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CONTENTS OF THE OCTOBER ISSUE

The Journal publishes not only papers read at meetings of the Society, but also papers on dairy technology by members and non-members

MEMORIAL TO DR A. T. R. MATTICK, C.B.E.

Readers of the *Journal of Dairy Research* will be pleased to know that the fund established to commemorate the work of Dr A. T. R. Mattick, C.B.E., a former editor of the *Journal* from 1933 to 1958 and distinguished Dairy Bacteriologist, is receiving generous support. The income from the memorial fund will be used to enable members of the research staff of the Institute to make visits, which would not otherwise be possible, to research centres in the United Kingdom or abroad or to specialist conferences. It might also be appropriate on occasion to invite a distinguished scientist to give a memorial lecture on a topic which would be of interest to people connected with dairying.

Former colleagues of Dr Mattick, and workers visiting the Institute during his term of office who have not been aware of the memorial fund, may still send their contribution to the Director, National Institute for Research in Dairying, Shinfield, Reading. Cheques should be crossed and made payable to 'N.I.R.D. Mattick Memorial Fund'.

Factors affecting the concentration of vitamins in milk

I. Effect of breed, season and geographical location on fat-soluble vitamins

By S. Y. THOMPSON, KATHLEEN M. HENRY and S. K. KON
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(*Received* 16 April 1963)

Summary. Two surveys were done, one in 1943–44 and one in 1958–60, of the seasonal variation in the concentration of vitamin A and carotene in milk fat from 13 different areas in Great Britain. In the 1958–60 survey α-tocopherol and vitamin D were also measured. The main purpose of the second survey was to find out if the marked swing from Shorthorns to Friesians, and the trend towards greater use of carotene-rich foods in winter feeds that occurred in the intervening 15 years, had affected the pattern of vitamin A activity. In addition, monthly samples of butter were obtained in 1958–59 from a single factory in New Zealand during a complete butter-making season (9 months) and were assayed for vitamin A, carotene, α-tocopherol and vitamin D. Three experiments were done to study the effect of breed on the vitamin A and carotene content of milk fat, and in one of them α-tocopherol and vitamin D were also determined.

The seasonal trends for vitamin A and carotene were essentially the same in both surveys. A peak occurred in May after the spring flush of grass and was followed by a decline with a second peak in the autumn, except in 1959 when both the summer and autumn were very dry. Potencies were higher in the south than in the north, where the differences between summer and winter were more marked. In winter, mean vitamin A potencies of 5·8 and 7·7 μg/g fat were found for northern and southern areas, respectively; the corresponding values for β-carotene were 2·0 and 4·5 μg/g fat. Mean summer values were 8·8 and 9·7 μg/g fat for vitamin A and 5·1 and 6·5 μg/g fat for carotene, equivalent to increases of about 52 and 26 % and about 155 and 44 %, respectively, over winter values. Differences in mean potency between areas are explained by length of grazing season and type of winter feed.

The concentration of vitamin A in the fat was essentially the same for Ayrshires, Friesians, Jerseys and Shorthorns but somewhat lower for Guernseys; that of carotene was higher for the Channel Island breeds, particularly Guernseys, than for the other breeds studied.

Compared with this country, concentrations of carotene were higher and of vitamin A lower in milk fat from New Zealand, probably because of the predominance of Jersey cows in New Zealand herds. The seasonal trends for these vitamins were similar in both countries, but the spring peaks were earlier, the autumn peaks later and the summer declines more pronounced in New Zealand than in this country.

Concentrations of α-tocopherol in British milk fat showed a trend similar to that found for both vitamin A and carotene and were again influenced by feeding practices.

Mean values of 23 and 29 μg α-tocopherol/g fat were found in the summer for northern and southern areas, respectively; the corresponding winter values were 15 and 23 μg/g fat. Mean values of about 30 μg/g fat were found, over a 28-month period, for the milk fat of each of the 3 breeds, Ayrshire, Friesian and Jersey. No seasonal trend was observed in the New Zealand samples; the mean value for the 9 months was 32 μg/g fat (range 24–39 μg).

Vitamin D potencies were higher in summer, when there is more sunshine, than in winter. The higher summer values in the northern than in the southern part of the country may have been related to a higher intake of vitamin D in the former from hay and pasture. Summer potencies of 0·44, 0·53 and 0·62 i.u. vitamin D/g fat, respectively, were found for Friesian, Jersey and Ayrshire milk; corresponding winter values were 0·06, 0·08 and 0·09 i.u./g fat. For the New Zealand butters, summer values were higher than in this country, about 0·7 compared with about 0·4 i.u./g fat; winter values were similar in both countries.

We have done 2 surveys of the effect of season and geographical location on the vitamin A and carotene content of bulk milk in Great Britain, the first from January 1943 to July 1944 (briefly reported at the 12th International Dairy Congress (Thompson, Ganguly, Mawson & Kon, 1949)) and the second from January 1958 to March 1960. Before 1943 it had been found that in individual herds in this country the vitamin A activity of milk fat is higher in summer than in winter because of the cow's greater carotene intake from fresh pasture (Booth, Kon, Dann & Moore, 1933; Mattick, 1937; Coward, 1938; Wilkinson, 1939; Morton, Lord & Goodwin, 1941) but there was no information about the country as a whole. The first survey was made to obtain more detailed information on seasonal variation in different parts of the country. Throughout that survey samples were also obtained from the Shorthorn and Guernsey cows of the Institute herd. Since 1948, increasing numbers of Friesians have been introduced into this herd, and between October 1956 and November 1957 a comparison was made of the vitamin A and carotene contents of the milk of 10 Shorthorn and 10 Friesian cows at Shinfield.

Already, before the first survey, a trend could be discerned towards greater use in winter of the carotene-rich foods, kale and silage, and later the marked swing began from Shorthorns to Friesians. The second survey was done to establish the effect of these changes on the pattern of seasonal fluctuations in the vitamin A and carotene contents of bulk milk. During this survey monthly samples of milk were also obtained from Ayrshire, Friesian and Jersey herds all maintained under similar conditions of feeding and management on a farm in Hampshire. They were kept in the fields in winter as well as summer and when pasture was insufficient they received kale and silage and small amounts of barley meal. Since the conditions of management on this farm thus resemble New Zealand rather than British farming practice, it was of considerable interest to compare the vitamin A potencies of milk fats from New Zealand with those of milk fats from the Hampshire farm. In New Zealand 85 % of the cows are Jerseys (New Zealand Department of Agriculture, 1952). Through the courtesy of the New Zealand Department of Agriculture (Dairy Division) in London, samples of butter were obtained monthly from a single large factory in the main

butter-producing area of the North Island during a complete butter-making season from August 1958 to April 1959.

In the second survey and in all experiments done during 1958–60 the vitamin D and α-tocopherol contents of the milk fat were also measured. Only a few isolated values for the α-tocopherol content of milk in this country are available in the literature before this period. Routine measurements were not begun until 1958 when a satisfactory and specific method was made available to us (Analytical Methods Committee, 1959). To the best of our knowledge no values for the vitamin D activity of New Zealand milk fat had been published, and only 2 limited surveys had been done previously in this country (Wilkinson, 1939; Henry & Kon, 1942).

EXPERIMENTAL

Collection of, and general information about, samples

1943–44 *and* 1958–60 *surveys*

The areas chosen as representative of dairy practice throughout Great Britain are shown in Fig. 1. The collecting depots (Table 1), with one exception, were large and served large populations. The exception, a town dairy in Liverpool (no. 14, 1943–44 survey), in which the cows were kept permanently indoors with no access to kale and grass silage, was chosen to represent a practice that was then almost confined to that city and has since become obsolete. Tables 2 and 3 give information on breed and feeding of cows, the number of producers and the daily turnover of the depots. As far as possible samples were obtained from the same areas in the 2 surveys. More detailed information about the grazing period and characteristic features of winter feeding was available in 1958–60 than in 1943–44. The increase in the gallonage of milk per producer that occurred between the 2 surveys is noteworthy and has been commented on by Foot (1961).

Samples of milk (1 gal) from the centres serving London (Table 1) were usually taken from rail tanks at London termini and dispatched from there. The remaining samples in both surveys were sent direct from the depots either as milk (1 gal) or cream (1 pint). In the 1943–44 survey riboflavin was determined in those samples received as milk and the results are reported separately in the accompanying paper (Thompson & Kon, 1964). In the 1958–60 survey, carotene, vitamin A, α-tocopherol and vitamin D were determined.

Friesian, Guernsey and Shorthorn herds at Shinfield

Monthly samples of morning and evening milk, mixed in equal proportions, were collected from Shorthorn and Guernsey cows at the time of the 1943–44 survey (expt. 1) and from Shorthorn and Friesian cows from October 1956 to November 1957 (expt. 2). Details of numbers of cows, milk yield and feeding are given in Table 4.

Ayrshire, Friesian and Jersey herds on the Hampshire farm

Monthly samples of morning and evening milk, mixed in equal proportions, were collected from the 3 herds on the Hampshire farm throughout the 1958–60 survey. Details of numbers of cows and of milk yield and feeding are given in Table 4 (expt. 3). For vitamin D tests a single winter sample of 15 gal was collected from each herd in

early March 1960 and a summer sample of 6 gal in July 1960. Butter was churned from each sample and stored at −20 °C until the tests could be done.

New Zealand butter samples

Samples of butter were received for each of the 9 months from August 1958 to April 1959. All samples were made in factory no. 1880 at Te Awamutu in the highly productive fertile Waikato district in the centre of the North Island. Adequate grazing is available there for the 9 months that cover the butter-making season, and no supplements are given. Some 10000 tons of butter are produced annually at this factory.

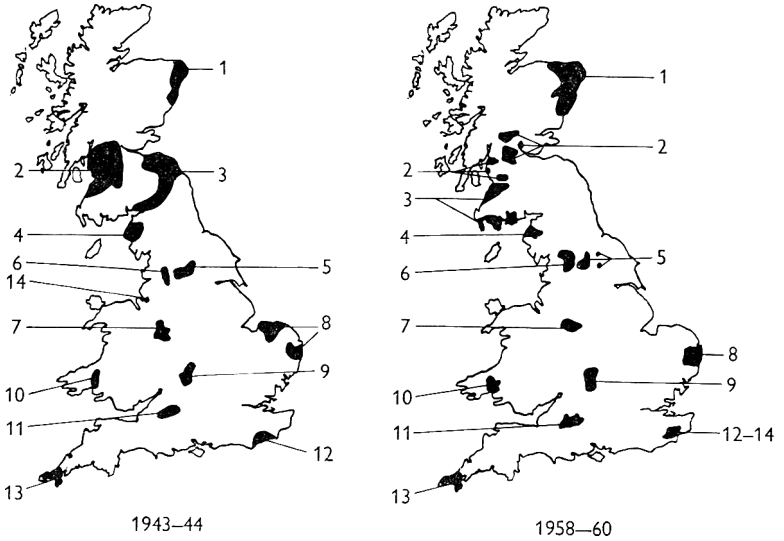

Fig. 1. Areas covered by the collecting depots in the 1943–44 and 1958–60 surveys. The numbers denote the depots as listed in Table 1.

Analytical techniques

1943–44 *survey*

Sampling and extraction of fat. From the milk samples a 90 ml portion was withdrawn for the measurement of riboflavin, and of fat by the Gerber method. The remainder was put through a cream separator and the cream was churned into butter which was stored at 2 °C until required for analysis, generally for not more than a few days. The samples received as cream were churned into butter and similarly stored. Occasionally, in hot weather, milk samples were sour on arrival and could not be separated for churning in the usual way. From them the fat was extracted by solvents, at first by the method of Henry, Kon, Gillam & White (1939) and later by that of Olson, Hegsted & Peterson (1939), a change which did not affect the results.

Measurement of vitamin A, total carotenoids and active carotenes. For the measurement of vitamin A and carotenoids the butter was melted at 45–50 °C, a quantity of the clear oil was pipetted off and 5–10 g was saponified as described by Gillam, Henry & Kon (1937) with the modification that 3 extractions with diethyl ether and 3 washings with water were used. At the beginning of the study the solvent was

removed from one-quarter of the final ethereal solution of the non-saponifiable residue, intended for measurement of vitamin A and total carotenoids, and the residue taken up in 5 ml of A.R. chloroform containing 1 % ethanol. The remainder, after evaporation, was taken up in 15–20 ml light petroleum (boiling range 40–60 °C) and was used for chromatographic separation of active carotenes. Later, as it was found possible to use a solution in *n*-hexane for the antimony-trichloride reaction, the procedure was modified as follows. The whole of the ethereal extract of the non-saponifiable residue from 5 g of butterfat was taken up in 20 ml of *n*-hexane (boiling range 67–69 °C) in a 20-ml volumetric flask with an additional 19 ml mark; two 0·5 ml portions were taken for duplicate measurement of vitamin A by the method since described by Thompson (1949) and the remainder was used for measurement of total carotenoids and for chromatographic separation of active carotenes which were collected in the same 20-ml volumetric flask and made up to 19 ml.

Table 1. *Depots from which samples were obtained in the* 2 *surveys*

Depot No.	Depot Name and location
1	Aberdeen and District Milk Marketing Board, Lilybank Creamery, Kitty-brewster, Aberdeen
2	Scottish Milk Marketing Board, Hogganfield Creamery, Glasgow
3	Scottish Milk Marketing Board, Restalrig Creamery, Edinburgh (1943–44 survey) Scottish Milk Marketing Board Creameries at Stranraer, Mauchline and Kirkudbright (1958–60 survey)
4	Milk Marketing Board, Aspatria Creamery, Aspatria, Cumberland
5	Leeds Industrial Co-operative Society Ltd, Leeds, Yorks
6	Dobson's Dairies Ltd, Barnoldswick, nr Colne, Lancs
7*	United Dairies Ltd, Uttoxeter, Staffs
8*†	United Dairies Ltd, Halesworth, Suffolk
9*†	United Dairies Ltd, Banbury, Oxon
10	United Dairies Ltd, Carmarthen, Wales
11*	Wilts United Dairies Ltd, Melksham, Wilts
12*	United Dairies Ltd, Mayfield, Sussex (1943–44 survey)
12†	United Dairies Ltd, Headcorn, Kent (1958–60 survey)
13*†	United Dairies Ltd, St Erth, Cornwall
14	J. L. Hogg, Fazakerley, Liverpool (1943–44 survey)
14†	United Dairies Ltd, Headcorn, Kent (Channel Island milk 1958–60 survey)

* Centres serving London 1943–44 survey.

† Centres serving London 1958–60 survey.

In measuring vitamin A with chloroform as solvent, 1 drop of acetic anhydride was added, but with *n*-hexane it was found necessary to add 5 drops to prevent cloudiness. The intensity and stability of the colour developed with the antimony trichloride reagent decreased with increasing quantities of acetic anhydride (Thompson, 1949).

The chromatographic method for separation of the active carotenes, predominantly β-carotene, was essentially that described by Thompson, Ganguly & Kon (1949) except that the pretreatment of the column with 8 % ethanol in *n*-hexane to decrease the adsorptive power of the alumina was omitted. Table 5 shows that the change from chloroform and light petroleum to *n*-hexane was without effect on values for vitamin A and for total and active carotenoids.

Losses of vitamin A and carotene during saponification and chromatography were each found to be about 7%.

1958–60 *survey*

Sampling and extraction of fat. Samples received as milk were separated in the Experimental Dairy of the Institute and transferred to our laboratory as cream. The

Table 2. 1943–44 *survey. Information about producers and herds supplying the different depots*

Depot		Producer				Carotene-rich foods in winter feed and approximate % D.M. of feeds supplied by them	
No.*	Mean turnover, gal/day	Approximate no.	Daily gal/producer	Composition of herds	Approximate grazing season		%
1	17000	500	34	Ayrshire and a few Shorthorn	May–October	Kale, silage	25
2	8800	430	20	Mainly Ayrshire	May–October	†	
3	1750	70	25	Ayrshire, Irish and other cross breeds	May–October	Kale, silage	5
4	23000	1200	20	Shorthorn, a few Shorthorn–Ayrshire cross and Friesian	April–October	Kale	5–10
5	17000	400	40	Shorthorn, a few Shorthorn cross, Ayrshire, Friesian	April–October	Kale	10
6	†	†	†	30% Ayrshire cross, 15% Friesian, 55% Shorthorn	April–October	Kale, silage	8
7	20050	756	26	10% Ayrshire crosses, 30% Friesian crosses, 60% Shorthorn	April–October	Kale	5
8	†	†	†	†	April–October	†	
9	11200	460	25	†	April–October	Kale, silage	5
10	16000	800	20	Mainly Shorthorns, a few Shorthorn crossed with Friesian or Jersey	April–October	Kale, silage	5
11	†	516	†	90% Shorthorn, 10% Channel Island	April–October	Kale, silage	15
12	16000	790	20	Shorthorn crosses, a few Ayrshire and Friesian	April–October	Kale	10
13	25200	1651	15	Guernsey, a few Friesian	March–November	Kale	10
14	120	1	120	37 cross-bred cows	No grazing	None	—

* See Table 1 and Fig. 1.
† No information available.

fat was extracted by adding 90 ml of 66% (v/v) aqueous alcohol and 200 ml light petroleum (boiling range 40–60 °C) to about 40 g of cream and rapidly mixing in a blender at 10000 rev/min under nitrogen. After removal of the petroleum fraction the aqueous ethanolic phase was extracted with a further 200 ml petroleum. The yield of fat was generally 15–20 g.

1	37000	395	93	30 % Ayrshire, 20 % Friesian, 50 % Ayrshire - Friesian cross	April (late)–October (early)	Kale, silage	50
2	19000	292	65	94 % Ayrshire, 6 % Friesian	May–October	Kale, silage (October–December) Silage (January–April)	20 20
3	87600	702	125	97 % Ayrshire, 3 % Friesian	April (middle)–October (middle)	Kale, silage (October–December) Silage (January–April)	15 10
4	39000	700	56	28 % Ayrshire, 24 % Friesian, 17 % Shorthorn, 31 % mixed	April (middle)–October (late)	Kale (November, December)	10
5	24000	740	32	9 % Ayrshire, 70 % Friesian, 20 % Shorthorn, 1 % other (pure or cross)	April (early)–November (early)	Kale, silage	37
6	17000	550	31	20 % Ayrshire, 30 % Friesian, 10 % Shorthorn, 40 % Shorthorn crossed Ayrshire or Friesian	May (early)–November (middle)	Kale, silage	25
7	32100	643	50	25 % Ayrshire, 53 % Friesian, 22 % Shorthorn	May (early)–November (early)	Kale, silage northern area southern area	 12 20
8	33900	599	57	78 % Friesian, 22 % mixed (Ayrshire Shorthorn, Channel Island)	April (end)–October (middle)	Kale Silage	25 20
9	22800	500	46	10–15 % Ayrshire, 65 % Friesian, < 10 % Shorthorn, < 5 % Channel Island	April (middle)–November (middle)	Kale (November, December) Kale, silage (January–April)	20 2·5–5
10	40200	1081	37	80 % Friesian, 10 % Shorthorn, 10 % mixed	May (middle)–October (middle)	Kale	5–12
11	42500	496	86	20 % Ayrshire, 55 % Friesian, 20 % Shorthorn, 5 % Channel Island	March (end)–November (end)	Kale (December, January)	50
12	20000	448	45	20 % Ayrshire, 34 % Friesian, 19 % Shorthorn, 22 % Channel Island, 5 % others	April (early)–October (middle)	Kale, silage	25
13	45000	1550	29	Guernsey with a few	March (early)–	Kale (October–mid-January)	30

Table 4. *Information on cows of different breeds at Shinfield and at the Hampshire farm*

Locality of farm	Expt. no.	Period	Cows: Breed	Cows: No.	Daily production, gal	Grazing season	Carotene-rich foods in winter feed and approximate % D.M. of foods supplied by them	%
National Institute for Research in Dairying, Shinfield, Berks	1	January 1943–July 1944	Guernsey	7	23	April–October (middle)	Kale	5
			Shorthorn	30	92			
	2	October 1956–November 1957	Friesian	10	35	April–October (middle)	Kale, silage	50
			Shorthorn	10	23			
Hampshire	3	January 1958–April 1960	Ayrshire	60	160	March (early)–November (late)	Kale, silage	80
			Friesian	60	230			
			Jersey	50	160			

Table 5. *Comparison of n-hexane (A) and chloroform (B) as solvent in the measurement of vitamin A by the antimony trichloride reaction and of carotene in 4 samples of milk fat*

Origin of sample	Vitamin A (μg/g fat)		Total carotenoids (μg/g fat)		Active carotenes (μg/g fat)	
	A	*B*	*A*	*B*	*A*	*B*
Depot 3*	9·4	9·2	1·46	1·44	1·26	—
Shinfield Shorthorn milk	4·4	—	3·27	3·27	—	—
Depot 13*	8·1	8·4	11·6	11·8	9·5	9·7
Shinfield bulk milk	9·4	9·2	7·5	7·6	6·1	6·4

* See Table 1.

Measurement of vitamin A, total carotenoids and active carotenes. Ten grams of the fat were saponified with 30 ml 5 % (w/v) pyrogallol in ethanol and 13 ml 60 % (w/w) KOH for about 10 min until all smell of volatile fatty acids had gone, and 140 ml distilled water were then added. After 3 extractions, each with 100 ml diethyl ether, the combined extracts were washed 4 times with 100 ml distilled water, or until the final washing was neutral to phenolphthalein. The extracts were then made up to 300 ml. 150 ml were used for measurement of tocopherol and 75 ml for measurement of vitamin A and separation of active carotenes as described for the 1943–44 survey (p. 5).

Measurement of tocopherols. The procedure was essentially that of the Analytical Methods Committee (1959). During the last few months of the survey the purification on Floridin Earth XS was replaced by the simpler purification on Decalso F, essentially as described by Crane, Lester, Widmer & Hatefi (1959). The method consisted of purifying the non-saponifiable residue on Floridin Earth or Decalso, the purification being followed by 2-dimensional separation on Whatman No. 4 chromatographic paper. The first solvent was 25 % (v/v) benzene in cyclohexane and the second solvent was 91 % (v/v) methanol or, later, 95 % (v/v) ethanol, which gives a better, though somewhat slower, separation. The colour formed with ferric chloride was read at 520 mμm either in a model D.U. Beckman spectrophotometer or in an Optica CF 4 DR recording spectrophotometer.

Biological estimation of vitamin D. Each month, after sufficient material had been removed for chemical analysis, the 6 individual cream samples from the northern depots (nos. 1–6) were bulked, churned into butter and stored at –20 °C to give one composite sample for the month. Every 2 months equal weights of the samples so obtained were bulked to form a composite bimonthly sample for vitamin D assay. Samples from the southern depots (nos. 7–13) were treated likewise. Of the New Zealand butters, the first 8 samples (August–March) were similarly paired, the 9th (April) being unpaired.

All butters were saponified and the non-saponifiable residue was assayed by the prophylactic method described by Henry & Thompson (1954); groups of 8 rats were used in litter-mate comparisons.

RESULTS

Vitamin A, total carotenoids and active carotene

Technique. Henry *et al.* (1939) showed that the concentration of total carotenoids in milk fat is slightly greater when the fat is obtained by solvent extraction of the milk than when it is obtained by churning. This finding was interpreted by Kon, Mawson & Thompson (1944) to mean that in milk fat the smaller fat globules contain a greater concentration of carotenoids, but not of vitamin A, than the larger ones. Subsequently, McGillivray (1957) confirmed the original observation, but showed that samples of cream obtained by centrifuging milk at different speeds of rotation all had the same concentration of carotenoids and vitamin A. Since solvent extraction of the fat-free milk yielded small quantities of carotenoids he concluded that they were bound to protein. However, no correction was made in the 1943–44 survey for the slightly higher carotenoid content of the few samples that were sour on arrival and had to be extracted with solvent instead of being churned.

Breed comparisons, Shinfield and Hampshire herds. Mean values for the concentrations of vitamin A and active carotenes in the milk fat of Ayrshire, Friesian, Guernsey, Jersey and Shorthorn cows and of α-tocopherol in that of Ayrshire, Friesian and Jersey cows are given in Table 6. The overall picture is that the concentration of vitamin A in the fat was essentially the same for Ayrshires, Friesians, Jerseys and Shorthorns but somewhat lower for Guernseys; that of carotene was higher for the Channel Island breeds, particularly Guernseys, than for the other breeds studied. These findings agree with those of Baumann, Steenbock, Beeson & Rupel (1934) who studied milk fat from groups of Ayrshire, Friesian, Guernsey and Jersey cows maintained under the same conditions.

Table 6. *Mean values for concentration of vitamin A, active carotenes and α-tocopherol in the fat of monthly milk samples of different breeds*

Expt. no.	Breed	Experimental period	Vitamin A, μg/g fat	Active carotenes: μg/g fat	Active carotenes: % of total carotenoids	α-Tocopherol, μg/g fat
		Whole survey				
1	Guernsey	January 1943–July 1944 (19 months)	5·1	12	76	—
	Shorthorn		7·7	5·5	81	—
2	Friesian	October 1956–October 1957 (13 months)	10	7·0	—	—
	Shorthorn		9·1	6·8	—	—
3	Ayrshire	January 1958–April 1960 (28 months)	9·1	6·4	76	30
	Friesian		10	5·8	77	27
	Jersey		9·6	8·3	75	29
		January–April only				
1	Guernsey	1943 and 1944	4·3	9·7	75	—
	Shorthorn		6·4	4·5	82	—
2	Friesian	1957	8·2	4·8	—	—
	Shorthorn		8·3	5·2	—	—
3	Ayrshire	1958, 1959 and 1960	10	6·2	73	29
	Friesian		11	6·5	74	29
	Jersey		10	8·1	69	31

Surveys in Great Britain and New Zealand. The results for the 2 British surveys are presented graphically in Fig. 2 (1943–44) and Fig. 3 (1958–60). The overall picture for Great Britain is that, although the vitamin A activity of milk fat was influenced by geographical location, the difference in the results of the 2 surveys were not marked either for individual areas or the country as a whole.

Figs. 2 and 3 show that, for the Scottish areas studied (nos. 1, 2, 3), concentrations of both vitamin A and carotene in the milk fat were rather higher in Aberdeen (no. 1) than elsewhere. During the winter months of January, February and March the concentration of total carotenoids was definitely higher in Aberdeen where the butter was more noticeably yellow and had a pink tinge. On inquiry we found that a substantial proportion of the cows' feed during these months consisted of yellow turnips. Chromatographic and spectroscopic analysis of the pigments of yellow turnip showed

that they contained lycopene, which was also found in the milk fat of cows given turnips and accounted for about 15% of the total carotenoids (Thompson & Kon, 1950). Otherwise the somewhat higher concentration of vitamin A and carotene in milk fat from Aberdeen than in that from other Scottish depots was probably a reflection of the greater use of carotene-rich foods, kale and silage, in that area (cf. Tables 2 and 3).

The results for the New Zealand samples are given in Fig. 2. Although only representative of the output of one factory during a single butter-making season, they are in reasonable agreement with results of large surveys done in New Zealand (cf. McDowell & McDowall, 1953).

α-Tocopherol

α-Tocopherol was determined only in those experiments done since 1958, so that a breed comparison is available only for the Ayrshire, Friesian and Jersey herds on the Hampshire farm. Table 6 shows that the mean α-tocopherol concentration in milk fat was essentially the same for these 3 breeds.

Seasonal changes in the α-tocopherol content of milk fats studied in the 1958–60 survey are shown in Fig. 3 and the values for the New Zealand samples in Fig. 2. On the whole, seasonal trends in this country in α-tocopherol resembled those for carotene and vitamin A. No seasonal trend was found for the samples of New Zealand milk fat.

Vitamin D

Tables 7, 8 and 9 give the vitamin D activity of the various milk fats tested.

The results of the 1958–60 survey are given in Table 7. The seasonal trends were in broad agreement with those found in an earlier survey done at this Institute (Henry & Kon, 1942) and also with those reported by Wilkinson (1939) for Scottish and Danish butters and by Bechtel & Hoppert (1938) for butters in Michigan, U.S.A. In general, potencies of the samples from the northern part of the country were higher than of those from the south, particularly in the summer months, May–August.

The potencies of the New Zealand samples (Table 8) did not differ greatly from those in the British survey. The summer values were rather higher and were maintained for a longer period than in this country. This finding is surprising since, with the greater amount of sunshine in New Zealand, we expected that winter potencies would be higher than here.

Table 9 gives the results for the summer and winter milk fats from the Ayrshire, Friesian and Jersey herds maintained on the Hampshire farm where the conditions of management were similar to those in New Zealand. The highest values, both in summer and in winter, were found for Ayrshires, and the lowest for Friesians. The small differences between the winter samples are obviously not significant, but further assays would be necessary to establish whether the larger differences between the breeds in summer are significant.

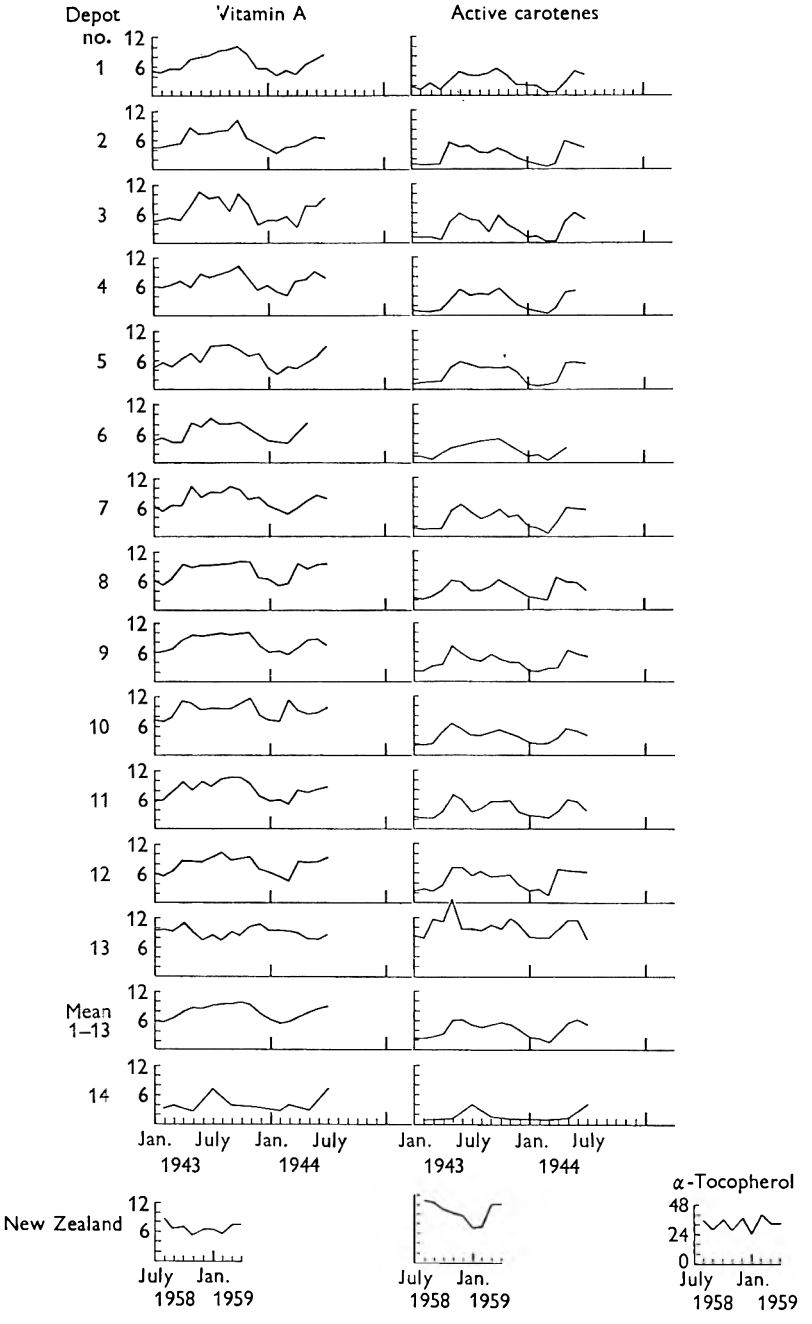

Fig. 2. (1) 1943–44 survey: seasonal variation in the concentrations of vitamin A and active carotenes in milk fat from depots in Great Britain listed in Table 1. (2) Seasonal variation in the concentrations of vitamin A, active carotenes and α-tocopherol of New Zealand milk fat 1958–59. Values in μg/g fat.

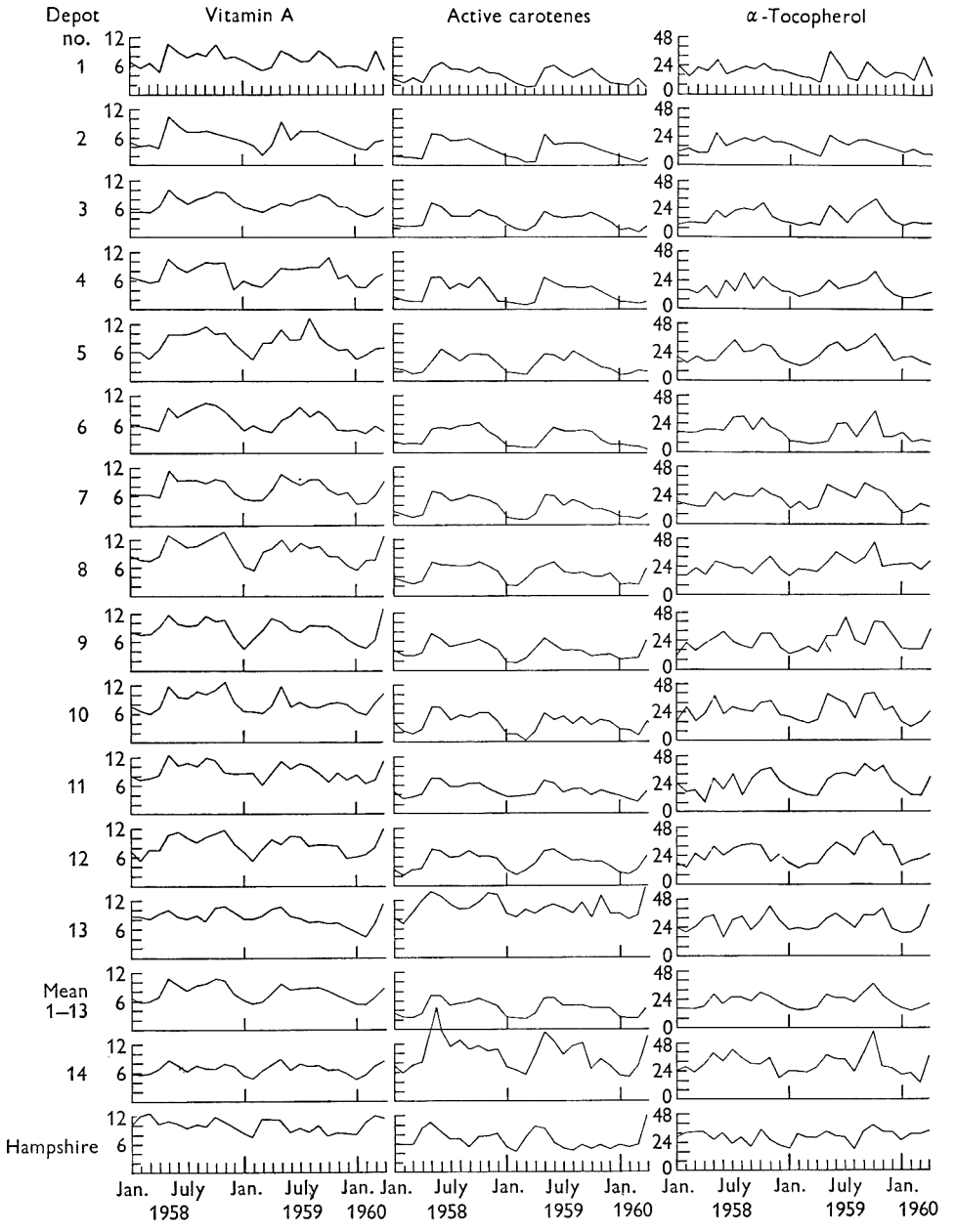

Fig. 3. (1) 1958–60 survey: seasonal variation in the concentrations of vitamin A, active carotenes and α-tocopherol in milk fat from depots in Great Britain listed in Table 1. (2) Corresponding values for a farm in Hampshire. Values in μg/g fat.

Table 7. 1958–60 *survey. Vitamin D activity (i.u./g fat, with true fiducial limits at P = 0·95) of pooled bi-monthly samples of milk fat from the northern and southern areas of the country*

	North*		South*	
Collection period	Value	Limits	Value	Limits
January, February 1958	0·08	0·07–0·10	0·08	0·06–0·09
March, April 1958	0·11	0·09–0·15	0·12	0·09–0·14
May, June 1958	0·37	0·27–0·54	0·27	0·20–0·37
July, August 1958	0·40	0·31–0·50	0·28	0·21–0·35
September, October 1958	0·17	0·11–0·24	0·14	0·09–0·21
November, December 1958	0·13	0·11–0·16	0·10	0·09–0·13
January, February 1959	0·23	0·16–0·36	0·20	0·14–0·30
March, April 1959	0·19	0·14–0·25	0·17	0·13–0·23
May, June 1959	0·51	0·37–0·69	0·39	0·28–0·53
July, August 1959	0·38	0·27–0·49	0·35	0·24–0·45
September, October 1959	0·20	0·17–0·24	0·25	0·21–0·30
November, December 1959	0·19	0·14–0·28	0·18	0·13–0·26

* North, Aberdeen to Yorkshire, depots 1–6, Table 1; south, Staffordshire to Cornwall, depots 7–13, Table 1.

Table 8. *Vitamin D activity (i.u./g fat, with true fiducial limits at P = 0·95) of New Zealand milk fat*

		Vitamin D	
Collection period	Equivalent months in Britain	Value	Limits
August, September 1958	February, March	0·23	0·19–0·29
October, November 1958	April, May	0·67	0·54–0·83
December 1958, January 1959	June, July	0·74	0·55–0·98
February, March 1959	August, September	0·51	0·38–0·67
April 1959	October	0·14	0·10–0·19

Table 9. *Vitamin D activity (i.u./g fat, with true fiducial limits at P = 0·95) of the fat of summer or winter milk of Ayrshire, Friesian and Jersey cows maintained on the Hampshire farm under the same conditions of feed and management*

	Winter (March 1960)		Summer (July 1960)	
Breed	Value	Limits	Value	Limits
Ayrshire	0·09	0·07–0·12	0·62	0·48–0·82
Friesian	0·06	0·05–0·08	0·44	0·34–0·54
Jersey	0·08	0·06–0·10	0·53	0·45–0·64

DISCUSSION

Vitamin A, total carotenoids and active carotenes

The results of these experiments confirm and extend earlier observations that the feed of the cow and, to a lesser extent, the breed, affect the carotenoid composition of the milk fat (Thompson & Kon, 1950). The carotenoids of milk fat consist largely of β-carotene with some xanthophylls (Palmer & Eckles, 1914). Varying small amounts of pigments, intermediate in chromatographic position between carotenes and xanthophylls may occur, depending on the feed and the breed of the cow, and one of these has been identified as the biologically inactive lycopene (Gillam & Heilbron, 1935).

In a careful investigation of the effect of the various acids present in silage on the carotenoids Quackenbush, Steenbock & Peterson (1938) showed that not only are breakdown products of xanthophylls formed which are difficult to separate from carotene, but that these pigments also appear in milk fat. Johnson, Peterson & Steenbock (1941) showed that the milk fat of cows on pasture contains 80 % β-carotene and 20 % non-carotene pigments, mainly xanthophylls, whereas in the milk fat of cows receiving phosphoric acid alfalfa silage only 65 % of the total pigment was β-carotene.

