Brit. J. Nutr. (1962), **16**, 65, *with* 1 *plate*

Studies on human lactation. Dietary nitrogen utilization during lactation, and distribution of nitrogen in mother's milk

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(*Received* 24 *April* 1961—*Revised* 30 *October* 1961)

Reports on correlations between dietary protein and the concentration of protein in human milk are contradictory (Gunther & Stanier, 1951; Kryzhanovskaya, 1953; DeLuca & Caruso, 1955; Gopalan, 1958). A few investigators have reported a favourable effect of dietary protein on the milk protein concentrations but no relation between the two has been observed by others. Sometimes, it has been asserted that the composition of milk is independent of the nutritional state of the mother (Walker, 1954; Gunasekara & Wijesinha, 1956).

In most of the studies referred to above, no distinction was made amongst the various components making up milk protein. These have been investigated by Lutz & Platt (1958). A few years earlier, a difference in the behaviour of casein and non-casein protein fractions of milk in the infant animal's stomach was observed (Platt, 1954). In view of this observation and considering the fact that in human milk the percentage of non-casein (whey) proteins is relatively high, we thought that the non-casein part of the protein might have some special physiological significance.

The investigation described here was planned to study the utilization of dietary protein nitrogen with special reference to the distribution of N in the milk of mothers on low-protein diets as well as after supplementation of such diets with protein of high biological value.

The major findings of this paper were presented at the Fifth International Congress on Nutrition (Cama & Deb, 1960).

EXPERIMENTAL

Selection of subjects. The subjects were lactating mothers in the poor socio-economic section of society in and around the city of Bangalore, the period of lactation varying from 2 to 10 months *post partum*. All consumed the same type of diet, with little variety throughout the period of study. Rice was the staple, with various pulses, a few vegetables, a small quantity of leafy vegetables and a meagre supply of milk. The blood haemoglobin levels were generally low and the blood protein concentrations were within the normal range. No sign of any acute vitamin deficiency was exhibited by any subject. For prolonged metabolic studies, twenty subjects were selected who were as similar as possible to one another in respect of age, height, weight and diet.

Dietary survey. The dietary surveys were carried out by the questionnaire method (Pasricha, 1958). The customs, economic conditions and dietetic habits of the subjects in this study were such that the composition of the dietary intake, particularly with respect to protein, remained fairly constant, which was verified in many subjects by duplicate dietary surveys conducted within a period of 3 months.

Protein supplementation. Dry casein in the form of sweetened biscuits was used to supplement the diets of the mothers for a period of 3 weeks or more at a time. Granules were made of a mixture of 85% casein, 14% sugar, 1% hydrogenated fat. These granules were pressed into biscuits each weighing approximately 5·7 g, which is equivalent to 5·0 g casein. The amount of supplementary protein was evaluated from the number of biscuits consumed.

Sampling of milk. Samples for analyses were collected by manual expression of almost equal quantities of milk from both breasts 4–5 h after the first feed of the infant in the morning. All mothers were allowed their usual morning meal 3 h before the time of collection. This procedure of obtaining milk samples has been used by earlier workers (Kon & Mawson, 1950; Gunther & Stanier, 1951; Belavady & Gopalan, 1959). The samples were brought refrigerated to the laboratory and were immediately analysed.

Analysis of milk. The total daily milk yield was determined for each subject by test weighing of the baby before and after each feed. Total N was measured by the micro-Kjeldahl method. Non-protein N (NPN) was measured in a sample of a 10% tungstate filtrate of milk. Lactose and fat were determined by the method quoted by Hawk, Oser & Summerson (1949).

The micro-Kjeldahl method was also used for N determination in urine, faeces and blood.

