubes was made to 5 ml. with distilled water, and 5 ml. of the double-strength basal medium were added to each tube. The baskets of tubes were covered with four layers of towelling and sterilized by heating in steam for 30 min.

The assay organism, *Lb. leichmannii*, was maintained as a stab culture in the single-strength basal medium supplemented with 0·01 mμg cyanocobalamin/ml. and with 2% agar. The culture was transferred monthly into liquid basal medium also containing 0·01 mμg cyanocobalamin/ml., incubated 24 h at 37° and then transferred back into the agar medium as a stab culture. Cultures were stored in the refrigerator.

The inoculum was prepared by transferring the culture from an agar stab into the liquid basal medium supplemented with 0·01 mμg cyanocobalamin/ml. After a 24 h incubation at 37°, 0·5 ml. of the culture was diluted to 50 ml. with sterile physiological saline, and one drop of this suspension added to each assay tube. The baskets of tubes were incubated at 37° for 72 h in a constant temperature water-bath. They were then steamed for 15 min and the growth responses read turbidimetrically in a Lumetron colorimeter. If the extracts were initially turbid, as for example with diluted whole milk, the growth responses were measured by titrating, with 0·1 N-NaOH, the acidity produced in each tube.

Since, over a certain range, the growth of the assay organism was proportional to the amount of cyanocobalamin present in the tubes, the concentration of 'vitamin B_{12}' in the milk extracts was calculated by comparing the growth with these extracts with the growth given by known amounts of cyanocobalamin.

2. *Assays with* Bacterium coli. The method of assay was based on the *Bact. coli* tube assay of Burkholder (1951) as described by Gregory & Holdsworth (1953).

3. *Assays with* Ochromonas malhamensis. The method of assay was as described by Ford (1953*b*).

RESULTS

The 'vitamin B_{12}' content of milk measured by three test organisms. The individual milks were extracted by autoclaving, at pH 4·6, in the presence of sodium cyanide (method 1). The more usual method 2 could not be used because *Ochromonas malhamensis* was inhibited by the presence of acetate in the medium. The extracts were assayed simultaneously in duplicate by *Lb. leichmannii*, *Bact. coli* and *Ochromonas malhamensis*. The mean results for the 'vitamin B_{12}' contents of the milks of different species are presented in Table 1. Since the values obtained with all three test organisms were similar, *Lb. leichmannii* was used, for the sake of convenience, as the test organism in subsequent assays.

Effect of method of extraction. The effect of different preliminary extraction procedures on the amount of 'vitamin B_{12}' measured in the milks by *Lb. leichmannii* is shown in Table 2. Recoveries of cyanocobalamin added before extraction by method 2 were between 85 and 110% for cow's, goat's, rat's and ewe's milk and the colostrum samples, but no recoveries (or responses) were obtained with extracts of sow's and

Table 1. *Mean values* (mμg/ml.) *for the 'vitamin* B_{12}*' content of the milk of several species of animal, measured by* Lb. leichmannii, Bact. coli *and* Ochromonas malhamensis

(The extracts were prepared by method 1.* The results are the mean values from two assays)

Species	*Lb. leichmannii* assay	*Bact. coli* assay	*Ochromonas malhamensis* assay
	Milk		
Cow	2·9	1·8	2·3
Goat	0·9	1·1	0·8
Woman	0·2	0·2	0·2
Sow	0·0	0·0	0·0
Rat	9·6	8·9	8·7
Ewe	10·0	8·2	10·3
	Colostrum		
Cow	3·9	3·2	4·0
Goat	7·1	6·2	6·3

* See p. 341.

Table 2. *Mean values* (mμg/ml.) *for the 'vitamin* B_{12}*' content of the milk of several species of animal, measured by* Lb. leichmannii *after different treatments of the samples*

(The results are the mean values from two assays)

Species	Treatment: Dilution with water	Cyanide extraction (method 2*)	Papain digestion (method 3*)	Alkali treatment
	Milk			
Cow	3·4	3·3	3·2	0
Goat	0·7	0·8	0·7	0
Woman	0	0	0·3	0
Sow	0	0	1·7	0
Rat	15·0	12·0	12·0	0·46
Ewe	8·0	7·0	7·0	0
	Colostrum			
Cow	2·4	2·7	2·9	0
Goat	6·0	5·0	5·0	0

* See p. 341.

human milk. *Lb. leichmannii* responded to all the milks digested with papain, and recoveries of the added vitamin were quantitative. Recoveries of cyanocobalamin added to the samples diluted with water were sometimes greater than 110%, indicating some stimulation of the growth of the assay organism by whole milk. Alkali treatment destroyed all the vitamin B_{12} activity of the milk extracts. The slight response given by the alkali-treated rat's milk may have been due to incomplete inactivation of the 'vitamin B_{12}' since the 'vitamin B_{12}' content of rat's milk is high.