The transfer of inactive carotenes to milk fat is affected by breed. Thompson & Kon (1950) reported that under identical conditions of feeding and management carotenoids of Guernsey milk fat contained on the average 75·5 % active carotenes (range 68–83 %) and Shorthorn milk fat 81·2 % (range 75–88 %). The Shorthorn milk fat gave as a rule a simple 2-zone chromatogram of xanthophylls and carotene, whereas Guernsey milk fat generally showed in addition 3 main bands between carotene and xanthophylls. As an example of this selectiveness Gillam & Kon (1940) showed that the Shorthorn cow effectively excludes lycopene, an isomer of carotene, from her milk, even when large quantities were given in the form of tomato purée. In contrast the milk of a similarly treated Guernsey cow contained lycopene, which at its highest concentration amounted to some 12 % of the total pigments of the milk (Thompson & Kon, 1950). In Aberdeen, where the breeds are predominantly Ayrshire or Ayrshire–Friesian cross, the finding that lycopene from yellow turnips gave a pink tinge to winter butter indicates that certain breeds other than Guernsey are also less selective than the Shorthorn.

It is evident from the experiments done at Shinfield in 1956–57 (Table 6) that the change from Shorthorn to Friesian cows could have only little or no effect on the total vitamin A activity of milk in this country. Nevertheless, in the 1956–57 experiment with Shorthorns the values for the carotene and vitamin A content of the milk were higher than in the similar experiment in 1943–44. They thus probably reflected the increased use on our farm of carotene-rich foods. However, despite the generally increased use of carotene-rich foods at the time of the 2nd survey (Tables 2 and 3), little difference in vitamin A activity of milk was found in the 2 surveys either for individual areas or the country as a whole (Figs. 2, 3 and Table 11). One possible explanation is that the increased use of carotene-rich foods was not sufficient to cause a significant increase in milk carotene and vitamin A. Alternatively, any goitrogenic effect of kale (Greene, Farran & Glascock, 1958) may have affected the utilization of carotene.

As seen from Table 10 between 1943 and 1958 the production of cabbage, kale, savoys and kohl rabi for fodder has increased by nearly 100 % in England and by some 50 % in Wales (Ministry of Agriculture and Fisheries, 1947; Ministry of Agriculture, Fisheries & Food, 1960). Kale constitutes some 90 % of this crop and is predominantly used in feeding dairy cows. Against this increase has to be put the increase in the dairy cow population of some 10 % and, particularly, the increase in milk production of the order of 50 % (Milk Marketing Board, 1960/61, 1962). The change between the surveys in methods of using kale, i.e. folding instead of carting, has probably had little effect on the consumption of the leafy part, the main source of carotene (C. Line, pers. comm.). Estimated figures for the production of grass silage show that there has been a 12- to 15-fold increase over the country as a whole

(Great Britain, Parliament, 1958). However, increases of this magnitude in the consumption of silage may not have been sufficient to influence sensibly the vitamin A and carotene content of milk (Thompson, 1959). Whatever the causes, the fact remains that we observed no differences in vitamin A and carotene levels in the 2 surveys and it seems that the situation would well repay more searching inquiry.

The results of the 2 surveys show that the total vitamin A activity of milk fat is higher in southern than in northern areas. This difference can be accounted for by differences in feeding practice. In the north of England milk from the large collecting depots of Aspatria (no. 4) in Cumberland and Barnoldswick (no. 6) on the Lancashire–Yorkshire border was consistently low in carotene and rather low in vitamin A and α-tocopherol. It is interesting that the area covered by Leeds (no. 5) produced milk somewhat richer in these vitamins than the adjacent area covered by Barnoldswick

Table 10. *Acreage under cabbage, kale, savoys and kohl rabi for fodder in England and Wales and in the counties supplying the depots in England and Wales*

Depot no.	County	1943*	1958†
4	Cumberland	2025	3501
5	Yorkshire	5732	6970
6	Lancashire	4954	2768
7	Staffordshire	5197	5575
8	Norfolk, Suffolk	18154	24870
9	Oxfordshire	4193	7394
10	Carmarthen	5075	4264
11	Wiltshire	6498	18206
12	Sussex, Kent	10773	15463
13	Cornwall	5590	16330
Whole of England		169731	324437
Whole of Wales		19476	29812

* Ministry of Agriculture and Fisheries (1947).
† Ministry of Agriculture, Fisheries and Food (1960).

(no. 6). A partial explanation may be that Aspatria and Barnoldswick cover hilly areas where pasture may be poor, whereas Leeds covers a less hilly district. In addition, farming practice in the area served by Aspatria included little kale or silage in the feed (cf. Tables 2 and 3). The absence of kale and silage in the diet of the cows in the Liverpool town dairy (Table 2, no. 14) was reflected in the very low concentrations of vitamin A and carotene in the milk (Fig. 2); the slight rise in July was probably associated with the inclusion of freshly cut grass in the feed.

Differences in the vitamin A and carotene concentrations in milk fat from areas south of Yorkshire (except for milk from Cornwall and the Channel Island milk from Kent) were small and similar in the 2 surveys. However, during the winter months of January, February and March, when the carotene intake is usually at its lowest, there were differences between areas that could be attributed to differences in winter feeding practices. For example, consistently lower values for vitamin A and carotene were found in milk fats from Carmarthen (no. 10) than in those from Melksham (no. 11), and this difference can be explained by the lower carotene intake during this period in the former area (Table 3). In summer, on the other hand, consistently higher values were found in Carmarthen, probably because of the more continuous growth of grass caused by a high annual rainfall (nearly 60 in).

Carotene concentrations were high in the milk of the Channel Island herds in Kent and in milk from Cornwall, where Guernseys predominate. In Cornwall the grazing season is longer than in other areas, grass is usually available until December and new growth starts in early March. As a result, the winter drop in carotene is less marked and of shorter duration there than in other areas (cf. Figs. 2 and 3). During the winter months of January, February and March values were consistently lower in Kent than in Cornwall though in summer the difference was less marked. Compared with the rest of the country the low vitamin A values in Kent were typical of Guernsey milk fat (cf. p. 10, and Table 6) and yet in Cornwall, with mainly the same breed, values for vitamin A were nearly equal to the mean value for the rest of the south of England. That these values were not lower was probably because of the longer grazing season and higher annual rainfall in Cornwall (47 in) than in Kent (35 in).

Some of the effects that can be attributed to the feed of the cow and the length of the grazing season (Tables 2 and 3) are brought out in Table 11. The differences between summer and winter were greater for northern than southern samples, particularly for carotene. The shorter grazing season in the north was barely sufficient to raise the summer level there above the winter level in the south. In both parts of the country concentrations of carotene and, more particularly, vitamin A were lower in the dry summers of 1943 and 1959 than in the very wet one of 1958 which was the wettest summer of this century so far, whereas 1959 was one of the driest. Low values were found in the winters of 1943–44 and 1959–60 that followed the hot dry summers, whereas the wet summer of 1958 caused an abundant growth of grass that was reflected in the higher values in the winter of 1958–59.

Figs. 2 and 3 show that peaks for both vitamin A and carotene occurred in May after the spring flush of grass, and that thereafter the values mostly declined. A similar observation for the Institute's Shorthorn and Guernsey herds was made by Kon in 1932–34 (reported by Mattick, 1937). In that survey the decline was more marked in the summer of 1933 that was dry than in that of 1932. This decrease was followed by a second peak in the autumn particularly in that of 1933 which was wet. In the surveys now reported a second peak was very obvious, after the wet summer, in October 1958; it was less obvious in 1943 when a dry summer was followed by autumn rain and absent in 1959 with its very dry summer and autumn. The mild winter of 1959–60 was followed by an early flush of grass so that an increase in potency of both vitamin A and carotene was already evident in March 1960.

The value of prolonged grazing is also shown by the high winter values for the herds on the Hampshire farm and for the New Zealand butter samples. The mean values for vitamin A and carotene of 9·7 μg and 6·8 μg/g fat, respectively, for the combined Hampshire herds were somewhat higher than the corresponding values of 8·5 and 5·8 (calculated from Fig. 3) for the rest of southern England and considerably higher than those of 7·2 and 3·5 for the northern part of the country. This situation can be explained by the higher carotene intake from grass on the Hampshire farm in the early winter, and it can be seen from Table 6 (expt. 3) that the mean winter values on this farm were essentially the same as those for the whole 28-month period of the experiment that included 2 summer periods. The carotene content of the milk on this farm showed no increase between January and March in 1958 and 1960 at a time

Table 11. *Mean values for the concentrations of vitamin A, active carotenes and α-tocopherol in milk fat in the northern and southern areas of the country during the summer and winter months together with the corresponding mean values for daily sunshine and monthly rainfall*

Season*	Vitamin A, μg/g fat		Active carotenes: μg/g fat		Active carotenes: % of total carotenoids		α-Tocopherol, μg/g fat		Sunshine, h/day		Rainfall, in/month	
	North†	South†	North†	South†	North†	South†	North†	South†	North†	South†	North†	South†
Summer 1943	8·6	9·3	4·6	6·2	84	82	—	—	4·88	5·88	3·25	3·02
Winter 1943–44	5·4	7·5	2·0	4·1	61	77	—	—	2·50	2·64	2·01	1·96
Summer 1958	9·4	10·5	5·6	7·1	77	77	23	27	4·53	4·72	3·27	3·83
Winter 1958–59	6·4	8·2	2·2	4·6	60	72	15	21	2·30	2·79	2·00	3·10
Summer 1959	8·5	9·4	5·0	6·3	80	76	24	31	5·50	7·11	1·87	1·75
Winter 1959–60	5·6	7·5	1·9	4·7	61	73	15	24	2·03	2·18	3·26	4·28
Mean 1943–44 (19 months)	6·7	8·2	3·1	4·2	69	79	—	—	3·69	4·26	2·63	2·49
Mean 1958–60 (28 months)	7·2	8·5	3·5	5·8	70	74	19	26	3·59	4·20	2·60	3·23

* Summer, May–October; Winter, November–April.

† North, Aberdeen to Yorkshire, depots 1–6, Tables 1, 2 and 3; south, Staffordshire to Cornwall, depots 7–13, Tables 1, 2 and 3.

when vitamin A values were increasing rapidly, whereas normally the contents of carotene and vitamin A increase together, as happened during these months in 1959 (Fig. 3). We have observed a similar lag with cows given large amounts of silage (Thompson, 1959).

Garrett & Bosshardt (1944) observed that when they gave stall-fed cows silage there was an increase in the vitamin A concentration in milk but little change in the concentration of carotene. A similar effect is evident from the earlier results of Peterson, Bohstedt, Bird & Beeson (1935), and from those of Trimberger *et al.* (1955). It has been suggested (Thompson, 1959) that, during silage-making, some colourless biologically active breakdown products of carotene are formed which are not measured in the carotene fraction. The finding by Zechmeister & Petracek (1956) and Budowski, Ascarelli, Gross & Nir (1963) of a biologically active coloured breakdown product of lutein suggests that the vitamin A activity of grass silage is greater than would be expected from its carotene content because of the presence of active breakdown products of carotenoids. Inspection of the results for the individual herds in Hampshire showed that, in 1958, the increase in vitamin A was most marked for the Ayrshire herd for which the only supplement was silage between January and March, whereas the Friesian and Jersey herds had a mixture of kale and silage.

It is evident that, at least in the south of England, a system of all-grass farming, similar to that practised in New Zealand, can achieve high concentrations of vitamin A and carotene in milk and reduce the winter drop.

The New Zealand (North Island) samples were very much richer in carotene than in vitamin A so that they resembled Guernsey rather than Jersey milk fat in this country (Table 6) and also in the United States (Baumann *et al.* 1934). McDowell & McDowall (1953) found that in butters from the South Island of New Zealand, where climatic conditions are somewhat similar to those in Hampshire, the seasonal fluctuations in vitamin A and carotene levels were much less marked than in those from the North Island and more akin to those found for Jersey milk in this country and the United States.

A second point of interest is that we confirmed the finding of McDowell & McDowall (1953) that in New Zealand there is a marked drop in the concentration of carotene in milk fat, and to a lesser extent of vitamin A, in the middle of the summer grazing season. We found a similar but less marked drop in this country (Figs. 2 and 3) particularly during the wet summer of 1958 when abundant grass was available.

Although the carotene intake in New Zealand is high throughout the summer, the carotene may be less well utilized from mature pasture (McGillivray, 1952; McDowall & McGillivray, 1963) owing to the influence of the composition of the plant lipids on the utilization of carotene (McGillivray & Worker, 1958). The same explanation may hold for the summer drop in this country. On the other hand, the decrease in the carotene content of pasture that can occur during a wet summer (Moon, 1939) may also contribute to the summer drop. However, in 1959 drought undoubtedly reduced the growth of grass and, consequently, the carotene intake in this country; this situation was reflected in the continuous decrease of both carotene and vitamin A concentrations in milk fat from the spring peak to the winter values.

Both breed and feed of cow affect the proportion of active carotenes in the total carotenoids of milk fat (Thompson & Kon, 1950). It is evident from our results that,

in this country as a whole, feed exerts a greater influence than breed. Compared with that of other breeds, Channel Island, particularly Guernsey, milk contains a lower proportion of active carotenes. Table 6 shows that, under similar conditions of feeding, the milk of Guernseys on the Institute farm contained a lower proportion of active carotenes both over the whole 19 months of the experiment and during the winter months of January–April. On the Hampshire farm, however, it was only during this winter period that the proportion of active carotenes in the total carotenoids of Jersey milk fat was appreciably lower than in that of Ayrshires or Friesians. As Channel Island cows constitute only a small proportion of the cow population, especially in the north, their influence in the surveys was small. Fig. 4 and Table 11 show that there was a seasonal variation in the proportion of active carotenes in milk fat and that this effect was more marked in the north than in the south. In winter, cows not only receive very much less carotene but also a greater variety of inactive carotenoids some of which, such as lycopene from yellow turnips, breakdown products of xanthophylls from silage or inactive carotenoids from hay (Kon & Thompson, 1940) are absorbed by the cow and secreted into the milk. The presence of these carotenoids was, no doubt, responsible for the lower percentage of active carotenes in winter milk, 62 % of the total in northern samples and 74 % in southern samples, as can be calculated from values in Table 11. It is noteworthy that in both surveys the lowest percentages were found in the Aberdeen samples, 25 % in March 1944 and 33 % in March 1958.

In the summer, in both northern and southern areas, cows receive mostly grass and from it large amounts of the active carotenoid β-carotene and the poorly absorbed xanthophylls so that the same proportion of active carotenes would be expected, and was found (about 80 %), in milk fat from both areas.

Surveys covering periods of 1–2 years, and showing the same trends as those found here, have been reported from the U.S.A. (Technical Committee in Charge of the Nation-wide Survey, 1947), Denmark (Fridericia, 1947), Sweden (Platon & Swartling, 1944), Holland (Kruisheer & den Herder, 1953) and for a herd of Ayrshire cows in Cheshire (Lord, 1945). Table 12 gives a summary of the total vitamin A potency of summer and winter milks studied in the different surveys. The summer and winter potencies quoted in Table 12 are not for the same months in the different countries and insufficient data were available to enable us to make the necessary recalculations. Nevertheless, it seems probable that the highest (New Zealand) and lowest (Denmark and Sweden) winter values reflect differences in the length of grazing season in these countries.

α-Tocopherol

Until the late 1950's no satisfactory method for the determination of tocopherols was available. It is not possible, therefore, to compare the results of our survey of 1958–60 with those of earlier work.

Table 11 shows that, in both summer and winter milks, the concentration of α-tocopherol was lower in northern than in southern samples. Over the whole country, summer milk was some 30 % richer in this vitamin than winter milk in consequence of the richness of grass in α-tocopherol. A similar increase was observed by Krukovsky, Whiting & Loosli (1950) with Brown Swiss, Guernsey and Jersey cows, but not with

Friesians for which the summer value was little higher than the winter one. As with carotene, the summer values in the north were similar to the winter values in the south. The fluctuations in tocopherol levels followed those of carotene more closely than those of vitamin A, a finding previously reported by Krukovsky *et al.* (1950). However, the seasonal differences were less for tocopherol than for carotene, particularly in the northern part of the country, probably because some winter feeds are relatively rich in tocopherols.

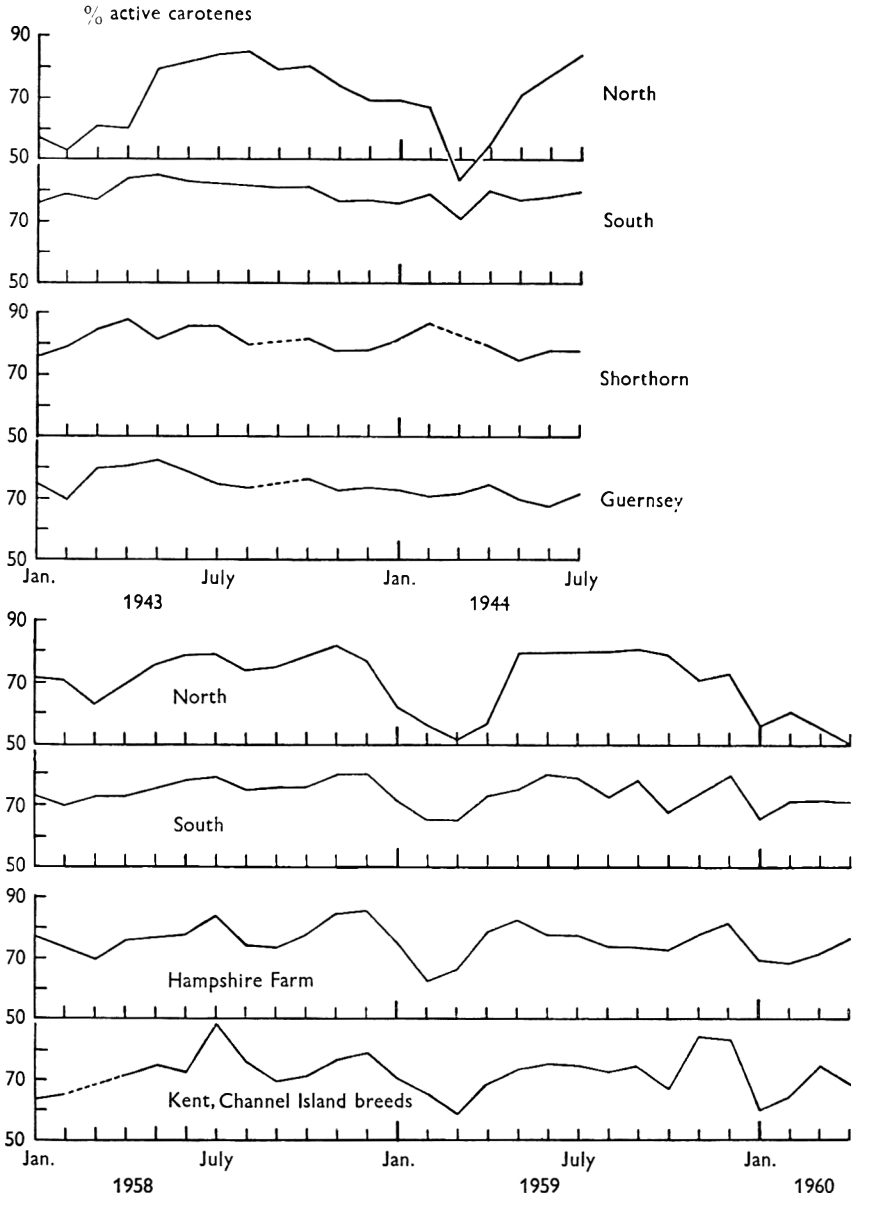

Fig. 4. Seasonal variation in the percentage of active carotenes in the total carotenoids of milk fat. Mean values for northern and southern areas of Great Britain in the 1943–44 and 1958–60 surveys, for Shorthorn and Guernsey cows at Shinfield, for the Hampshire farm and for Channel Island breeds in Kent.

Moreover, in January, February and March of each year the samples from the Aberdeen and Glasgow areas contained, in addition to some 12–15 μg α-tocopherol/g fat, a small proportion (3–5 μg) of ζ-tocopherol. This tocopherol was also observed in February 1958 in the milk fat of cows receiving brewer's grains at this Institute. The main source of ζ-tocopherol for dairy cattle is distiller's or brewer's grains. These are often used in winter feeds in areas where such materials are available. We have already pointed out that the composition of the carotenoids in the feed can alter the composition of the carotenoids of milk fat; in the same way it now seems that a similar phenomenon may occur with the tocopherols.

Blaxter, Brown & MacDonald (1953) found that mangolds depress the tocopherol level in milk, but they are unlikely to do so in practice as only small quantities are now fed. Krukovsky, Trimberger, Turk, Loosli & Henderson (1954) found higher tocopherol levels in winter milk when silage rather than when hay was given.

Surveys, lasting for 1 year, in which monthly samples of butter were analysed for

Table 12. *Comparison of vitamin A potencies (i.u./lb) of butter in different countries*

Country	Winter	Summer
New Zealand	19000	15000
Great Britain	10500	15000
Sweden	6800	11000
Denmark	7500	15000
U.S.A.*	9500	15600
Holland	10700	16800

* Recalculated, using the factors 1 μg vitamin A = 3·33 i.u., 1 μg carotene = 1·67 i.u.

tocopherol have been reported from Canada (Anglin, Mahon & Chapman, 1955) and Holland (Kruisheer, 1956). In Canada the range of potencies was 10–50 μg tocopherol/g fat and in Holland 10–40 μg/g fat. Classified into summer and winter values, as was done in our survey, the respective mean values were 38 and 23 μg/g fat in Canada and 25 and 16 μg/g fat in Holland compared with 26 and 19 μg/g fat in this country.

In the New Zealand butter samples analysed at this Institute the range was similar to that reported by McGillivray (1956), 24–39 μg/g fat, but there was no seasonal trend. The mean value of 32 μg/g fat was similar to that found for summer butter in the south of England (cf. Table 11).

Vitamin D

The vitamin D potencies of the samples of milk fat from northern depots were generally higher than for the southern ones (Table 7). This finding was unexpected as there had been more sun in the south than in the north (cf. Table 11). Further, more light of the vitamin D-forming wavelengths (Abrams, 1952) would penetrate the atmosphere in southern than in northern latitudes. Ashbel (1961) points out, however, that radiation in the south of England is reduced by industrial pollution. It is well known that the absorptive effect of impurities increases with decreasing wavelength. Although exposure of the cow to sun- and sky-shine is the main factor

in raising the vitamin D potency of milk to summer levels, it has been found that when cows are exposed to the summer sun, the milk of those given hay and other winter feeds contained more vitamin D than that of animals on pasture (Campion, Henry, Kon & Mackintosh, 1937). It is known that quickly growing herbage is a negligible source of vitamin D, whereas hay may contain appreciable quantities; moreover, marked variations may occur in the vitamin D activity of pastures and hay (Henry, Kon, Thompson, McCallum & Stewart, 1958). Thus differences in the maturity of the herbage, and the shorter grazing season in the north with concomitant greater use of hay may contribute to the differences in the vitamin D potency of milk from the 2 areas.

Although summer values of the New Zealand milk fat were somewhat higher and were maintained for a longer period than in Great Britain, the differences were less marked than we had expected from the difference in geographical latitude, in which the Waikato district corresponds to the Mediterranean. We have previously found values of 0·4 and 0·6 i.u. vitamin D/g fat for butters produced, respectively, in April and June in south India (Henry & Kon, 1954), values that are similar to those found in this country. Our findings would indicate that latitude may influence the vitamin D potency of milk to a smaller extent than was previously thought.

Although our values for the vitamin D potency of the milk fat of the 3 breeds studied (Table 9) are too few to establish whether the breed differences were significant, the higher value obtained for Jersey than for Friesian milk in summer agrees with the findings of Wallis (1944) who, in more detailed experiments, found some 20% more vitamin D in Jersey than in Friesian milk fat.

The predominance of Jerseys in New Zealand compared with that of Friesians in Great Britain may thus make some small contribution to the rather higher summer potencies in New Zealand.

These studies lasting several years, and covering most of Great Britain and a New Zealand creamery, would not have been possible without the help of many people. It is with pleasure and gratitude that we acknowledge here the collaboration of the persons or organizations listed below in their capacities at the time of the surveys.

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Factors affecting the concentration of vitamins in milk

II. Effect of breed, season and geographical location on riboflavin

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Summary. In a survey done in 1943–44 there was no seasonal difference in the riboflavin content of milk from 10 different areas in England and Wales that could have been associated with the change from stall to pasture feeding, and mean values were about 160 μg/100 ml for both the periods November–April and May–October. However, values for the 6 months August–January were higher (about 175 μg) than for the 6 months February–July (about 150 μg). Over a 12 months' period the mean riboflavin contents of Shorthorn and Guernsey milk from the Institute's farm were 142 and 191 μg/100 ml, respectively.

The effect of season and geographical location on the concentration of vitamin A and carotene in milk fat has been studied in 2 surveys done in 1943–44 and in 1958–60 (Thompson, Henry & Kon, 1964). Riboflavin measurements were made in milk from 10 of the collecting depots in the 1943–44 survey and were summarized by Kon (1945). These results, together with some obtained for milk of the Institute's Guernsey and Shorthorn herds (Thompson *et al.* 1964) are now reported in full.

EXPERIMENTAL

Source of samples

Details of the collection of milk samples in the 1943–44 survey and from the Institute's Guernsey and Shorthorn herds are given in the accompanying paper (Thompson *et al.* 1964). The survey milks were obtained from those depots from which the samples were received as milk, i.e. Leeds (no. 5), Barnoldswick (no. 6), Uttoxeter (no. 7), Halesworth (no. 8), Banbury (no. 9), Carmarthen (no. 10), Melksham (no. 11), Mayfield (no. 12), St Erth (no. 13) and Liverpool (no. 14). No special precautions were taken to protect the milk from light in the routine handling at the depot up to the time of sampling. Thereafter the samples were protected.

Measurement of riboflavin

Riboflavin was measured by the fluorimetric method of Emmerie (1938), the treatment with potassium permanganate and hydrogen peroxide being omitted. The fluorescence of a suitable dilution of the methanol filtrate was measured in a Cohen

type fluorimeter (Henry, Houston, Kon & Osborne, 1939) and more recently in a fluorimeter of greater sensitivity equipped with a photo multiplier (Thompson, unpublished).

RESULTS

The results are given in Fig. 1. Those for the survey show that, unlike with the vitamin A and carotene contents (Thompson *et al.* 1964), there was no marked

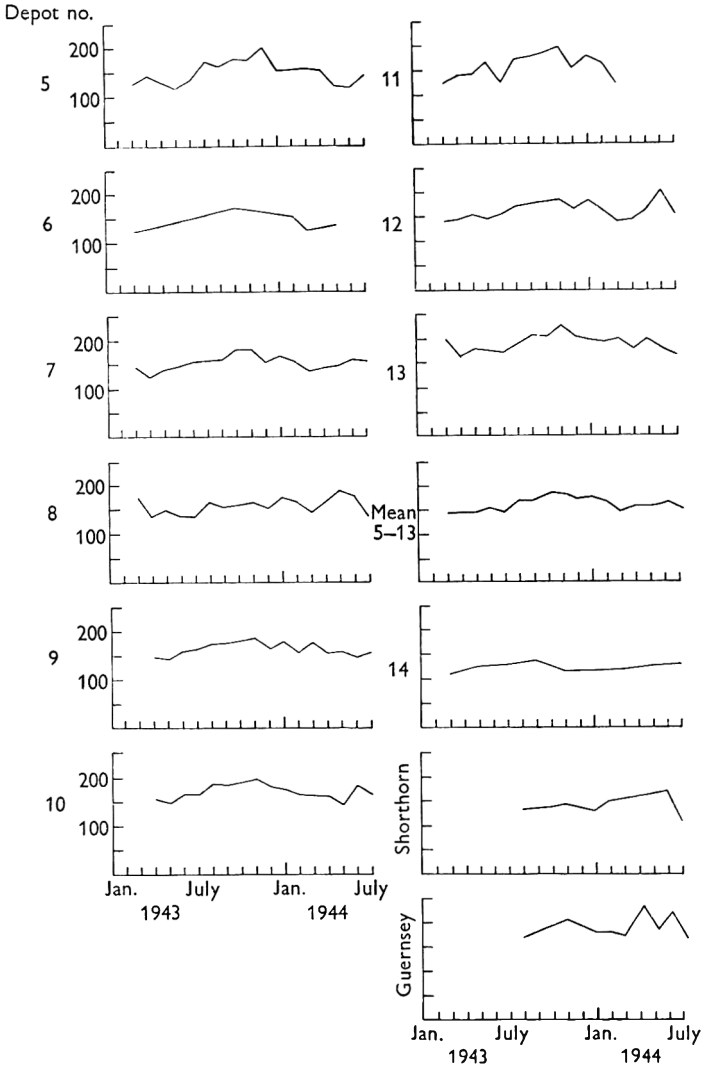

Fig. 1. The riboflavin content of milk from depots 5–13 (see Table 1 in Thompson, Henry & Kon, 1964) in England and Wales, and of milk from Shorthorn and Guernsey cows of the Institute herd (μg/100 ml milk).

variation in the riboflavin content of milk attributable to season or geographical location. Although fewer values were available for the breed comparison than for the survey, it is clear that the concentration of riboflavin was higher in Guernsey than in Shorthorn milk, the means being 191 and 142 μg/100 ml, respectively. In this

connexion it is noteworthy that milk from Cornwall, where Guernseys predominate, also contained on average 191 μg/100 ml. Values for the town dairy in Liverpool were low, the mean being only 123 μg/100 ml. For the country as a whole the values ranged from 170 to 184 μg/100 ml from August to January and from 144 to 165 μg/100 ml from February to July.

DISCUSSION

The relationship between the concentration of riboflavin in fodder and in milk is far from clear. The cow undoubtedly obtains riboflavin not only from the food but also by endogenous synthesis in the rumen (Wegner, Booth, Elvehjem & Hart, 1941). McElroy & Goss (1940) showed that a cow receiving a diet almost free from riboflavin produced milk containing normal concentrations of this factor. However, it has been reported that summer milk contains some 20–50 % more riboflavin than winter milk (Houston, Kon & Thompson, 1940*a*; Whitnah, Kunerth & Kramer, 1938) and that this increase is due to pasture feeding (Houston, Kon & Thompson, 1940*b*; Virtanen & Holmberg, 1938; Hand & Sharp, 1939). Evidence for and against this effect of pasture has subsequently been put forward by many investigators (e.g. Theophilus & Stamberg, 1945; Sharp, Shields & Stewart, 1945). It may well be that the synthesis of riboflavin in the rumen is affected by the diet, and our finding of very low concentrations in milk of stall-fed cows in the Liverpool town dairy might perhaps be explained in this way. Results from our laboratory are inconsistent. Results of the 1943–44 survey show that over the country as a whole the riboflavin content of milk did not increase when cows went out to pasture. However, values for the 6 months August–September, with peak values in October and November, were higher than for the next 6 months. In experiments already reported for milk from 2 dairies in Reading (Kon & Thompson, 1953), an increase was noted between March and April 1949, with peak values in July and September. However, in a similar experiment (Kon & Thompson, 1953) lasting for 12 months from March 1950, the riboflavin concentration in the milk remained constant throughout. The sum total of evidence for and against the effect of pasture (*see* reviews by Kon, 1940, 1943, 1949) lends support to the suggestion of Kramer, Dickman, Hildreth, Kunerth & Riddell (1939) that climatic and seasonal factors may be of more importance than the diet.

Our observation at Shinfield that Guernsey milk is richer than Shorthorn milk in riboflavin confirms the findings of many workers (*see* Kon, 1940, 1949; Kon & Henry, 1954) that Channel Island milk contains more of this factor than milk of other breeds. Hand & Sharp (1939) found mean values for the periods of stall and pasture feeding of 228, 198, 212, 183 and 153 μg riboflavin/100 ml for the milk of Jersey, Guernsey, Brown Swiss, Ayrshire and Friesian cows, respectively. Their value for Guernsey milk is very close to our value of 191 μg/100 ml and that for Friesian milk is similar to our Shorthorn value of 142 μg. These breed differences have led to the suggestion by some authors (e.g. Whitnah *et al.* 1938) that the riboflavin content of milk is positively correlated with fat content.

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With 2 plates

Action of rennin on α-, β- and γ-caseins

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Summary. Whole acid casein and α-, β- and γ-caseins were isolated and treated with rennin. Chemical, chromatographical and electrophoretical analyses were done on the caseins and on the products obtained from them after rennin treatment. Three different fractions were obtained which had some specific protective activity for the Ca-sensitive forms of α-, β- and γ-casein, respectively, and which differed in their chemical, chromatographical, and electrophoretical properties.

It is suggested that each casein fraction in milk is associated with its own protective fraction which when released by the action of rennin, allcws the casein to clot.

It is now generally accepted that casein contains in addition to the 3 main fractions, α, β- and γ-casein, a fourth fraction generally called κ-casein which is Ca-insensitive and rennin sensitive, and which protects the casein against flocculation by calcium (Waugh & von Hippel, 1956; Swaisgood & Brunner, 1962; Payens, 1961).

On the addition of rennin to whole-casein solutions the protective fraction is attacked and the α-, β- and γ-casein clots in the presence of Ca^{++}. It has been suggested that the κ-casein fraction of whole-casein is associated exclusively with the α-casein. Cherbuliez & Baudet (1950) and Alais, Mocquot, Nitschmann & Zahler (1953) even concluded that only α-casein is altered by rennin, whereas β- and γ-casein are not attacked but are merely co-precipitated unchanged along with the altered α-casein in the presence of Ca^{++}. In this connexion, however, it should be noted that the definition of γ-casein is not precise. Wake & Baldwin (1961) stated that there was uncertainty about the definition of γ-casein and Neelin, Rose & Tessier (1962) appear to be the only investigators to investigate γ-casein using starch gel electrophoresis.

Different κ-preparations show differences in their electrophoretical and sedimentational properties, in their phosphorus content, and in their stabilizing power towards the casein fractions in the presence of Ca^{++}. Swaisgood & Brunner (1962) concluded from electrophoretical and sedimentational observations that κ-casein may be either heterogenous or that there may exist polymeric species of κ-casein induced by the experimental technique. Furthermore, they suggested that scme κ-casein may also be associated with the β-casein fraction.

Wake & Baldwin (1961), using starch gel electrophoresis of α- and β-caseins, found 'a spread-out material, *presumably* κ-casein', with bands between 0·41 and 0·65 (numbered by their relative distance from the starting slot). Electrophoresis of para

κ-casein normally resulted in a diffuse pattern covering bands of 0·18, 0·34 and 0·41, but occasionally produced bands between 0·41 and 0·65. They suggested, therefore, that the κ-casein preparations examined may be heterogenous. The possibility of the existence of more than one rennin-sensitive substrate is also implied from the work of Jollès & Alais (1961), who found that various glycomacropeptides are released during the action of rennin on whole-casein.

In our investigations we have concentrated upon two main points: the behaviour and properties of whole-, α-, β- and γ-casein after the action of rennin on them, and the behaviour and the properties of the protective casein fractions released by rennin. These protective fractions are referred to as k-caseins and should not be confused with the κ-casein which can be obtained without first treating casein with rennin.

MATERIALS AND METHODS

The α- and β-caseins were prepared by the urea method and γ-caseins were prepared by both the urea method and by the alcohol method (Hipp, Groves, Custer & McMeekin, 1952). A yield of 150, 60 and 3 g of purified α-, β- and γ-caseins, respectively, were obtained.

Analyses by paper electrophoresis were done in a LKB cell as described by Sode-Mogensen & Lahav (1960).

Paper chromatography was performed, using *n*-propanol – 1 % NH_4OH (2:1, v/v) as solvent for 24 h at 16 °C with descending technique on Whatman No. 1 paper. The paper was dried at room temperature, sprayed with 0·4 % ninhydrin in water-saturated *n*-butanol and heated for 30 min at 80 °C. It was then sprayed on both sides with water and treated with a copper reagent (1 ml saturated $Cu(NO_3)_2$ and 0·2 ml of 1 % HNO_3, 100 ml 95 % ethanol).

P contents were determined by an adaptation of the method of Fiske & Subarrow (as described in Hawk, Oser & Summeson, 1954). N contents were determined by the micro-Kjeldhal method (*Methods of Analysis*, 1960).

Rennin reaction. The rennin was a highly purified crystalline product kindly supplied by Dr N. J. Berridge of the National Institute for Research in Dairying, Shinfield, Reading, England.

Enzyme concentrations used for the preparation of para-caseins and the rennin-sensitive fractions were adjusted so that they would complete the primary stage of the rennin action in about 3 and 14 h, respectively, as measured by the N soluble at pH 4·7, and in 2 % and in 12 % trichloroacetic acid (TCA, Fig. 1). The prolonged period of the rennin action was arranged to enable the reaction to be easily followed at short intervals by chromatography and other analyses.

One and 5 ml of an aqueous solution of the rennin (0·23 mg enzyme/100 ml water) were added to each of two 100 ml quantities of 1·7 % whole, α-, β- and γ-casein solutions prepared by the technique described by Sode-Mogensen & Lahav (1960) and adjusted to pH 6·4 (i.e. 0·02 or 0·1 μg of crystalline rennin per ml casein solution). The mixtures were incubated at 30 °C for 3 and 14 h, respectively.

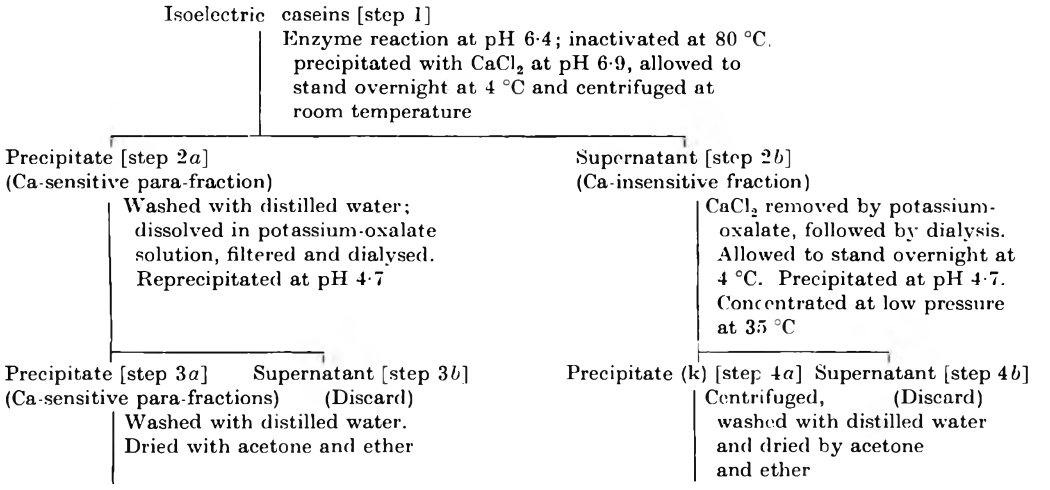
In order to keep the reaction products in a fluid state for analyses, the experiments were carried out in the absence of Ca^{++}, and the various solutions treated later with Ca^{++} to separate the sensitive and insensitive fractions.

Fractionation of the Ca-sensitive para-fractions and the Ca-insensitive fractions

At the end of the primary reaction the samples were heated to 80 °C in order to inactivate the enzyme, and $CaCl_2$ was added to give a final concentrate of 0·2 M while maintaining the pH at 6·9. After standing overnight at 4 °C the samples were warmed to room temperature and the Ca-sensitive fractions removed by centrifuging for 2 h at 1490 *g*. The precipitates were washed with distilled water, suspended in 100 ml of distilled water and the Ca^{++} precipitated with potassium oxalate. The calcium oxalate was removed by filtration and the excess oxalate by dialysis.