Fractionation of the proteins. Casein N and non-casein N were determined by the isoelectric precipitation method (Rowland, 1938), slightly modified as follows. Milk was diluted four times with distilled water, the pH was adjusted to 4·6 with dilute acetic acid and the solution was centrifuged after incubation at 38° for 20 min. A distinct separation of fat on the top and casein sediment at the bottom with a clear translucent whey serum between the two layers was thus obtained. After removal of the fat layer, the clear whey serum was carefully decanted and filtered through a fluted filter-paper. N was measured in a portion of this filtrate (milk whey serum) by the micro-Kjeldahl method. The following calculation was then used: If total N = X, total NPN (estimated in the tungstate filtrate) = Y and total milk whey serum N (after removal of casein) = Z, then

$$\text{total protein N} = X - Y,$$
$$\text{total casein (curd) N} = X - Z,$$
$$\text{total non-casein (whey) N} = Z - Y.$$

Electrophoretic separation of proteins. The agar electrophoretic technique (Giri, 1956) was used for the resolution of milk and blood proteins

About 30 μl of breast milk were slowly and gradually placed on a small strip of filter-paper 2·0 cm × 0·2 cm and the whole amount was then allowed to dry on the

strip before an electric fan. The dried strip was then slowly and repeatedly stirred in cold diethyl ether with three changes of ether during the process. The strip was then dried again.

The casein sediment obtained in the fractionation of milk proteins mentioned above was washed twice with water (made to pH 4·6 with a drop or two of dilute acetic acid). The casein thus obtained was dissolved in 0·01 N-NaOH so as to give a solution with a protein concentration of about 5%. About 10 μl of the casein solution were allowed to dry on a small strip of filter-paper, as with whole milk. Similarly, paper strips containing 60 μl of milk whey serum and about 10 μl of a clear blood serum from the mother were prepared. The strips were then placed on separate glass plates containing a thin sheet of 1% agar gel made up in the usual manner with veronal buffer of pH 8·6. The electrophoresis was carried out at 300, 275 and 250 V, consecutively, for 1·5 h at each voltage, as it assisted separation of the proteins from the place of origin. The mean current strength per plate was 6 mA, with veronal-HCl buffer of pH 8·6 and ionic strength 0·05. The plates were dried and stained with amido-black 10B (E. Merck and Co., Germany) and the stained bands were scanned in a photovolt densitometer (Photovolt Corporation, U.S.A.). The relative concentrations of the electrophoretically separated protein bands were calculated on the assumption of proportionality between the densitometer areas and the dye-binding capacity of the various protein bands.

RESULTS AND DISCUSSION

Dietary intake and initial composition of milk. The dietary intakes of the subjects are summarized in Table 1. It is evident that by comparison with the recommended allowances, the energy and protein intakes were strikingly poor.

Table 1. *Mean daily dietary intakes of twenty lactating Indian mothers compared with recommended allowances*

	Indian mothers		Recommended allowance	
Nutrient	This study (value and range)	Gopalan & Belavady (1960)	India*	U.S.A.†
Fat (g)	35 (26–45)	—	—	—
Carbohydrate (g)	342 (290–370)	—	—	—
Protein (g)	41 (34–50)	43	112	98
Energy (kcal)	1850 (1700–2025)	1860	2700	3300

* Indian Council of Medical Research: Nutrition Advisory Committee (Aykroyd, Patwardhan & Ranganathan, 1956).

† National Research Council (1958).

Table 2 shows the composition of the milk of the mothers, compared with values reported in the U.S.A., U.K. and Africa. It will be seen that the fat and total protein contents in the milk of the Indian mothers studied by us were low in comparison.

Table 2. *Composition of human milk*

Constituent	Indian* (this study)	American (Macy, 1949)	British (Kon & Mawson, 1950)	African Bantu (Walker, 1954)
Fat (g/100 ml)	2·99 (2·4–3·8)	4·5	4·78	3·90
Lactose (g/100 ml)	6·88 (6·8–7·1)	6·43	6·95	7·10
Protein (g/100 ml)	0·88 (0·70–1·18)	1·06	1·16	1·35
Non-protein nitrogen (mg/100 ml)	37·0 (30–55)	35	—	—

* Value and range.

Effect of protein supplement on N balance and milk composition. Because of the individual differences in the composition of milk, the results are given of longitudinal studies on single subjects so that the effects, if any, of the dietary supplement can be more clearly evaluated (Tables 3 and 4). The results were similar in all subjects.

