The total content of vitamin D in human milk and cow's milk

BY E. LEERBECK AND H. SØNDERGAARD
National Food Institute, 19 Mørkhøj Bygade, 2860 Søborg, Denmark

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1. It was shown that the water-soluble fraction of cow's milk and human milk did not possess significant antirachitic activity on rats.
2. Whole cow's milk was found to contain 38 i.u. vitamin D/l. Whole human milk contained 15 i.u. vitamin D/l, of which 12 i.u. derived from the lipid fraction.
3. Thus the values found were much lower than the 204 i.u./l in cow's milk and 950 i.u./l in human milk given in the literature based on chemical determination of the sterol sulphate content.

The content of vitamin D in cow's milk for human consumption is given in different tables of nutrient values to be approximately 5–35 i.u./l (Ege, 1970; Souci *et al.* 1973; Helms, 1978; Paul & Southgate, 1978), and accordingly milk is not considered to be a significant source of this vitamin. In human milk the content has likewise been found to be fairly small. Harris & Bunker (1939) thus found a content of 4·2 i.u./l. Contents of vitamin D of a magnitude of 10 i.u./l have been reported by Polskin *et al.* (1945) and Escudero *et al.* (1947), while Hartman & Dryden (1965) give the value 22 i.u./l as the mean of eight values from non-specified references.

These values must be assumed to correspond to the amount contained in the lipid fraction of milk. However, Sahashi *et al.* (1967) have isolated water-soluble vitamin D from cow's milk and from human milk in quantities corresponding to much higher values, i.e. in cow's milk 204 i.u./l in addition to 36 i.u./l in fat-soluble form, while in human milk the concentration corresponded to 950 and 15·7 i.u./l. In a subsequent work (Sahashi *et al.* 1969) the authors have determined the biological activity.

Le Boulch *et al.* (1974) have likewise isolated and, by means of methylene blue reaction, approximately determined water-soluble vitamin D (cholecalciferol sulphate) in cow's milk and human milk. In cow's milk they found an amount of cholecalciferol sulphate corresponding to 4·4 μg cholecalciferol/l and in the lipid fraction an amount of cholecalciferol and 25-hydroxycholecalciferol corresponding to 71 i.u./l. In human milk they found 10 μg cholecalciferol as sulphate and in the lipid fraction 69 i.u./l. These authors state that if the water-soluble form of vitamin D is biologically active, whole milk must have a greater antirachitic effect than hitherto assumed.

Using the chemical method devised by Sahashi *et al.* (1967) Lakdawala & Widdowson (1977) have determined vitamin D sulphate in human milk and found from 10·0 to 17·8 μg/l. Values from 4·5 to 10·5 μg vitamin D sulphate/l obtained by chemical determination have been reported by the Department of Health and Social Security (1977).

These results must give rise to considerations about the correctness of the statements made hitherto with regard to the content in milk of the vitamin in question. They will be of special importance to the evaluation of apparently suboptimal intakes of vitamin D. O'Hara-May & Widdowson (1976) found, in an examination of two groups of Asian boys of which one group showed signs of rickets while the other did not, that the calculated vitamin D intake is slightly less than 40 i.u./boy per d when the content of water-soluble cholecalciferol is excluded and 140 i.u./boy per d when it is included. In relation to the intake of vitamin D by breast-fed Asian infants doubts have been expressed regarding the value of water-soluble vitamin D. Porter (1978) considered that the biological activity of vitamin D sulphate had not

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been demonstrated but that it would explain the fact that breast-fed babies seldom develop symptoms of rickets.

Consequently we have attempted to determine the vitamin D activity of cow's milk and human milk experimentally in order to ascertain whether the so-called water-soluble vitamin D possesses significant activity. As regards both categories of milk the examination comprised milk as received and milk which we subjected to various treatments.

MATERIALS AND METHODS

Whole milk (35 g fat/l) and skim milk (1 g fat/l) were bought from retail dealers. The samples in Expts K2, K6 and K7, respectively, were bought in the months November, February and March. The samples of human milk were obtained in the months November and March (Expts H1 and H2, respectively) and were reported to have been collected during the period from the fifth to the eighth day after parturition and to derive from normal women with a normal intake of vitamin D. The content of fat was determined by a standard procedure (Association of Official Analytical Chemists, 1975). The two samples of human milk examined were found to contain 34 g fat/l each.

Determination of the vitamin D content in the samples was made using a biological assay according to Pharmacopoea Nordica (1964) using eight to ten rats/dose. According to this method the response to the treatment is determined by taking X-ray photographs of the proximal ends of the tibia and is designated by the values 1–12 (Bourdillon's scale). The supplements of vitamin D (standard and sample) were fed separately. In Expts K1 and K2 the whole-milk sample was fed in the form in which it was purchased, while for the remaining experiments with cow's milk pretreated samples were fed. Pretreatment of the milk was carried out according to the following procedures:

The unsaponifiable residue was prepared by saponification of the sample with ethanolic potassium hydroxide (150 g/l) for 30 min and subsequent extraction with peroxide-free diethyl ether. The diethyl ether was evaporated in vacuum.