The resultant solutions were brought to pH 4·7 with 0·1 N-HCl in order to precipitate the Ca-sensitive para-fractions. The precipitates were washed with distilled water and dried with acetone and ether. Removal of Ca^{++} from the supernatants obtained in the preparation of the Ca-sensitive fractions was carried out in a similar way. The decalcified supernatant, containing the Ca-insensitive fraction, was cooled

Table 1. *Preparation of the calcium-sensitive para-caseins and their protective fractions (k fractions)*



overnight at 4 °C, acidified to pH 4·7 and concentrated *in vacuo* at 35 °C to complete the precipitation. The precipitate formed was centrifuged, washed with distilled water, and dried with acetone and ether.

For control purposes, the same procedures were applied to whole-, α-, β- and γ-caseins without the initial rennin treatment.

Analyses for P and N were carried out on the supernatants (Table 1, step 2*b*) before precipitating the Ca-insensitive fractions, and on the isolated fractions (Table 1, step 4*a*).

For electrophoretic and chromatographic analyses, as well as for the behaviour of the fractions towards Ca^{++} and rennin, the dried Ca-sensitive caseins and Ca-insensitive fractions were dissolved in 0·1 N-NaOH and the pH adjusted to 6·7 and 6·4, respectively. The line of application in the electrophoretic patterns was taken as zero point and the distance from it of each band was noted.

RESULTS AND DISCUSSION

The action of rennin on caseins

The soluble N released by the action of rennin on whole-casein and soluble at pH 4·7, or in 2 or 12 % TCA (Fig. 1) was in the range found by Alais *et al.* (1953) and the shape of the curves for N soluble at pH 4·7 and in 12 % TCA resembles those obtained by Beeby & Nitschmann (1963). It confirms the view that 'the NPN/time curves show clearly that a specific reaction is involved which terminates before visible clotting occurs and which differs distinctly from the slow but general proteolytic breakdown effected by the enzyme' (Alais *et al.* 1953). The relatively high amount of N soluble in 12 % TCA and the nearly horizontal course of the linear part

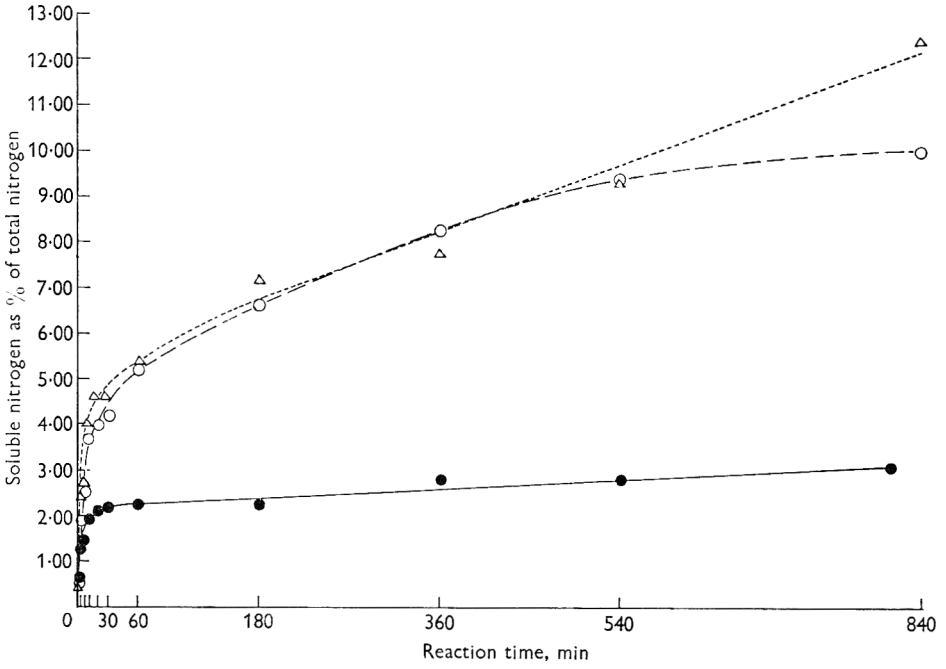


Fig. 1. The rate of release of soluble nitrogen in a 1·8 % solution of whole-casein containing 0·1 μg of rennin per ml (30 °C and pH 6·4). ○, Nitrogen soluble at pH 4·7; ●, nitrogen soluble in 12 % TCA; △, nitrogen soluble in 2 % TCA.

of this curve from the turning point until 3 and 14 h, respectively, show that the amount of NPN soluble in 12 % TCA formed a relatively high proportion of the total NPN during the preparation of the Ca-insensitive fractions.

It appears, therefore, that the rennin action is limited mainly to the primary stage only, and the fractions obtained in the supernatants (Table 1, step 2*b*), as well as the percentage of P and N of the different Ca-insensitive fractions (Table 3) are not due to a non-specific proteolysis.

The chromatographic analysis of samples taken during the 3 and 14 h periods, respectively, support this opinion. The chromatograms of the supernatants from the α- and γ-fractions (Table 1, step 2*b*) showed no decomposition spots at the rennin

concentrations used. However, although the supernatant of the β-fraction gave no decomposition spots at 3 h, 8 decomposition spots were found at 14 h. This finding is consistent with that of Cerbulis, Custer & Zittle (1960) who also found that β-casein was hydrolysed to a considerable extent by rennin, but contradicts that of Nitschmann & Keller (1955) and Keller (1954) who concluded that rennin did not hydrolyse β-casein appreciably (cited by Cerbulis *et al.* 1960).

Effect of Ca^{++} on the Ca-sensitive and Ca-insensitive fractions

The sensitivity of the fractions to precipitation by Ca^{++} was tested on 1 % solutions at pH 6·7. To each, separately an equal volume of 0·1 M-$CaCl_2$ was added. Only the sensitive para-caseins solutions gave heavy precipitates immediately after the addition of Ca^{++}, while, as expected, the insensitive fractions did not precipitate.

Table 2. *Composition of the different casein fractions upon reaction of rennin during 14 h*

Type of casein	P %	N %	P/N	% Phosphorus released	% Nitrogen released
Whole-acid casein	0·85	15·70	0·0539	6·00	11·17
α-Casein	0·97	15·69	0·0618	3·35	7·34
β-Casein	0·65	15·40	0·0423	2·17	10·00
γ-Casein	0·15	15·66	0·00945	5·84	22·18

Table 3. *Composition of the different Ca-insensitive fractions obtained from casein with and without rennin treatment*

	With rennin treatment				Without rennin treatment			
	k_w*	k_α	k_β	k_γ	κ_w*	κ	κ_β	κ_γ
% P	0·36	0·38	0·17	0·04	0·500	0·87	—	—
% N	15·70	15·67	15·66	15·68	14·49	15·54	—	—
P/N	0·0229	0·0242	0·0109	0·00255	0·0345	0·0560	—	—

* k_w, k_α, k_β, k_γ were the fractions obtained from whole-, α-, β- and γ-casein, respectively.

Effect of rennin on the Ca-sensitive and insensitive fractions

Rennin solution (1 p rennin to 2000 p casein) was added to duplicate samples of 3 solutions: (*a*) mixture of 1 % Ca-sensitive and 1 % Ca-insensitive fraction plus an equal volume 0·04 M-$CaCl_2$; (*b*) 1 % Ca-insensitive fraction plus an equal volume 0·04 M-$CaCl_2$; (*c*) 0·5 % Ca-sensitive fraction. In each case the final pH was adjusted to pH 6·4 and the samples were incubated at 30 °C for 3 and 14 h, respectively. Only the mixtures of the Ca-sensitive + Ca-insensitive fractions, and the solution of the Ca-insensitive fractions clotted on the addition of rennin, while the Ca-sensitive fractions did not clot in the absence of Ca^{++} even after 14 h.

Protective action of the Ca-insensitive fractions on the para-caseins

The ability of the Ca-insensitive fractions to protect the para-caseins from coagulation by Ca^{++} was assessed as follows: 1 % solutions of the sensitive fractions and 0·1–1·0 % solutions of the insensitive fractions were made at pH 6·7. Samples of each of the sensitive para-fractions were treated with various quantities of its

'own' as well as of the other insensitive fractions, mixed well and an equal volume of 0·1 M-$CaCl_2$ added (Pilson, Henneberry & Baker, 1960). No precipitation occurred at the concentration ratios noted in Table 4.

Characteristics of fractions obtained from caseins after rennin treatment

The procedure of fractionation of the Ca-insensitive fractions used in our experiments (Table 1) involved treatment with 0·2 M-$CaCl_2$ which would itself be expected to free protective factors from casein. This possibility was investigated in control experiments in which rennin was omitted but the same fractionation procedure used. From Table 3 it can be seen that no protective fractions were obtained from β- and γ-caseins without rennin treatment. Protective fractions κ_w and κ_α were, however, obtained from whole-casein and α-casein without rennin treatment. They differed distinctly from those obtained with rennin treatment in P content, P/N ratio (Table 3) and in their electrophoretic properties (Plate 1*f*, *h*; Plate 2*b*, *c*). From

Table 4. *The ratio by weight of the Ca-sensitive casein fractions to the Ca-insensitive fractions, at which precipitation in the presence of Ca^{++} was just prevented*

	Ca-sensitive whole-casein	α-Casein	β-Casein	γ-Casein
k_w*	4·5	5·0	4·0	5·0
k_α	5·0	4·0	4·0	4·0
k_β	1·0	1·0	5·0	1·25
k_γ	2·0	2·0	3·0	4·5

* k_w, k_α, k_β, k_γ were the Ca-insensitive fractions obtained from whole-, α-, β- and γ-casein, respectively.

Plate 2 it is seen that κ_w gave a diffuse band with partial separation into 2 components as obtained by Libbey & Ashworth (1961) under similar conditions. κ_α gave a diffuse band which was similar to that reported by Wake & Baldwin (1961) and to one prepared by us from a κ-casein kindly given by Dr C. Alais. In contrast, the protective *k*-fractions obtained from the rennin-treated caseins gave well-defined and reproducible bands on electrophoresis (Plate 1*f*, *h*).

In the electropherograms of the supernatant of whole-casein treated with rennin (Plate 1*f*) there were several faint, but nevertheless clear and reproducible bands designated k_1, k_2, k_3 whose positions coincided with those present in the supernatants of the different casein fractions after treatment with rennin (Plate 1*f*, area 4, 6·5, 7–8). It would, therefore, appear that they are not impurities arising from the method of preparation.

When the isolated *k* preparations were examined electrophoretically, components other than those represented by bands k_1, k_2, k_3 were found. This was doubtless the result of using high concentrations of the fractions concerned.

k_α-casein contained two clearly defined negatively charged components, one corresponding in position to k_1 and the other located near the origin (Plate 1*h*, areas 4, 9·5–10). A component similarly located was also found on the starch gel electropherogram of Wake & Baldwin (1961).

k_β-casein contained only one negatively charged component corresponding to band k_2 of whole-casein (Plate 1*j*, area 6·5). Libbey & Ashworth (1961) also observed such a band. It cannot yet be said with certainty that it is a component specifically

associated with β-casein because decomposition products were detected chromatographically in the supernatant of the β-fraction. Also viscosity measurements of the β-casein mixed with rennin indicated a considerable amount of degradation, which could be accounted for only if the protective fraction amounted to about 7 % of the β-casein (Dr Oosthuizen, Shinfield, Reading, pers. comm.).

k_γ-casein gave 3 clear bands on electrophoresis. The major component (Plate 1*l*, area 7–8) was identical in position with band k_3 (Plate 1*f*), but relatively much higher in concentration. The difference in intensities of the bands reflected the low concentration of γ-casein (about 6 %) found in whole-casein. Two other components of k_γ-casein (Plate 1*l*, area 3–3·5, 6–6·5) were perhaps, for the same reason, not detectable in the electropherogram of the *k*-fraction of whole-casein. However, one of these components (area 6–6·5) had a position similar to that of k_β and may, therefore, be only a contaminant.

In fact the concentration of *k*-casein associated with the γ-fraction appeared to be high. Also the amount of soluble nitrogen (22·18 %) released when rennin acted on γ-casein was high (Table 2). These observations agree with the findings of Wake (1959) and Hipp, Groves & McMeekin (1961) that fractions rich in κ-casein released large amounts of NPN.

Positively charged components were found in the preparations of para-caseins, para α-casein, para β-casein and of the *k*-fractions of whole-, α- and β-casein.

Preliminary results indicate that these components are polypeptide in nature. They have a protective action on the Ca-sensitive fractions and may be considered as forming part of the rennin-sensitive *k*-fractions.

The various *k*-caseins differed greatly in their P content (Table 3). The P content of k_α (0·38 %) conforms with that of the κ-casein of Hipp *et al.* (1961) (0·35 %), while the P content of k_β was similar to that reported by Waugh (1958) for κ-casein (0·19 %).

As seen from Table 4 the specificities of the protective fractions in stabilizing the Ca-sensitive fractions were also different. For example, whereas both k_α and k_γ had a powerful stabilizing effect on α- and γ-casein, respectively, only k_α had also a good stabilizing effect on whole-casein.

As expected all 3 para-caseins were sensitive to Ca^{++}, while their *k*'s were insensitive. On recombining the para-caseins with the appropriate protective fraction, they became stabilized against Ca^{++}, but when rennin was added to the reconstituted fractions soluble N was released and clotting occurred in the presence of Ca^{++}. These observations contradict those of Cerbulis, Custer & Zittle (1959) who maintained that 'α-casein is the only individual component of casein showing the clotting reaction'. The further release of soluble N on treating the reconstituted fractions with rennin is, however, in accord with the results of Beeby & Nitschmann (1963) using a heat reconstituted casein complex.

On the basis of the above findings, we suggest that casein contains several different Ca-insensitive fractions, each principally associated with its 'own' casein fraction α, β and γ.

By treating whole-casein with rennin, each one of the fractions α, β and γ releases its 'own' protective fraction(s). These protective fractions differ characteristically in their electrophoretical and chemical properties, and in their ability to stabilize the different casein fractions. They may be identical with those protective fractions designated

by various investigators as α_2, α_3, λ, m, z or generally as κ, and each one is specific for its 'own' fraction α, β and γ (McMeekin, Hipp & Groves, 1959; Hipp, Groves & McMeekin, 1961; Waugh & von Hippel, 1956; Linderstrøm-Lang, 1925; Long, Vanwinkle & Gould, 1958).

Another explanation (Lindqvist, pers. comm.) of these findings is, however, possible, namely that the protective fractions do not belong specifically to the respective casein fractions, but are protein breakdown products of a later rennin action. Many peptides, especially phosphopeptones, have similar protective properties (Reeves & Latour, 1958; Schipper, 1961). The clotting of the mixtures of para-casein and protective fraction on the addition of rennin would then be explained by a further splitting and inactivation of the protecting peptones.

In any case, it is evident that the products obtained by us are able to act as protectors and are very reproducible.

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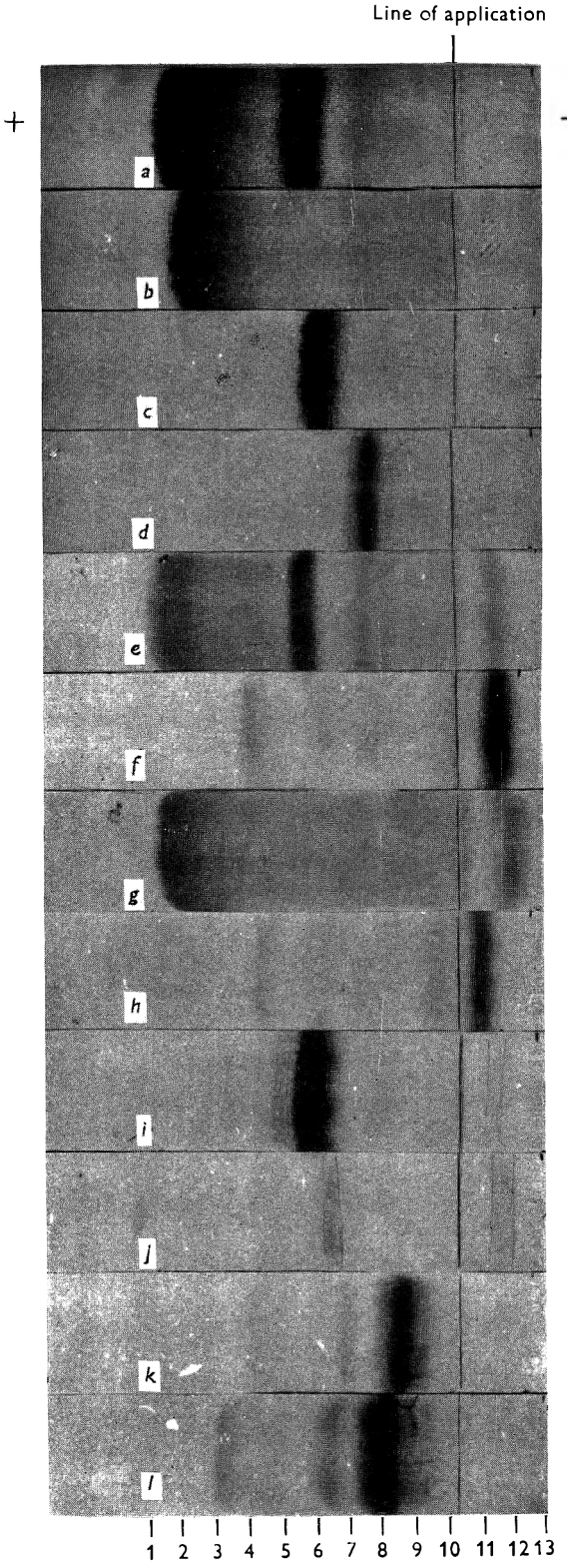

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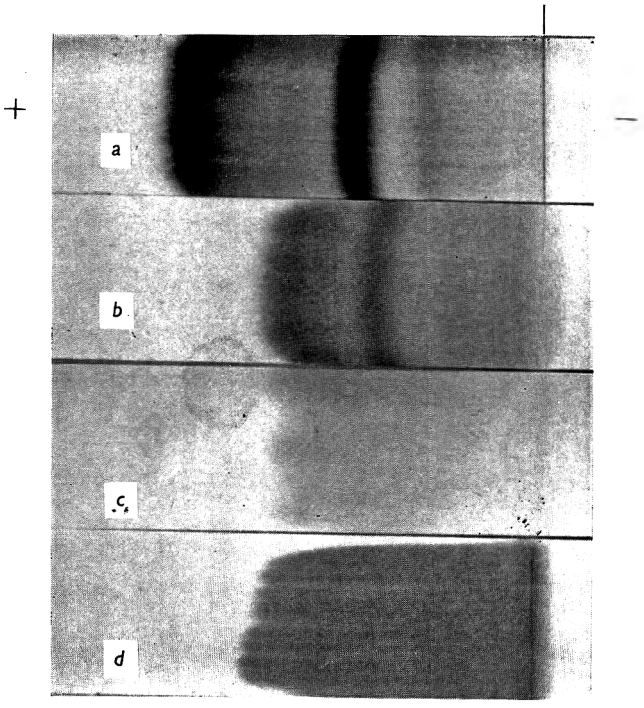
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EXPLANATION OF PLATES

Plate 1

Paper electrophoresis of whole acid casein, its purified fractions (α, β, γ), and of rennin-treated whole acid casein, its 3 purified para-fractions and their Ca-insensitive fractions (k fractions), respectively. *a*, Whole acid casein; *b*, α-casein (area: 1–4); c, β-casein (area 5–6·5); *d*, γ-casein (area: 7–8); *e*, whole para-casein; *f*, k-fractions of whole-casein (area: 4; 6·5; 7–8); *g*, para-α-casein; *h*, k_α fraction (area: 4); *i*, para-β-casein; *j*, k_β fraction (area 6·5); *k*, para-γ-casein; *l*, $k_{\gamma 1}$-, $k_{\gamma 2}$- and $k_{\gamma 3}$-fractions (area 3, 6·5, 7–8). Method of electrophoresis: Sode-Mogensen & Lahav (1960). The whole area of mobilities has been divided into 13 parts; for each fraction a certain area is characteristic.

Plate 2

Paper electrophoresis of whole-casein and of the Ca-insensitive fractions (κ) obtained from whole- and α-caseins without rennin treatment. *a*, Whole-casein; *b*, κ_w-casein; *c*, κ_d-casein; *d*, κ-casein (supplied by Dr C. Alais). Method of electrophoresis: Sode-Mogensen & Lahav (1960).

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The free and masked sulphydryl groups of heated milk and milk powder and a new method for their determination

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Summary. A new method is described for the determination of the free and masked sulphydryl (–SH) groups in heated milk and milk products. The procedure is based on the use of the specific –SH reagent *p*-chloromercuribenzoate, and utilizes another specific –SH reagent, 5,5′-dithiobis(2-nitrobenzoate), as an indicator producing yellow colours which are matched with the aid of standard glasses in the Lovibond Comparator.

The method was applied to heated skim-milks and a series of commercially prepared milk powders. Measurements were made in particular of the –SH content of UHT-treated milk. The changes in the –SH content of such milks on storage were studied; these changes could not be explained by a reversal of the denaturation of the whey proteins, although evidence was obtained that small amounts of heat-denatured whey protein can revert to the native state.

The sulphydryl (–SH) groups of milk are located chiefly in β-lactoglobulin. In unheated milk they are masked, but they become free and highly reactive when milk is heated and are then involved in changes in flavour (Hutton & Patton, 1952), affect the heat stability of milk (Rose, 1962) and appear to be of importance in the rate of clotting of milk by rennet (Kannan & Jenness, 1961).

Several methods have been used for the estimation of the –SH groups in milk. The widely used amperometric titration method has presented difficulties in its application to milk (Burton, 1959), difficulties which are also apparent from the recent work of Yoshino, Wilson & Herreid (1962). The thiamine disulphide method of Harland & Ashworth (1945) is rather elaborate and time-consuming. Several techniques based on reagents specific for –SH groups, such as that of Fridovich & Handler (1957) using *p*-chloromercuribenzoate, and of Ellman (1959) using 5,5′-dithiobis(2-nitrobenzoate), were found unsuitable for use with milk, and this led to the development of a simple method capable of yielding accurate results which is described below. It has been applied to heated skim-milk and to commercially prepared milk powders; it has also been used to measure the changes in the –SH content of UHT-treated milk on storage.

DETERMINATION OF –SH GROUPS

Principle of the method

A series of tubes is prepared containing increasing volumes of a solution of *p*-chloromercuribenzoate (PCMB), a specific reagent for –SH groups. Milk and a buffer solution are added to each tube, followed by a solution of 5,5′-dithiobis(2-nitrobenzoate) (DTNB). This reagent, developed by Ellman (1959), produces a yellow colour in those tubes in which –SH is in excess of PCMB with an intensity of colour proportional to the excess. The colour intensity is measured in the Lovibond Comparator in reflected light and, from a graph of intensity against PCMB-concentration, the equivalence point is readily determined. Application of this method provides a measure of the free –SH groups. The total –SH content (i.e. the sum of masked and free –SH groups) is determined in the same manner, but after addition of a denaturing agent, such as urea.

Reagents and apparatus

To avoid possible contamination from trace metals, all solutions must be made up in de-ionized distilled water.

PCMB. The reagent is purified by Boyer's (1954) method of dissolving it in dilute alkali, centrifuging the cloudy solution, and precipitating PCMB from the clear supernatant liquid with a few drops of concentrated hydrochloric acid. The precipitate is spun down, washed with water, spun down again and redissolved in dilute alkali. This procedure is repeated until the dissolved precipitate forms a clear solution. A standard solution is prepared in pyrophosphate-acetate buffer, pH 7·0 ($Na_4P_2O_7.10H_2O$,8·92 g/l; glacial acetic acid, 1·4 ml/l). The standard solution of PCMB should be approximately 1 mM and is prepared either directly and standardized by measuring the extinction of a 1:50 dilution in water at 232 mμ (ϵ = 16500), or by dilution of an approximately 40 mM solution of PCMB, standardized by iodine titration as described by Boyer (1954). The standard solution must be kept in the dark and should be discarded if a precipitate forms.

DTNB. The compound can be purchased (Aldrich Chemical Co., Milwaukee, U.S.A.) or prepared according to the procedure of Ellman (1959). Solution *A*: 10 mM-DTNB in 0·1 M-phosphate buffer, pH 7·0 ($Na_2HPO_4.12H_2O$, 21·9 g/l; $NaH_2PO_4.2H_2O$, 6·07 g/l). Solution *B*: 1 ml of solution *A* diluted to 10 ml with water. The solutions keep for 1 week in the refrigerator.

Thiourea. 0·5 g in 100 ml water.

Buffer. The solution contains sodium citrate, tribasic, 43·26 g/l, citric acid monohydrate 0·62 g/l, sodium veronal 4·13 g/l, and sodium azide 0·044 g/l. The pH of the buffer is measured and, if necessary, adjusted to 8·0 ($\pm$ 0·1) with N-NaOH or HCl.

Urea-buffer. 40 g urea and 30 ml of the above buffer solution are mixed and warmed to 37 °C in a water-bath to dissolve the urea. The solution is held at that temperature, and must be prepared fresh daily.

Apparatus. A Lovibond 'all purposes' Comparator complete with stand for work in reflected light is used with the disk No. 4/27 (obtainable from Tintometer Ltd, Salisbury) specially made for the new method. The 7 glasses of the disk match solutions containing 0–6 μM–SH.

A water-bath capable of being maintained at 37 °C.

A 2-ml micro-burette, graduated to 0·01 ml.

A supply of test tubes conforming to British Standard 625:1959, nominal size 150/16, marked at 10 ml, with rubber stoppers to fit.

Procedure for milk

Preliminary test

Before a sample of milk of unknown –SH content is analysed for either free or total –SH, an approximate estimation must be made, using a series of tubes containing 0–0·6 ml PCMB solution at 0·1-ml intervals; an additional tube with 1 ml of PCMB is prepared to serve as the blank. The procedure given below for adding milk, buffer and DTNB solution to the tubes and measuring the colour developed is then followed. The result of the preliminary test permits selection of a narrow range of volumes of PCMB suitable for the accurate determination of the –SH groups; generally, a range covering 0·12 ml at 0·02-ml intervals is required, but in severely heated samples it is necessary to increase both the range and the interval. With other samples, the precision can be increased by reducing the interval to 0·01 ml.

(*a*) *Determination of total –SH*

To 100 ml of the milk sample, add 0·5 ml thiourea solution, mix, and allow the mixture to stand for not less than 10 min. If the preliminary test shows the presence of free –SH, this step may be omitted.

To a series of test tubes add increasing volumes of the PCMB solution from the micro-burette over the predetermined range, making sure that the last tube (blank) contains an excess of PCMB solution.

To each tube add 3 ml of the mixture of milk and thiourea solution, and mix. Add urea-buffer solution to the 10 ml mark, and mix. Finally, add 0·1 ml of DTNB solution *A* and mix again. Measure the colour in each tube 1–5 min later against the blank in the Lovibond Comparator, and record the apparent tint of each tube, interpolating colour values where necessary.

Plot the readings against the volumes of PCMB, as shown in Fig. 1. Draw the best straight line through the points: the intercept on the abscissa gives the equivalent volume of PCMB. Then

$$-\text{SH concentration (mM)} = \text{PCMB concentration (mM)} \times \frac{\text{equivalent volume of PCMB}}{\text{volume of milk}}.$$

If the readings are repeated within the time limits given above, the equivalence point should not vary by more than 0·005 ml.

(*b*) *Determination of free –SH*

To a series of test tubes add increasing volumes of PCMB solution, as described under (*a*).

To each tube add 7 ml of buffer followed by 3 ml of milk, and mix. Immediately add 0·1 ml of DTNB solution *B* and mix again, starting a stop-watch when adding DTNB to the first tube. Measure the colour in each tube after 1 min as quickly as possible, and repeat the measurements at least twice at intervals of 1–2 min, noting

the time of starting each set of readings. Plot each set of readings against the volumes of PCMB solution. The apparent equivalent volume of PCMB will be seen to increase with time and must be extrapolated back to the time of the first addition of DTNB; to do this, the apparent equivalent volumes are plotted against the time of starting each set of readings. The concentration of free –SH groups is then calculated as in the determination of total –SH.

Modified procedure for milk powders

A special problem is presented by the analysis of milk powders, since the free –SH groups tend to be lost rapidly when the powders are dispersed in water in the conventional manner. The difficulty can be circumvented by adding a small fixed amount of powder to each tube used in the test, a procedure producing higher and

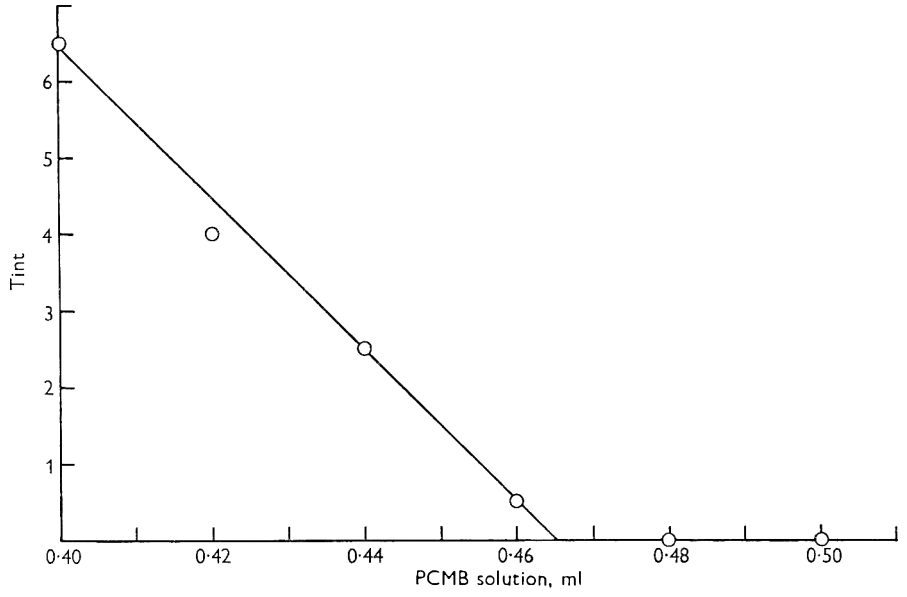

Fig. 1. Determination of the equivalent volume of *p*-chloromercuribenzoate (PCMB) solution. The equivalent volume is given by the intercept on the abscissa and is 0·465 ml in the example shown.

more consistent results than any method of preparation involving dispersion of the powder in bulk. For this purpose, the procedure is modified as follows. After adding PCMB to the tubes, add either 10 ml of a mixture of 7 parts urea-buffer solution and 3 parts water for total –SH, or 10 ml of buffer for free –SH. Add to each tube 0·27 g of skim-milk powder or 0·39 g of whole milk powder, stopper the tubes and shake to dissolve the powders. Add DTNB and complete the analysis as described for milk. Unlike milk samples, some milk powders when analysed for total –SH have been found to produce a rise in the equivalence point resembling that which normally occurs only in the determination of free –SH groups. No reason for this is known, but with such powders, it is necessary to extrapolate back to the time of addition of DTNB, as described for the determination of free –SH groups. It is convenient to express the results in the same units as those used for liquid milk. If it is assumed that the

weights of milk powder given above are equivalent to 3 ml of milk on a basis of the reconstitution of 9 g of skim-milk powder or 13 g of whole milk powder to 100 ml results may be expressed as mM–SH.

Notes on the procedure

The citrate-veronal buffer serves not only to adjust the final pH to the range 7·5–8·0 for the reaction of DTNB with –SH groups, but also, through the clearing action of citrate on the casein of milk, to increase considerably the apparent intensities of the yellow colours. In condensed and in sterilized milk, little clearing action occurs; as a result, the steepness of the slope is reduced when readings are plotted as in Fig. 1, and the accuracy of the determinations is decreased. The turbidity caused by the fat droplets in cream or homogenized milk produces a similar but less marked effect.

As mentioned above, thiourea need not be added to samples containing free –SH groups. In the absence of free –SH, thiourea must be added to suppress the effect of an oxidizing agent in milk; if thiourea is omitted in the analysis of a sample not containing free –SH, the apparent value of the total –SH is lowered by 2–8 %.

The mode of action of the azide present in the buffer is uncertain, but its omission led to low and erratic results in the analysis of unheated milk samples.

It is essential to complete the analysis within the time limits given, since about 8 min after the addition of DTNB all tubes start to develop a yellow colour, so that reproducible equivalence points can no longer be obtained.

The precision of the method is limited by the accuracy with which colours can be matched with the disk. This causes an uncertainty in determining the point of intercept and hence the equivalence point. Expressed as a volume of 1 mM-PCMB the uncertainty appears to be of the order of 0·005 ml; in turbid solutions, it is larger for the reasons given above. Except in solutions of very low –SH concentration, this volume is independent of the amount of –SH groups present, so that the percentage error is reduced in the analysis of samples of high –SH content. In solutions low in –SH ($< 0{\cdot}02$ mM), the error is increased because of the weakness of the tints developed; with such solutions, no more than an estimate of –SH content can be made.

Tests of the method

The accuracy of the method for determination of total –SH was tested with the aid of crystalline β-lactoglobulin, which is known to contain two –SH groups per molecule. A solution of β-lactoglobulin was prepared and its concentration determined by measurement of the extinction at 280 mμ, using a value of 35,500 for the molecular weight and of 9·5 for the specific extinction coefficient of a 1 % solution. The concentration was 0·0694 mM which is equivalent to 0·139 mM–SH. Direct determination of the –SH groups gave the same value of 0·139 mM–SH. Determinations were also made on fresh skim-milk and heated skim-milk (85 °C for 15 min) with and without the addition of amounts of the β-lactoglobulin solution sufficient to increase the –SH concentration by 0·139 mM. The results are shown in Table 1; it is seen that the recovery of –SH groups was within 2 % of the expected value.

As β-lactoglobulin is known to account for practically all the –SH groups in milk, the reliability of the method was further tested by comparing the total –SH content

of the milk of 5 individual cows with the –SH content calculated from the β-lactoglobulin concentrations as determined by the method of Aschaffenburg & Drewry (1959). The results are shown in Table 2, in which the results of the nitrogen determinations have been converted to equivalent –SH concentrations on the basis of 2 groups per molecule (molecular weight, 35,500) and a nitrogen content of 15·6 %. Considering the possible errors in the determination of β-lactoglobulin nitrogen, agreement was fair, with an average discrepancy of 0·010 mM.

It proved much more difficult to devise tests for the accuracy of the determination of free –SH groups, which require the addition of reactive –SH compounds such as cysteine. In experiments of this nature recovery was erratic and always low. Contrary to those of small –SH compounds, the –SH groups of the large molecule of bovine serum albumin were found to provide a suitable basis for testing. When a solution of this protein of known –SH content was added to skim-milk, recovery was 95 %.

Table 1. *Recovery of –SH groups added as a β-lactoglobulin solution to skim-milk and to heated skim-milk*

(–SH groups added, 0·139 mM.)

	Total –SH content (mM)	
	Found	Recovered
Unheated milk with added β-lactoglobulin	0·262	0·138
Unheated milk without added β-lactoglobulin	0·124	–
Heated milk with added β-lactoglobulin	0·232	0·137
Heated milk without added β-lactoglobulin	0·095	—

Table 2. *–SH content of milks from individual cows*

(Comparison of the total –SH content measured by the new method with the –SH content calculated from determination of the β-lactoglobulin nitrogen.)

	Total –SH content (mM)	
Cow	Measured	Calculated
Brilliant	0·112	0·125
Begonia 2	0·114	0·114
Bride 11	0·185	0·174
Grouse 2	0·205	0·190
Glee 11	0·221	0·208

APPLICATIONS OF THE METHOD

Heated skim-milk

Fig. 2 shows the results of determinations of the free and total –SH content of portions of bulk skim-milk heated for 15 min to a series of temperatures. The samples were heated in test tubes rotated in a constant-temperature water-bath, except that those at the highest temperature (100 °C, nominal) were immersed stationary in a large beaker of boiling water. The general shape of the curves obtained by the new method is the same as that obtained by Hutton & Patton (1952) by argentometric titration. There was virtually no liberation of –SH groups below 70 °C; above that

temperature the increase in free –SH was marked, as a result of the denaturation of β-lactoglobulin. The drop in both free and total –SH at the higher temperatures was probably caused by oxidation, as air was not excluded from the tubes during the heating process. It will be noted that although β-lactoglobulin must have been completely denatured at the highest temperature of heating, the gap between the two curves did not close, suggesting that a small proportion of the –SH groups remained masked.

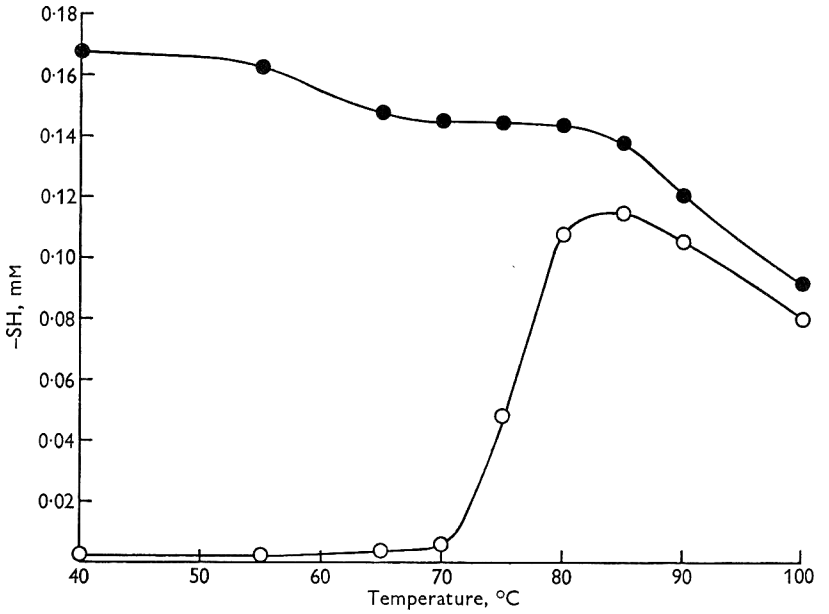

Fig. 2. –SH content of skim-milk heated at various temperatures for 15 min. ○—○, Free –SH; ●—●, total –SH.

UHT-treated milk

No information is available on the effect of UHT treatment on the –SH groups of milk, and in view of the increasing importance of treatments based on this process its effect on milk –SH groups and subsequent changes on storage have been studied. Some data on the –SH content of milk heated to temperatures above 100 °C have been provided by the results of Harland, Coulter, Townley & Jenness (1955), but these were obtained in plant differing considerably from present-day UHT plants. In our experiments, samples were taken from the line of a commercial plant immediately after passing through the UHT heater. The samples were cooled rapidly and distributed in a series of screw-cap bottles, without complete exclusion of air. Some bottles were refrigerated whilst others were left at room temperature. The results of free and total –SH determinations (the average of 2 runs) at various times after the heat treatment are shown in Fig. 3. Before heating, the milk had no free –SH groups and was 0·139 mM in respect to total –SH. Immediately after heating, nearly all the –SH groups were free. There was a slow decline in total and free –SH after storage in the cold, but at room temperature both total and free declined rapidly; the very

marked effect of storage temperature on the rate of disappearance of –SH groups is difficult to explain.