Table 3 shows the effect of protein supplementation on the composition of milk and on the distribution of N in milk, urine, and faeces. With the initial low intake of N, the total N excreted in the urine and faeces was less than the intake, but if the N output in milk is also taken into consideration, the total excretion was more than the intake. It is reasonable to assume that the subjects were in marginal equilibrium at the initial N intake. The weights of the subjects did not decrease, and on supplementation there was no appreciable increase. An increase in the protein intake did not lead to an increase in the total N content of the milk, although on account of the low initial content of total N with this subject it was expected that there would be such an increase. The yield of milk also was not increased. There was, however, a change in the fat content of the milk, a finding which is in agreement with those of other authors (Macy, 1949).

Table 4 shows that supplementation of the diets with casein brought about a striking change in the relative proportions of non-casein and casein fractions of the milk of six typical subjects. In the milk of average or normally fed mothers in the West, the whey : curd ratio has been reported to be 1·5 (Beach, Bernstein, Hoffman, Teague & Macy, 1941). The mean value found by us for all those mothers on low-protein diets was approximately 1·15, which indicates a very low level of the whey protein fraction. Table 4 also shows that this ratio changed to about 1·50 or more as the mother's diet was supplemented with large quantities of good-quality protein. When the casein supplement was withdrawn the ratio in the milk tended to revert to its original value, as observed with all six subjects.

Another relevant observation was that the albumin concentration in the blood serum of the mother rose and fell concomitantly with the high or low protein content of her diet (Table 4). The significance of these two observations is discussed later.

Electrophoretic pattern of the proteins of human milk. Heyndrickx & De Vleeschauwer (1952), applying moving boundary electrophoresis to dialysed and centrifuged milk samples, reported the presence of six or seven fractions of milk proteins, whereas Bosticco & Ubertalle (1954) and DeLuca & Cutroneo (1955), using paper electro-

Table 3. *Effect of protein supplementation of the diet on the composition of milk and the concentrations of nitrogen in urine and faeces of one Indian mother*

Protein intake (g/day)	Weight of mother (lb)	Period of lactation (days *post partum*)	Yield of milk (ml/day)	Composition of milk				Urinary N (g/day)	Faecal N (g/day)
				Fat (g/100 ml)	Lactose (g/100 ml)	Total protein N (mg/100 ml)	Non-protein N (mg/100 ml)		
46 (45–47)	99	48–109	588 (576–600)	2·94 (2·5–3·4)	6·85 (6·8–6·9)	137·5 (125–160)	36·5 (35–40)	4·75 (4·5–5·0)	2·30 (2·0–2·6)
60	99	110–132	596 (588–600)	3·66 (3·5–3·8)	6·87 (6·8–6·9)	132·0 (128–138)	40	7·06 (6·8–7·4)	2·20 (1·8–2·5)
46 (45–47)	100	133–174	600	3·06 (2·8–3·3)	6·88 (6·8–6·9)	128·6 (125–136)	36 (30–42)	4·65 (4·5–4·8)	2·45 (2·3–2·6)
70	100	175–195	580 (576–588)	3·56 (3·4–3·7)	6·96 (6·9–7·1)	131·0 (128–137)	40 (38–40)	8·46 (7·8–9·0)	2·36 (2·0–2·9)
46 (45–47)	100	196–240	595	3·02 (2·8–3·5)	6·89 (6·8–7·0)	134·5 (125–145)	32·5 (30–35)	4·80	2·30

Table 4. *Variation in the composition of the milk and blood proteins of Indian mothers with different levels of protein intake*