Ultrafiltration experiments. When the ultrafiltrates were diluted before assay to the same extent as the whole milks, no 'vitamin B_{12}' was found. A slight amount of activity could be detected, however, in the undiluted ultrafiltrates but since this activity was stable to treatment with alkali, it was probably due to deoxyribosides. It

seems, therefore, that 'vitamin B_{12}' occurs in a bound form in the milk of the cow, goat, pig, rat, sheep and woman and in colostrum from the cow and goat.

When cow's milk was extracted by methods 2 or 3 before ultrafiltration, from 50 to 75% of the 'vitamin B_{12}' present in the milk could be ultrafiltered.

Determination of 'vitamin B_{12}' in unheated cow's milk. Three samples of raw milk were tested. With two of the diluted samples added directly to the assay tubes *Lb. leichmannii* showed no response, and when 0·02 mμg cyanocobalamin/ml. was added aseptically to one of the samples before assay the response was erratic and only 22–53% of the added vitamin was measured. With the third sample of milk, *Lb. leichmannii* gave a response, but the 'vitamin B_{12}' content calculated from the different assay levels decreased from 2·2 to 0·5 mμg/ml. with increasing amounts of milk added to the assay tubes. The recovery of cyanocobalamin added aseptically to the milk before assay also decreased with increasing amounts of raw milk in the assay tubes. When the milk was heated with the basal medium in the assay tubes before the tubes were inoculated, these effects were not observed. The same 'vitamin B_{12}' content was calculated from each assay level. These findings suggest, therefore, that raw milk contained substances that inhibited the growth of *Lb. leichmannii*.

DISCUSSION

Ochromonas malhamensis gave approximately the same result as the other two assay organisms for the 'vitamin B_{12}' contents of the milks of the different species tested. If any of the other vitamin B_{12}-like compounds had been present in any quantity, the results obtained by the *Ochromonas malhamensis* assay would have been lower than those obtained with *Lb. leichmannii* or *Bact. coli* since *Ochromonas malhamensis* is specific for cyanocobalamin (Ford, 1953*b*). The results, therefore, indicate that the 'vitamin B_{12}' activity of the milks studied is due mainly to cyanocobalamin. Chromatographic evidence confirms this finding for cow's milk, in which Holdsworth (1953) detected by paper chromatography only a trace of factor A accompanying the large cyanocobalamin spot.

Since all three assay organisms gave similar results, *Lb. leichmannii* was used for further studies on the 'vitamin B_{12}' content of milk. It had the advantage over the other two organisms in that it could be used to assay turbid extracts such as whole milk and it did not require shaking during incubation.

The experiments in which cow's milk, collected aseptically, was assayed unheated, proved unsatisfactory. The unheated milk contained inhibitory substances that interfered with the assay. Therefore some preliminary treatment of the milk was necessary in order to obtain reliable results with the microbiological procedures. The simplest method of treating the milks, and the one used by Collins *et al.* (1951), is dilution with water, addition of the diluted whole milks to the assay tubes containing the basal medium and finally sterilization by heating in steam for 30 min. Under the conditions of assay described here, this treatment did not make the cyanocobalamin of either sow's milk or human milk available to the assay organism. However, Collins *et al.* (1951) found an average of 1·05 mμg 'vitamin B_{12}' per ml. sow's milk. The assay of sow's milk was repeated here by the method of Collins *et al.* (1951) using the medium

of Thompson, Dietrich & Elvehjem (1950) and sterilizing the assay tubes by autoclaving at 15 lb. pressure for 3 min. *Lb. leichmannii* was still unable to respond to the 'vitamin B_{12}' in sow's milk. The reason for this discrepancy therefore remains unexplained.

A more usual method for preparing samples for cyanocobalamin assay is treatment with cyanide in order to release the vitamin from bound forms and to convert any hydroxocobalamin into the more stable cyanocobalamin. However, this treatment (method 2) did not release the cyanocobalamin from its bound forms in human and sow's milks. Digestion with cyanide-activated papain or with trypsin was necessary before the cyanocobalamin in these milks could be measured quantitatively. Enzyme digestion did not increase the value obtained for the cyanocobalamin content of milk from the cow, goat, ewe and rat or of colostrum from the cow and goat. Therefore the cyanide treatment was adequate for measuring cyanocobalamin in these milks. Since the clear extracts obtained by this method enabled growth responses to be measured turbidimetrically, it is preferable to using diluted whole milks, especially as these appear to have a slight stimulating effect on *Lb. leichmannii*.