The gap between total and free –SH in the samples stored at 4 °C changed little on storage, but in samples kept at room temperature the gap widened, indicating a rise in masked –SH groups, an observation suggesting the possibility that some denatured β-lactoglobulin had reverted to the native state. An attempt to investigate this possibility was made with the aid of the turbidity test of Aschaffenburg (1947). Although originally developed for sterilized milk control, this test was found to be applicable to the estimation of the concentration of undenatured whey proteins in samples of less severely heated milk. For this purpose, the test was applied to a

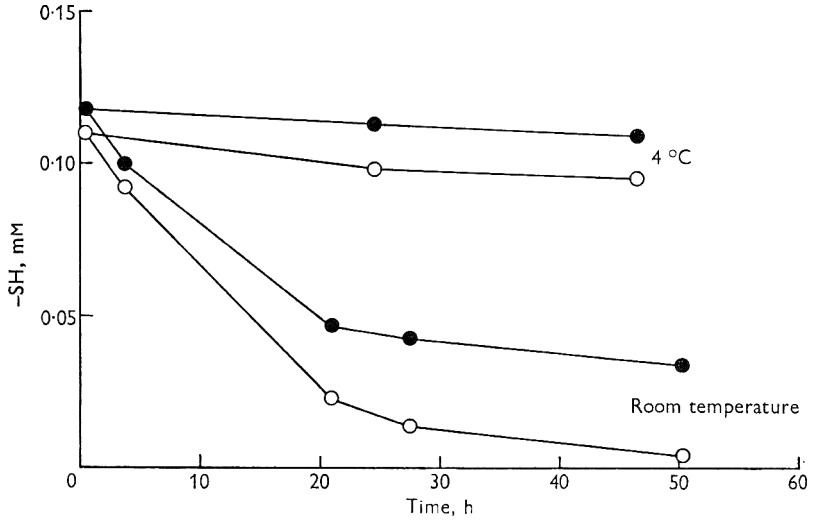

Fig. 3. Changes in the –SH content of UHT-treated milk on storage at either 4 °C or room temperature. ○—○, Free –SH; ●—●, total –SH.

series of mixtures of unheated milk and milk heated sufficiently strongly to give no turbidity, so as to form a series of tubes of increasing turbidity. The turbidity of an unknown sample could then be matched with this series. In mixtures containing up to 5 % unheated milk, differences in turbidity corresponding to 0·5 % unheated milk were distinguishable.

Application of this method to samples of UHT-treated milk produced turbidities equivalent to those of mixtures containing up to 3 % unheated milk. That the whey proteins present in these samples still contained undenatured β-lactoglobulin was shown by paper electrophoresis of the soluble whey proteins by the method of Aschaffenburg & Drewry (1957); in addition to the α-lactalbumin band, weak bands of β-lactoglobulin A and B still appeared in their usual positions. The turbidities of UHT milk were strongest in the most rapidly cooled samples; this is not surprising, since the milk at the outlet of the UHT plant was still at 80 °C.

A small increase in turbidity was observed in samples of UHT milk held at room temperature; in one experiment, the initial turbidity matched that of heated milk containing 2·5 % of unheated milk, but 24 h later, when the test was repeated, the turbidity matched that produced by 3 % unheated milk. The high temperatures used

in UHT plants are not essential for this effect: similar small increases were observed in samples of milk held at room temperature after they had been heated in boiling water for up to 12 min. These results provide evidence for the reversion of small amounts of whey protein to its native state. However, the extent of reversion was too small to account for the increase in amounts of masked –SH groups, which, as shown in Fig. 3, rose from less than 10 % to more than 20 % of the total –SH present in the milk before heating. Since such a rise clearly could not be caused by the reversion of denatured β-lactoglobulin to the native state, it must have been due to changes in the state of the denatured protein.

Milk powders

Since the heat treatment of milk in the manufacture of milk powder varies considerably, a series of spray- and roller-dried powders made at different factories was analysed shortly after manufacture. The results are given in Table 3. The total –SH content of the milk before heating was not measured but was probably in the

Table 3. *Sulphydryl content of milk powders*

(Results expressed as mM –SH on a basis of the reconstitution of 9 g of skim-milk powder or 13 g of whole milk powder to 100 ml. 'Brief' heating means that the time of passage through the heater was short but not known.)

	Heat treatment before evaporation	Free –SH groups (mM)	Total –SH groups (mM)
Spray-dried skim-milk powders:			
A	79 °C/20 sec	0	0·118
B	88 °C/brief	0·016	0·124
C	135 °C/brief and 85 °C/30 sec	0·064	—
Roller-dried powders:			
D (skim-milk)	74 °C/10 min and 88 °C/30 sec	0·083	0·094
E (skim-milk)	88 °C/brief and 91 °C/2 min	0·093	0·108
F (skim-milk)	104 °C/brief	0·084	0·092
F (whole milk)	104 °C/brief	0·082	0·101

range 0·14–0·16 mM; if so, a marked drop in total –SH occurred in all the powders. With spray-dried powders it seems clear that the concentration of free –SH depended on the temperature of pre-heating, and tended to rise with increasing temperature. This did not apply to the roller-dried powders, where most of the –SH groups were in the free state, regardless of the temperature of pre-heating. Slight decreases in both free and total –SH content were observed over a period of 4 or 5 months, during which the powders were kept at room temperature, in closed, but not hermetically sealed, tins. In sealed tins at 37 °C, no change in the –SH content of whole milk powder was noted in 3 months by Harland, Coulter & Jenness (1952).

DISCUSSION

In choosing reagents for a new analytical method, specificity is of great importance. PCMB has the desired specificity for –SH groups and is readily available; and although it requires purification before use, its solubility properties make this an easy task. DTNB has proved a useful indicator, even though, as a disulphide compound, it is

sensitive to the oxidizing and reducing agents in milk. The effects of these agents have been minimized, but could not be eliminated completely; they would not easily be recognizable in a straightforward titration method, but are detected readily when a series of tubes is used to determine the equivalence point, as proposed in the new method.

The effect of one of the oxidizing agents present in unheated milk is neutralized by the addition of thiourea; absence of thiourea from a sample without free –SH groups leads to a low result for total –SH. The oxidizing agent responsible for this loss of –SH groups is thought to be dehydroascorbic acid, formed from ascorbic acid by the action of light. Thiourea, at the concentration specified, was the only one of many compounds tested that was capable of neutralizing the effect of the oxidizing agent without reducing DTNB or reacting with PCMB. Since thiourea has no effect on the rapid decline of free –SH groups in milk powders dispersed in water by conventional methods, such losses must be ascribed to another oxidizing agent.

In the determination of free –SH groups, extrapolation of the apparent equivalent volumes of PCMB to zero time is necessary, since the yellow colours increase slowly with time. The increase is caused by 3 factors. First, reducing agents in milk act on DTNB, an effect which has been minimized by keeping the concentration of DTNB as low as possible. Secondly, there is a slow reaction of the masked –SH groups of β-lactoglobulin with both DTNB and PCMB. Thirdly, a factor of minor importance, PCMB and the reduced form of DTNB combine slowly to form a compound with a faint greenish-yellow colour which tends to alter the tint, although it scarcely affects the intensity.

Since virtually all the –SH groups of milk arise from one of the milk proteins, β-lactoglobulin, the course of the liberation by heat of the –SH groups must follow that of the heat-denaturation of this protein. The heat-denaturation of β-lactoglobulin, like that of most other proteins, is characterized by a very large temperature coefficient, as indicated by the steep rise in free –SH between 70 and 80 °C shown in Fig. 1. In view of this large temperature coefficient, it was surprising to find small amounts of residual native β-lactoglobulin in UHT-treated milk; from the effect of heating at temperatures between 70 and 80 °C it can be calculated that, at 135 °C, 99·9% of the β-lactoglobulin should be denatured in much less than 1 sec. The survival of small amounts of native protein, which is supported by the results of Harland *et al.* (1955), suggests that the mechanism of the denaturation reaction differs at the higher temperature, a point of considerable interest in view of the prevailing trend towards higher temperatures and shorter times in the heat treatment of milk.

Residual masked –SH groups have been found even when milk has been heated to temperatures of 100 °C or above. These masked groups do not arise either from residual undenatured β-lactoglobulin or from denatured β-lactoglobulin that has reverted to the native state. How they are formed is not known, but interaction with other proteins is probably not involved; in heated solutions of pure β-lactoglobulin Larson & Jenness (1952) observed that, although all of the protein was denatured, about a fifth of the total –SH groups were not susceptible to atmospheric oxidation in spite of exposure to the air for 48 h, and it seems likely that this inertness of –SH groups to atmospheric oxidation is similar to the failure of the masked groups to react with –SH reagents. In their reactions with –SH reagents, these masked groups are indistinguishable from those of native β-lactoglobulin.

Although the experiments on reversion of whey proteins to the native state showed this process to be quite insufficient to account for the changes in the concentration of masked –SH groups in stored UHT-treated milk, the fact that some reversion of whey proteins in the native state does occur is of importance, and seems comparable to the reactivation of milk alkaline phosphatase after similar heat treatment (Lyster & Aschaffenburg, 1962). In both instances, only 1 %, or less, of the protein present before heating appears to return to its native state. With the enzyme, direct observation of the increase in the amount of reactivated protein is possible by measurement of the specific enzymatic activity; however, with the whey proteins, it has not so far been possible to measure directly the increase in the amount of any one particular protein. This problem must await the development and application of sensitive methods specific for the individual whey proteins.

The results given in Table 3 show that the –SH content of spray-dried powders reflects the degree of pre-heating. The –SH groups of spray-dried whole milk powders have been shown to be correlated with keeping quality by Harland *et al.* (1952), using as a measure of –SH content the thiamine disulphide reducing substances, which may be taken as equivalent to the free –SH groups in our method. In powders with relatively high free –SH content, these authors found that the development of fat peroxides and the loss of flavour score during 10 weeks' storage were inhibited, a finding of considerable interest in view of the practical importance of the keeping quality of milk powders. The study of these, and other similar problems, has been hindered in the past by the elaborate nature of the analytic procedures involved, and it is hoped that the method described in this paper will remove this limitation to the applications of the measurement of the –SH groups of milk.

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Skin structure and milk production of British dairy cattle

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SUMMARY. The possibility of using a skin measurement as an index of potential milk-producing capacity in cattle was re-examined. Skin specimens were obtained by biopsy from 7 dairy herds, and each of 5 anatomical measurements was correlated with (1) milk yield and (2) butterfat percentage.

Only one of the pooled within-herd relationships was statistically significant. This relationship indicated that within the breeds studied, cows with the least hair follicle depth would be expected to give the highest milk yield. It is suggested that a more extensive investigation of this relationship might yield results of practical value in assessing milk-producing capacity and in progeny testing.

Burcev (1937) reviewed reports of a correlation between the number of sweat glands per unit area of skin in the ear of the cow and the milk yield, and in a study of some 500 Red German cattle confirmed the existence of such a correlation. Findlay, Goodall & Yang (1950), however, found that sweat gland number per unit area varied with age and between different parts of the ear in Ayrshire cattle and concluded that such sweat gland counts could not possibly be used as an indication of milk-producing capacity. In these earlier studies only sweat gland number per unit area of skin was considered, but recently the close phylogenetical relationship between the mammary gland and the glands of the skin (Carlisle, 1954; Linzell, 1959) led us to examine the feasibility of using some form of skin measurement as an index, however rough, of potential milk production.

MATERIALS AND METHODS

Skin specimens from 5 dairy herds from different parts of Britain were used in the initial investigation. Details of these herds (I–V) are given in Table 1. From one of them (no. IV) skin specimens were obtained again 2 years later and the cows used on that occasion were regarded as herd IV*a*. All these herds, with the exception of IV*a*, were sampled in winter.

The animals in these herds (I–V) varied considerably in age, and the management conditions for the different herds were not all the same. Two further herds (nos. VI

and VII) were therefore chosen. All the animals in these herds were of approximately the same age and were kept under exactly the same conditions of feeding and management. The animals were divided into groups each of which was sired by the same bull. These herds, details of which are given in Table 1, were sampled in summer.

Duplicate skin specimens of a uniform size (5 mm in diameter) were obtained by biopsy from the midside region of each animal using a high speed punch technique (Findlay & Jenkinson, 1960). These specimens were fixed in 5% formol-saline and sections 300–350 μ in thickness, containing numbers of undamaged sweat glands, were cut parallel to the direction of hair growth and prepared for examination using a modification (Nay, 1959) of the method of Nay & Hayman (1956).

The following measurements were made on the skin specimens from each animal:

(*a*) *Hair follicle depth* (*FD*)

Hair follicle depth was defined as the perpendicular distance between the base of an active hair follicle and the surface of the skin. Giant follicles which were seated deep in the reticular layer and represented some 4–6% of all the follicles present (Hayman & Nay, 1961) were ignored. Ten follicles in each section were measured and the mean value taken as the typical follicle depth.

(*b*) *Sweat gland length* (*L*)

The length of the fundus (secretory portion) of each of 10 glands in each section was measured and the mean value calculated.

(*c*) *Sweat gland diameter* (*D*)

The diameter of the secretory portion of each of the same 10 glands in each section was also measured.

From these estimates of length and diameter the following were calculated:

(*d*) *Sweat gland shape* (*L*/*D*)

This was expressed as sweat gland length divided by sweat gland diameter, and is a measure of the extent to which animals tend to have simple sac-like or tubular glands.

(*e*) *Sweat gland volume* (*V*)

By considering the sweat glands as cylindrical vessels the glandular volume was estimated using the formula $\pi(D/2)^2 L$.

These skin measurements were compared with 2 measures of milk production, namely:

(1) *Milk yield* (*MY*)

The total amount of milk (kg) produced in the first 305 days of the first lactation was used. Where a lactation did not extend to 305 days the value taken was the total yield for the whole lactation. The first lactation has been shown (Mahadevan, 1951*a*) to be a reliable index of the potential milk-producing capacity of a dairy cow.

(2) *Butterfat percentage* (F %)

This quantity was also obtained from records of the first lactation compiled in the standard way by the Scottish Milk Records Association and the Milk Marketing Board. No records of butterfat percentage were available for herd V.

Statistical analysis of the results was made on an electronic digital computor ('Silliac').

Table 1. *Details of the herds studied*

Location of herd	Experimental number	Breed	Number of animals sampled
West of Scotland Agricultural College, Ayr	I	Friesian	28
University of Durham, Cockle Park Research Farm, Morpeth	II	Jersey	32
Agricultural Research Council, Institute of Animal Physiology, Babraham, Cambridge	III	Dairy Shorthorn	15
Hannah Dairy Research Institute, Ayr	IV	Ayrshire	16
Hannah Dairy Research Institute, Ayr	IV*a*	Ayrshire	24
Agricultural Research Council Field Station, Compton	V	Ayrshire	8
British Oil and Cake Mills Ltd, South Cathkin Farm, Glasgow	VI	Ayrshire	37
British Oil and Cake Mills Ltd, Selby, Yorkshire	VII	Friesian	20
		Total	180

RESULTS

The mean and standard deviations of all 7 measurements under consideration are given in Table 2. The correlation coefficients between each of the skin measurements and the 2 measures of milk production are shown in Table 3. The relationships between the 5 skin measurements will be discussed in a later publication. A pooled correlation coefficient combining the correlation coefficients within herds was calculated in each instance using the Z-transformation method of Fisher (1948) (Table 4). For this calculation the data for herd IV*a* were omitted except where stated in Table 4.

DISCUSSION

As shown in Table 3 no statistically significant correlations were found between each of the sweat gland measurements and either the milk yield or the butterfat percentage, except for a negative correlation between sweat gland length and milk yield for herd IV and a positive correlation between sweat gland length and butterfat percentage for herd IV*a*. The pooled within-herd correlations of milk yield and of butterfat percentage with each of the sweat gland measurements did not vary significantly from zero as shown in Table 4.

In one herd (no. III) a significant positive correlation was found between hair follicle depth and butterfat percentage (Table 3). However, the pooled within-herd correlation was not significant (Table 4). A negative correlation between hair follicle depth and milk yield was found for all but one herd (no. IV) as shown in Table 3. When this herd was sampled again 2 years later 8 additional animals were present

and some of the original animals had been replaced. A non-significant negative correlation was then found (no. IV*a*). Non-significant negative correlations between hair follicle depth and milk yield were found for herds VI and VII, which were kept under standard conditions, and significant negative correlations were found for herds I and III (Table 3). Similarity of age and management of the animals, therefore, did not improve the correlation between hair follicle depth and milk yield.

The pooled within-herd correlation between milk yield and hair follicle depth was highly significant (Table 4). This indicates that within the breeds studied, cows with the least hair follicle depth would be expected to give the highest milk yield. Nay & Hayman (1963) have shown that hair follicle depth varies with season, being less in

Table 3. *Correlation coefficients between each of the various skin measurements, and the milk yield and the butterfat percentage*

	Herd	Hair follicle depth	Sweat gland				Milk yield
			Length (*L*)	Diameter (*D*)	Shape (*L*/*D*)	Volume	
			Milk yield				
I	Friesian	−0·43*	+0·11	+0·08	+0·08	+0·13	—
II	Jersey	−0·19	0·00	+0·04	−0·04	+0·03	—
III	Dairy Shorthorn	−0·63*	−0·32	−0·05	−0·25	−0·24	—
IV	Ayrshire	+0·15	−0·50*	−0·09	−0·43	−0·32	—
IV*a*	Ayrshire	−0·31	−0·24	−0·17	−0·06	−0·23	—
V	Ayrshire	−0·46	−0·43	−0·08	−0·56	−0·22	—
VI	Ayrshire	−0·16	+0·04	−0·11	+0·19	−0·04	—
VII	Friesian	−0·01	−0·08	+0·20	−0·15	+0·09	—
			Butterfat %				
I	Friesian	−0·05	−0·14	−0·02	−0·13	−0·12	−0·33
II	Jersey	+0·18	−0·03	+0·06	−0·09	+0·04	−0·25
III	Dairy Shorthorn	+0·52*	+0·44	+0·08	+0·36	+0·34	−0·55*
IV	Ayrshire	+0·41	+0·45	+0·04	+0·44	+0·24	−0·11
IV*a*	Ayrshire	+0·35	+0·45*	+0·19	+0·26	+0·33	−0·09
V	Ayrshire	—	—	—	—	—	—
VI	Ayrshire	−0·10	−0·22	−0·18	−0·09	−0·21	−0·25
VII	Friesian	+0·22	+0·17	−0·02	+0·18	+0·05	−0·33

* $P < 0·05$.

summer than in winter. One-third of the animals in herd IV*a* were sampled in summer only. The measurements of hair follicle depth obtained for these animals were, therefore, increased by approximately 5% to represent the expected winter values and the pooled within-herd correlation recalculated. The significance of the pooled within-herd correlation, however, was not affected by this adjustment for seasonal changes.

A highly significant negative pooled within-herd correlation (−0·30) between milk yield and butterfat percentage was obtained confirming many previous findings of a similar nature, e.g. Mahadevan (1951*b*). The opposite signs of the pooled correlation coefficients between hair follicle depth and milk yield and between hair follicle depth and butterfat percentage were therefore to be expected.

Herd	Hair follicle depth, mm	Sweat gland: Length (L), μ	Sweat gland: Diameter (D), μ	Sweat gland: L/D (shape)	Sweat gland: Volume, $\mu^3 \times 10^6$	Milk yield, kg	Butterfat, %
Friesian, I	1·56 ± 0·14	1014 ± 145	133 ± 10	7·65 ± 1·02	14·3 ± 3·7	4180 ± 775	3·92 ± 0·28
Jersey, II	1·39 ± 0·11	755 ± 115	115 ± 12	6·58 ± 1·02	8·1 ± 2·3	2880 ± 525	5·44 ± 0·58
Dairy Shorthorn, III	1·75 ± 0·14	1103 ± 130	109 ± 8	10·21 ± 1·31	10·3 ± 2·1	3705 ± 645	3·78 ± 0·32
Ayrshire, IV	1·52 ± 0·12	765 ± 114	130 ± 15	5·94 ± 0·81	10·4 ± 3·2	3840 ± 770	3·98 ± 0·39
Ayrshire, IV*a*	1·46 ± 0·15	780 ± 102	129 ± 15	6·11 ± 0·76	10·4 ± 3·2	4175 ± 855	3·97 ± 0·37
Ayrshire, V	1·81 ± 0·18	987 ± 154	141 ± 19	7·02 ± 0·72	16·2 ± 7·4	3430 ± 350	—
Ayrshire, VI	1·36 ± 0·12	833 ± 114	125 ± 15	6·71 ± 0·89	10·5 ± 3·4	3880 ± 825	3·69 ± 0·32
Friesian, VII	1·42 ± 0·11	825 ± 116	126 ± 12	6·62 ± 1·15	10·4 ± 2·7	4700 ± 780	3·64 ± 0·19

Table 4. *Pooled within-herd correlations between each of the skin measurements and the milk yield and the butterfat percentage*

	Correlation of milk yield with:		Correlation of butterfat percentage with:
FD	−0·23**†		FD + 0·13 (0·1 < P < 0·2)
	−0·28***	(Using data for herd IV*a* in place of those for herd IV)	
	−0·24 **	(Including data for both herds IV and IV*a*)	
L	−0·08 (NS)		L +0·02 (NS)
D	+0·01 (NS)		D −0·03 (NS)
L/D	−0·06 (NS)		L/D +0·04 (NS)
Volume	−0·03 (NS)		Volume 0·00 (NS)

† Significance indicates whether or not the correlation differs from zero. NS, $P > 0·1$; **, $P < 0·01$; ***, $P < 0·001$.

FD, follicle depth; L, sweat gland length; D, sweat gland diameter.

Finzi & Cenni (1962) in a study of Friesian cows measured the thickness of the skin over 3 areas of the body with a gauge and compared the results obtained with the milk production of the animals. The skin thickness of 1 area, the mammary gland, gave a statistically significant negative correlation with milk yield. The correlation coefficients, one of which was positive, obtained for the other 2 areas were not statistically significant. The overall negative correlation coefficient between milk yield and mean skin thickness obtained by these workers provides a possible parallel to the present findings.

No physiological explanation for the negative relationship between hair follicle depth and milk yield has yet been found. Moreover the correlations calculated are for phenotypic characteristics, while those characteristics important for breeding are genetic. However, with a sample of only 156 animals, one of the present correlations was highly significant and another (that between hair follicle depth and butterfat percentage) approached significance. These facts encourage the supposition that further exploration of these correlations and a search for others may yield results of practical value in determining the potential milk-producing capacity of heifers, and might therefore be of assistance in the choice of bulls for progeny testing.

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The effect of restricted grazing during a single day upon milk yield, milk composition and butterfat characteristics of Jersey cows

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Summary. The effects of depriving cows of pasture, for part or all of a single interval between the morning and evening milkings, on milk yield and composition have been investigated. The yields of milk and solids-not-fat, but not of fat, were reduced. The characteristics of the butterfat obtained at the first 2 milkings following grazing restriction differed markedly from those of cows allowed to graze normally.

The changes observed suggested that the grazing restriction led to a temporary reduction in the supply of volatile fatty acids from the rumen and an increased utilization of fatty acids from body fat by the mammary gland.

The effects of underfeeding on the yield and composition of the milk of dairy cows has been the subject of a number of studies at the Dairy Research Institute, New Zealand (see reviews by Campbell & Dolby, 1953, 1959). Under controlled grazing the intake of cows may fluctuate widely over short periods because of changes in the availability or palatability of pasture. It therefore seemed important to extend these previous investigations, which were chiefly concerned with underfeeding for periods of several weeks, by studying the effects of restricting the access of cows to pasture for periods of several hours.

MATERIALS AND METHODS

Design of experiments

Expt. 1 was completed during December 1958 with 18 pedigree Jersey cows of mixed ages. The cows were divided into blocks of 3, according to milk yield and calving date in August and September 1958, and allocated at random within these blocks to three 6-cow treatment groups, *A*, *B*, and *C*. For a preliminary period of 5 days, all the cows were allowed to graze at will between milkings. Between the morning milking which marked the end of day 5 and the next afternoon milking, the first milking of day 6, two groups (*B* and *C*) were subjected to grazing restriction. The control group *A* was allowed to graze normally. Group *B* cows were allowed to graze for 2 periods, each of 1 h, immediately following the morning milking and

immediately before the afternoon milking, but were held in a concrete yard for the rest of the interval. Group *C* cows were held in the yard for the whole interval.

In expt. 2, carried out in October and November 1959, 30 pedigree Jersey cows of mixed ages were arranged in 5 blocks on the basis of milk yield and calving date and allotted at random within these blocks to one of six 5-cow sets. These cow-sets were allocated at random to the rows of an orthogonal pair of Latin squares. With this design all the cows were subjected in successive periods to the 3 treatments *A*, *B* and *C* described above for expt. 1. The design allowed the estimation of residual effects of treatments applied in the previous period. Fourteen days elapsed between the days on which grazing was restricted in successive periods. The cows had calved between 4 May and 27 June 1959 (1 block), and between 1 July and 4 August 1959 (4 blocks).

Grazing behaviour

In expt. 1, the activity of the cows at pasture was observed at 10-min intervals and classified as 'grazing', 'ruminating', or 'loafing', on 3 days (a total of 72 h) in the preliminary period and continuously for 72 h from the beginning of the treatment interval.

Milk recording, sampling and analyses

In both experiments the cows were milked twice daily. In expt. 1 milking began at 5.15 a.m. and 3.30 p.m. and in expt. 2 at 5 a.m. and 4 p.m.

The milk yield was recorded for each cow at each milking, and a sample of milk taken for estimation of fat and total solids content: in expt. 1 for 10 milkings before the application of the grazing treatment and for the 8 milkings immediately following the treatment interval; in expt. 2 for 4 milkings before and 8 milkings after treatment.

The total solids content of the milk was estimated by the gravimetric method and fat content by the Gerber method. The solids-not-fat (SNF) content was obtained by difference.

Butterfat sampling and analyses

At the times of sampling for milk analysis, bulk samples of the milk from groups *A* and *C* in expt. 1 and from each of the six 5-cow sets in expt. 2 were collected. Creams separated from these samples were churned, as described by Dolby (1954), and butterfats were isolated from the butter by melting, decanting and filtering.

Refractive index was read in an Abbé refractometer at 40 °C and softening point was measured by the falling ball procedure (Barnicoat, 1944). Iodine (by the Wijs method), saponification and Reichert values were determined by British Standard Methods (British Standards Institution, 1958).

Statistical analyses

In expt. 1 the overall effects on the milk yield and composition were examined by analyses of covariance (Snedecor, 1946). The corresponding values for the preliminary period were employed as concomitant variables to adjust the post-treatment values for differences between animals.

In expt. 2 the overall effects of the treatments on milk yield and composition were

examined in analyses of variance which allowed separation of direct and residual effects (Cochran, Autrey & Cannon, 1941).

In both experiments additional analyses of variance (Snedecor, 1946) were made on the data for individual milkings in the post-treatment period with the object of detecting milking-to-milking changes resulting from the treatments. In particular the magnitude of the interaction between treatments, milking intervals and days ($T \times M \times D$) was examined.

A somewhat similar form of analysis was employed to examine the interaction of treatment and days in respect of the grazing behaviour data in expt. 1.

The absence of replication in expt. 1 precluded statistical analysis of the results for butterfat characteristics. In expt. 2 the Latin square design was ignored and the data for the characteristics of butterfat were examined by analyses of covariance. Two analyses were completed for each characteristic. In one the mean values for the 4 milkings immediately after the treatment interval were examined; in the other the values for the subsequent 4 milkings were examined. In both analyses data obtained in the preliminary period were used as the independent variables.

RESULTS

Effect of treatment on grazing behaviour (expt. 1)

The effective length of the night interval (from the end of the afternoon milking to the end of the morning milking) was approximately 800 min, and of the day interval 600 min. In the course of a day approximately 40 min were accounted for in movement between the milking shed and pasture. When observed during the preliminary period, on average, each cow in the course of the night interval expended 220 min in grazing and 350 min in ruminating, and during the day interval, 270 min in grazing and 190 min in ruminating.

The effect of experimental restriction of grazing time over a 1-day interval on the subsequent grazing behaviour was examined by testing the significance of the interaction of treatments by days for each of the day and night grazing and ruminating times. The interaction was not significant for day grazing time (with the experimental interval excluded) or for night ruminating time ($P > 0{\cdot}50$ in each case). The interaction was significant in the analysis for day ruminating time ($P < 0{\cdot}01$) and in that for night grazing time ($P < 0{\cdot}05$). Examination of the means for treatment groups within days indicated that in the interval when grazing time was experimentally restricted the ruminating times of groups B and C were reduced; in the following night interval the grazing time of these groups increased, and in the succeeding day interval there was a corresponding increase in the time spent in rumination.

Effects on milk yield and composition

The effects of restriction of grazing on milk yield composition in the following 4-day period are shown in Tables 1 and 2. In both experiments the mean yields of milk and SNF were lower for the restricted groups, but these yields did not differ significantly between treatments B and C. In both experiments, the mean values for fat yield and fat content did not differ significantly. In expt. 2, but not in expt. 1,

the SNF content of the milk of the cows subjected to treatment *C* was significantly lowered.

The mean values for individual milkings obtained in expt. 2 are shown in Fig. 1. Analyses of variance of milk yield, fat content and SNF content were completed for the milkings following the experimental grazing interval. In both experiments the

Table 1. *The effect of restricted grazing on a single day on the mean milk yield and composition for the 4-day period following restriction (expt. 1)*

(Each treatment group contained six cows.)

Treatment	Milk yield, lb/day	Fat yield, lb/day	SNF yield, lb/day	Fat content, %	SNF content, %	Milk serum SNF‡ content, %
A. Control	27·0	1·33	2·45	5·02	9·05	9·51
B. Partial restriction	25·2	1·32	2·29	5·03	9·07	9·60
C. Complete restriction	25·8	1·28	2·36	5·09	9·12	9·59
			Significances of differences§			
Between treatments	*	NS	†	NS	NS	NS
A v. B and *C*	*	NS	*	NS	NS	NS
B v. C	NS	NS	NS	NS	NS	NS

‡ Given by $\frac{\text{SNF} \times 100}{100 - \text{fat } \%}$.

§ Significances of difference between means were determined after adjustment for differences in the preliminary period by analysis of covariance. The means shown are adjusted means (Snedecor, 1946). NS, $P > 0{\cdot}1$; †, $P < 0{\cdot}1$; *, $P < 0{\cdot}05$.

Table 2. *The effect of restricted grazing on a single day on the mean milk yield and composition: for the 4-day period following restriction (expt. 2)*

(Data from 30 cows included in each mean.)

Treatment	Milk yield, lb/day	Fat yield, lb/day	SNF yield, lb/day	Fat content, %	SNF content, %	Milk serum SNF‡ content, %
A. Control	30·7	1·63	2·87	5·38	9·36	9·89
B. Partial restriction	29·8	1·64	2·77	5·53	9·32	9·86
C. Complete restriction	29·4	1·60	2·71	5·49	9·25	9·79
			Significance of differences§			
Between treatments	†	NS	*	NS	*	†
A v. B and *C*	*	NS	**	NS	NS	NS
B v. C	NS	NS	NS	NS	NS	NS
A v. B	NS	NS	NS	NS	NS	NS
A and *B v. C*	NS	NS	NS	NS	*	*

‡ Given by $\frac{\text{SNF} \times 100}{100 - \text{fat } \%}$.

§ Means and tests of significance of differences calculated after elimination of residual effects of treatments in previous periods (Cochran, Autrey & Cannon, 1941). NS, $P > 0{\cdot}1$; †, $P < 0{\cdot}1$; *, $P < 0{\cdot}05$; **, $P < 0{\cdot}01$.

interactions between milking interval, days and treatments were significant for milk yield, fat content and, in expt. 2 but not in expt. 1, for SNF content.

In expt. 1 the overall reduction in milk yield of the restricted groups in the post-treatment period (Table 1) was due to the lowered milk yield at the first (*B*) or first

and second (*C*) milkings following the restriction of grazing. The situation with regard to fat content in this experiment was confused by unexplained variations in the fat content of the control group and by the occurrence of oestrus in 2 of the cows in group *C*.

In expt. 2 (Fig. 1) the milk yield at the 2 milkings following the experimental interval was lowered in groups *B* and *C*. The fat contents for the groups did not differ significantly at the first milking after the grazing restriction, but at the second milking the fat contents for both restricted groups were significantly higher than that of the control group. The small overall reduction in SNF content for treatment *C* (Table 2) was mainly the result of lower values for this treatment, in comparison with both the other treatments, at both milkings on the 2nd post-experimental day and on the first milking of the 3rd post-experimental day (Fig. 1).

Effects on butterfat characteristics

In both experiments marked changes occurred in butterfat characteristics. The results of examination of the butterfat from bulked milk from all the cows in each of treatment groups *A* and *C* during expt. 1 are shown in Fig. 2. The maximum effect in all the indices occurred at the second milking following the grazing restriction, and the difference had decreased markedly but had not disappeared entirely by the fourth milking (7 a.m. in the figure).

The results obtained in expt. 2 were, for the most part, similar to those in expt. 1. In Fig. 3 each point represents the mean of 6 separate determinations on butterfat from the bulked milk of 5 cows. The changes in the indices for treatment *B* were less marked than in those for treatment *C*. The reaction of softening point was not the same as in expt. 1. The apparent increase in softening point for group *C* at milkings 3 a.m. and 4 p.m. (Fig. 3) was an artifact created by the particular method of graphical representation. This effect was not apparent in the mean values for the 4 milkings (3 p.m.–4 a.m.) after adjustment by analysis of covariance (Table 3).

The changes in butterfat characteristics in expt. 2 are shown in Table 3 as the mean values for the first and second groups of 4 milkings after the treatment interval. The butterfat characteristics of the 2 restricted groups of cows were, in some respects at least, still different from those of the control cows on the third and fourth days following the experimental interval.

DISCUSSION

In expt. 1, the decrease in grazing time produced by the experimental restriction was greater than the compensating increases in grazing time observed in the subsequential night interval. This suggests that over the whole 24-h period the cows in groups *B* and *C* ingested less than the control cows. On the other hand, the fall in ruminating time observed in the experimental interval was matched in magnitude by the rise during the following day interval. This could indicate that the intake of the restricted groups over the 24 h equalled that of the control group. If ruminating time is a better measure of feed intake than grazing time (cf. Hancock, 1953), it is reasonable to assume that in expt. 1 at least, the cows subjected to a grazing restriction in the day interval probably ate as much over the total 24 h as the control cows.

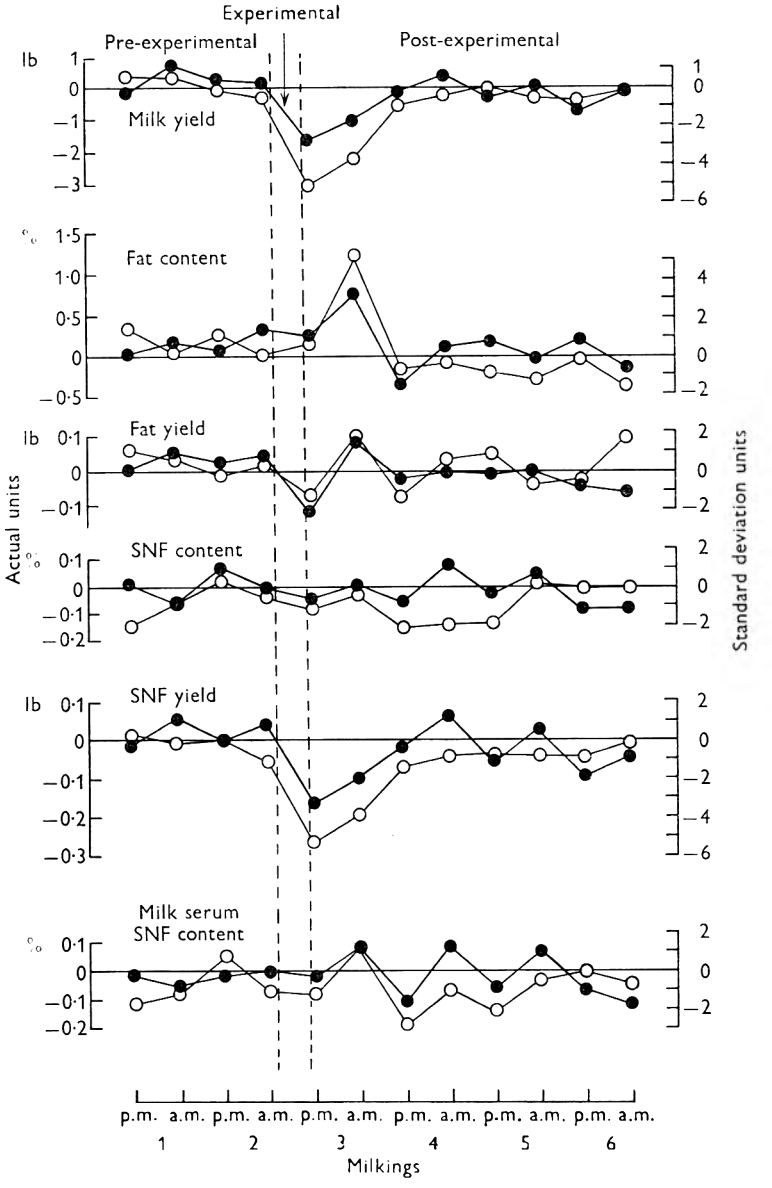

Fig. 1. Changes in milk yield and composition (expt. 2). ●—●, Partial grazing restriction (group *B*); ○—○, complete grazing restriction (group *C*). Each point is the mean of 30 observations expressed as a deviation from the overall mean for the appropriate milking interval (a.m. or p.m.) in the control group (shown ———). The variation (within each milking interval) from day to day in the control group is the basis of the standard deviation units shown on the right-hand ordinate.

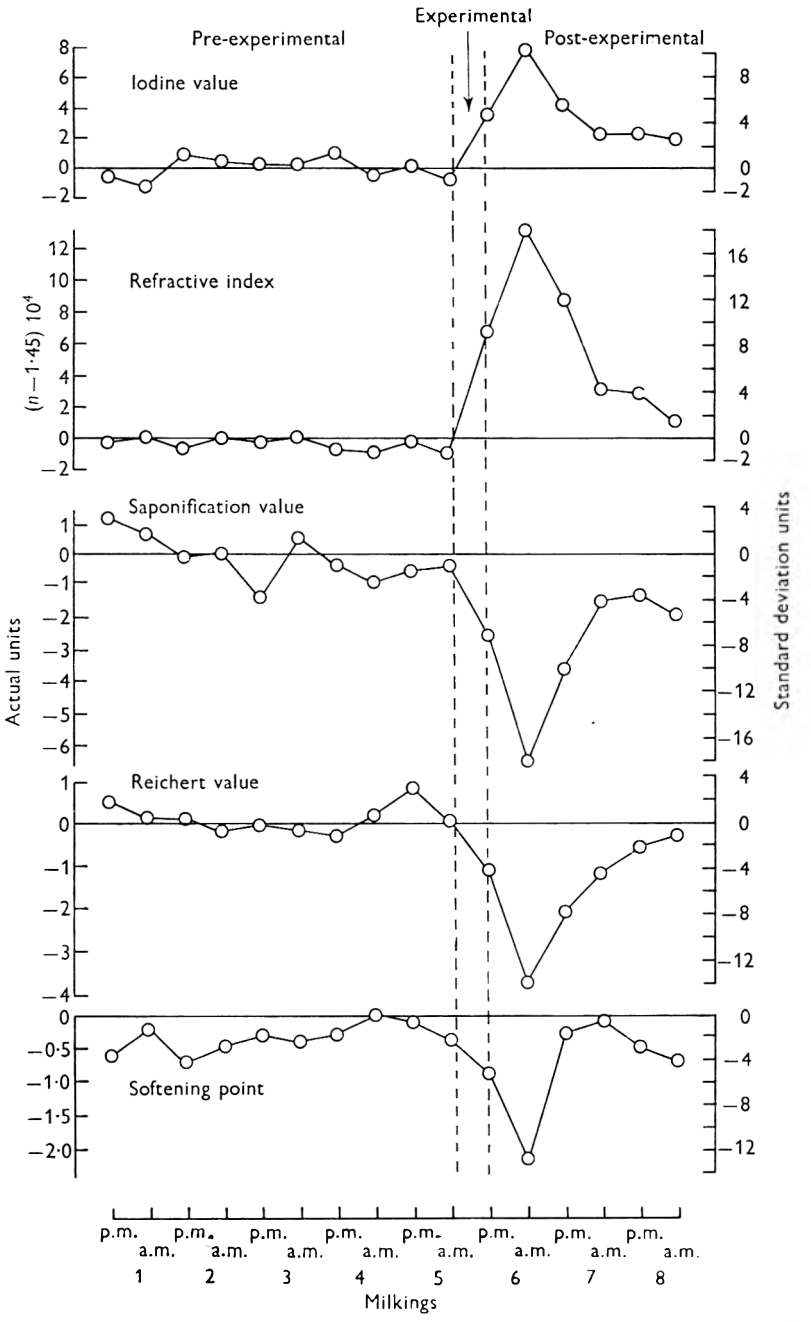

Fig. 2. Changes in butterfat characteristics (expt. 1). ○—○, Complete grazing restriction (group *C*). Each point is the mean of duplicate determinations on the bulk milk of 6 cows expressed as a deviation from the mean for the appropriate milking interval (a.m. or p.m.) in the control group (shown ———). The variation (within each milking interval) from day to day in the control group is the basis of the standard deviation units shown on the right-hand ordinate.