Subject no.	Weight of mother (lb)	Period of lactation (days *post partum*)	Protein intake (g/day) Total	From casein supplement	Period of supplementation (weeks)	Yield of milk (ml/day)	Content in milk (mg/100 ml) Total N	Casein N (curd)	Non-casein N (whey)	Ratio, whey: curd	Fraction I of milk* (%)	Content in blood (g/100 ml) Total protein	Albumin	Globulin
1	99	40–109	45	0	—	588	180	65	75	1·16	3·30	6·98	3·66	3·32
	99	110–132	60	15	3	596	172	54	85	1·57	7·64	7·10	3·85	3·15
	100	133–174	45	0	—	600	168	62	70	1·13	3·05	7·00	3·60	3·40
	100	175–195	70	25	3	582	171	54	86	1·59	7·87	7·20	3·90	3·30
2	105	30– 75	40	0	—	526	180	66	74	1·13	3·30	6·65	3·28	3·37
	105	76– 98	60	20	3	515	190	56	88	1·56	7·60	6·74	3·40	3·34
	106	99–164	40	0	—	525	185	65	75	1·16	3·60	6·70	3·30	3·40
	105	165–196	70	30	4	530	188	55	85	1·55	7·50	6·80	3·52	3·28
3	107	39– 88	35	0	—	500	166	61	72	1·18	3·85	7·00	3·50	3·50
	107	89–122	60	25	5	522	153	46	74	1·59	7·90	7·05	3·65	3·40
	107	123–160	35	0	—	540	150	54	66	1·21	4·10	7·00	3·54	3·46
	107	161–183	75	40	3	540	147	45	74	1·61	8·16	7·00	3·68	3·32
	107	184–218	35	0	—	540	150	55	65	1·19	3·90	7·00	3·55	3·45
	107	219–260	60	25	6	525	150	46	70	1·52	7·20	7·15	3·72	3·45
4	106	34– 62	40	0	—	390	195	66	82	1·24	4·48	6·98	3·60	3·38
	106	63– 89	60	20	4	435	177	54	85	1·58	7·80	7·05	3·72	3·33
	107	90–126	40	0	—	420	184	68	80	1·18	3·80	7·00	3·65	3·35
	107	127–150	60	20	4·5	435	176	54	84	1·57	7·67	7·10	3·78	3·32
5	100	65–100	40	0	—	435	157	58	68	1·19	3·93	6·18	3·25	2·93
	100	101–119	60	20	2·5	450	150	45	70	1·53	7·30	6·30	3·45	2·85
	100	120–186	40	0	—	450	150	54	66	1·22	4·17	6·25	3·37	2·88
	100	187–220	60	20	5	435	155	48	75	1·56	7·60	6·40	3·50	2·90
6	99	34– 69	40	0	—	410	162	60	72	1·20	4·00	6·66	3·39	3·27
	99	70– 90	60	20	3	410	172	54	83	1·55	7·55	6·80	3·52	3·28
	98	91–134	40	0	—	400	175	64	76	1·18	3·80	6·80	3·40	3·40
	99	135–180	60	20	6·5	415	165	50	80	1·60	8·00	6·85	3·60	3·25

* See p. 71.

phoresis, found three peaks in the milk. By the paper-electrophoresis method, casein, lactoglobulin and immuno-globulin fractions of milk were identified by Sumtsov (1956), whereas Gügler, Bokelmann, Dätwyler & Muralt (1958) identified only two fractions, of which one was antigenically similar to blood serum albumin and the other corresponded to the serum globulin.

The agar electrophoretic pattern of human milk and the resulting densitometric curve are shown in Pl. 1 *a*, *b*. For comparison, the electrophoretic patterns of the total milk proteins and of milk casein (Pl. 1 *c*, *d*) were obtained on the same plate and under identical experimental conditions. Similarly, the proteins from the milk whey serum and milk casein have been differentiated in Pl. 1 *e*, *f* and the comparison of the proteins from the mother's blood serum and milk is illustrated in Pl. 1 *g*, *h*.

As evident from Pl. 1, good resolution of the milk proteins was obtained by the agar electrophoretic method, and five fractions can be distinctly observed. These five peaks in Pl. 1 *b* are designated fractions I, II, III, IV and V in the order of decreasing mobility.

From Pl. 1 *c*, *d* and Pl. 1 *e*, *f* it is clearly seen that fractions I, II, III and V are those of the whey proteins and fraction IV is that of casein. It may be that fractions I, II, III and V are those of the albumins and globulins of the milk serum. A comparison of the blood serum protein and the milk protein of the mother (Pl. 1 *g*, *h*) further supports the assumption that fraction I of the milk protein is of the nature of milk serum albumin. In analogy to the previously reported observation from immune studies (Gügler *et al.* 1958) that a fraction of milk is antigenically similar to the mother's serum albumin, it is rational to identify this fraction I of milk as the milk serum albumin fraction. The relative percentage of this fraction was found to vary from 2·5 to 11 in the different subjects studied by us.