In Expt K3 the unsaponifiable residue from skim milk was given dissolved in vegetable oil. In Expts K4 and K5 the skim milk was given in the form of powder obtained by freeze-drying (1 g powder corresponded to 10·4 ml milk and was found, by analysis, to contain (g/kg): 6·9 fat, 10·6 phosphorus). In the Expts K4 and K5 the animals given doses of standard vitamin D received compensatory supplements of phosphate corresponding to the amounts of P contained in the samples administered to the animals that were given doses of the samples.

In Expts K6 and K7 the pretreatment of 7 l whole milk involved the preparation of a fraction corresponding to that isolated by Le Boulch *et al.* (1974): 1 l milk was concentrated to 300 ml by evaporation under reduced pressure and 900 ml ethanol was added. The mixture was filtered and the filtrate was evaporated to dryness. 1 g powder corresponded to 21·4 ml milk and contained (g/kg) 30 fat, 2·3 P. The fat was determined by a standard procedure (Schweizerische Lebensmittelkommission, 1967).

The first sample of human milk (Expt H1) was examined without pretreatment. The second sample (Expt H2) was given untreated as well as after fractionation. The lipid fraction and the unsaponifiable residue from the water-soluble vitamin D sulphate fraction were obtained from the fractionated milk as described by Sahashi *et al.* (1967) with the exception that the unsaponifiable residue of the vitamin D sulphate fraction was dissolved in vegetable oil.

Because of the low content of P in human milk it was not considered necessary to give compensatory supplements of P with the standard vitamin D doses.

Table 1. *Vitamin D content of cow's milk, determined by biological assay*

Expt	Test dose		Extent of healing (mean value)	Vitamin D content (i.u./l)
K1	Standard* (i.u./d)	0·25	8·1	
		0·10	3·2	
	Whole milk (ml/d)	1·5	1·0	Not calculated
	Negative control	0	1·0	
K2	Standard (i.u./d)	0·25	8·2	
		0·10	3·5	
	Whole milk (ml/d)	8·0	10·0	} 38
		4·0	4·4	
		2·0	2·3	
	Negative control	0	2·0	
K3	Standard (i.u./d)	0·12	7·9	
		0·06	3·4	
	Solution in vegetable oil of the unsaponifiable residue of skim milk (mg/d)†	60	1·1	Not calculated
		30	1·4	
	Negative control	0	1·0	
K4	Standard (i.u./d)	0·08	10·3	
		0·04	9·4	
	Freeze-dried skim milk (g/d)‡	1·0	9·4	} 4 or less
		0·5	6·6	
	Negative control	0	2·0	
K5	Standard (i.u./d)	0·04	8·8	
		0·02	7·6	
	Freeze-dried skim milk (g/d)‡	0·8	6·3	} *c.*1·5
		0·4	5·8	
	Negative control	0	3·0	
K6	Standard (i.u./d)	0·10	10·3	
		0·04	6·1	
	Powder fraction of whole milk (g/d)§	1·0	11·5	
		0·5	11·0	10·9
	Negative control	0	2·0	
K7	Standard (i.u./d)	0·12	10·6	
		0·06	5·7	
	Powder fraction of whole milk (g/d)§	0·30	3·9	7·1
		0·15	2·7	
	Negative control	0	1·3	

* WHO International Standard.
† 50 g of the solution in oil corresponded to 460 ml milk.
‡ 1 g powder corresponded to 10·4 ml milk.
§ Prepared according to Le Boulch *et al.* (1974) (for details, see page 8); 1 g powder fraction corresponded to 21·4 ml milk.

RESULTS

Cow's milk. In Expt K1 the level of whole-milk dose was based on the assumption that the vitamin D content was 100–200 i.u./l, but no curative effect was observed in the experimental animals compared with negative controls, so that a quantitative result was not obtained (Table 1).

In Expt K2 doses were increased, resulting in values for the extent of healing that made calculation of antirachitic activity possible. Antirachitic activity corresponded to 38 i.u.

Table 2. *Vitamin D content of human milk, determined by biological assay*

Expt	Test dose		Extent of healing (mean) value)	Vitamin D content (i.u./l)
H1	Standard* (i.u./d)	0·16	11·3	
		0·08	8·6	
	Human milk, whole (ml/d)	0·8	2·5	About 20
		0·4	1·7	Not calculated
		0·2	1·7	
		0·1	1·9	
	Negative control	0	2·0	
	Standard (i.u./d)	0·12	7·8	
		0·06	2·7	
	Human milk, whole (ml/d)	3·4	1·4	Not calculated
	Negative control	0	1·5	
H2	Standard (i.u./d)	0·12	8·1	
		0·06	3·3	
	Human milk:			
	Whole (ml/d)	5·0	4·9	15
	Fractionated†			
	Lipid fraction (mg/d)	200	4·1	12
	Sulphate fraction (mg/d)	10	2·4	Not calculated
		270	2·8	
	Negative control	0	2·5	

* WHO International Standard.