The experiments described in this paper were designed to study the effect of different assay organisms and different extraction methods on the measurement of 'vitamin B_{12}' in milk from different animal species. Therefore only a few samples of milk from each species were examined. Most of the results quoted fall within the range of values found by Collins *et al.* (1951) for the vitamin B_{12} content of milk of various species. The exception was the single sample of ewe's milk which gave a higher value (7·0 mμg/ml.) than that found by these authors (1·0–2·0 mμg/ml.) but a lower value than the one reported by Sreenivasamurthy *et al.* (1950) (14 mμg/ml.). The value for cow's colostrum (2·9 mμg/ml.) is much lower than that reported by Anthony, Couch, Rupel, Henderson & Brown (1951) (28–79 mμg/ml.) and by Collins, Boldt, Elvehjem & Hart (1953) (8–16 mμg/ml.) for the colostrum of Friesian cows. The cyanocobalamin content of rat's milk was within the range found by Meyer, Thompson & Elvehjem (1951).

Gregory *et al.* (1952) reported that the cyanocobalamin naturally present in sow's milk and cow's milk occurred in a bound form, since it could not be ultrafiltered from these milks. The ultrafiltration experiments described in this paper have shown that the vitamin also occurs in a bound form in the milk of the woman, goat, rat and ewe and in colostrum from the cow and goat.

Further details of the work on the bound form of cyanocobalamin in milk will be published separately.

SUMMARY

1. Colostrum of the cow and goat, and milk of the cow, goat, woman, sheep, pig and rat have been assayed for 'vitamin B_{12}' by three different assay organisms (*Lactobacillus leichmannii*, *Bacterium coli* and *Ochromonas malhamensis*). Various methods for preparing the milk samples for assay were compared.

2. The vitamin B_{12} activity of the milks was due almost entirely to cyanocobalamin. In all the species of milk tested the cyanocobalamin was present in a bound form.

The author wishes to express her thanks to Miss O. Digby for her technical assistance, to Dr D. Morris and the staff of the British Hospital for Mothers and Babies, Woolwich, for the samples of human milk, and to all members of the N.I.R.D. staff who have helped in the collection of milk samples. Thanks are especially due to Dr J. E. Ford, Dr E. S. Holdsworth and Dr S. K. Kon for their helpful advice and criticism.

REFERENCES

Anthony, W. B., Couch, J. R., Rupel, I. W., Henderson, M. B. & Brown, C. (1951). *J. Dairy Sci.* **34**, 749.
Braude, R., Coates, M. E., Henry, K. M., Kon, S. K., Rowland, S. J., Thompson, S. Y. & Walker, D. M. (1947). *Brit. J. Nutr.* **1**, 64.
Burkholder, P. R. (1951). *Science*, **114**, 459.
Collins, R. A., Boldt, R. E., Elvehjem, C. A. & Hart, E. B. (1953). *J. Dairy Sci.* **36**, 24.
Collins, R. A., Harper, A. E., Schreiber, M. & Elvehjem, C. A. (1951). *J. Nutr.* **43**, 313.
Folley, S. J., Ikin, E. W., Kon, S. K. & Watson, H. M. S. (1938). *Biochem. J.* **32**, 1988.
Ford, J. E. (1953*a*). *Nature, Lond.*, **171**, 148.
Ford, J. E. (1953*b*). *Brit. J. Nutr.* **7**, 299.
Gregory, M. E., Ford, J. E. & Kon, S. K. (1952). *Biochem. J.* **51**, xxix.
Gregory, M. E. & Holdsworth, E. S. (1953). *Biochem. J.* **55**, 830.
Henry, K. M. & Kon, S. K. (1952). *J. Dairy Res.* **19**, 216.
Holdsworth, E. S. (1953). Unpublished.
Meyer, M. L., Thompson, H. T. & Elvehjem, C. A. (1951). *J. Nutr.* **45**, 551.
Shive, W., Ravel, J. M. & Harding, W. M. (1948). *J. biol. Chem.* **176**, 991.
Skeggs, H. R., Nepple, H. M., Valentik, K. A., Huff, J. W. & Wright, L. D. (1950). *J. biol. Chem.* **184**, 211.
Sreenivasamurthy, V., Nambudripad, V. K. N. & Iya, K. K. (1950). *Indian J. Dairy Res.* **3**, 179.
Temple, P. L. & Kon, S. K. (1937). *Biochem. J.* **31**, 2197.
Thompson, H. T., Dietrich, L. S. & Elvehjem, C. A. (1950). *J. biol. Chem.* **184**, 175.