Effect of protein supplement on milk and blood proteins. The variation in fraction I of milk protein has been found to be correlated with the variation in the whey:curd ratio of the milk. Further, when the variation in the relative percentage of this fraction was studied in the same mother with different intakes of protein (Table 4), it was found that with the increase in the whey : curd ratio of the milk due to supplementation, the relative amount of fraction I also increased; at the same time, there appeared to be a little increase in the absolute concentration of the blood albumin of the mother. Similarly, when the whey:curd ratio decreased on withdrawal of the protein supplement, the value for blood albumin also reverted to almost its initial, lower figure.

These results indicate the possibility that the milk protein fraction I, identified as the milk serum albumin (Table 4 and Pl. 1), is directly derived from the increased albumin concentration of the blood resulting from dietary supplementation. Recent work on the biosynthesis of milk proteins indicates that they are largely (nearly 90–95%) synthesized in the mammary gland from the amino acids, but that some of the whey proteins are derived from extra-mammary sources and may enter the milk in a preformed state (Campbell & Work, 1952; Askonas, Campbell & Work, 1954; Askonas, Campbell, Humphrey & Work, 1954; Askonas, Campbell, Godin & Work, 1955).

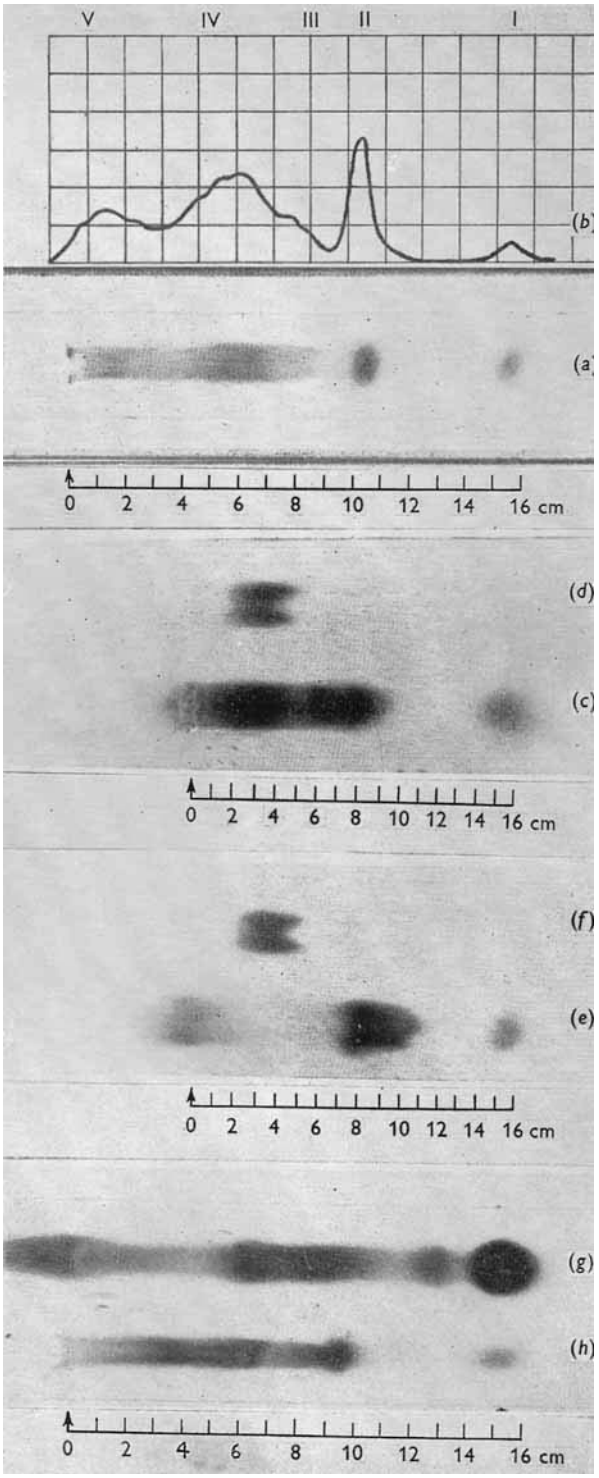
The observed changes in the relative proportions of the different milk proteins when the protein content of the diet was altered may be explained by a mechanism such as the following. The subjects studied by us having been adapted to a low-protein diet for a long period before and after lactation, had just the maintenance level of serum albumin with a decreased turnover rate, i.e. the concentration of serum albumin was not enough to contribute to the synthesis of other tissue proteins either directly or indirectly. The tissue proteins were being synthesized, in situ, from the free amino acid pool in equilibrium with the circulating free amino acids of blood. Under these circumstances, the relative formation of the different fractions of milk protein are likely to be influenced. Larson & Gillespie (1957) found that, in the cow, casein is almost completely synthesized in situ from the tissue amino acid pool, whereas a part of the whey proteins, e.g. milk serum albumin and the immuno-globulin, enter the milk preformed from the blood stream—evidently from the blood proteins. In subjects with low protein intakes, the blood albumin is possibly in a limiting condition as stated earlier; consequently, the proportion of casein in the milk of these mothers is relatively high, resulting in a low whey:curd ratio. With dietary protein supplementation the turnover rate of serum albumin increases (Jeffay & Winzler, 1958), and in our experiment also there was a perceptible increase in the serum albumin. This small increase in albumin above the maintenance level might then directly or indirectly have stimulated the formation of those milk proteins that were not derived from the amino acid pool or that entered the milk in a preformed state from the blood.