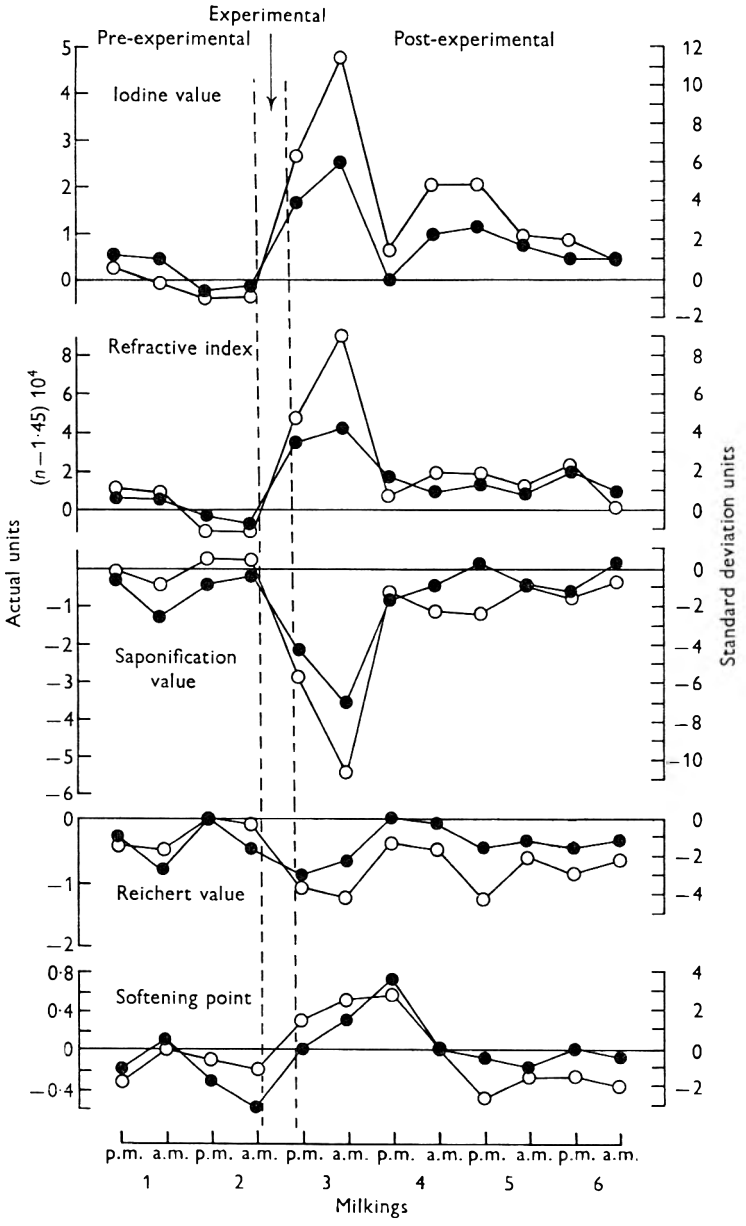

Fig. 3. Changes in butterfat characteristics (expt. 2). ●—●, Partial grazing restriction (group *B*); ○—○, complete grazing restriction (group *C*). Each point is the mean of determinations on the bulk milk of six 5-cow sets, expressed as a deviation from the mean for the appropriate milking interval (a.m. or p.m.) in the control group (shown ———). The variation (within each milking interval) from day to day in the control group is the basis of the standard deviation units shown on the right-hand ordinate.

Table 3. *The effect of restricted grazing on a single day on butterfat characteristics (mean values for 4 milkings) (expt. 2)*

Treatment	Iodine value		Refractive index $(n_D^{40} - 1{\cdot}45)10^5$		Saponification value		Reichert value		Softening point, °C	
	A‡	B§	A	B	A	B	A	B	A	B
A. Control	32·5	32·3	304	303	235·0	235·2	31·7	32·0	32·99	32·88
B. Partial restriction	33·5	32·8	315	307	233·5	235·1	30·9	31·4	33·01	32·65
C. Complete restriction	34·8	33·3	335	311	232·8	234·5	30·4	30·9	32·97	32·36
					Significance of differences‖					
Between treatments	**	†	**	NS	**	NS	**	**	NS	**
A v. B and *C*	**	*	**	NS	**	NS	**	**	NS	**
B v. C	**	NS	**	NS	NS	NS	†	†	NS	†

‡ A is the mean for 4 milkings immediately following grazing restriction (3 p.m.–4 a.m. inclusive).

§ B is the mean for 4 milkings following A (5 p.m.–6 a.m. inclusive).

‖ Significance of differences between means determined after adjustment for differences in the preliminary period by analysis of covariance. The mea shown are adjusted means (Snedecor, 1946). NS, $P > 0{\cdot}1$; †, $P < 0{\cdot}1$; *, $P < 0{\cdot}05$; **, $P < 0{\cdot}01$.

It is unsafe, in either experiment, to conclude that the effects observed were due to a reduction in feed intake over the 24 h.

In general, the effects of grazing restrictions on milk yield, milk composition and butterfat characteristics were similar in the 2 experiments. In expt. 1 the effects of the treatments were confused by the occurrence of oestrus in 2 cows in group *C* on days 6 and 7. The affected data were replaced by 'missing plot estimates' (Cochran & Cox, 1957), but this means that the mean values for milk yield and composition for milkings at the crucial period were based with this group on only 4 cows. In expt. 2 the animals were all pregnant and, in addition, the effects of differences between cows at individual milkings were eliminated by the experimental design. Accordingly more weight must be placed on the results of the second experiment than on those of the first.

The changes in milk yield and composition could not be explained simply in terms of alterations in the efficiency of milk withdrawal. There may have been some interference with release or transport of oxytocin (see Cross, 1961) leading to less efficient removal of milk at the first milking following the grazing restriction. However, although the changes in fat yield at the first 2 milkings after restriction might reflect such an effect, the absence of a fall in fat content at the first milking and of a rise in milk yield at the second milking indicated that secretory activity of the mammary gland had been altered.

The changes in butterfat characteristics indicated an increase in the degree of unsaturation, and a decrease in the proportion of short-chain fatty acids in the milk fat of the restricted groups. A similar effect was reported by Eckles & Palmer (1916), in the milk of cows on a subnormal plane of nutrition, and by Smith & Dastur (1938) and McClymont (1951) in fasted cows. These effects on milk fat have been related (see Rook, 1959) to the concentration of volatile fatty acids in the rumen. Since there was no significant overall reduction in the amount of fat produced by the restricted cows, reduction in the availability of volatile fatty acids, particularly acetate, must have been counteracted by increased utilization of fatty acids from depot fat for milk fat synthesis. If, as suggested by Annison & Lewis (1959), fluctuations in the production of volatile fatty acid in the rumen lead to inefficient utilization of acetate, in particular, it is not necessary to postulate a reduction of the 24 h production of volatile fatty acid.

Presumably the cows held in the yard were subject to emotional stress. This could have caused increased release of adrenocorticotrophin (Harris, 1955), increased output of adrenal corticosteroids (Astwood, 1955) and a raised level of circulating lipid in the blood (Bossak, Wang & Adlersberg, 1956). Such effects could have contributed to the changes observed in butterfat characteristics in the present experiments. This aspect will be discussed in a later paper.

Although the effects of the grazing restriction upon butterfat characteristics diminished rapidly, there were detectable differences between the control and restricted groups in certain indices on the 3rd and 4th post-experimental days. These effects did not decrease steadily and cannot be ascribed entirely to the existence of residual milk.

If the lower SNF content of the milk of the *C* treatment group observed in expt. 2 represented a real effect it is difficult to explain. The effect is consistent (cf. Rook &

Balch, 1959) with an increase in the proportion of acetic to propionic acid in the rumen, but such a change in volatile fatty acid proportions is not consistent with the persisting effect on butterfat characteristics.

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The effect of adrenocorticotrophic hormone on the yield, composition and butterfat properties of cow's milk

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Summary. A single injection of 100 i.u. ACTH given to milking cows had no statistically significant effect on the yield or composition of the milk. A long-acting preparation of ACTH, when given to cows in single doses of 200 and 400 i.u., depressed milk yield and raised the butterfat content of the milk. There was no significant change in the SNF content of the milk but the SNF content of the milk serum was increased. Three injections of 300 i.u. of long-acting ACTH given at 24-h intervals reduced yields of milk, fat and SNF and increased the fat and SNF contents of the milk.

Small but generally consistent changes occurred in the butterfat characteristics of the milk of all groups treated with ACTH. There was a decrease in the iodine number and refractive index and a rise in the softening point of the fat in all the experiments. The Reichert value of the butterfat was lowered by single or multiple injections of the long-acting preparation. The carotene content of the butterfat was lowered by injections of the long-acting ACTH preparation but the vitamin A content was unaffected.

The effect of restricting grazing, during a single day, on the yield and composition of cow's milk and on the butterfat properties was investigated by Munford, Campbell, McDowall & Davey (1964). The present experiments are an extension of that work. The changes in milk and butterfat they reported may have partly resulted from nervous stress in the cows caused by the change in routine when they were confined to a concrete yard. The effects of stress in animals are believed to be consequences mainly of the release of adrenocorticotrophin (ACTH). For this reason the effects of injections of ACTH on milk yield and composition and on the properties of the butterfat of dairy cows were examined.

Expt. 1 was conducted in December 1959. Two further studies, expt. 2, with higher dose rates of ACTH and expt. 3, with repeated injections of ACTH, were made in December 1960 and December 1961, respectively.

MATERIALS AND METHODS

Experimental animals

In all the experiments the animals used were pedigree Jersey cows of various ages which had calved during July and August of the particular year.

In both expts. 1 and 2 fifteen cows were grouped into blocks of 3 according to milk yield, calving date and age; and within these blocks cows were allocated at random to one of 3 treatment groups, *A*, *B* or *C*. In expt. 3 ten cows were grouped into blocks of 2 using the same criteria as for expts. 1 and 2, and within these blocks, cows were allocated at random to treatment groups *A* and *B*. In each of the 3 experiments there were thus 5 cows in each treatment group.

In all the experiments the cows were grazed as a group for 5 days before injection and then for the duration of the experimental period. Ample leafy pasture was available during expts. 1 and 2, but for expt. 3 the pasture was shorter and contained more stemmy material.

The cows were milked as a group twice daily at 5.0 a.m. and 3.0 p.m.

ACTH preparations

The ACTH used in expt. 1 was an aqueous solution, 'Cortrophin' (Organon Laboratories Ltd). The ACTH used in expts. 2 and 3 was a long-acting form

Table 1. *Injection treatment of cows in expts.* 1–3

Expt.	Group	Injections: Content	Injections: No.
1	*A*	Physiological saline	1
	B	100 i.u. ACTH	1
	C	200 i.u. ACTH	1
2	*A*	Physiological saline	1
	B	200 i.u. ACTH (ZN)	1
	C	400 i.u. ACTH (ZN)	1
3	*A*	Physiological saline	3‡
	B	300 i.u. ACTH	3‡

‡ At 24 h intervals.

'Cortrophin ZN' (Organon Laboratories Ltd), stated by the makers to reach maximum activity within 8 h of injection, with a possible duration of activity of 24–72 h.

Doses of ACTH and application of treatments

All ACTH injections were given subcutaneously immediately following the morning milking. In all the experiments the 5 cows in the control groups (treatment *A*) were injected with 10 ml physiological saline, equivalent to the volume of medium injected into the ACTH-treated animals. The treatments given in the 3 experiments are shown in Table 1.

The levels in expt. 1 were chosen on the basis of previous work by Flux, Folley & Rowland (1954). The intervals between injections in expt. 3 were chosen on the basis of work by Brush (1960), who reported that blood corticosteroid levels reached a maximum 3–6 h after injection of 160–200 i.u. of long-acting ACTH, and returned to near normal within 24–48 h. In addition, Flux *et al.* (1954) with a different long-acting form of ACTH observed that the depression in the number of circulating eosinophils was at a maximum after about 24 h. It is suggested from this evidence that blood

corticosteroid levels may be maintained at a reasonably high level by repeated injections of long-acting ACTH at 24 h intervals.

Milk recording and milk and butterfat sampling and analysis

In expt. 1 milk yields were recorded and samples of milk for fat and total solids estimations were taken for 2 days before the injection of ACTH, and in expts. 2 and 3 for 3 days before the first injection. In expts. 1 and 2 sampling continued for 4 and 5 days after injection, respectively, and in expt. 3 for 5 days following the last injection. Composite samples of milk for each treatment group were obtained at these same milkings for centrifugal separation of the cream and preparation of butterfat samples.

The methods of butterfat analyses were as described by Munford *et al.* (1964).

In expts. 2 and 3 the carotene and vitamin A in the butterfat were estimated by the procedure of McDowell (1949).

Statistical analysis

In all the experiments the significance of differences between treatments for milk yield and composition was examined by analysis of covariance using the values for the preliminary period as the independent variable. In expt. 2 the multiple range test of Duncan (1955) was used for testing the significance of differences between

Table 2. *The effect‡ of single injections of* 100 *and* 200 *i.u. of ACTH on milk yield and composition* (*expt.* 1)*: adjusted mean values§ for the* 3*-day period following the injection*

Treatment	Milk yield, lb/day	Fat yield, lb/day	SNF yield, lb/day	Fat content, %	SNF content, %	Serum SNF content, %
A. Control injection	27·7	1·48	2·55	5·25	9·25	9·72
B. 100 i.u. ACTH	27·1	1·42	2·54	5·35	9·38	9·85
C. 200 i.u. ACTH	26·6	1·40	2·49	5·29	9·36	9·91

‡ Tests of significance were made by calculating F ratios from the adjusted mean squares. There were no significant differences between the adjusted means for any measure ($P > 0{\cdot}1$).

§ The adjusted mean values were obtained by analyses of covariance with values for the preliminary period as the independent variable.

adjusted means for individual treatment groups. Because separate samples were not taken from individual cows for butterfat analyses, the results for fat characteristics could not be analysed statistically.

RESULTS

Milk yield and composition

The single injections of 100 and 200 i.u. ACTH in expt. 1 (Table 2) had no statistically significant effect on milk yield, fat and solids-not-fat (SNF) content, or on the yield of fat and SNF. In expt. 2 (Table 3) the milk yield was lowered by both the 200 and 400 i.u. dose levels and the effect of the higher dose level was more marked than that of the lower level. Fat content was raised by both the 200 and the 400 i.u. levels but the increase with the 200 i.u. level was not statistically significant. There were no significant effects at either dose level on SNF content, SNF yield and fat yield.

The effects of repeated doses of 300 i.u. ACTH in expt. 3 are shown in Table 4. A significant reduction in milk yield was detected following the second injection and a considerable reduction occurred following the third injection. From the 4th day following the first injection there was a slight recovery in milk yield, but the yield was still markedly depressed 1 week after the first injection. There was a marked increase in butterfat content on the day following the second injection and like that on milk yield this effect was still apparent 1 week after the first injection. The differences between the adjusted mean values for fat yield were of similar magnitudes on the 1st and subsequent days (see Table 4) but the variability within the groups was such that these differences were not always significant.

In Tables 3 and 4 the increases in the SNF content of the milk of cows treated with ACTH were not significant. The differences were apparently partly obscured by changes in fat content and, when calculated on a fat-free basis (serum SNF content),

Table 3. *The effect of single injections of* 200 *and* 400 *i.u. of ACTH on milk yield and composition (expt.* 2)*: adjusted mean values*‡ *for the* 3*-day period following the injection*

Treatment	Milk content, lb/day	Fat yield, lb/day	SNF yield, lb/day	Fat content, %	SNF content, %	Serum SNF content, %
A. Control injection	27·7	1·48	2·61	5·29	9·39	9·94
B. 200 i.u. ACTH	26·2	1·53	2·45	5·88	9·42	10·01
C. 400 i.u. ACTH	23·9	1·46	2·29	6·12	9·54	10·17
			Significance of differences§			
A v. B	*	NS	NS	**	NS	NS
A v. C	**	NS	NS	**	NS	*
B v. C	**	NS	NS	NS	NS	NS

‡ The adjusted mean values were obtained by analyses of covariance with values for the preliminary period as the independent variable.

§ Obtained by applying Duncan's range test when the analysis of covariance indicated that there were significant differences among the adjusted means. NS, $P > 0{\cdot}05$; *, $P < 0{\cdot}05$; **, $P < 0{\cdot}01$.

they were more marked and were statistically significant for the 400 i.u. dose level (Table 3) and for the group given repeated doses (Table 4). There was a general and steady reduction in SNF yield from the day following the second injection in expt. 3.

Butterfat characteristics

The effects of ACTH upon butterfat characteristics are shown in Fig. 1 (expts. 1 and 2) and Fig. 2 (expt. 3). In both figures the values for the ACTH-treated cows are shown as deviations from the values for each milking for the control cows. To aid visual appraisal of relative changes in different measures the scales for all measurements were made proportional to their normal standard deviations between days within milking intervals (a.m. and p.m.) obtained from the values for the control groups and averaged over the 3 experiments. In Fig. 1 average values (from the two ACTH-treated groups) are shown for each experiment.

In all the experiments the iodine value and refractive index of the butterfat were lowered by treatment with ACTH. Saponification value appeared to be unaffected by ACTH, but Reichert values were reduced by single or repeated injections of the

long-acting preparation of ACTH (expts. 2 and 3). Softening point rose following injection of ACTH in expts. 1 and 3 and possibly, but to a much smaller extent, in expt. 2. The carotene content of the butterfat appeared to be lowered by treatment with ACTH (expts. 2 and 3), but vitamin A content was unaffected.

Table 4. *The effect of* 3 *injections of ACTH* (*each of* 300 *i.u.*) *on milk yield and composition* (*expt.* 3)*: adjusted mean values for the period following the first injection*

Treatment	Milk yield, lb/day	Fat yield, lb/day	SNF yield, lb/day	Fat content, %	SNF content, %	Serum SNF content, %
		1st day				
A. Control injections	25·2	1·60	2·44	5·58	9·61	9·90
B. After the first injection of 300 i.u. ACTH	23·3	1·25	2·22	5·59	9·58	10·16
Difference (*A* − *B*)	1·9	0·35	0·22	−0·01	0·03	−0·26
		2nd day				
A. Control	25·4	1·52	2·41	5·61	9·44	9·98
B. After the second injection of ACTH	20·8	1·25	1·96	6·78	9·55	10·27
Difference	4·6	0·27	0·45**	−1·17†	−0·11	−0·29
		3rd day				
A. Control	25·8	1·52	2·48	5·52	9·39	10·00
B. After the third injection	18·7	1·19	1·78	7·23	9·85	10·55
Difference	7·1**	0·33†	0·70**	−1·71**	−0·46	−0·55*
		4–7th days				
A. Control	24·8	1·36	2·34	5·43	9·43	9·81
B. After the third injection	17·2	1·04	1·66	6·39	9·75	10·32
Difference	7·6**	0·32*	0·68**	−0·96*	−0·32	−0·51**

The adjusted mean values were obtained by analyses of covariance with values for the preliminary period as the ndependent variable. Tests of significance were made with F ratios calculated from the adjusted mean squares. †, $P < 0{\cdot}1$; *, $P < 0{\cdot}05$; **, $P < 0{\cdot}01$.

DISCUSSION

The single injections of ACTH in expts. 1 and 2 did not depress the milk yield to the extent shown by Cotes, Crichton, Folley & Young (1949), Flux *et al.* (1954), Shaw, Chung & Bunding (1955) and Brush (1960). Nor did the response between individual cows in any of the present experiments vary to the extent reported by Flux *et al.* and by Brush. The repeated injections in expt. 3 caused a greater depression in milk yield than the single injections, a result which was more in keeping with those of the above workers. Cows of the Jersey breed were used in the present experiments, whereas the previous reports referred to cows of various other breeds. The Jersey breed may be less sensitive to exogenous ACTH, in terms of its effect on milk yield. However, possible effects of variations in potency and type of preparation of ACTH used by different workers cannot be excluded. In the present study there were differences in the response to 200 i.u. ACTH in expts. 1 and 2. In the latter experiment the fall in milk yield and the rise in fat percentage were more marked. This may have resulted from differences in the effectiveness of the preparations of ACTH used, or from differences in the responsiveness of the 2 groups of cows or from both.

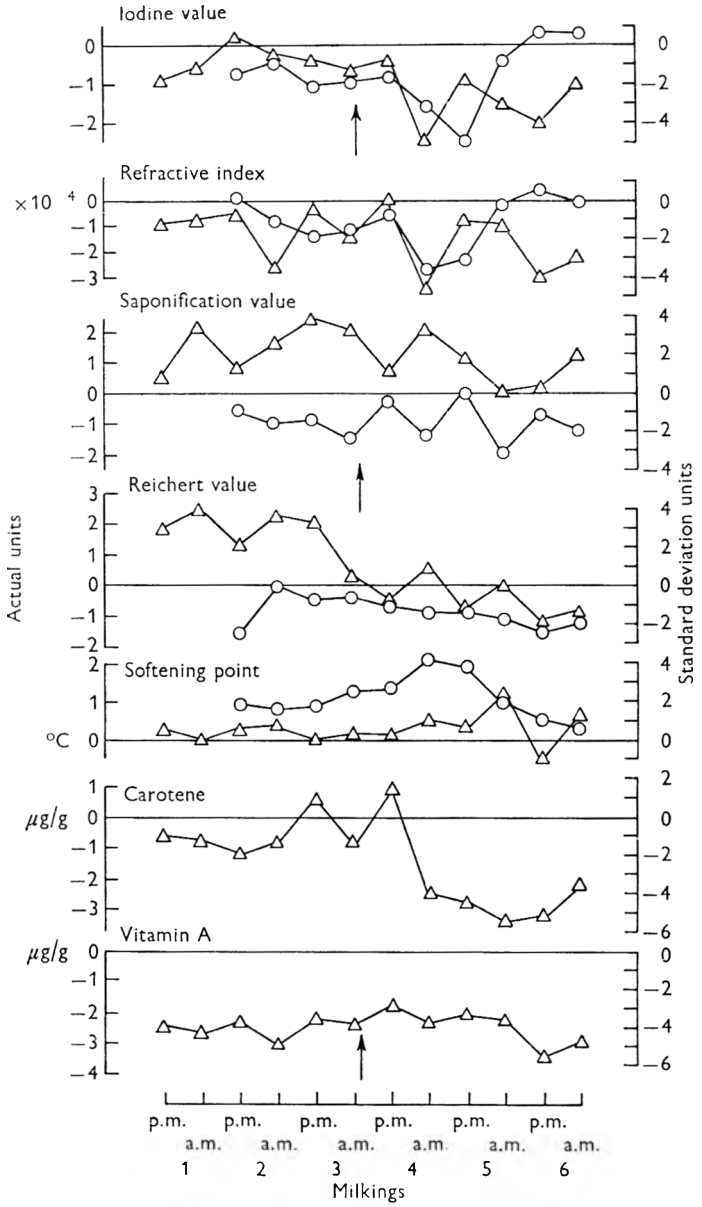

Fig. 1. The effect of single injections of ACTH on the characteristics of the butterfat of cows. ○—○, Expt. 1, average of 2 groups injected with 100 or 200 i.u. ACTH; △—△, expt. 2, average of 2 groups injected with 200 or 400 i.u. ACTH; ↑, injection of ACTH at this time. Values for each milking are shown as a deviation from the value for the appropriate control group for that milking. The standard deviation units are described in the text.

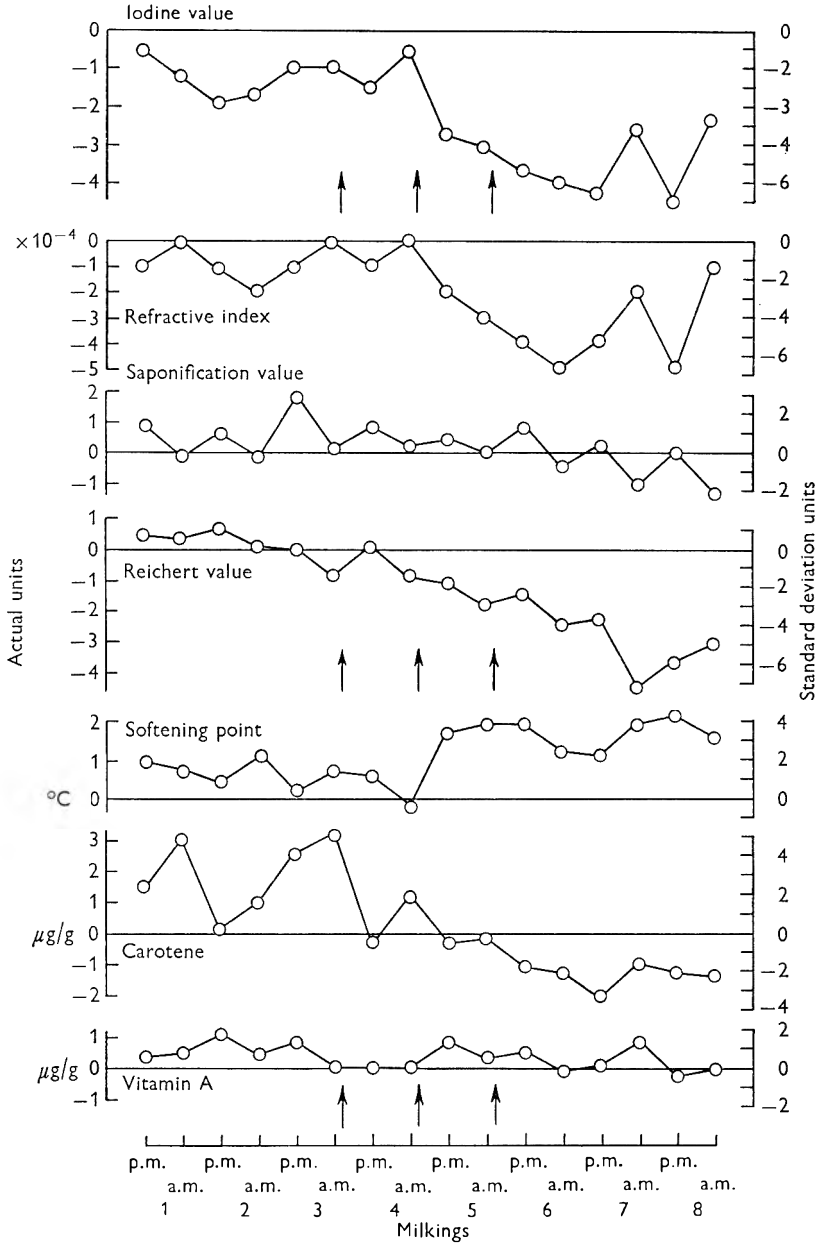

Fig. 2. The effect of repeated injections of ACTH (3 times 300 i.u.) on the characteristics of the butterfat of cows (expt. 3). ↑, Injection of 300 i.u. of ACTH. Values for each milking are shown as a deviation from the value for the control group for that milking. The standard deviation units are described in the text.

The changes in milk composition observed in expts. 2 and 3—a rise in the fat and SNF contents—were similar to those observed by Flux *et al.* (1954). In the present study the effect of ACTH on fat content was more marked than on SNF content and accordingly fat synthesis, as indicated by fat yield, was less affected than was synthesis of the other milk constituents (see Table 4).

Although a decrease in milk yield and an increase in fat content occurred during underfeeding (e.g. Flux & Patchell, 1954), the rise in SNF content found in the present study was not consistent with underfeeding and the changes found in butterfat characteristics were in no way similar to those reported as a result of restricted grazing (Munford *et al.* 1964). The changes in butterfat characteristics in the present experiments were not large, being smaller than those generally found in the butterfat obtained from a group of cows at different seasons of the year (Cox & McDowall, 1947). However, the changes, when obvious, were in a similar direction in all the experiments and the decrease in iodine value, reflecting the presence of a higher proportion of saturated fatty acids, is in agreement with the results of Chung & McKeeney (1957). That there was a slightly greater proportion of higher fatty acids in the butterfat of ACTH-treated cows is confirmed by the small decreases in Reichert value found in expts. 2 and 3.

In rodents ACTH may cause increased release of free fatty acids from adipose tissue, but the extent of the effect may be modified by other endocrine factors and by the dietary status of the animal (see Winegrad, 1962; Levin & Farber, 1952). The results of the present study cannot be explained simply in terms of such an effect. Greater availability of fatty acids from depot fat and hence greater utilization of them by the mammary gland for lipogenesis would tend to increase rather than decrease the iodine value of the butterfat in ACTH-treated cows. The effects observed here are perhaps more in line with what might be expected if, as suggested by *in vitro* studies with corticosteroids in rodents (see Folley, 1953, 1956), ACTH had interfered with normal lipogenesis in the mammary gland. It is clear that further work is necessary to establish the effects of ACTH in ruminants with respect to its effects on blood lipids and the synthetic activity of the mammary gland.

The apparent fall in the carotene content and the relative stability of the vitamin A in the milk of the treated cows cannot be explained easily. Clark & Coburn (1955) obtained a decrease in the amount of vitamin A in the livers of rats treated with ACTH. McGillivray (1961) reported an initial rise followed by a fall in blood vitamin A alcohol, and a fall in the esterified form in the liver of rats treated with ACTH. Thompson, Ganguly, Mawson & Kon (1949) showed that most of the vitamin A in cow's milk is in the esterified form. From this evidence it might be expected that the vitamin A content of the milk of cows injected with ACTH would be lowered rather than the carotene content. However, ACTH may have affected the absorption of carotene by the mammary gland or it may have influenced the mechanism of conversion of carotene to vitamin A.

Our experiments may have failed to simulate the suggested stress effect when cows were confined to a concrete yard for a day as in the experiments of Munford *et al.* (1964). ACTH may not be the only endocrine factor involved in such stress and the effects of any release of ACTH may have been modified by the mild degree of underfeeding in the previous experiment. Nevertheless it does not appear likely that stress

could have contributed significantly to the effects of restricted grazing on the yield and composition of the milk and on the butterfat properties observed in the earlier work (Munford *et al.* 1964).

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The effect of fatty acids on the metabolism of lactic acid streptococci

I. Inhibition of bacterial growth and proteolysis

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Summary. The early loss of viability of *Streptococcus cremoris* strain C13 in Cheddar cheese was investigated. The growth of this strain was markedly inhibited by cheese extracts containing unesterified fatty acids of which oleic acid was the major inhibitory constituent active against the coccus. This acid was found to accumulate in cheese early in the ripening process and may be responsible for the early loss of viability of strain C 13 in cheese.

Early loss of viability of a starter organism in cheese could result in a low peptidase activity by limiting the number of bacterial cells present. The subsequent accumulation of unhydrolysed bitter peptides would produce a bitter flavour.

In Cheddar cheese, protein hydrolysis is carried out by proteolytic enzymes derived mainly from rennet extracts and from bacteria (see Mabbitt, 1961). As rennet extracts do not exhibit peptidase activity it is considered that peptidases in cheese are derived solely from micro-organisms, in particular the lactic acid streptococci (Amundstad, 1950; Stadhouders, 1960).

It has been shown that one cause of bitter flavour in cheddar cheese is the accumulation of bitter-tasting peptides (Emmons, McCugan & Elliott, 1960). Certain strains of lactic acid streptococci e.g. *Str. cremoris*, strains C13, HP and K (Emmons, McCugan, Elliott & Morse, 1962) were found to be associated with this defect and produced both a low amino nitrogen and a bitter flavour in the cheese. It was suggested that these strains are deficient in the enzymes responsible for hydrolysing the bitter peptides.

The potential concentration of bacterial peptidases in the cheese contributed by a particular strain of lactic acid streptococci will be determined by the number of cells of that strain which is present. Therefore any substance which inhibits the growth of the starter organisms during either the manufacture or the ripening of the cheese would reduce the actual number of bacterial cells present and consequently the peptidase activity. Furthermore, the presence of any substance in cheese which selectively inhibits the peptidases could result in a bitter flavour.

It has been shown that unesterified fatty acids accumulate in Cheddar cheese during ripening (Peterson, Johnson & Price, 1949). These substances have also been shown

to inhibit starter growth (Costilow & Speck, 1951) and the protease activity of *Bacillus subtilis* and *Streptomyces griseus* (Kondo, 1962).

This report presents a study of the effect of fatty acids on the growth of various strains of lactis acid streptococci, and suggests a relationship between inhibition of the growth of lactic acid streptococci by fatty acids and the development of bitter flavour in Cheddar cheese.

MATERIALS AND METHODS

Micro-organisms. The starter organisms used were *Str. cremoris* strains K, C 13, HP (known to cause bitterness in cheese), and the non-bitter strains E 8, ML 1, R 1 (Emmons *et al.* 1962), *Str. lactis* strains C 2, C 6, C 10 and *Str. diacetilactis* strains DRC 1, DRC 2, DRC 3.

For the preparation of inocula, each strain was grown in sterile skim-milk for 16 h at 30 °C.

Quantities of bacterial cells were prepared by growth in a synthetic medium (pH 6·5) containing tryptone (Difco), 30 g; yeast extract (Difco), 10 g; lactose, 5 g; K_2HPO_4, 5 g; meat extract, 2 g; NaCl, 3 g and water to 1000 ml.

Acetone-dried cells were prepared according to the method described by Colowick & Kaplan (1955) and suspended in 0·1 M phosphate buffer, pH 7·0 (25 mg cells/ml) before use.

Cell-free extracts were prepared by grinding bacterial cells, which had been washed twice with 0·9 % saline, with alundum (60 mesh) and extracting the ruptured cells with 0·1 M phosphate buffer, pH 7·0.

Viable cell counts were carried out according to the method of Miles & Misra (1938).

Estimation of proteolytic activity. Proteolysis was determined by estimating the acid-soluble tyrosine and tryptophane (Hull, 1947) liberated during incubation of casein with cell-free extracts or acetone-dried cell suspensions in the presence and absence of fatty acids. The reaction mixture contained 0·5 ml 0·1 M phosphate buffer, pH 7·0 with or without fatty acid, 2·0 ml of a 3 % casein (B. D. H., Hammarsten) solution freshly prepared in 0·1 M phosphate buffer, pH 7·0 and 0·5 ml acetone-dried cell suspension or cell-free extract. Incubation was at 30 °C.

Determination of nitrogen. Total nitrogen was estimated by a semi-micro Kjeldahl procedure (Pregl, 1945).

Determination of total free fatty acids in cheese. The total non-esterified fatty acid content of cheese was determined by a modification of Dole's Method for estimation of non-esterified fatty acids in plasma (Trout, Estes & Friedberg, 1960). The cheese was ground with twice its weight of washed sand, and 6 g of the cheese–sand mixture was shaken mechanically for 10 min with 40 ml extraction mixture consisting of *iso*propyl alcohol-*n*-heptane-1 N-H_2SO_4 (40:10:1, by vol). Two phases were formed by the addition of 24 ml heptane and 16 ml water and the mixture was shaken for a further 10 min. The two phases were allowed to separate in a stoppered measuring cylinder and a sample of 5 ml of the heptane layer was removed for titration of the non-esterified fatty acids. To remove interfering substances, particularly lactate, the sample was transferred to a glass-stoppered test tube, an equal volume of 0·05 % H_2SO_4 in water added and the mixture shaken vigorously for 5 min. After separation of the two phases by centrifuging, 3 ml of the washed heptane phase was transferred

to a test tube containing 1 ml of titration mixture (0·01 % thymol blue and 90 % ethanol in water). The mixture was titrated with 0·1 N-NaOH to a green-yellow end-point. During the titration a constant stream of nitrogen was passed through the mixture. The alkali was standardized against stearic acid in heptane. The remaining heptane phase was then completely removed from the alcohol–water phase which was re-extracted with a volume of heptane equal to the original phase. The acidity of a washed sample of the second heptane phase was determined as before. The extraction and titration procedure was standardized using pure solutions of capric and stearic acids. Recoveries of capric and stearic acids ground into cheese–sand mixtures were 80 and 106 %, respectively.

Gas–liquid chromatographic analysis of the non-esterified fatty acids from cheese

The combined heptane phases obtained as described above were shaken with 0·05 N-NaOH in 50 % ethanol (Borgstrom, 1952). The alkaline ethanol phase, now containing the fatty acids as sodium salts, was washed twice with petroleum ether to remove any neutral fat. The ethanol phase was then acidified with 1 N-H_2SO_4 and the fatty acids re-extracted into petroleum ether. The petroleum ether was evaporated under a stream of nitrogen at approximately 50 °C, and the residual fatty acids were converted to methyl esters by treatment with a solution of diazo-methane in diethyl ether. A sample of 0·025 μl of the ether solution of methyl esters was analysed on a gas–liquid chromatograph. A 6 ft glass column (0·4 mm diameter), packed with 12 % polyethylene glycol on 80/100 mesh Gas Chrom P. (purchased premixed from the Applied Science Laboratories Inc., State College, Pennsylvania U.S.A.) was used at 172 °C with an argon ionization detector and a gas flow rate of 36 ml/min. The apparatus was standardized with samples of methyl esters of fatty acids obtained from the National Institutes of Health, Bethesda, U.S.A.

Assay of inhibition by fatty acids. The inhibition of the growth of lactic acid streptococci by fatty acids was determined by growing cultures in sterile skim-milk containing varying concentrations of different fatty acids. Each test sample contained an inoculum of approximately 30,000 cells in 2 ml skim-milk. The acidity produced after incubation for 16 h at 30 °C was taken as a measure of the growth of the organism. No change in the pH of the test medium was taken as 100 % inhibition. The acidity produced by the control culture was taken as zero inhibition. Half maximum acid production was called a 50 % inhibition.

Cheese samples. Cheddar cheese of varying maturity (1·5, 3, 4 and 5 months) selected at random, were kindly supplied by Holdenson and Nielson Pty. Ltd. Melbourne.

RESULTS

As shown in Fig. 1 the growth of *Str. cremoris* strain C 13, was completely inhibited by concentrations of capric acid above 1·56 mM while those below 0·2 mM did not inhibit growth. The concentration which produced a 50 % inhibition of growth was determined as shown in Fig. 1. All inhibition assays were carried out in this way.

The growth of 6 strains of *Str. cremoris*, 3 strains of *Str. lactis* and 3 strains of *Str. diacetilactis* was examined in the presence of each of 8 saturated fatty acids of

increasing chain length and one unsaturated fatty acid. These were butyric (C 4), caproic (C 6), caprylic (C 8), capric (C 10), lauric (C 12), myristic (C 14), palmitic (C 16), stearic (C 18) and oleic (C 18:1) acids.