† Prepared according to Sahashi *et al.* (1967); 200 mg lipid fraction corresponded to 5·75 ml milk. The quantities of sulphate fraction refer to unsaponifiable residue from 740 ml milk dissolved in 40 g oil.

vitamin D/l, slightly more than the amounts of vitamin D normally stated for the lipid fraction of milk. However, the higher vitamin D activity was not, or not exclusively, due to the occurrence of vitamin D in the aqueous phase of milk, because the P content in milk has an antirachitic effect for which no compensation was made.

In order to assess vitamin D activity in the water-soluble fraction of cow's milk an experiment using skim milk was carried out (Expt K3).

As it was not possible to administer sufficiently large doses to the animals, the milk was saponified using alcoholic potassium hydroxide to release vitamin D from any vitamin D sulphate present. The vitamin D released was then extracted by diethyl ether which in turn was evaporated in vacuum. The residue was dissolved in vegetable oil. Values for extent of healing were far below the standard range, and antirachitic activity could not be calculated.

In Expts K4 and K5, in which skim milk was administered as a freeze-dried powder, a value of not more than 4 i.u. vitamin D/l was obtained from two determinations which did not suggest the presence of considerable amounts of water-soluble vitamin D.

Finally, in Expts K6 and K7 an attempt was made to demonstrate vitamin D activity in the whole-milk fraction prepared according to Le Boulch *et al.* (1974). From two determinations values obtained for vitamin D activities were approximately 11 and 7 i.u./l respectively, i.e. a mean value of 9 i.u./l or 0·23 μg cholecalciferol/l. It was not possible to demonstrate any activity that corresponded, even approximately, to that of 4·4 μg cholecalciferol sulphate/l found by Le Boulch *et al.* (1974).

Human milk. In Expt H1 (Table 2) the milk doses were chosen on the assumption that the milk did contain a large vitamin D activity. If, for example, the extent of healing of the animals given 0·4 and 0·2 ml milk were of the same magnitude as that of the animals given

0·16 and 0·08 i.u., respectively, the calculated content of vitamin D activity would be 400 i.u./l. However, in the actual assay the extent of healing observed corresponded to only about 0·016 i.u. for the highest dose (0·8 ml milk) and consequently only an approximate value of 20 i.u. vitamin D/l could be estimated. From the same sample a greater dose (3·4 ml/d) was also given, but in this instance the extent of healing was so low that it was impossible to obtain a definite value.

In Expt H2 administration of 5 ml milk gave a vitamin D activity corresponding to 15 i.u./l. The lipid fraction showed a vitamin D activity corresponding to 12 i.u./l, while the sulphate fraction showed very little curative effect even when a large dose was given, thus a value for vitamin D could not be obtained.

The accuracy at our bioassays may be stated to approximately ± 25 % in the normal cases where at least two standard doses and two test doses are supplied and the responses for the high doses (standard and test) are of the same magnitude and likewise for the low doses. In Expt K2 the fiducial limits ($P < 0{\cdot}05$) have been calculated to 82–123 % of the found value. However, in Expts K4 and K5 the conditions for the usual statistical treatment of the data were not fulfilled and the fiducial limits were not calculated according to the method. In the cases where the response from only one test dose was available the accuracy has not been calculated.

DISCUSSION

The results reported by Sahashi *et al.* (1967), Le Boulch *et al.* (1974), Lakdawala & Widdowson (1977) and the Department of Health and Social Security (1977) have by other authors been assumed to demonstrate that cow's milk and human milk contain an amount of active vitamin D which is around ten times higher than that obtained previously (Jelliffe & Jelliffe, 1978; Brostrøm, 1978). However, Lakdawala & Widdowson (1977) were most cautious in inferring that the aqueous material had antirachitic activity.

We have found that, in rats, neither cow's milk nor human milk has a significantly higher antirachitic activity than the lipid fraction. Cholecalciferol sulphate isolated by chromatography in the previously mentioned studies was found in greater amounts than that corresponding to the antirachitic activity found by us, therefore we must assume that the greater part must have been present in a form biologically inactive to rats, if the substance isolated by Sahashi *et al.* (1967) and Le Boulch *et al.* (1974) in fact was vitamin D sulphate. We were unable to release vitamin D by saponification of the aqueous phase of milk which should have been expected according to the chemical properties of sterol sulphates. The finding of cholecalciferol sulphate in milk (Sahashi *et al.* 1967; Le Boulch *et al.* 1974) could be because of less specific methods. Thus Hollis *et al.* (1979) by means of HPLC to a detection limit of 1 μg/l failed to detect vitamin D sulphate in either human milk or bovine milk.

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