The concomitant increase in the percentage of milk protein fraction I along with the rise in serum albumin content suggests that fraction I is directly derived from the blood albumin. Since the total protein N did not increase with dietary supplementation, the observed increase in the whey N was associated with a decrease in the relative concentration of casein N. Under these circumstances, the whey:curd ratio of milk increases appreciably. Thus, when the existing low-protein diets of the Indian mothers were supplemented with protein of high biological value, the whey:curd ratio of the milk increased to values similar to those reported in the milk of average or better-fed mothers in the West, whose diets usually include liberal amounts of high-quality proteins. The whey:curd ratio and not the total protein content of the mother's milk is consequently a better criterion of the nutritional state of the mother.

Further work is in progress to assess the effect of different dietary proteins in varying quantities on the whey:curd ratio of the mother's milk as well as to record the magnitude of this ratio in the milk of different species of animal.

SUMMARY

1. The composition of milk of poor Indian mothers on a marginal diet has been compared with that of the milk of healthy mothers of other countries. The subjects were twenty normal lactating mothers on a habitual low-protein intake; their diets were periodically supplemented with casein. Determinations of the fat, protein, casein and non-casein fractions of protein, non-protein N and lactose were made by chemical and electrophoretic methods. Dietary supplementation with protein did not appear to



increase the total protein concentration of the milk but the quality of the milk protein was changed.

2. When the protein intake of the mother was raised, there was a concomitant rise in the albumin content of her blood and milk serum. The whey:curd ratio of the milk was thus increased on a high-protein diet. The possibility is discussed that a portion of the whey protein fraction of milk may be derived directly from the blood.

Thanks are due to Raptakos Medical Research Board, Bombay, for a financial grant to one of the authors (A.K.D.).

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EXPLANATION OF PLATE

Densitometric curve and agar electrophoretic patterns of human milk, its casein and whey proteins and of human blood serum proteins. Milk and blood were obtained from the same subject; casein and whey proteins are compared with that sample of human milk from which they had been isolated.

Arrow shows origin. (*a*), (*c*), (*h*), electrophoretic patterns of human milk; (*b*), corresponding densitometric curve; (*d*), (*f*), electrophoretic pattern of casein of human milk; (*e*), electrophoretic pattern of whey proteins of human milk; (*g*), electrophoretic pattern of blood serum—the fastest-moving fraction occupies the same position as fraction I of milk.

Printed in Great Britain