As shown in Fig. 2 the overall inhibitory effects of the individual saturated fatty acids on all 3 streptococcal groups were very similar. Inhibition became more pro-

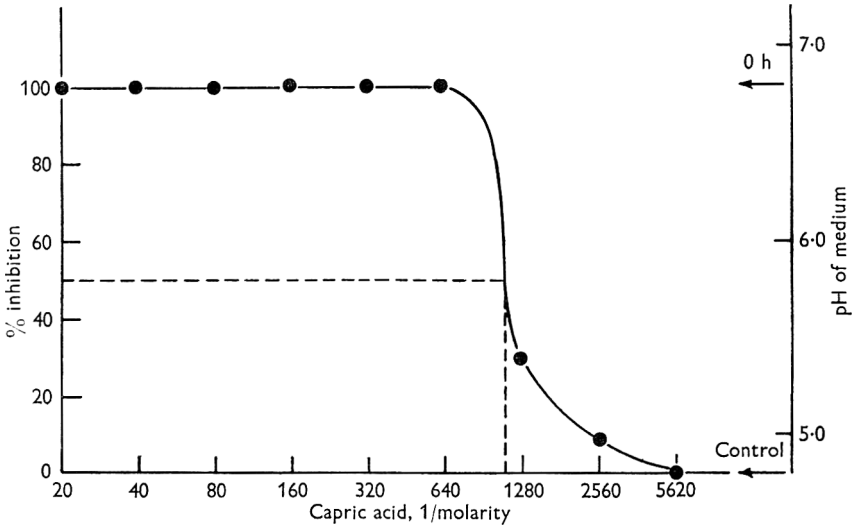

Fig. 1. Inhibition of the growth of *Str. cremoris* strain C 13 by capric acid. An inoculum of approximately 30,000 cells was added to each sample of sterile skim-milk (final volume 2 ml) containing the appropriate concentration of capric acid. The acidity produced after incubation for 16 h, at 30 °C, was taken as a measure of the growth of the organism.

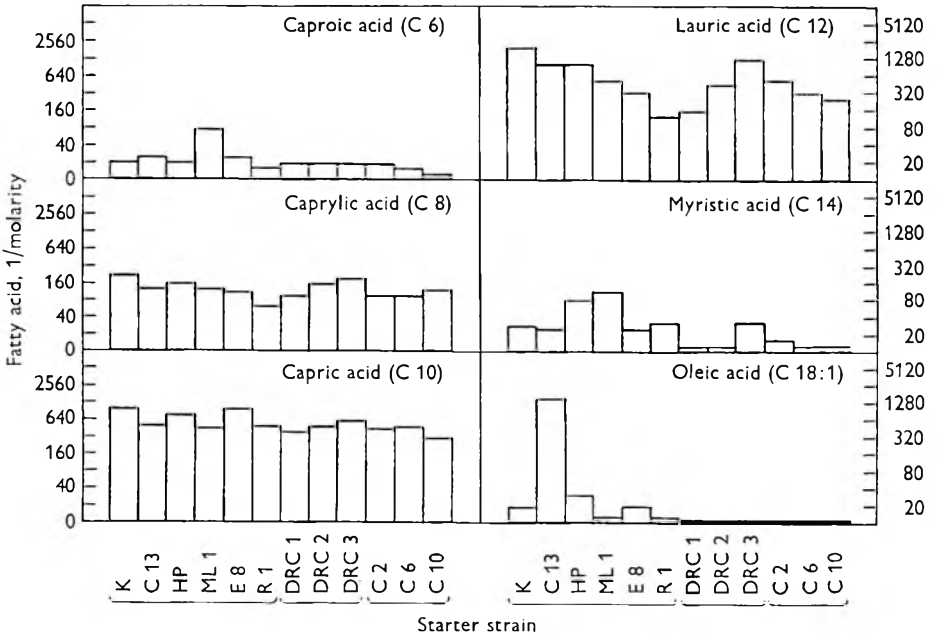

Fig. 2. Concentrations of fatty acids required to produce 50% inhibition of the growth of different strains of lactic acid streptococci. For experimental details see Fig. 1.

nounced with increase in the chain length of the fatty acids up to C 10 or C 12 depending on the strain. Inhibition by myristic (C 14) was considerably less, while palmitic (C 16), stearic (C 18) and butyric (C 4) had no effect on growth. However, differences among the streptococcal groups were found when they were tested against the unsaturated fatty acid oleic acid. This fatty acid did not inhibit any of the *Str. lactis* or *Str. diacetilactis* strains while the *Str. cremoris* strains showed varying degrees of sensitivity to it. In particular *Str. cremoris* strain C 13 was markedly inhibited.

The total unesterified fatty acid content of Cheddar cheese of different ages is shown in Fig. 3. As may be seen the concentration of the unesterified fatty acids/g

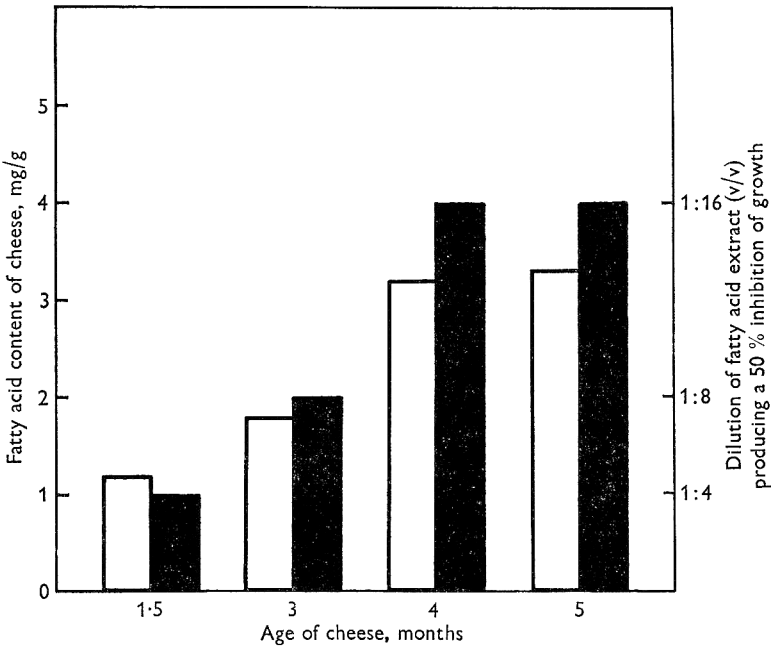

Fig. 3. Concentration and inhibitory activity of fatty acids extracted from cheese of varying maturity. □, Total concentration of fatty acids in cheese; ■, dilution of the fatty acid extracts required to produce 50 % inhibition of the growth of *Str. cremoris* strain C 13. The 1:2 dilution of each extract contained half the fatty acid concentration present in each sample of cheese. For experimental details see Fig. 1.

of cheese increased with increasing age of the cheese. The inhibitory activity of varying dilutions of the fatty acid extracts prepared from the cheese was determined using *Str. cremoris* strains C 13 and ML 1. The 1:2 dilution represented half the fatty acid concentration found in the cheese. As shown in Fig. 3 the titre of the inhibition of strain C 13 followed very closely the concentration of the fatty acids in the extracts. Strain ML 1 was not inhibited at all.

Analysis of the unesterified fatty acid extracts by gas chromatography showed that the saturated inhibitory fatty acids comprised approximately 16 % of the total, and oleic acid approximately 31 %. Table 1 gives the distribution of these fatty acids in Cheddar cheese, 1·5 to 5 months in age. Caproic acid was detected only in very small amounts and therefore accurate values for its concentration were not obtained. The

relative proportions of the individual fatty acids were approximately the same in all the samples tested and were similar to those of milk fat (Hilditch, 1941). This finding suggests a lack of specificity of the milk lipases in cheese.

The inhibition studies already described were carried out using small inocula. Fig. 4*a* shows the inhibitory effect of capric acid on the growth of *Str. cremoris* strain C 13, using a 5% inoculum, thereby more closely approximating to the cell numbers initially found in cheese. As may be seen, the capric acid reduced the viable numbers of the organism and did not merely slow down the rate of reproduction. In the control tube the viable cell count was at a maximum 12 h after inoculation,

Table 1. *Concentration (μg/g) of individual unesterified fatty acids in cheese at different stages of maturation*

	Age of cheese (months)			
Fatty acid	1½	3	4	5
Caprylic	6	11	13	17
Capric	23	38	48	66
Lauric	45	45	71	96
Myristic	136	153	288	337
Oleic	377	564	935	954

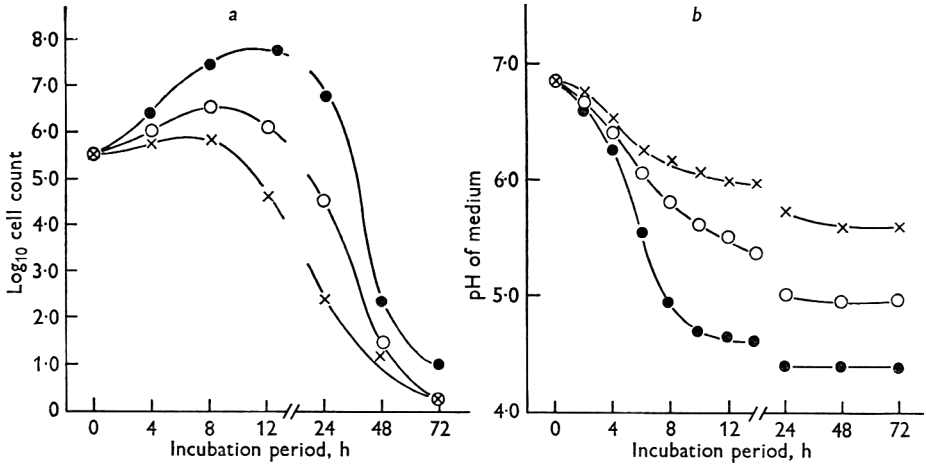

Fig. 4. The effect of capric acid on (*a*) the growth of and (*b*) acid production by *Str. cremoris* strain C 13. ●, Control; ○, 1·56 mM capric acid; ×, 3·13 mM capric acid. Milk containing a 5% inoculum of the test organism was used as growth medium.

at which time the pH of the medium had dropped to 4·65 (Fig. 4*b*). At the same point in the time course the viability of the cultures containing capric acid at concentrations of 1·56 and 3·13 mM had already decreased markedly, while the pH had not fallen below 5·45 and 5·95, respectively. Thus, the inhibition of growth was not due to excess acidity and the capric acid appeared to be bactericidal.

Figs. 5*a* and *b* show the inhibitory effect of capric and oleic acids on the proteolytic activity of acetone-dried whole cells and cell-free extracts of *Str. cremoris* strain C 13. However, the concentrations of these fatty acids found necessary to inhibit proteolysis (8·3 mM) were greatly in excess of those which inhibited growth and also in

excess of concentrations found in the cheese examined. It is unlikely, therefore, that fatty acids in cheese inhibit the proteolytic activity of enzymes released from autolysed cells.

DISCUSSION

It is well known that the growth of many micro-organisms, including lactic and bacteria, is inhibited by the addition of fatty acids to the growth medium (see Nieman, 1954). Costilow & Speck (1951) studied the effect that varying degrees of rancidity in milk had on the growth of *Str. lactis* strain H-1-10, and they found that the degree of inhibition of this strain was directly proportional to the extent of lipolysis which had occurred in the milk. Of the fatty acids which can be released from milk fat, they subsequently showed that caprylic, capric and lauric acids inhibited the growth of this strain. The inhibition produced by myristic acid was less pronounced while butyric, caproic, palmitic, stearic, arachidic, oleic, linoleic and linolenic acids had no effect.

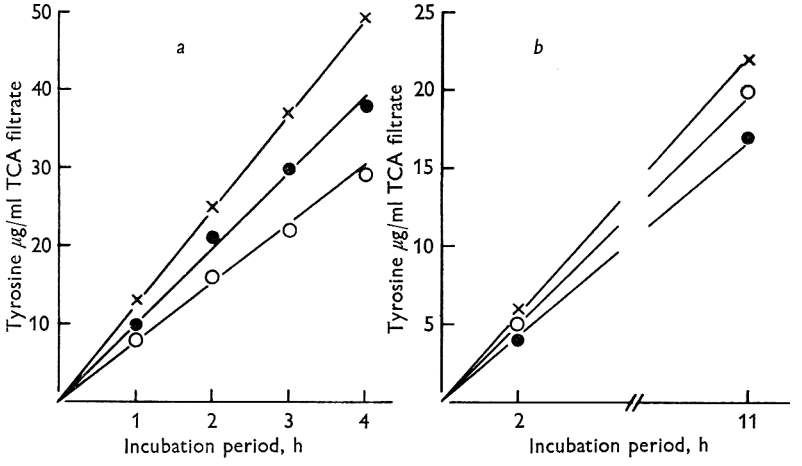

Fig. 5. Effect of capric and oleic acids (8·3 mM) on the proteolytic activity of *Str. cremoris* C 13 as measured by the acid soluble tyrosine liberated from casein by (*a*) suspension of acetone dried cells, (*b*) cell free extract. ×, Control; ○, capric acid; ●, oleic acid.

In the present investigation the growth responses of strains belonging to the species *Str. cremoris*, *Str. lactis* and *Str. diacetilactis* to individual saturated fatty acids were similar. However, differences became apparent in the presence of oleic acid. All the *Str. cremoris* strains showed varying degrees of sensitivity to oleic acid while all the strains of *Str. lactis* and *Str. diacetilactis* were completely insensitive, even to high concentrations of the acid.

Furthermore from the response of *Str. cremoris* strain C 13 to capric acid (Fig. 4) it appears that inhibitory levels of fatty acids in the growth medium quickly reduce the viability of the culture.

Whether fatty acids ever reach concentrations in cheese milk sufficient to cause starter slowness has not, as yet, been established. However, where milk is stored for prolonged periods before manufacture into cheese under conditions favouring lipolytic activity (Herrington, 1954), it seems highly probable.

In the present investigation attention was directed to the appearance of fatty acids in cheese after manufacture. The fatty acid extracts obtained from all the cheeses examined were very inhibitory to *Str. cremoris* strain C 13 but inhibition of *Str. cremoris* strain ML 1 could not be detected by the methods used. As both these strains, ML 1, and C 13, were inhibited to approximately the same degree in the presence of saturated fatty acids in milk it seems almost certain that the inhibition of C 13 by the cheese extracts was due to oleic acid only. The concentration of oleic acid in the youngest cheese (6 weeks) was approximately 400 μg/g cheese. Since levels of 50–100 μg oleic acid/ml of milk were sufficient to inhibit strain C 13 it is evident that oleic acid may reach levels in cheese sufficient to inhibit this strain very early in the ripening process. As all 3 species of lactic acid streptococci were equally inhibited by the inhibitory saturated fatty acids, it appears that these fatty acids are not responsible for the early loss of viability of *Str. cremoris* in cheese.

Evidence that inhibition of strains of *Str. cremoris* does occur in cheese is obtained from the investigations of Dawson & Feagan (1957) who studied the survival rates of lactic acid streptococci in Australian cheddar cheese. They found that most strains of *Str. cremoris* lost viability during the first 8 weeks of ripening while the viability of strains of *Str. lactis* and *Str. diacetilactis* persisted for periods of up to 20 weeks. Similar results were obtained by Perry (1961) with New Zealand cheddar cheese.

Moreover, Dawson & Feagan (1957) showed that *Str. cremoris* strain C 13 lost viability completely within 8 days of manufacture while *Str. cremoris* strain ML 1 was still viable after 77 days.

Estimates of bacterial populations in cheese have usually been based on viable cell counts. Non-viable cells, and therefore the total number of cells, of any given strain in cheese cannot be estimated by this method. Cell viability does, however, indicate that cell reproduction is still possible. Once viability is lost no further reproduction of the cell can occur. Therefore, cheese in which viability is lost shortly after manufacture might be expected to contain fewer bacterial cells than cheese in which viability is still present several months after manufacture.

As lactic acid streptococci are considered to be the major source of peptidases in cheese (Stadhouders, 1960) the early mortality of a starter strain in the cheese would prevent the accumulation of a relatively large total cell mass and therefore of a high level of peptidase activity.

In view of the early loss of viability in cheese by *Str. cremoris* strain C 13 it is not surprising to find that cheese made with this organism has a relatively low content of amino N and a bitter flavour, that is, it has low peptidase activity (Emmons *et al.* 1962). On the other hand, *Str. cremoris* strain ML 1 which survives for a longer period in the cheese (Dawson & Feagan, 1957) produces a higher level of amino N and no bitter flavour (Emmons *et al.* 1962).

This combination of early loss of viability with a low amino N content is also found in cheese manufactured with another 'bitter' strain, *Str. cremoris* strain HP (Dawson & Feagan, 1957; Emmons *et al.* 1962). However, the factor responsible for the early loss of viability (36 days) by this strain is not yet apparent as only very high concentrations of oleic acid inhibit its growth.

Other theories have been advanced to explain low peptidase activity and bitterness in cheese. Both Czulak (1959) and Emmons *et al.* (1962) have suggested that the

so-called 'bitter' strains lack enzymes which are specific for hydrolysing the bitter-tasting peptides.

It appears, however, that the early loss of viability of a starter culture in the ripening cheese will prevent the accumulation of a relatively large total cell mass, and therefore decrease the peptidase activity and result in a bitter flavour. Thus any factor which reduces the period of viability of a starter strain in cheese is likely to produce bitterness.

Thanks are due to Dr F. D. Collins of this Department, for advice on fatty acid analyses and techniques of gas chromatography. We also wish to thank Mr N. O'Sullivan for technical assistance.

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The effect of fatty acids on the metabolism of lactic acid streptococci

II. Resistance of a variant of *Streptococcus cremoris* strain C 13

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Summary. It was previously found that low concentrations of oleic acid in the growth medium inhibited the growth of *Streptococcus cremoris* strain C 13. However, a variant of this strain has now been isolated which is capable of growth in relatively high concentrations of oleic acid. This was achieved by the extended incubation of inocula of strain C 13 in milk containing various concentrations of oleic acid.

It has been shown that the growth of *Str. cremoris* strain C 13 is inhibited by low concentrations of oleic acid in the growth medium (Anders & Jago, 1964). As oleic acid accumulates in cheese early in the ripening process it was suggested that inhibition by oleic acid may be responsible for the early loss of viability (Dawson & Feagan, 1957) and the low peptidase activity (Emmons, McCugan, Elliott & Morse, 1962) of strain C 13 in cheese. Strains of *Str. lactis* and *diacetilactis* which are not inhibited by oleic acid (Anders & Jago, 1964) remain viable in cheese for periods of up to 20 weeks after manufacture (Dawson & Feagan, 1957).

It was considered possible that strains of lactic acid streptococci, capable of tolerating relatively high concentrations of oleic acid, may have arisen by the evolution of resistant variants. This hypothesis was tested by incubating *Str. cremoris* strain C 13 for a prolonged period in milk containing various concentrations of oleic acid and isolating variants of this organism which possessed a high degree of resistance to oleic acid.

METHOD AND RESULTS

For the preparation of inocula *Str. cremoris* strain C 13 was grown in sterile skim-milk for 16 h at 30 °C. Inocula of this culture were added to tubes containing various concentrations of oleic acid in sterile skim-milk (final volume 2 ml, concentration of inoculum 1 %). The tubes were incubated at 30 °C for 14 days. Growth of the culture was shown by acid production which resulted in coagulation of the growth medium.

The degree of inhibition of the growth by oleic acid was determined by the method previously described (Anders & Jago, 1964).

When *Str. cremoris* strain C 13 was grown in milk containing various concentrations

of oleic acid, growth occurred randomly in only 10 tubes out of 60 (Table 1). In all the tubes in which growth occurred the concentration of oleic acid was above 0·78 mM. Coagulation occurred in these tubes from the 4th to the 8th day but the time lag bore no relation to the concentration of oleic acid. After 14 days the cultures in those tubes showing no growth were tested for viability in the absence of oleic acid and all were found sterile.

The cultures which had grown in the presence of oleic acid were subcultured into milk containing the concentration of oleic acid in which the organism had initially grown. At first the rate of growth was slow, but with repeated transfers it gradually

Table 1. *The isolation of a variant of* Str. cremoris *strain* C 13 *capable of growth in the presence of oleic acid*

Concentration of oleic acid mM	Coagulation time, days									
50	—	—	—	—	—	—	—	—	—	7
25	—	8	—	—	—	7	—	8	—	—
12·5	—	7	—	—	—	—	—	—	—	—
6·25	—	6	—	—	—	—	10	—	—	—
3·13	—	6	—	—	—	—	—	—	—	—
1·56	—	—	—	—	—	—	6	—	4	—

Inocula of *Str. cremoris* strain C 13 were incubated in milk containing various concentrations of oleic acid as indicated. The values given represent the time in days required for coagulation of the culture in each tube. Tubes in which no coagulation occurred are indicated by (—).

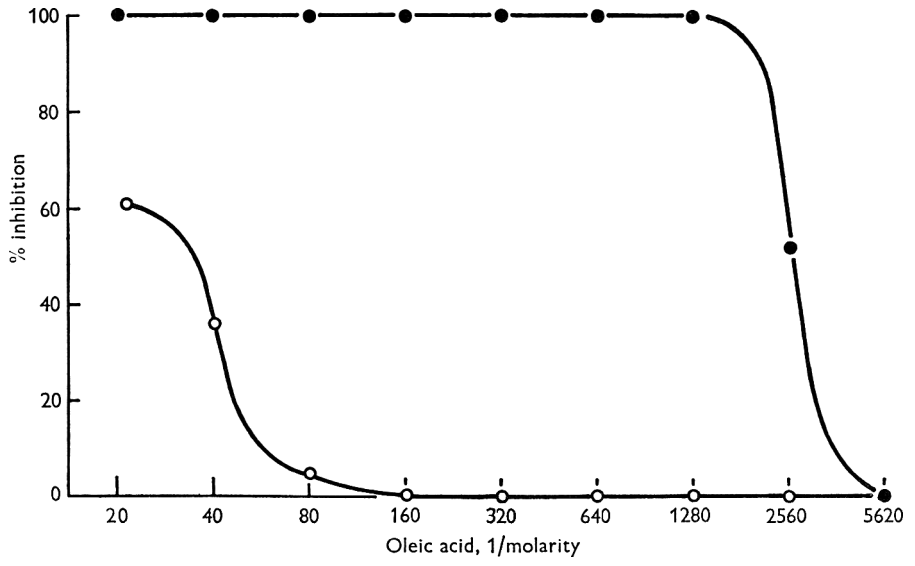

Fig. 1. Inhibition of the growth of *Str. cremoris* C 13 and an oleic-acid resistant variant in skim-milk containing added oleic acid. Inoculum, 15000 cells/ml; incubation 16 h at 30 °C; ●, strain C 13; ○, resistant variant.

increased and finally approximated that of the controls. Furthermore, some but not all the cultures which had established tolerance to low concentrations of oleic acid

became tolerant to higher concentrations by repeated subculture into milk containing the higher concentration.

Using this technique, it was finally possible to obtain an almost normal rate of growth of the variant of *Str. cremoris* strain C 13 in 0·05 M oleic acid, the highest concentration used. A comparison of the growth of this variant with that of the original strain in different concentrations of oleic acid is shown in Fig. 1.

The identity of this strain as a variant of *Str. cremoris* strain C 13 was confirmed by morphological examination and phage typing. It was found to have the same morphology and to be susceptible to the same specific phages as the original strain.

The capacity of this variant strain to grow in the presence of oleic acid appeared to be a permanent characteristic since it was not lost during a period of 3 months in which it was sub-cultured daily into milk in the absence of added oleic acid.

Both the variant and the original C 13 strains were examined for inhibition by the other C 18 unsaturated fatty acids, linoleic and linolenic acids. As shown in Fig. 2 the variant strain was less sensitive than the original strain at the lower concentrations of these acids.

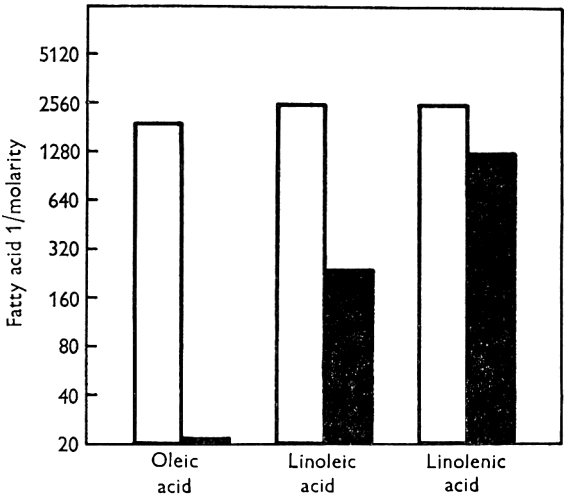

Fig. 2. Concentrations of unsaturated C 18 fatty acids required to produce 50 % inhibition of the growth of both the variant and original cultures of *Str. cremoris* strain C 13. Inoculum, 15000 cells/ml; incubation 20 h at 30 °C; □, strain C 13; ■, resistant variant.

Prolonged incubation of the original *Str. cremoris* strain C 13 in concentrations of linoleic acid inhibitory to growth resulted in a complete loss of viability of the organism. No variants resistant to this fatty acid were observed.

DISCUSSION

The survival of a bacterial culture in an environment containing a substance which normally inhibits its growth may be due either to the induction of enzymes capable of metabolizing the inhibitor, or to the selective growth of individual cells possessing these enzymes constitutively. However, induction of specific enzymes usually occurs in all the cells of an inducible bacterial culture and the high levels of these enzymes

are not maintained in the prolonged absence of the specific inducer (Gale, 1947; Jacob & Wollman, 1961).

In this investigation, a variant of *Str. cremoris* strain C 13 was isolated which grew in the presence of high concentrations of oleic acid, a substance which markedly inhibited the growth of the parent strain. The random appearance of these resistant colonies together with the subsequent retention of the resistance suggested that a mutant had arisen.

A similar finding was reported by Kodicek (1956) who observed the appearance of a mutant of *Lactobacillus casei* in cultures grown in the presence of high concentrations (300 μM) of linoleic acid. This mutant, resistant to linoleic acid, was also resistant to oleic acid but not to linolenic acid. The parent strain was inhibited by all 3 acids.

In the present investigation the resistance to inhibition by oleic acid shown by the variant strain of culture C 13 was accompanied by an increased resistance to both linoleic and linolenic acids, which, however, was evident only at low concentrations of these acids. Higher concentrations were inhibitory.

The availability of a variant of *Str. cremoris* strain C 13 resistant to inhibition by oleic acid will allow a more accurate determination of the influence of oleic acid on the viability of this strain in cheese. If oleic acid is responsible for the production of a bitter flavour in cheddar cheese, manufactured with *Str. cremoris* strain C 13, by causing early loss of viability of the organism (Dawson & Feagan, 1957) and a low peptidase activity (Emmons *et al.* 1962), then the use of a variant resistant to oleic acid should produce a non-bitter cheese. This aspect is under investigation.

We wish to thank Mr N. O'Sullivan for technical assistance.

This investigation was supported by a grant from the Australian Dairy Produce Board which also awarded a Senior Postgraduate Studentship to one of us (R.F.A.). This assistance is gratefully acknowledged.

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A new laboratory method for preventing bacteriophage attack on cheese starter streptococci

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Summary. A method of preventing the action of bacteriophages on starters in cheese milk is described. Direct intramuscular injection of high titre phage preparation into a cow resulted in the appearance in the blood serum and milk of a high concentration of antibody. Even after considerable dilution this antibody is capable of neutralizing the homologous phage and serologically related phages. Methods of concentrating the antibody-containing milk were examined with a view to commercial application of the method in the protection of starter bacteria from phage attack.

Whitehead & Cox (1935) first reported the observation that bacteriophage was responsible for the failure in acid producing activity of a culture of *Streptococcus cremoris* used as a cheese starter. Since that time the importance of bacteriophages as contaminants in dairy factories and their role in slow acid development by lactic cultures has received much emphasis (Whitehead, 1953; Babel, 1955, 1962; Crawford, 1962; Collins, 1962).

The problem in commercial practice is to devise means for excluding bacteriophages from the starter culture and cheese milk or for inhibiting their action. The solution of this problem is particularly important in New Zealand where cultures of pure strains of *Str. cremoris* or *Str. lactis* are used as cheese starters.

Procedures for preventing bacteriophage contamination depend on the use of aseptic techniques in the preparation and propagation of the mother cultures and bulk starter (Whitehead & Hunter, 1945; Lewis, 1956). Procedures for limiting the development of bacteriophage have assumed more importance in recent years because of the realization that bacteriophage probably exists as an airborn infection in every factory, and that the human factor militates against the effective prevention of contamination of the bulk starter at all times in all factories. Babel (1962) describes the various methods introduced in recent years in attempts to control bacteriophage. Particular attention has been given to the use of media low in calcium (Collins, Nelson & Parmalee, 1950; Reiter, 1956) and more recently to the addition of calcium binding agents to media (Galesloot, 1959; Hargrove, McDonough & Tittsler, 1961; Kadis & Babel, 1962).

All of the methods suggested for preventing bacteriophage attack on starter organisms in milk suffer from certain limitations, both with regard to degree of protection and to ease of commercial application.

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In the course of experiments in which immune sera from phage-infected rabbits were used to determine phage relationships, it was found, as would be expected, that addition of the appropriate serum to cheese milk containing bacteriophage resulted in neutralization of the phage and consequently prevented phage attack on the appropriate starter which was subsequently added. Serum, containing antibodies for the particular bacteriophage injected into the rabbit, would also prevent attack on the starters by phages serologically related to the injected phage.

This paper describes the extension of this method of protection to its logical conclusion. It was postulated that antibodies to phage, produced by injection of the phage into the cow, would pass directly into the milk. Any serologically related phage gaining access to the milk would be neutralized and starters added later would thus not be liable to phage attack.

EXPERIMENTAL

Bacterial cultures

The strains of bacteria used in both the preparation of the phages and the neutralization tests were obtained from the culture collection of *Str. cremoris* and *Str. lactis* strains normally supplied as cheese starters from the Institute. The cultures were maintained by daily transfer (1 % inoculum) in autoclaved skim-milk and incubated at 22 °C.

Bacteriophages

The phages used, originally isolated from cheese whey, were maintained as whey filtrates (Seitz) at 4 °C. Phage assay was carried out according to the method of Whitehead, Hunter & Cox (1952). For the purpose of injecting the cows and rabbits a high-titre ($3{\cdot}9 \times 10^9$ plaque-forming units/ml) stock preparation of phage kh was prepared by the method of Whitehead & Bush (1957).

A Spinco model L centrifuge was used in the preparation of a phage kh suspension containing very little foreign protein material. The broth suspension was centrifuged at 3000 *g* for 20 min to deposit non-phage protein. The supernatant was pipetted off and then centrifuged at 47 000 *g* for 60 min. The supernatant was discarded and the phage, sedimented by the second centrifugation, was resuspended in Ringers solution. This process was repeated 3 times.

Media

Lactose-yeast-phosphate agar (LYPA) (Robertson, 1960, after Hunter, 1946) was used for phage assays and the neutralization tests. The corresponding broth (LYPB) used in the preparation of stock phages and for dilution purposes in the neutralization tests had the same composition, except for a double proportion of phosphate and the absence of agar.

Antisera

(1) *Rabbits*

Antisera against the stock phage races were prepared in the following manner. Rabbits were injected subcutaneously with 5 ml of high-titre phage preparation (broth suspension) every other day for a total of 15 injections. One week after the last injection, a test rabbit was bled from the ear vein and a phage neutralization

test (Rountree, 1949) was performed on the serum. If the test gave a satisfactory neutralization titre, 15 ml of blood were taken by cardiac puncture from each rabbit. This withdrawal was repeated 7 days later and after another 7 days the rabbits were exsanguinated. The sera from the 3 bleedings were stored at 4 °C, in screw-cap bottles, for a period of 3 months with no loss in titre.

(2) *Cows* (*3-year-old Jerseys*)

(*A*) *Intramammary injection.* Cows were given injections into the udder tissue, of 5 ml of kh phage every other day for a total of 10 injections. Samples of blood from the mammary vein and milk from all 4 quarters were taken at intervals to follow the build-up in antibody during the injection programme, and stored at 4 °C. The antibody level was identical in the milk samples from each quarter of the udder, and the stage of milking at which the milk samples were taken (fore-milk, mid-milk or strippings) had no effect on the antibody level.

(*B*) *Intramuscular injection.* Cows were given injections of kh phage into the semitendinosus muscle of the pelvic limb. The frequency of injection and the volume of phage suspension administered were variables within the group of experimental cows. Milk samples and samples of blood from the jugular vein were taken at regular intervals during and after the injection period.

Reaction of the cows to phage injection

Precautions were taken to inject the animals with bacteria-free phage preparations; the injection programme had no apparent effect on the general health and milk-producing capacity of any of the experimental cows. Viable bacteriophage was not detected in the milk at any stage subsequent to injection of the phage. Consequently the milk could be supplied to the cheese factory during the injection period.

Preparation of whey

The preparation of whey, which retains the neutralizing power of the milk against phage antigens, was as follows. The casein was precipitated by addition of 10 % lactic acid and the whey, held at 40 °C, was adjusted to pH 5·0 by the addition of 2 N sodium hydroxide. The sample was then cooled to room temperature and passed through a Seitz filter to give a clear filtrate.

Phage-neutralization test

Neutralization constants were determined by a modification of the method described by Rountree (1949) after Burnet (1933). Tenfold serial dilutions of the immune serum, milk, or whey were made in broth and an equal volume of diluted phage added to each of a series of tubes for each whey dilution. The phage dilution used was that which had been previously shown to give a countable number (50–200) of plaques when 0·05 ml quantities were plated. The phage-antibody mixtures were incubated in different experiments at 30, 22 and 45 °C. Duplicate tubes were withdrawn at intervals, immersed in cold water, and 0·05 ml samples plated on the appropriate propagating strain of streptococcus. After incubation overnight at 30 °C the number of plaques was counted.

Phage-antibody reaction in milk

10 ml quantities of sterile skim-milk were dispensed in tubes. Starter KH (0·6 % addition), phage kh (1000 plaque-forming units) and immune whey (0·3 or 1 % addition of a sample having a neutralization titre of 200) were added to each tube, at various times, to test the effectiveness of antibody-containing whey in overcoming different forms of phage contamination, as indicated by the presence or absence of clotting in each tube after incubation at 22 °C for 18 h. Control milk tubes contained starter and starter plus phage, respectively. Each tube showing clotting was subsequently subcultured to a fresh milk tube and incubated at 22 °C for a further 18 h to test for starter survival and activity. The tests were carried out in triplicate.

Heat and pH *tolerance*

Heat tolerance. 2-ml quantities of the immune whey were dispensed in small soft-glass tubes, the tubes sealed and immersed in a water bath held at 72 °C. At 5-min intervals, triplicate tubes were removed from the holding bath and cooled to room temperature by immersion in cold water before proceeding with the neutralization tests.

pH *tolerance.* 2-ml quantities of the whey were dispersed in tubes and the pH adjusted, within the range of 3·5–7·0, by addition of 10 % lactic acid or 2 N sodium hydroxide, and distilled water, to give identical final volumes in each tube. Neutralization tests were carried out on tenfold dilutions of each adjusted whey.

Evaporation of immune milk

Condensed skim-milk was prepared by evaporation of immune skim-milk to 40–45 % total solids in a Luwa laboratory thin-layer evaporator at temperatures below 72 °C.

Freeze-dried whey

Freeze-dried whey was obtained using an Edwards Centrifugal Freeze Dryer and stored at 4 °C in sealed soft-glass tubes. Neutralization tests were carried out on tenfold dilutions of the freeze-dried whey resuspended in broth.

Precipitated whey powder

On addition of 2 N sodium hydroxide to the whey the whey proteins precipitated at pH 6·2. This precipitate was collected and dried at 30 °C. For use in neutralization tests the whey powder was resuspended in broth at pH 5·0.

RESULTS

Factors determining antibody level in milk and blood

Milk and blood samples collected from the experimental cows prior to injection of phage did not contain phage, phage antibodies or other phage-inhibiting agents.

The effect of a number of variations in the injection procedure on the rate of antibody production and final antibody titre is illustrated in Table 1. In the first place, it is evident that both intramammary and intramuscular injections are equally

effective in the promotion of an immune response. Secondly, the higher the titre of the phage suspension injected, the greater the response.

Table 2 illustrates the effect on antibody level of variations in the number of injections and the period over which they are administered. It is seen that a series of small injections given over a period of 3 weeks is more effective than a single massive dose. Administration of 1 booster injection, when the antibody level begins to fall off, is sufficient to restore the antibody titre to its former level.

Table 1. *Effect of variations in the injection procedure on rate of antibody production and final antibody titres*

Site of injection	Titre of phage, plaque-forming units/ml	Neutralization titres of milk samples taken 1 day after injection*			
		2nd	5th	8th	10th
Intramammary	$3{\cdot}9 \times 10^9$	120	600	1000	1000
Intramuscular	$3{\cdot}9 \times 10^9$	120	800	1000	1000
Intramuscular	$4{\cdot}4 \times 10^5$	40	200	500	500

* Each cow given 10 injections on alternate days. Neutralization titre expressed as the reciprocal of that dilution which gave approximately 80 % reduction in the plaque count as compared with that given by controls consisting of phage diluted with normal cow serum or milk.

Table 2. *Effect on antibody level of variations in the number of injections and the period over which they are administered*

Treatment	Neutralization titres; no. of weeks after administration of final injection									
	1	2	3	4	5	6	7	8*	9	10
1 injection (200 ml)	600	600	600	400	200	100	100	100	600	600
10 injections (20 ml each) on alternate days	1000	1000	1000	1000	600	500	200	200	1000	1000

* Booster injection (30 ml) given 8 weeks after the last of the initial injections.

Phage-neutralizing capacity of different fractions

Milk and blood samples were withdrawn from 1 of the experimental cows (which had received 10 small injections) at a stage when the antibody level had reached its maximum titre. A comparison of antibody level in the blood serum and in various milk fractions before and after a number of treatments is given in Table 3. It will be seen that, prior to treatment, the various milk fractions possess identical neutralizing abilities. The antibody titre in the blood serum was, however, higher. During a brief mastitis infection of the udder of one of the experimental cows an increase in the level of antibody in the milk was apparent.

Pasteurization (72 °C for 1 min) of the immune skim-milk had no effect on antibody level. Higher temperatures resulted in marked denaturation of the antibodies. Of the 3 concentrating treatments, milk evaporation and precipitation of whey proteins resulted in some loss of neutralizing ability, whilst freeze-drying of the whey had no apparent effect.

Characteristics of the phage-antibody reaction

The reaction between the phage and antibody proceeds at a readily measurable rate function dependent on the concentrations of the reactants and the temperature. The influence of temperature on the neutralization reaction between immune whey and phage kh is illustrated in Fig. 1. As would be expected the rate of reaction increases with elevation of temperature. The neutralization constants (K) at 30, 37 and 45 °C were 26·8, 53·7 and 80·6 min^{-1}, respectively.

Table 3. *Phage antibody level in different fractions of milk and blood before and after various treatments*

Fraction	Neutralization titre
Raw whole milk	1000
Raw skim-milk	1000
Cream (30 % fat)	1000
Acid whey	1000
Blood serum	1200
Pasteurized skim-milk (72 °C for 1 min)	1000
Evaporated skim-milk:	
30 % solids	1000*
45 % solids	800*
Reconstituted freeze-dried whey	1000
Reconstituted alkali-precipitated whey protein	600

* Value corrected to allow for change in volume.

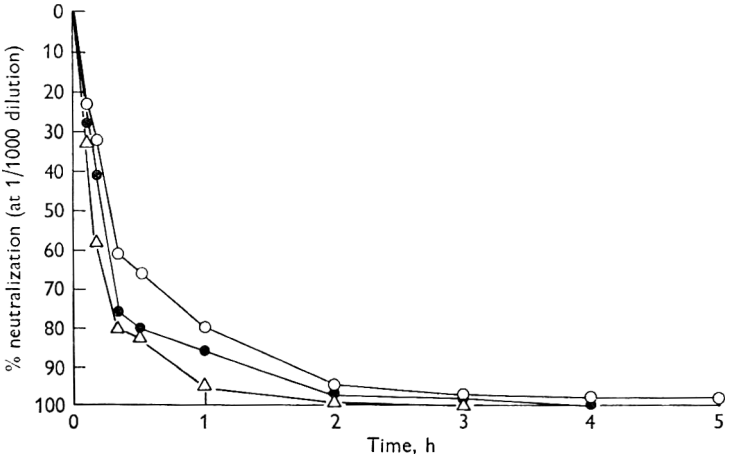

Fig. 1. The effect of temperature on the rate of the neutralization reaction between immune whey and phage kh. ○, 30 °C; ●, 37 °C; △, 45 °C.

Phage-antibody reaction in milk

An attempt was made in this experiment to simulate, in the laboratory, conditions likely to obtain in a factory during the cheese-making process. An examination was made of the effectiveness of antibody-containing whey in overcoming different forms of phage contamination. The conditions investigated were:

(1) The addition of immune whey to phage-contaminated milk before addition of starter.

(2) The addition of phage to milk containing starter and antibody.

(3) The addition of immune whey to milk containing starter and phage.

In each case the accepted criterion of effectiveness of the immune whey in preventing phage attack was the occurrence of clotting, in a defined time, in tubes of milk treated in these ways.

The results given in Table 4 show that 1 % addition of immune whey (with a neutralization titre of 200) to phage-contaminated milk 10 min before addition of starter, to starter-containing milk at the same time as phage, and to starter-containing milk 30 min after phage contamination occurs, will prevent bacteriophage attack on the starter if the contamination is of the order of 1000 plaque-forming units or less in 10 ml milk.

Each positive tube clotted again after subculture to fresh milk and further incubation at 22 °C.

Table 4. *Effect of addition of immune whey to milk at various times before or after addition of phage and starter*

Milk treatment*	Immune whey: % addition	Clotting
Whey and phage added	0·3	+
30 min before starter	1·0	+
Whey and phage added	0·3	−
10 min before starter	1·0	+
Whey, phage and starter	0·3	−
added at same time	1·0	+
Whey added 10 min after	0·3	−
phage and starter	1·0	+
Whey added 30 min after	0·3	−
phage and starter	1·0	+

+, Clotted; −, no clot.

* Neutralization titre of immune whey = 200. Phage concentration in milk = 100 plaque-forming units/ml.

Heating and pH *tolerance trials*

In an effort to characterize further the phage-antibody reaction the effect of holding the whey at 72 °C for different periods was studied (triplicate determinations). The results (Fig. 2) indicate that the velocity of inactivation of the antibody was at its greatest in the initial stages; the rate of inactivation decreased as the percentage neutralization approached zero.

Within the range pH 3·5–7·0 alteration of pH had no effect on the activity of the antibody or on the rate of the reaction.

Specificity of the phage-antibody reaction

Antigenic analysis

Separation of bacteriophages into groups on the basis of degrees of neutralization in homologous and heterologous antisera is a very reliable method of establishing relationships among them (Adams, 1959). To investigate serological relationships among the stock bacteriophages which attack KH and a few other strains of starter streptococci each phage was tested against homologous, heterologous and composite

rabbit antisera at 30 °C. The results, expressed in terms of neutralization titres, are recorded in Table 5. Each phage was neutralized by the homologous and composite antisera. There was no cross-neutralization by heterologous antisera in the case of phages e_8 and br_4 indicating that each of these phages is antigenically distinct from the 7 phages against which each one was tested. Phages kh, ml_3, ml_8, ml_1, hp and c_{13},on the other hand, are evidently related to 1 or more of the other phages.

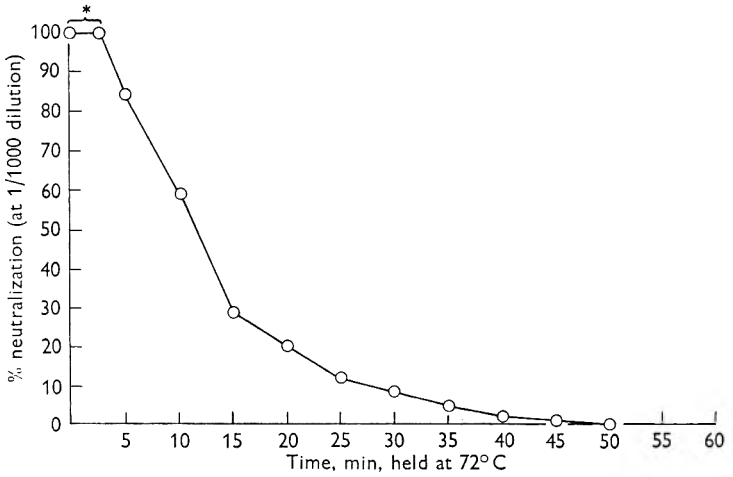

Fig. 2. Rate of inactivation of phage antibody in immune whey at 72 °C. ○, Neutralization titre expressed as a % of that of unheated sample; *, time required for tube contents to reach 72 °C.

Table 5. *Specificity of the phage-antibody reaction: cross-neutralization tests*

	Neutralization titres									
	Rabbit serum									
	Antisera									Cow's milk.
Phage	kh	ml_3	ml_8	ml_1	hp	c_{13}	e_8	br_4	Composite antiserum*	Antiphage kh whey
kh	5 000	100	200	100	0	500	0	0	1 000	1 000
ml_3	200	1 000	0	0	0	0	0	0	100	600
ml_8	1 000	0	1 000	0	10	0	0	0	100	600
ml_1	1 000	0	0	1 000	0	0	0	0	1 000	800
hp	0	10	0	0	10 000	0	0	0	10 000	0
c_{13}	2 000	0	0	0	0	5 000	0	0	1 000	800
r_6	—	—	—	—	—	—	—	—	—	400
tr	—	—	—	—	—	—	—	—	—	0
r_1	—	—	—	—	—	—	—	—	—	0
z_8	—	—	—	—	—	—	—	—	—	0
e_8	0	0	0	0	0	0	5 000	0	100	0
br_4	0	0	0	0	0	0	0	1 000	100	0
ml_2	—	—	—	—	—	—	—	—	—	100
am_1	—	—	—	—	—	—	—	—	—	0
am_2	—	—	—	—	—	—	—	—	—	0
am_3	—	—	—	—	—	—	—	—	—	200
us_3	—	—	—	—	—	—	—	—	—	0
k	—	—	—	—	—	—	—	—	—	0

0, no neutralization (or negligible); —, undetermined.

* Composite antiserum was prepared by injecting rabbits with a mixture of all of the stock phages. Phages kh, ml_3, ml_8 and ml_1 attack the starter KH.

In order to determine whether it was effective in neutralizing heterologous phages the whey, containing kh antibody, was tested against all the stock laboratory phages. It may be seen from Table 5 that the whey neutralized phages kh, ml_1 and c_{13} at similar dilutions, reacted less strongly with phages ml_3, ml_8, r_6, ml_2 and am_3 and had no detectable effect on the other phages.

DISCUSSION

The study reported in this communication has shown that antibodies induced by intramuscular injection of phage into a cow are excreted in the milk and are effective in neutralizing the homologous phage even after considerable dilution with normal milk. Results show that the antibodies respond to heat treatment in the characteristic manner of normal whey proteins. The specificity of these antibodies, their thermal sensitivity, and the fact that their presence in the milk was induced by injection of homologous antigen, indicate that they are classical antibodies.

From a practical point of view it is suggested that milk from treated cows could be used for the preparation of a freeze-dried whey which could be added, in appropriate quantities, to the milk used for the mother culture or bulk starter. This immune whey will neutralize any homologous phage present in the milk and effectively protect the starter which is subsequently added.

It is evident that milk containing antibody can be pasteurized without impairing its antibody potency and evaporation of the milk results in only a small reduction in neutralizing efficiency. As an alternative to the use of freeze-dried whey advantage could, therefore, be taken of the fact that manufacturers can produce low-heat, non-fat dry milk solids in which less than 20% of the serum protein is denatured. Clearly they will also be able to prepare whey powder containing essentially unimpaired antibody.

The problem of strain specificity, which was early viewed as a possible drawback, has been partially resolved by the demonstration that the antibody will, in some degree, neutralize closely related phages and will therefore probably neutralize any variant of the injected phage which might arise by mutation. In unpublished work on mutations in phages, it has been found that variants arising as a result of mutation are serologically related to the original phage, although antiserum to the original phage generally neutralizes that phage more rapidly than the mutant phage. However, such difficulties emphasize that it would be wise to reserve judgement on the practical efficacy and widespread applicability of the use of immune whey to protect these starters until trials in cheese and casein factories have been conducted and evaluated.

It is a great pleasure to acknowledge many stimulating discussions with Dr W. A. McGillivray and Dr P. S. Robertson. The author is indebted to Mr T. Lynch and other members of the Massey University College farm staff for their supervision of the feeding and milking of the cows.

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Some bound forms of pantothenic acid in the milk of normal dairy cows

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Summary. The levels of coenzyme A were measured in the milk of normal dairy cows during the first 20 weeks of lactation. Estimations were made on milk samples obtained from some cows in their first lactation and from older cows. The microbiological method described by Brown (1959) for the estimation of bound forms of pantothenic acid was used, but considerable modification of this method was necessary to avoid elevated results due to the stimulatory action of free pantothenic acid on the response of the assay organism. Appreciable amounts of coenzyme A were excreted in the milk of cows in their second or subsequent lactations during the first 12–16 weeks after parturition, peak levels being reached after the end of the 1st week. In comparison, only small amounts of coenzyme A were excreted in the milk of cows in their first lactation. Attempts to detect the presence of phosphopantetheine in milk were unsuccessful.

Introduction

Bach & Hibbitt (1959) suggested that the levels of coenzyme A may be deranged in certain tissues of cows suffering from primary ketosis. In view of the importance of this cofactor in intermediary metabolism, as shown by many workers, (e.g. Lipmann, 1954), it was considered desirable to investigate its suspected loss in the milk of cows in early lactation.

Apart from the studies of Lauryssens, Peeters & Donck (1956) there appears to have been little previous work carried out on the levels of coenzyme A in cow's milk. Many workers have shown the presence of pantothenic acid in milk, but it was Gregory, Ford & Kon (1958) who showed that the highest concentrations of this vitamin were present in the milk of cows early in lactation.

For the extraction of pantothenic acid from cow's milk, Gregory *et al.* (1958) heated the diluted milk in a steam bath for 10 min. However, in a few of their preliminary experiments, the milk samples were treated with intestinal phosphatase and a chicken liver enzyme as described by Neilands & Strong (1948). Using this procedure the free pantothenic acid level of the milk was increased by approximately 10% which suggested the presence of bound forms of the vitamin.

In view of these findings, it was of interest to determine the coenzyme A content of milk and for this purpose a microbiological method was developed.

METHODS

Animals

Three cows in their first lactation and 5 cows in their second or subsequent lactations were used for this experiment. All animals were of the Ayrshire breed and formed part of a dairy herd where milk recording was practised. The animals were steamed up for 6 weeks before calving and the calves were removed from their dams 24 h after parturition.

During the course of the experiment (May–October 1962), the animals were grazed on 2–5 year leys with a high clover content. A balanced concentrate ration was given only to the higher yielding animals at the beginning and end of the experimental period when the available grass was limited.

Milk sampling

Milk samples were collected at varying intervals during the first 20 weeks of lactation, the first samples being collected 24–48 h after calving. Approximately 30–50 ml of freshly secreted milk was removed from the 4 quarters of each cow within 10 min of the completion of the evening milking. The individual quarter samples were pooled and plunged immediately into a boiling water-bath and boiled for 5 min to destroy the endogenous phosphatases. After cooling, the samples were stored at −30 °C until estimated. All estimations were commenced within 24 h of sample collection.

Coenzyme A estimation

Coenzyme A was estimated by a method based on a procedure in which the growth response of *Lactobacillus helveticus* (N.C.I.B. 80) to pantetheine was measured. Pantetheine is utilized by this organism as a growth factor, whereas coenzyme A, dephosphocoenzyme A and phosphopantothenic acid are not utilized. Phosphopantetheine and pantothenic acid are utilized only in relatively high concentrations, but have been shown by Craig & Snell (1951) to stimulate the growth response of *L. helveticus* to pantetheine. Therefore, it was necessary to modify this method since coenzyme A in milk was estimated in the presence of pantothenic acid.

Coenzyme A, dephosphocoenzyme A and phosphopantetheine, when incubated with alkaline phosphatase, are all degraded to pantetheine (Brown, 1959), so that any growth response of a bacterial culture to an assay mixture after such enzymic treatment must be due to (*a*) the presence of free pantetheine and/or (*b*) the presence of degraded coenzyme A, dephosphocoenzyme A, or phosphopantetheine. On the other hand, acid phosphatase treatment can only produce pantetheine from phosphopantetheine (Brown, 1959). In view of a lack of response after acid phosphatase treatment, only alkaline phosphatase was used, and the increased growth response after such enzymic treatment was considered to be due to coenzyme A and dephosphocoenzyme A.

The stimulating action of phosphopantetheine and pantothenic acid (Craig & Snell, 1951) on the growth response of *L. helveticus* to free pantetheine had to be allowed for in the procedure. This was achieved by adding aliquots of the milk extract before phosphatase treatment to tubes already containing graded amounts of phosphatase-

treated coenzyme A. The volumes added were the same as the volumes of phosphatase-treated material added to the 'unknown' assay tubes (Table 1). In this way, any free pantetheine, or any stimulating action on the growth response due to phospho-pantetheine or pantothenic acid could be disregarded and the growth response of the 'unknown' assay series compared directly with the so-treated coenzyme A standard.

Preparation of samples for assay

After thawing, 100 ml of the milk to be assayed was added to an equal volume of distilled water. The pH was adjusted to 4·7 with HCl and the precipitated casein removed by filtration. The clear filtrate was readjusted to pH 8·2 with NaOH, placed in a boiling water-bath for 5 min and refiltered. The volume of the clear filtrate was then reduced by half in a rotary evaporator at 25 °C. The resulting solution (*A*) was divided; one aliquot was treated with alkaline phosphatase and the other was incorporated into tubes already containing graded amounts of coenzyme A (Table 1).

Table 1. *The volumes of various solutions added to the assay tubes for the estimation of coenzyme A in milk*

Milk extract tubes			Standard coenzyme A tubes			
Basal medium, ml	Phosphatase-treated milk extract, ml	Water, ml	Basal medium, ml	Phosphatase-treated coenzyme A, ml	Milk extract, ml	Water, ml
5·0	0·25	4·75	5·0	0·2	0·25	4·55
5·0	0·50	4·5	5·0	0·4	0·5	4·1
5·0	0·75	4·25	5·0	0·6	0·75	3·65
5·0	1·0	4·0	5·0	0·8	1·0	3·2
5·0	1·25	3·75	5·0	1·0	1·25	2·75

Phosphatase treatment of the samples

1·0 ml 0·2 M bicarbonate buffer pH 8·5 ($NaHCO_3/Na_2CO_3$) and 10 mg of purified alkaline phosphatase (Light & Co.) dissolved in 0·5 ml water were added to 20 ml of solution *A* in a boiling tube.

The tubes were plugged with cotton wool and incubated at 37 °C in a constant temperature water-bath with gentle agitation. After 3 h, incubation was terminated by plunging the tubes into a boiling water-bath for 5 min to inactivate the added phosphatase. After cooling, the incubation mixture was adjusted to pH 6·8 with HCl and the volume made up to 25 ml with water. Portions of this solution were added to the assay tubes as shown in Table 1.

Preparation of standard coenzyme A for assay

To 15 ml water containing 1·0 ml 0·2 M bicarbonate buffer pH 8·5 were added 20 μg coenzyme A (Sigma, 75 %) dissolved in 0·2 ml water. After adjusting the pH to 8·5, 10 mg purified alkaline phosphatase was added and the mixture incubated for 3 h at 37 °C. The phosphatase activity was destroyed by boiling, and after cooling, the pH was adjusted to 6·8 and the volume made up to 25 ml so that 1·0 ml of the standard solution was equivalent to 0·8 μg coenzyme A.

Preparation of the inoculum

The inoculum of *L. helveticus* was prepared in 1-oz McCartney bottles containing 5 ml basal medium (Craig & Snell, 1951), and 50 μg of D-calcium pantothenate dissolved in 5 ml water. At this concentration of pantothenate the organisms showed a vigorous growth response. After sterilization the medium was inoculated from the stock culture and incubated at 37 °C for 24 h when a substantial growth was observed. The culture was centrifuged, the supernatant poured off, and the organisms re-suspended in 10 ml of 0·9 % sterile saline.

Assay procedure

The assays were carried out by adding 5-ml portions of double strength basal medium (Craig & Snell, 1951), to 1-oz McCartney bottles. Portions of phosphatase-treated milk, untreated milk and standard phosphatase-treated coenzyme A were added to the bottles of the 'unknown' and standard series as shown in Table 1.

It can be seen that the volumes of the milk extract not treated with phosphatase added to the standard assay bottles were exactly the same as the volumes of the phosphatase-treated milk extracts in the 'unknown' assay bottles. Water was added to the assay bottles to make the total volume up to 10 ml. After sterilization, the assay bottles were inoculated with one drop of the saline suspension of *L. helveticus* described above, and incubated at 37 °C for 48 h. The lactic acid formed in the standard and 'unknown' assay bottles was titrated against 0·1 N–NaOH using brom-thymol blue as indicator.

It may be seen from the typical dose–response curves shown in Fig. 1 that the addition of the untreated milk extract to the bottles already containing graded amounts of phosphatase-treated coenzyme A had a considerable stimulating effect on the growth response of the organism. Therefore, by adopting this procedure, a direct comparison could be made between the coenzyme A standard and the coenzyme A present in the milk.

To calculate the coenzyme A content of the milk, the log of the dose was plotted against the log of the response for both the standard coenzyme A and the milk samples. Parallel lines were produced as shown in Fig. 2; a lack of parallelism indicated an invalid assay. For the response represented by any point on the log response axis within the range of the parallel lines, the log of the concentration of coenzyme A in the milk extract (μg/ml) = log dose(ml) of the standard coenzyme A solution − log dose(ml) of the milk extract + log concentration of the standard (μg/ml). To calculate the concentration of coenzyme A in the milk, allowances were made for the dilution of the milk sample during the preparation and phosphatase treatment of the milk extract, and the 75% purity of the coenzyme A used as a standard.

It was realized that phosphopantetheine may be present in the milk in addition to coenzyme A and dephosphocoenzyme A, and after its conversion to pantetheine by alkaline phosphatase it would be estimated as coenzyme A. Therefore, in a preliminary investigation on the milk of 2 cows in early lactation, the growth response of *L. helveticus* was measured after the milk samples had been treated with acid phosphatase. As described above, treatment with this enzyme only results in pantetheine formation from phosphopantetheine. Therefore, by subtracting the resulting growth

response of the organism to a milk extract not receiving any phosphatase treatment from the growth response after acid phosphatase treatment, the phosphopantetheine level of the milk could be calculated. Using this procedure, however, no phosphopantetheine was detected in either milk sample.

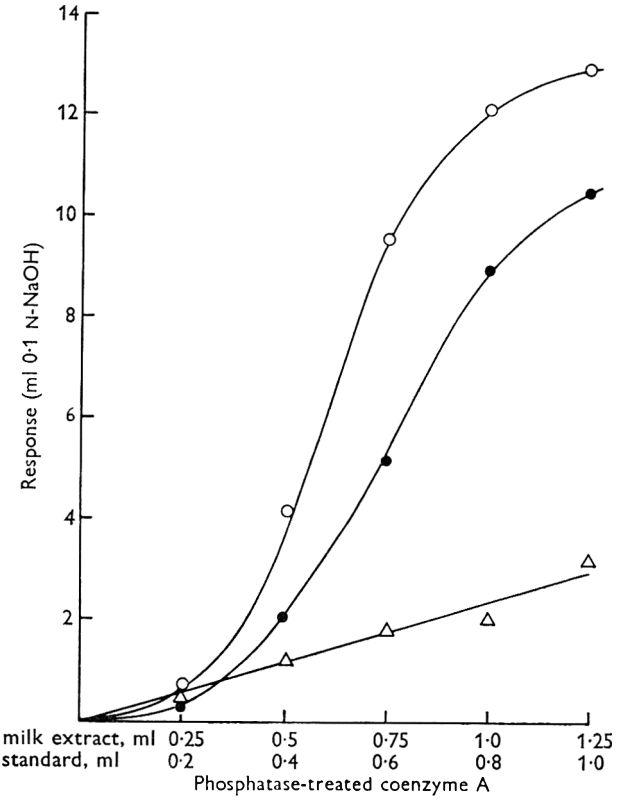

Fig. 1. Typical curves showing the effect of phosphatase-treated coenzyme A (△—△), phosphatase-treated milk extract (●—●), and mixtures of phosphatase-treated coenzyme A and milk extract (○—○) on the growth of *L. helveticus*.

RESULTS

The daily excretion of coenzyme A in the milk of 8 normal dairy cows, during the 1st week of lactation, is shown in Fig. 3. In spite of the variation in the coenzyme A excretion rates of the individual animals it can be seen that after the 1st week of lactation considerable quantities of the cofactor are excreted in the milk. However, in the first few days after calving the coenzyme A excretion was low, a finding which is compatible with those of Gregory *et al.* (1958) who could detect only low levels of pantothenic acid in the colostrum. Later on in lactation, some animals excreted as much as 30–50 mg coenzyme A per day, whereas others excreted no more than 2–8 mg coenzyme A per day throughout the whole 20-week experimental period. It is noteworthy that the animals excreting the most coenzyme A per day were all in the older age group, having had at least 1 previous lactation. On the other hand, the coenzyme A excretion in the milk of the first lactation cows was without exception

low in comparison, and showed only a slight decline during the experimental period. After 12–16 weeks of lactation the excretion of coenzyme A in the milk of cows of all ages remained steady at 2–6 mg/day.

Although maximum milk yields are usually attained within the first 6 weeks of lactation (Hammond, 1952), the increased excretion of coenzyme A in the milk observed at the beginning of lactation is not simply the result of higher milk production. Increased concentrations were found during this period, which, if plotted against time (Fig. 4), showed a similar picture to that seen in Fig. 3. The very

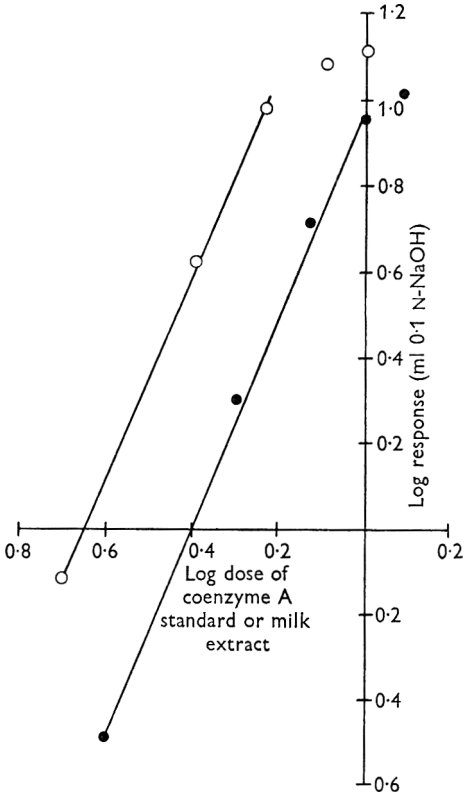

Fig. 2. A typical logarithmic graph of the response of *L. helveticus* to a phosphatase-treated milk extract (●—●), and to a mixture of the untreated milk extract and phosphatase-treated coenzyme A (○—○).

high concentrations of coenzyme A (0·9–1·72 μg/ml) attained after the 1st week of lactation in the milk of some of the older cows appeared to fall to a steady concentration of 0·1–0·3 μg/ml by the 12th–16th week. The first lactation cows on the other hand who also showed elevated milk coenzyme A levels at the beginning of lactation never exceeded 0·63 μg coenzyme A per ml.

DISCUSSION

Lipmann, Kaplan, Novelli, Tuttle & Guirard (1947) showed that pantothenic acid was formed when coenzyme A was treated with a chick liver enzyme and alkaline phosphatase. These 2 enzymes were also used by Neilands & Strong (1948) to release

pantothenic acid from natural materials, but unfortunately they showed a high pantothenic acid blank. In 1958 Gregory *et al.*, who also used this method of Neilands & Strong for the extraction of free pantothenic acid from milk, found that their results were approximately 10% higher than in the experiments in which enzymes were not used. In 1956, Lauryssens *et al.* found 0·5 Lipmann units of coenzyme A per ml of milk using a modification of the Kaplan & Lipmann (1948) method. However, no indication is given of the period after parturition when the samples were collected.

In view of these experiments by other workers, it was not surprising that coenzyme A was found in the milk samples described in this present study, particularly in view of the increased concentrations of coenzyme A in mammary tissue early in

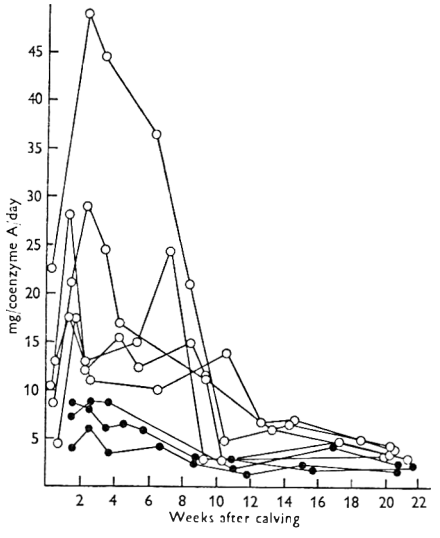

Fig. 3

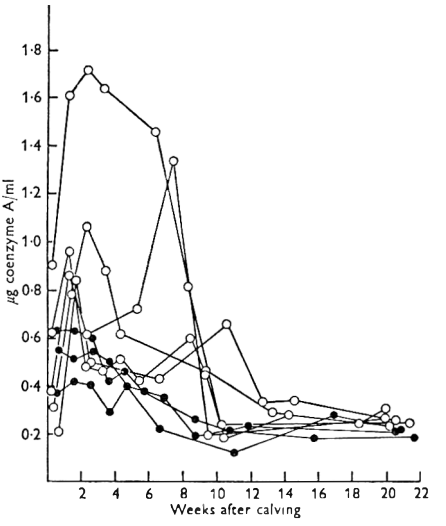

Fig. 4

Fig. 3. The quantity of coenzyme A excreted per day in the milk of normal dairy cows. ○—○, Animals in their second or subsequent lactation; ●—●, animals in their first lactation.

Fig. 4. The concentration of coenzyme A in the milk of normal dairy cows. ○—○, Animals in their second or subsequent lactation; ●—●, animals in their first lactation.

lactation (Lauryssens *et al.* 1956; Ringler, Baker & Nelson, 1954). Furthermore, it is a plausible argument that the elevated tissue concentrations of coenzyme A would result in the presence of the cofactor in the milk due to a loss of some of the glandular cell cytoplasm in the process of secretion and the presence of whole or damaged tissue cells in the milk.

The levels of coenzyme A in the colostrum and milk of the animals examined can be regarded as compatible with the findings of Gregory *et al.* (1958). These workers, in a study of the levels of members of the vitamin B complex in the milk of cows at various stages of lactation, found low levels of pantothenic acid in the colostrum which increased to a maximum by the end of the first 7–10 days of lactation. This high concentration of pantothenic acid in the milk gradually declined to a steady level of approximately 2–3 μg/ml. The coenzyme A levels behaved in a similar way, the maximum levels (1·72 μg/ml) being attained within the first 14 days of lactation

falling to a steady level (0·1–0·3 μg/ml) by the 16th week. The milk concentration of coenzyme A, however, although elevated at the beginning of lactation was not so high in the first-calf cows as in the older animals.

With reference to the previous suggestion of Bach & Hibbitt (1959) that primary bovine ketosis may be associated with low levels of coenzyme A in certain tissues of the body, it is noteworthy that the excretion of coenzyme A in the milk of normal cattle is greatest during the first few weeks of lactation when the incidence of primary ketosis is highest (Leech, Davis, Macrae & Withers, 1960). Furthermore, it has been shown by Leech *et al.* (1960) that the incidence of primary ketosis is much higher in cows after the first lactation. This may have some bearing on the different excretion rates of coenzyme A observed in the milk of the cows of the 2 age groups studied.

The technical assistance of Miss Sara White is gratefully acknowledged.

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Nutritive properties of freshly prepared and stored evaporated milks manufactured by a normal commercial procedure or by reduced thermal processes in the presence of nisin

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Summary. From a batch of raw milk, evaporated milk was prepared commercially by 3 procedures: *A*, a normal commercial procedure involving holding the filled cans at a maximum temperature of 113 °C for 15 min; *B*, with nisin added and holding at a maximum temperature of 105 °C for 15 min; *C*, with nisin added and holding at a maximum temperature of 113 °C for 3 min. The content of *B* vitamins in the raw and evaporated milks was measured microbiologically; the nutritive value of the proteins was determined in rat tests.

In milk *A*, 83 % of the vitamin B_{12}, 38 % of the vitamin B_6 and 20 % of the thiamine were destroyed during processing. In milk *B*, the losses were 67, 30 and 19 %, respectively, only the loss of vitamin B_{12} being significantly lower than in milk *A*. In milk *C*, the losses were 67, 23 and 14 %, respectively, each of them being significantly lower than in milk *A*. There was no further loss of thiamine on storage of the milks for 12 months at 4 °C, but at room temperature and at 37 °C further losses occurred in all the milks. Similarly, the vitamin B_6 activity of the milks decreased on storage, the loss being greatest at 37 °C, but also detectable at 4 °C. No change in the vitamin B_{12} content occurred on storage, and no losses of biotin, nicotinic acid, pantothenic acid or riboflavin were detected during manufacture or storage of the evaporated milks.

The biological value and true digestibility of the proteins of the evaporated milks were slightly lower than for the raw milk. Neither nisin treatment nor storage at room temperature for a year affected these characteristics.

One of the properties of nisin, a metabolite produced by certain group-N lactic streptococci, is that of inhibiting clostridia and certain other food-spoilage bacteria. The facts that nisin occurs naturally in some foods (such as milk and cheese), is readily destroyed by digestive enzymes and is not used therapeutically, make it suitable for food preservation. The properties of nisin and its suitability as a food additive are discussed in a detailed review by Hawley (1958).

The Preservatives in Food Regulations (Great Britain, Parliament, 1962) now make it permissible for cheese, clotted cream and certain canned foods to contain nisin. One advantage of adding this antibiotic to foods to be preserved by canning is that

the severity of the heat treatment during sterilization can be reduced without increasing the risk of bacteriological spoilage (Campbell, Sniff & O'Brien, 1959). The normal heat sterilization processes used commercially in the manufacture of canned evaporated milk destroy some of the thiamine and a high proportion of the vitamin B_6 and vitamin B_{12} (see Kon, 1960). Thus it became of interest to determine whether this destruction would be reduced with the less severe heat treatment made possible by the use of nisin. We have, therefore, compared the nutritive properties of evaporated milks prepared in a full-scale plant by a normal commercial procedure and by 2 reduced thermal processes in the presence of nisin. Results are now reported for the B-vitamin content and for the biological value of the proteins of the raw milk and of the evaporated milks, freshly prepared and after storage at different temperatures for periods up to 12 months.

EXPERIMENTAL

Preparation and treatment of the evaporated milks

After removal of a 6-gallon portion that was kept chilled until delivered to Shinfield, a batch of raw milk was evaporated in the usual way on a commercial plant with disodium phosphate added as a stabilizer during the process. The evaporated milk was fortified with 0·56 mg ascorbic acid/g. One-third of the milk was measured into 6-oz cans and sterilized by the normal procedure of holding at 113 °C for 15 min (milk *A*). Nisin (Nisaplin, Aplin and Barrett Ltd, Yeovil, Somerset, 10^6 units/g) was added to the remainder at a level of 100 units/g of evaporated milk, half of which was sterilized (again in 6-oz cans) at a reduced temperature of 105 °C for the normal time of 15 min (milk *B*) and half at the normal temperature of 113 °C for a reduced time of 3 min (milk *C*). It should be emphasized that these conditions for the experimental milks *B* and *C* were chosen arbitrarily. The Preservatives in Food Regulations (Great Britain, Parliament, 1962) do not permit the addition of nisin under these conditions, since its use in canned goods is restricted to food that has been 'sufficiently heat processed to destroy any *Clostridium botulinum* in that food or container, or which has a pH of less than 4·5'.

The conditions of heating for the control (*A*) and experimental (*B* and *C*) milks may be expressed in terms of '*F*' values (Ball & Olson, 1957). In calculating '*F*' values it has been assumed that the temperature within the can was identical with that in the heating environment, an assumption close to the truth for evaporated milk. '*F*' values used in this experiment were: milk *A*, 3·0; milk *B*, 0·5 and milk *C*, 1·2.

The chilled raw milk and the cans of evaporated milk were delivered to Shinfield on the day after manufacture. To prevent spoilage, and to facilitate handling in the microbiological tests, the raw milk was freeze-dried within 2 days of arrival. Half of this dried milk was stored in screw-topped jars at 4 °C, and the remainder in polythene bags in an airtight tin at − 20 °C. Twenty-five cans of evaporated milk *A* were opened, the contents mixed well, freeze-dried and stored in screw-topped jars at 4 °C. Twenty-five cans each of milks *B* and *C* were treated similarly. All these samples were freeze-dried within a week of manufacture. The B-vitamin content and the biological value of the proteins of the milks so prepared were measured by the methods described below.

To study the effect of storage on the B-vitamin content, 16 cans each of evaporated milks *A*, *B* and *C* were kept at 4 °C, room temperature (about 20 °C) and 37 °C. After 6, 9 and 12 months, 4 cans from each treatment were opened and the contents mixed well and freeze-dried. The dried samples were stored in screw-topped jars at 4 °C and their B-vitamin content was measured within a few weeks. In each assay a sample of freeze-dried raw milk was included. At the end of the 12-month storage period, when all the vitamin assays had been completed, mean values for each vitamin in the raw milk were calculated. The percentage losses of the vitamins in the normal and nisin-treated evaporated milks were then calculated in terms of the mean values for the raw milk.

To study the effect of storage on the nutritive value of the proteins, 25 cans of each of the evaporated milks *A*, *B* and *C* were kept at room temperature for 12 months. The contents were then freeze-dried and stored in polythene bags in airtight tins at − 20 °C until rats were available for the tests. These stored milks were then compared with the original raw milk which had been stored as a freeze-dried powder at − 20 °C during this same period. Under these conditions of storage no decrease in the nutritive value of the proteins of the raw milk occurred.

In all, 96 cans of each of the evaporated milks were used in this experiment and in none of them was any bacterial spoilage observed. The nisin-treated evaporated milks were in our opinion paler in colour and had less cooked flavour than the control evaporated milk.

Assay methods

Vitamins

The contents of biotin, nicotinic acid, pantothenic acid, vitamin B_6 (as pyridoxal) and vitamin B_{12} in the milks were determined as described by Gregory, Ford & Kon (1958) but with 0·5 g freeze-dried milk in place of the liquid volumes specified. The method of assay for riboflavin was that described by Gregory *et al.* (1958) except that the extract was diluted to contain 0·05 μg riboflavin/ml and the medium of Roberts & Snell (1946) was used. Thiamine was measured with *Lactobacillus fermenti* as described by Banhidi (1958).

Proteins

The biological value and true digestibility of the proteins of the raw and evaporated milks were determined by the balance-sheet method of Mitchell (Mitchell, 1923–24; Mitchell & Carman, 1926), as modified in this laboratory (Henry, Kon & Watson, 1937; Henry, Kon, Lea & White, 1947–48; Henry & Kon, 1956). The basal 'protein-free' diet is described by Henry & Toothill (1962); a supplement of fat-soluble vitamins was given once a week. Quantities of the freeze-dried milks calculated to supply about 8% protein replaced an equal weight of rice starch in the basal diet. The milks were tested in 2 experiments: (1) raw milk and the 3 freshly prepared evaporated milks, (2) raw milk stored at − 20 °C and the 3 evaporated milks that had been stored for 12 months at room temperature.

RESULTS

Table 1 gives the concentrations of thiamine and of vitamin B_6 and B_{12} in the raw milk and in the freshly prepared evaporated milks together with the percentage losses, relative to the raw milk, due to processing. Some 20% of the thiamine was lost in milks *A* and *B*, but a significantly smaller loss of 14% ($P < 0.05$) was found for milk *C*. The losses of vitamin B_6 were 38% in milk *A*, 30% in milk *B* and 23% in milk *C*, but only in milk *C* was the loss significantly lower ($P < 0.05$) than in milk *A*. Losses were much more marked for vitamin B_{12}; some 83% of the vitamin was destroyed in milk *A*, whereas in both nisin-treated milks the loss, though still high, was significantly reduced to 67% ($P < 0.001$).

Table 1. *Losses of thiamine, vitamin* B_6 *and vitamin* B_{12} *during the manufacture from raw milk of evaporated milk prepared by a normal commercial procedure* (*A*) *or by reduced thermal processes in the presence of nisin* (*B*, *C*)

(Figures in parentheses are the numbers of assays.)

Type of milk	Thiamine		Vitamin B_6 (as pyridoxal)		Vitamin B_{12}	
	μg/g D.W.	% loss	μg/g D.W.	% loss	μg/g D.W.	% loss
Raw	3·42 (7)	—	2·61 (6)	—	0·0334 (7)	—
Evaporated						
A, Normal treatment; heated at 113 °C for 15 min	2·72 (7)	20	1·62 (6)	38	0·0058 (7)	83
B, With nisin; heated at 103 °C for 15 min	2·78 (7)	19 NS	1·84 (6)	30 NS	0·0112 (7)	67***
C, With nisin; heated at 113 °C for 3 min	2·93 (7)	14*	2·02 (6)	23*	0·0111 (7)	67***

NS Not significantly different from milk *A*. * Significantly different from milk *A* ($P < 0.05$). *** Significantly different from milk *A* ($P < 0.01$).

The effects on these vitamins of storing the cans of milk at 3 different temperatures for periods of up to 12 months are shown in Fig. 1. Losses of thiamine remained at about the same level of 10–30% when the cans were stored at 4 °C. At room temperature there was some evidence of increasing loss of thiamine with time. At 37 °C losses were greater and more rapid. No statistical analysis of these results was possible because there were not enough determinations. However, it can be seen that during storage the rate of loss was similar for the 3 milks.

In all 3 milks there was a progressive loss of vitamin B_6 during storage, and the loss increased markedly with increasing temperature. At all 3 storage temperatures the difference between the milks became less marked during the 12-month storage. No further loss of vitamin B_{12} occurred during storage irrespective of temperature.

The levels of biotin, nicotinic acid, pantothenic acid and riboflavin were, respectively, 0·36, 5·50, 25·0 and 12·7 μg/g of freeze-dried raw milk. There were no significant losses of these 4 vitamins either during the manufacture of the evaporated milk or during subsequent storage of the cans for 12 months at 4 °C, room temperature or 37 °C.

Table 2 gives the biological values and true digestibilities of the proteins of the evaporated milks, both freshly prepared and after storage for a year, and of the raw milk. The results for the biological values are not clear-cut; in the freshly prepared samples values for milks *A* and *C* were significantly lower than for the raw milk whereas in the stored samples the value for milk *B* was significantly lower than for the other

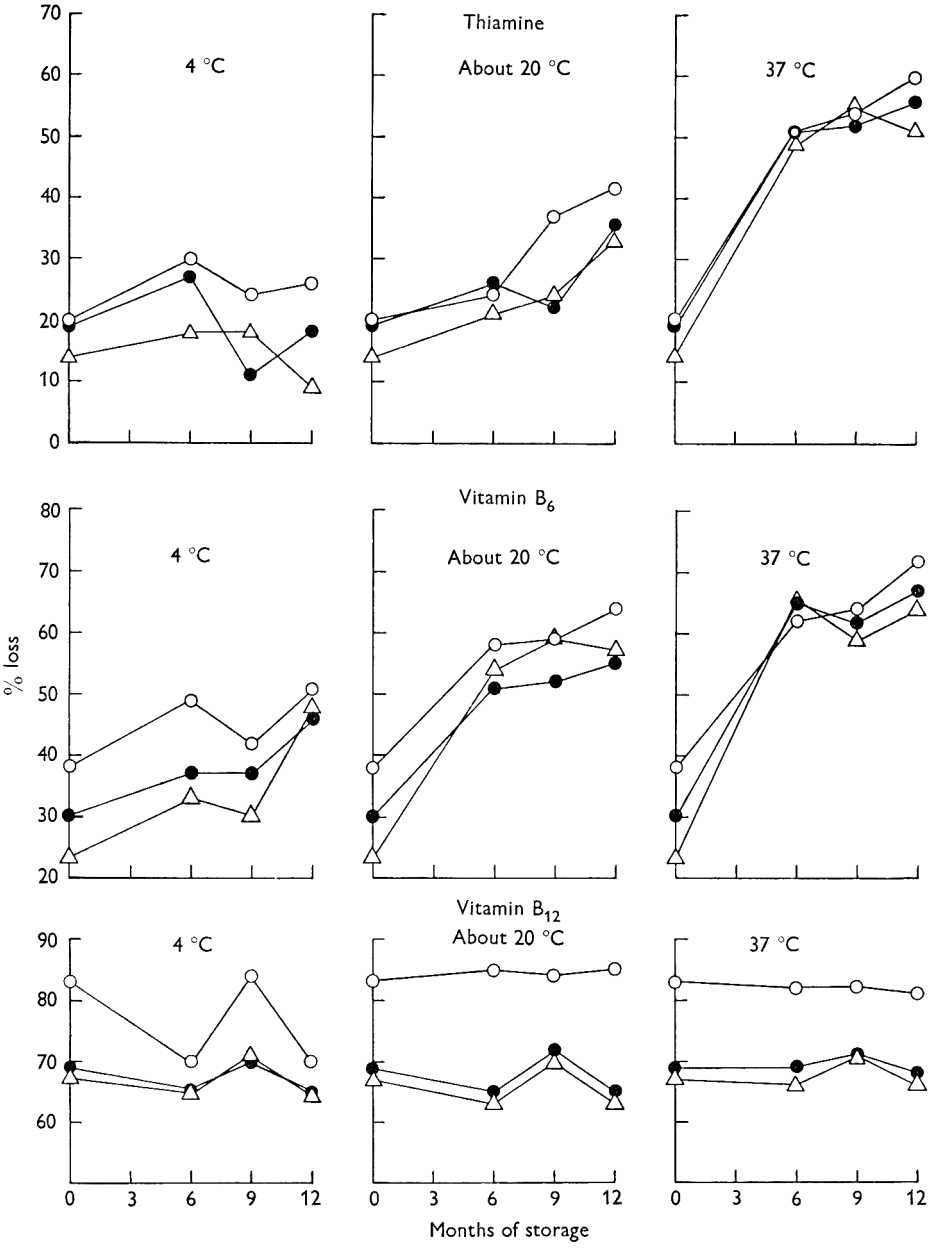

Fig. 1. Changes during storage at 4 °C, room temperature or 37 °C in the thiamine, vitamin B_6 and vitamin B_{12} contents of evaporated milk prepared by a normal commercial process or by 2 reduced thermal processes in the presence of nisin. ○—○, Normal treatment; ●—●, nisin added, heated at 105 °C for 15 min; △—△, nisin added, heated at 113 °C for 3 min.

3 milks. In both the fresh and stored evaporated milks the true digestibility of the proteins of milk *A* was significantly lower than that of raw milk; the addition of nisin (milks *B* and *C*) counteracted this loss. No direct statistical comparison is possible between the fresh and stored evaporated milk. However, all the differences were small.

Table 2. *Biological value and true digestibility of the proteins of raw milk and of evaporated milks prepared from it by a normal commercial procedure or by reduced thermal processes in the presence of nisin*

(Mean values for groups of 12 rats.)

Expt.	Milk tested	Biological value	True digestibility
1	Raw	91·6	96·2
	Evaporated: freshly prepared		
	A, Normal treatment, heated at 113 °C for 15 min	88·2	93·3
	B, With nisin, heated at 105 °C for 15 min	90·0	95·5
	C, With nisin, heated at 113 °C for 3 min	88·9	95·7
	S.E.M.	±0·58	±0·37
2	Raw: stored at −20 °C for 12 months	90·0	94·7
	Evaporated stored at room temperature for 12 months:		
	A, Normal treatment, heated at 113 °C for 15 min	89·5	91·1
	B, With nisin, heated at 105 °C for 15 min	86·3	93·2
	C, With nisin, heated at 113 °C for 3 min	89·7	93·9
	S.E.M.	±0·68	±0·62

DISCUSSION

The finding that 83 % of the vitamin B_{12}, 38 % of the vitamin B_6 and 20 % of the thiamine is lost during the manufacture of evaporated milk, by the standard commercial heat-treatment, confirms earlier results from this laboratory (Chapman *et al.* 1957; Gregory, 1959; Davies, Gregory & Henry, 1959) and others (see McGillivray & Gregory, 1962). The results of the study presented here show that a reduced heat treatment in the presence of nisin caused a smaller initial loss of these vitamins. Heating at the normal temperature (113 °C) for a shorter time (3 min) was less harmful than the treatment with a reduced temperature (105 °C) for the normal time (15 min).

Our observation that further losses of vitamins B_1 and B_6 occurred during storage of the evaporated milks, particularly at the higher temperature of 37 °C, confirms our earlier findings with evaporated milk (Davies *et al.* 1959) and that of Mijll Dekker & Engel (1952) and of Causeret, Hugot, Goulas-Scholler & Mocquot (1961) who studied in-bottle sterilized milk. These losses on storage were not prevented by nisin.

Manufacture of evaporated milk by a normal procedure (milk *A*) caused a small loss in both the biological value and true digestibility of the milk proteins. These losses were similar to those observed in milk subjected to in-bottle sterilization (Henry & Kon, 1938; Henry & Porter, 1959; Hugot & Causeret, 1962) and somewhat greater than those previously found by us for evaporated milk (Henry, Houston,

Kon & Thompson, 1944). Nisin treatment prevented the loss in true digestibility of the proteins of evaporated milk in both the freshly prepared and the stored samples, but no consistent effect on the biological value was observed. Although no direct comparison is possible between the fresh and stored samples it is clear that storage of evaporated milk for a year at room temperature has no appreciable effect on the nutritive value of its proteins, an observation in agreement with that of Hugot & Causeret (1962) for sterilized milk stored for 6 months in the dark at 20 °C.

Therefore, any small beneficial effect of the procedures involving the use of nisin on the nutritive properties of evaporated milk are too slight to be of significance with a normal mixed human diet. However, the improved appearance and palatability of the nisin-treated milk may render it more attractive to the consumer than evaporated milk prepared in the conventional way.

We are indebted to United Dairies Ltd for help in planning this experiment and in particular for preparing the evaporated milks. We acknowledge the assistance of Miss H. Furneaux, Miss M. V. Chapman and Miss E. Mason, and express our thanks to Dr S. Y. Thompson for freeze-drying the milks and to Mr J. H. Prentice for help with some of the statistical analyses.

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With 2 plates

An electron microscope study of the internal structure of casein micelles

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Summary. A study of casein micelles was made with the electron microscope, using very thin sections cut from micelles embedded in Araldite. The micelles appear to be built up of units that are approximately spherical, about 100 Å in diameter and of about 300000 molecular weight.

The technique of electron microscopy has been used by a number of workers to study the shape and size distribution of the casein micelles in milk. Nitschmann (1949) investigated the size and shape of micelles in skim-milk and Hostettler & Imhof (1951) made a similar study which also reported on the physical changes caused by rennet treatment. The size distributions of the micelles in cow, goat and human milk were studied by Knoop & Wortmann (1960) and Adachi (1963) studied the effects of different alkaline earth cations on the size of the micelles in caseinate solutions. Knoop & Wortmann (1960) prepared their samples for observation by embedding the fixed micelles in methacrylate and cutting sections; the other three workers used the techniques of shadowing with evaporated carbon and metal films. From the foregoing studies it can be concluded that the shape of the micelles is spherical and that the concentration and type of alkaline earth cation markedly affect the size distribution of the micelles. However, since the discovery of κ-casein and its role in stabilizing the other caseins in milk (Waugh & von Hippel, 1956) it has become of interest to study the physical organization of the protein particles within the micelle. If electron microscopy is to be of use for this purpose the thickness of the sections used must be comparable to the diameter of the particles making up the micelles. From what is known regarding the combining proportions of α and κ-caseins (Waugh & von Hippel, 1956) and the molecular weight of κ-casein (Beeby, 1963) it was estimated that sections about 100 Å thick would be required. The problems involved in preparing and examining such thin sections have been overcome and in this paper we present, it is believed for the first time, micrographs showing the internal structure of the casein micelle.

Materials and methods

The milk was fresh raw milk from which fat had been removed as described by Hill (1963). In order to fix the micelles a beaker containing 50 ml skim-milk was placed in a closed container in an oven at 50 °C, along with a crystal of osmium

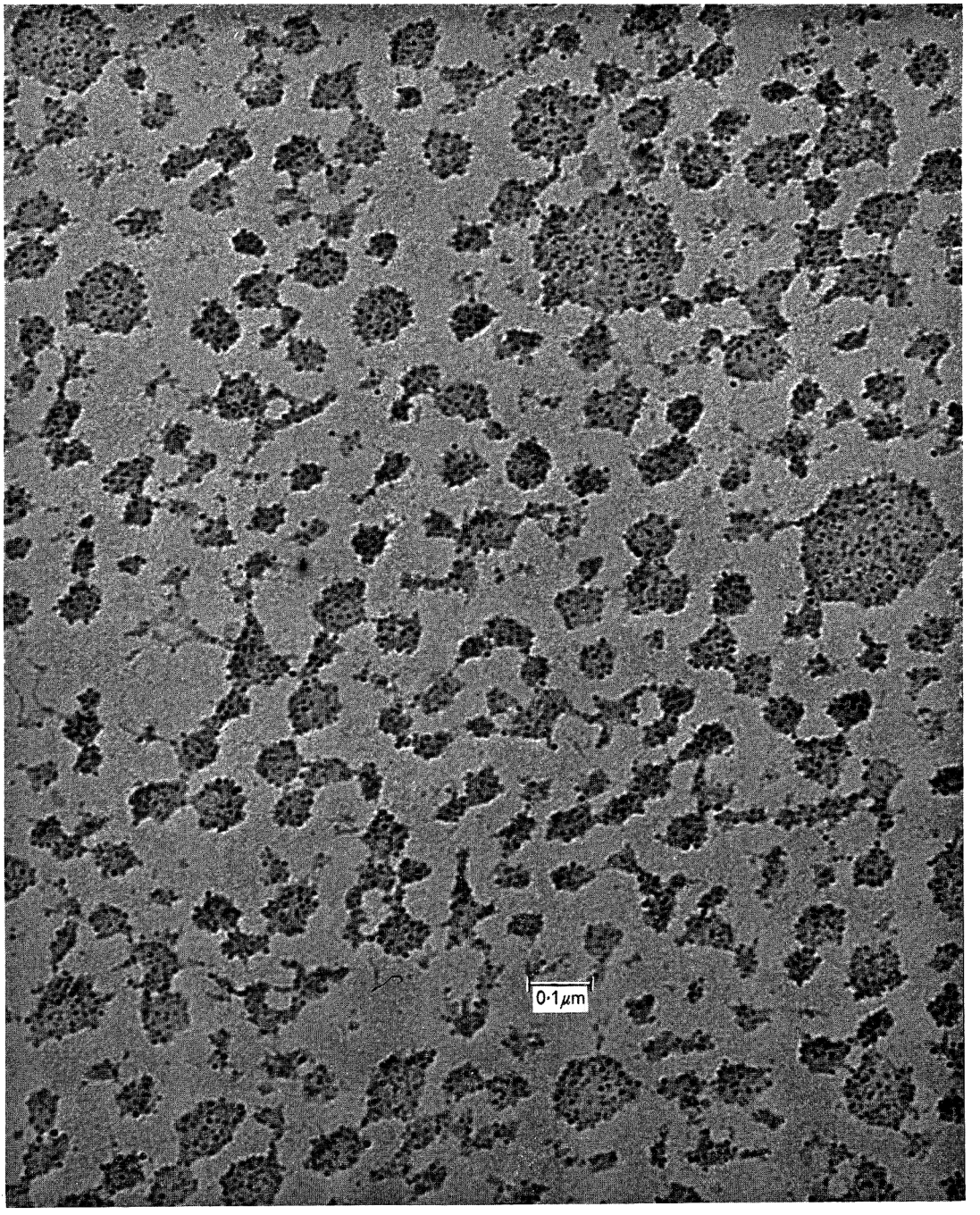
tetroxide which was allowed to evaporate into the milk for 23 h. The sample was then diluted to 180 ml with distilled water and the fixed casein micelles were spun down in the Spinco Model L ultracentrifuge at 60000 *g*/for 1 h. The sedimented micelles were redispersed in distilled water, and progressively dehydrated by spinning down in successive solutions of 25, 50 and 100% ethanol. The aggregates were then suspended in a little propylene oxide and embedded in an Araldite mixture of 50% hardener No. 964 and 50% casting resin M containing 4% accelerator (C.I.B.A. Ltd). The castings were hardened by holding them at 48 °C for 72 h.

Sections were cut with a diamond knife on an LKB ultrotome Type 480 TA; for examination in the electron microscope they were suspended over holey formvar films in a manner similar to that described by Harris (1962). The electron microscope was an Akashi Tronscope Type 50 E 1. A preliminary voltage stabilizer was used and the electron beam was collimated with apertures of 50 μ diameter.

RESULTS AND DISCUSSION

Plates 1 and 2 show micrographs of sections of micelles at a total magnification of 100000. The particles of which the micelles are composed can clearly be seen to be approximately spherical in shape, and they are reasonably uniform in size. The section illustrated in Plate 1 does not appear to be much greater than 100 Å in thickness, since only one layer of the particles is visible. In a section of this thickness some of the embedded particles will be complete, while others will have been cut away to different extents. This would leave spherical caps of different sizes and electron densities embedded in the section, and this effect rather than variations in extent of staining is thought to be the reason for the variations in apparent electron density of the particles shown in Plate 1. The densest particles are about 100 Å in diameter. The section shown in Plate 2 appears to be rather thicker so that the proportion of complete particles is greater and the density of staining appears to be uniform; on the other hand, the resolution between particles is reduced. If the thickness of the section were much greater, it is likely that detail would be completely lost. The apparent elliptical shape of the micelles of Plate 2 was caused by mechanical distortion of the section during preparation. As can be appreciated, to cut, mount and keep intact in the beam sections as thin as 100 Å presents considerable difficulty. The distribution of micelle diameters is similar to that reported by the workers quoted earlier, the range being from 400–3000 Å with an average size of about 800 Å.

Waugh (1958) has suggested that α_s-, β- and κ-casein molecules are rod-like in shape with lengths of 150–215 Å and diameters of about 16 Å. These dimensions were calculated from molecular weights and frictional ratios, the molecular weight of κ-casein being taken as 16000. Waugh & von Hippel (1956) had shown that κ-casein could interact with 4 times its own weight of α_s-casein and had suggested that the probable configuration of the α-κ-casein complex was that the rodlike α_s molecules were aligned parallel to a central κ-casein molecule. The part of the κ-casein molecule acted on by rennin was presumed to project beyond the surrounding α_s molecules. These suggestions would imply a molecular weight of about 80000 for the α_s-κ complex and a minimum length in excess of 200 Å.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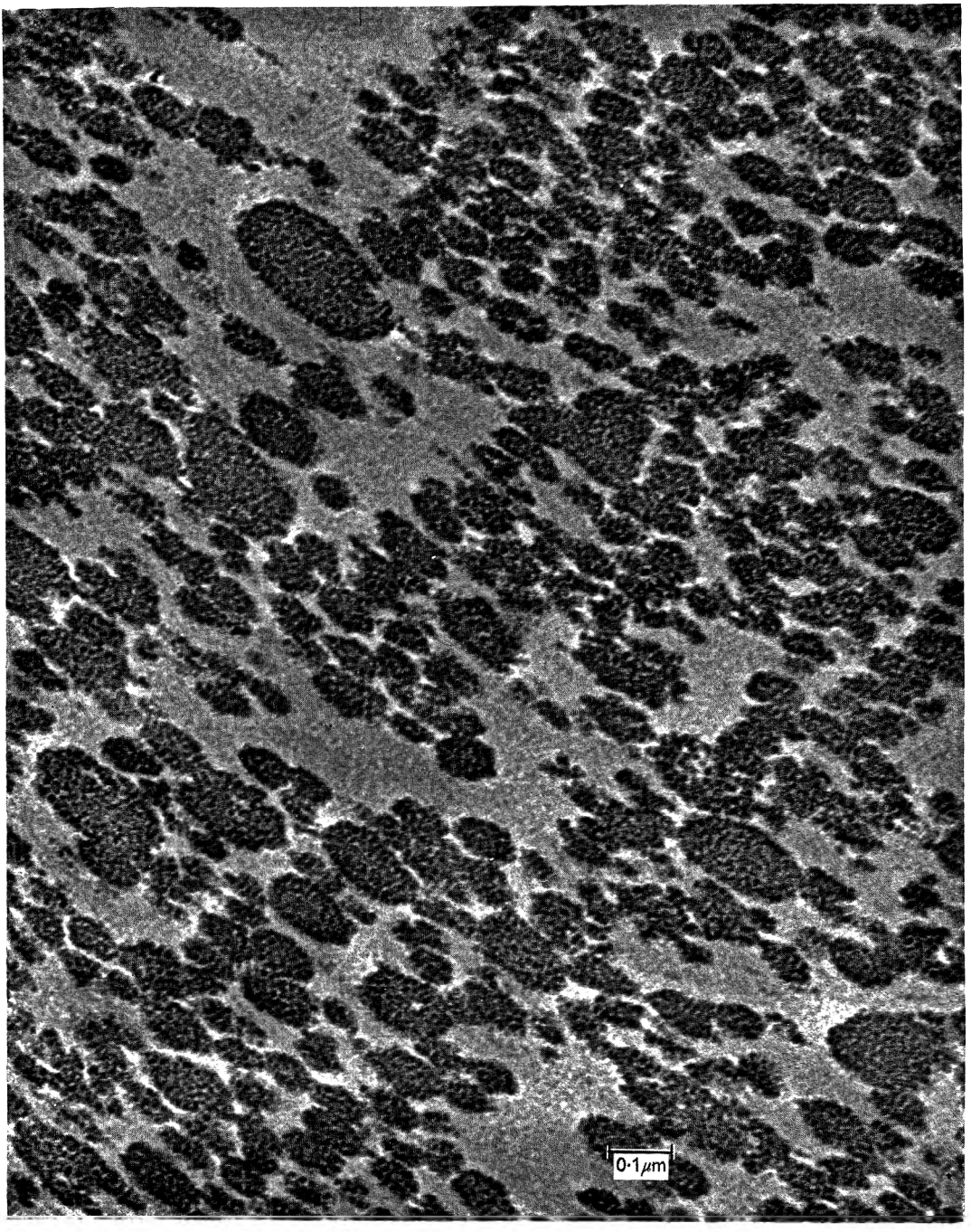

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On the other hand, Beeby (1963) has suggested that κ-casein is a complex of 3 different proteins and that it has a total-molecular weight of about 50000. The α_s-κ-casein complex would, on this basis, have a molecular weight of about 250000. Since the molecular weight of α_s-casein is probably in the range 24000–30000 (Waugh, 1958; Manson, 1961) this would mean that 6–8 molecules of α_s-casein would be associated with each κ-casein molecule, and in milk there would also be several loosely associated β-casein molecules. The total molecular weight of the α_s-κ+β-casein complex would therefore be about 300000. In view of the number of molecules in the complex and in view of the many different types and sites of interaction between the casein molecules, the most probable shape of the complex would be spherical, and a spherical protein molecule of this weight would have a diameter of about 90 Å (Birbeck, 1961). It is not necessary to have a part of the κ-casein projecting in order to allow reaction with the rennin, all that is needed is the folding of the molecules so that the affected part of the κ-casein lies on the surface of the complex.

The concept of an approximately spherical α-κ+β-casein complex of about 300000 molecular weight as the building unit for the micelles is reasonably in accord with the experimental evidence. The evidence does not support the suggestion that the complex is rod-like, with a length of over 200 Å. In fact, among the many samples examined there was no evidence for a rod-shaped particle at all. Although the resolution of the electron microscope used is not better than 15–20 Å, and there is, therefore, some uncertainty as to the actual size of the particles, the agreement between the calculated and observed sizes is quite good. The suggestion that the micelle is built up from units which are roughly spherical in shape and about 100 Å in diameter can therefore be made with some confidence.

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EXPLANATION OF PLATES

Plate 1

Electron micrograph of thin sections of casein micelles embedded in Araldite. Magnification ×100000.

Plate 2

Electron micrograph of thin sections of casein micelles embedded in Araldite. Magnification ×100000.

Printed in Great Britain

A note on the serological typing of some strains of *Streptococcus cremoris*

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The serological identity of group N streptococci was established by Shattock & Mattick (1943), who discovered that *Streptococcus lactis* and *Str. cremoris* had a common antigen, which differentiated them from the rest of the Lancefield groups of streptococci. Although phage typing of these organisms has met with some success (Nichols & Hoyle, 1949; Whitehead & Bush, 1957) attempts to type these organisms with rabbit antisera so far have failed (Briggs & Newland, 1953).

Cow's milk is known to contain 'natural' antibodies against many bacteria including lactic streptococci (McPhillips, 1958). Although the titre of these antibodies is low (maximum 1:16 in normal milk), Portmann & Auclair (1959) attempted to group 15 strains of lactic acid streptococci into 5 groups by agglutination tests in raw milk. The possibility of typing these organisms with immune whey was therefore investigated.

Organisms. 19 strains of *Str. cremoris* were obtained from Dr H. R. Whitehead, Palmerston North, New Zealand (HP, OP_4, TEM_2, RW, YP_2, R_6, K, UD_5, FH, AM_1, HS_2, D_9, TR, D_4, ML_2, R_1, ML_1, KH, E_8) and maintained by frequent subculturing in skim-milk at 22 °C.

Specific antibodies in cow's blood and in colostrum were prepared by infusing the udders of healthy, dry cows in calf at 4-weekly intervals with a suspension of organisms in physiological saline-O.D. = 0·4, 1 cm light path (Kerr, Peerson & Rankin, 1959). After parturition, blood samples were taken and the colostrum collected. Clear whey was obtained by the method of Hall & Learmouth (1933) and, when containing specific antibodies to the strain infused, is referred to as 'immune whey'.

Agglutination test. Bacterial suspensions were prepared from cultures grown in 0·2% glucose Lemco broth for 6 h at 30 °C. The washed and killed suspensions (0·25% formaldehyde) were inoculated into tubes containing immune whey or blood antisera diluted in powers of 2 in saline. Agglutination tests were read after 2 h at 55 °C and again after overnight incubation at 37 °C.

Agglutination absorption was performed with unformalized suspensions and absorptions were allowed to proceed for 2 h at 42 °C, one absorption being usually adequate. Colebrook's (1935) 'five tube' titration was followed.

Although the immunization was done by infusion of the udder, the blood titre proved to be higher than the whey titre in the case of HP (Table 1). Immune whey containing HP antibodies was absorbed with each strain and then tested for agglutination against all other strains.

Table 2 shows that the strains can be divided into 4 groups on the basis of their absorption patterns. Group I strains absorbed all the antibodies from the immune whey; group II strains absorbed all the antibodies except those reacting with group I strains; group III strains removed all the antibodies except those reacting with groups I and II strains, and group IV strains failed to absorb antibodies reacting with groups I, II and III, absorbing only those against their own group IV. This suggests a multiplicity of antigenic components, group I having an antigenic pattern *ABCD* (4 antigens), group II strains having 3 antigens BCD, group III strains having 2 antigens *CD* and group IV having only 1 antigen *D*.

Table 1. *Agglutination titres of unabsorbed immune wheys (and blood antiserum) against* Str. cremoris

	Agglutination titre* of immune wheys				
Strain	HP	R_6	K	E_8	TR
HP	6 (8)	4	7	4	7
OP_1	5 (8)	6	7	4	7
TEM_2	5 (7)	3	7	4	7
RW	4 (7)	7	7	4	7
YP_2	5 (7)	5	7	4	7
R_6	5 (4)	5	7	4	7
K	1 (2)	4	7	2	6
FH	5 (6)	4	7	4	7
UD_5	1 (0)	0	0	0	1
AM_1	4 (6)	3	7	4	7
HS_2	4 (6)	3	7	4	7
E_3	2 (6)	5	6	4	6
D_9	5 (6)	6	7	4	7
TR	3 (6)	5	7	4	9
D_4	5 (6)	4	7	4	9
ML_2	2 (5)	6	6	4	6
R_1	2 (5)	6	6	4	6
ML_1	2 (4)	4	6	4	6
KH	2 (5)	0	0	2	6

* Titre 1 ≡ 2^5, titre 2 ≡ 2^6, etc.; figures in parentheses refer to blood serum.

The weak cross reactions observed between the whey absorbed with group I strains (including the homologous strain HP) and the group IV strains, strongly suggest that these reactions were due to naturally occurring antibodies present in the colostrum. The other cross agglutinations observed may well be due to the same cause.

The blood serum of the cow, taken at the time of the collection of the colostrum, gave identical results.

Absorption tests (Table 3) with immune whey TR using only representative strains of each group show that strain TR possesses a type antigen of its own which is absorbed only by the homologous strain. In addition, the antigenic patterns suggested in Table 2 are confirmed, except for an anomalous weak reaction between ML_2 and the immune whey absorbed with group II.

After absorption with group I or group II strains, both containing antigens *C* and *D* which are also contained by the group III strain TR, only the homologous strain TR was agglutinated. However, after absorption of the whey with group IV strain, which contains only antigen *D* and can only absorb antibodies against this group,

Table 2. *Agglutination reactions of HP immune whey after absorption by various strains of* Str. cremoris

Strain agglutinated	Agglutination titre															
	Group I			Group II			Group III						Group IV			
	HP	OP_4	TEM	RW	YP_2	R_6	FH	AM_1	HS_2	D_9	TR	D_4	ML_2	R_1	ML_1	KH	E_8
HP	−	−	−	++	++	++	++	++	++	++	++	++	++	++	++	++	++
OP_4	−	−	−	++	++	++	++	++	++	++	++	++	++	++	++	++	++
TEM	−	−	−	++	++	++	++	++	++	++	++	++	++	++	++	++	++
RW	−	−	−	−	−	−	++	++	++	++	++	++	++	++	++	++	++
YP_2	−	−	−	−	−	−	+	++	+	++	++	++	++	++	++	++	++
R_6	−	−	−	−	−	−	++	++	++	++	++	++	++	++	++	++	++
FH	−	−	−	+	+	−	−	−	−	−	−	−	++	++	++	++	++
AM_1	−	−	−	+	+	−	−	−	−	−	−	−	++	++	++	++	++
HS_2	−	−	−	−	−	−	−	−	−	−	−	−	++	++	++	++	++
D_9	−	−	−	+	+	−	−	−	−	−	−	−	++	++	++	++	++
TR	−	−	−	−	−	−	−	−	−	−	−	−	+	++	++	++	+
D_4	−	−	−	−	+	−	−	−	−	−	−	−	++	++	++	++	+
ML_2	+	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−
R_1	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
ML_1	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
KH	+	+	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−
E_8	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

++, Full titre or 1 tube less; +, 2 tubes less (up to 75 % decrease of agglutination according to Colebrook, 1935); −, 5 tubes less (75–96·8 % decre of agglutination) or negative at 1:32 dilution.

strains of groups I–II were still agglutinated because antibodies to antigen *C* remained.

Absorption tests with immune wheys of representative strains of groups II and IV were designed to confirm the grouping of anomalous strains but proved to be unsuccessful. It appears that 'natural' or 'non-specific' antibodies were present at concentrations which completely obscured the results.

Table 3. *Agglutination reactions of TR immune whey*

Strains agglutinated	Agglutination after absorption with strains			
	I HP	II R_6	III TR	IV ML_2
HP	–	–	–	+
R_6	–	–	–	+
TR	++	++	–	++
ML_2	–	+	–	–

++, Strong agglutination; +, weak agglutination; –, no agglutination.

Table 4. *Action of phage races on strains of* Str. cremoris*

Bacterial strain	Phage races																	
	hp	op_4	tem_2	rw	yp_7	r_6	k	fh	ud_5	am_1	hs_2	e_8	d_9	tr	d_4	ml_2	r_1	ml_1	kh
HP	5	4	4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
OP_4	3	5	3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TEM_2	4	A	5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
RW	–	–	–	5	5	5	4	–	–	–	–	–	–	–	–	–	–	–	–
YP_7	–	–	–	5	5	5	5	–	–	–	–	–	–	–	–	–	–	–	–
R_6	–	–	–	–	4	5	–	–	–	–	–	–	–	–	–	–	–	–	–
K	–	–	–	–	–	–	5	–	–	–	–	–	–	–	–	–	–	–	–
FH	–	–	–	–	–	–	–	5	4	3	3	–	4	A	–	–	–	–	–
UD_5	–	–	–	–	–	–	–	3	5	2	3	–	5	3	–	–	–	–	–
AM_1	–	–	–	–	–	–	–	–	–	5	4	5	3	2	–	–	–	–	–
HS_2	–	–	–	–	–	–	–	A	–	3	5	3	3	A	–	–	–	–	–
E_8	–	–	–	–	–	–	–	A	–	5	5	5	2	–	–	–	–	–	–
D_9	–	–	–	–	–	–	–	–	–	–	–	–	5	5	4	–	–	–	–
TR	–	–	–	–	–	–	–	–	–	–	–	–	3	5	4	–	–	–	–
D_4	–	–	–	–	–	–	–	–	–	–	–	–	4	5	5	–	–	–	–
ML_2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	4	5	–	–	–
R_1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	5	–	–
ML_1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	5	–
KH	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	5

5, max. titre; 4, 10^{-1}–10^{-2}; 3, 10^{-3}–10^{-4}; 2, 10^{-5} or more of max. titre; A, adaptation.

* Compiled from Whitehead & Bush (1957). Results based on inhibition in liquid and solid media have been removed from the original table because it is now known that such inhibition is due to phage lysis (Reiter & Oram, 1963).

The grouping based on agglutination with immune whey (Table 2) is in close agreement with that based on the phage relationships of the same organisms (Table 4) reported by Whitehead & Bush (1957); a different grouping was obtained only in the case of strain E_8 but no agglutination grouping of strains K and UD_5 was possible because of their weak or negative reaction with unabsorbed whey (Table 1).

Further work is clearly justified to investigate the agreement noted between the 2 methods and to investigate the purified antigens, which have been identified as polysaccharides (Oram & Reiter, unpublished).

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With 1 plate

A new method for the preparation of an immunologically homogeneous β-casein

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Summary. The preparation of an immunologically homogeneous β-casein is described, involving several separations by chromatography on diethylaminoethyl cellulose with urea-imidazole buffer at pH 7, followed by Tris buffer at pH 8·2. 30 % of the β-casein present in the skim-milk is obtained by this method. β-casein gives a single band in urea-starch gel electrophoresis when it is obtained from the milk of cows homozygous for this character.

INTRODUCTION

β-casein is one of the major proteins of milk since it represents 25 % of the total milk proteins or about 30 % of the whole casein (Ribadeau-Dumas, Maubois, Mocquot & Garnier, 1963). It was one of the first caseins to be prepared in a reasonable state of purity by Warner (1944) and by Hipp, Groves, Custer & McMeekin (1952), although this latter preparation was shown by Garnier, Ribadeau-Dumas & Gautreau (1962) to contain at least 2 minor contaminants. Recently 2 other methods were proposed: Groves, McMeekin, Hipp & Gordon (1962) prepared β-casein with a very low yield (0·5–1 % of total β-casein in milk) which still presented slight impurities; Aschaffenburg (1963) proposed a method of preparation capable of giving a good yield (30 %) of β-casein, but free only of major impurities such as α_s- and κ-caseins.

A technique is described for the preparation of β-casein which also gives a good yield. The β-casein obtained is free from any contaminants which may be detected by such sensitive methods as starch-gel-urea electrophoresis or immunoelectrophoresis.

Aschaffenburg (1961) has recently observed genetic variants of β-casein. Using the technique presented here, pure genetic variants of β-casein may be prepared providing that the starting material is obtained from cows which are homozygous for β-casein. The chemical differences observed between β-casein variants will be published later.

EXPERIMENTAL

Principle

A homogeneous material is obtained, amounting to 30 % of the β-casein contained in whole casein, by chromatographic separations on DEAE cellulose in urea-imidazole buffer and then in Tris buffer.

Acid casein precipitation

Whole casein is prepared from the skim-milk of individual Friesian cows previously typed for their genetic β-casein variants. Casein is first precipitated from milk at pH 4·5 and then twice at pH 4·7. After each precipitation the casein is carefully washed 3 times with distilled water and dissolved at pH 7 by slowly adding N-NaOH. The solution of whole casein at pH 7 is freeze-dried.

Chromatography in urea-imidazole buffer

Essentially the same technique as the one previously described by Ribadeau-Dumas (1961) and Ribadeau-Dumas *et al.* (1963) is used except that a linear salt gradient is employed.

Whatman DEAE cellulose powder DE 50 is sieved to select particle sizes between 250 and 120 mesh. A column 3 × 16–18 cm is prepared and equilibrated with buffer I (0·01 M imidazole-4·5 M urea-pH 7). Two grams of freeze-dried whole casein are dissolved in 35–40 ml of buffer I and applied to the column. The column is eluted first with buffer I and then with a linear salt gradient up to 0·6 M-NaCl at the rate of 80 ml/h.

The linear salt gradient is obtained by putting 2 l of buffer II (0·02 M imidazole-3·3 M urea-pH 7) into the mixing vessel and 2 l of buffer II containing 0·6 M-NaCl into the other vessel.

10-ml fractions are collected and the crude β-casein, which corresponds to the first major peak, is recovered (see Fig. 1) and dialysed against 10 l of 0·05 M-NaCl solution at 4 °C for about 20 h.

Finally, the column is washed overnight at 80 ml/h with 0·25 N-NaOH, regenerated with 0·1 M piperazine-HCl (pH 4·4)–20 % NaCl for 24 h and re-equilibrated with buffer I till the pH rises to 7. This step requires about 48 h.

The 4·5 and 3·3 M urea solutions are treated before use with activated charcoal to remove material absorbing at 280 mμ. Activated charcoal (1 g/l) is added to the urea solutions, and after stirring for 30 min the solutions are filtered, first on paper, then on a 3 × 10 cm column of DEAE cellulose. They are then made up to 0·01 or 0·02 M imidazole, and the pH is adjusted to 7 with concentrated HCl.

Chromatography in Tris buffer

After dialysis the crude β-casein is precipitated at pH 4·7 with 0·1 N-HCl at room temperature, washed once with distilled water, dissolved in 25 ml of Tris buffer I (0·01 M tris (hydroxymethyl) aminomethane adjusted to pH 8·2 with concentrated HCl). During dissolution the pH is maintained at pH 8·2 with 0·25 N-NaOH. The solution is then applied to a 3 × 15 cm Whatman DEAE cellulose column, similar to the one described above, previously equilibrated with Tris buffer I. The column is eluted first with Tris buffer I then with an exponential salt gradient at the standard rate of 80 ml/h.

The exponential salt gradient is obtained by adding a solution of Tris buffer II (0·02 M Tris, pH 8·2) with 0·6 M-NaCl dropwise to the mixing chamber containing 2 l of Tris buffer II. Finally the column is washed with 0·25 N-NaOH and then with 0·1 M piperazine-HCl (pH 4·4)–20 % NaCl, as for chromatography in urea, and re-equilibrated with Tris buffer I.

10-ml fractions are collected and the purified β-casein is recovered in the salt eluate as shown in Fig. 2 while the tailing is carefully discarded. The β-casein is precipitated at pH 4·5 with 0·1 N-HCl at room temperature, washed once with distilled water, dissolved in 25-ml Tris buffer I and rechromatographed a second time in Tris buffer as above. The final β-casein is precipitated at pH 4·5 with 0·1 N-HCl, washed 3 times with distilled water, and freeze dried or alternatively dried with absolute ethanol (3 times) and ether (3 times).

Ribadeau-Dumas *et al.* (1963) found that whole casein contained about 30 % β-casein. The yield of β-casein by the method described here varies between 80 and 115 mg/g of whole casein, i.e. approximately 30 % of the theoretical yield.

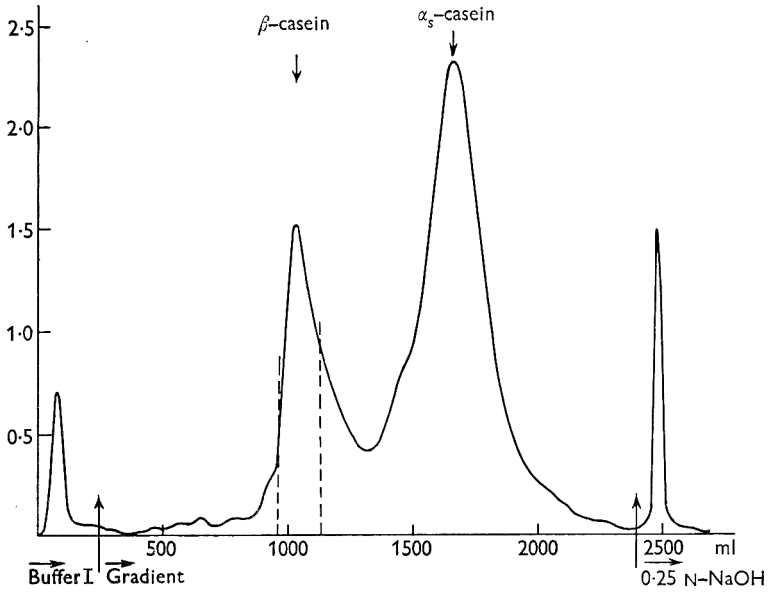

Fig. 1. Chromatography in urea-imidazole buffer at pH 7 of whole casein from the milk of 1 cow (β-caseins A and B) on DEAE cellulose.

Analytical methods

Immuno-electrophoresis was carried out according to Grabar & Williams (1955) under the conditions described by Garnier *et al.* (1962) in veronal buffer $\mu = 0{\cdot}025$, pH 8·2 and using immune rabbit serum prepared against whole casein.

Starch-gel electrophoresis was run at pH 8·6 in 7 M urea by the method of Wake & Baldwin (1961), and at pH 3·8 in 5 M urea by the method of Groves *et al.* (1962). The gels were prepared with 'Starch-Hydrolysed', obtained from the Connaught Medical Research Laboratories, Toronto, Canada.

Ultracentrifugation was performed on a mixture of β-caseins A and B in veronal buffer-NaCl ($\mu = 0{\cdot}1$) at pH 7·78 according to Sullivan *et al.* (1955) at 3 °C in a Spinco model E apparatus. The concentration of β-casein was 0·95 % (w/v).

Phosphorus determinations were made using the method of Bamann, Novotny & Rohr (1948). Nitrogen was determined by a micro-Kjeldahl method (Ogg, 1960).

Sialic acid was determined by the thiobarbituric acid method of Warren (1959). The reference sample was prepared from whole casein according to Ribadeau-Dumas & Alais (1961).

Dry weight determinations were made by drying under vacuum with P_2O_5 at room temperature for 24 h.

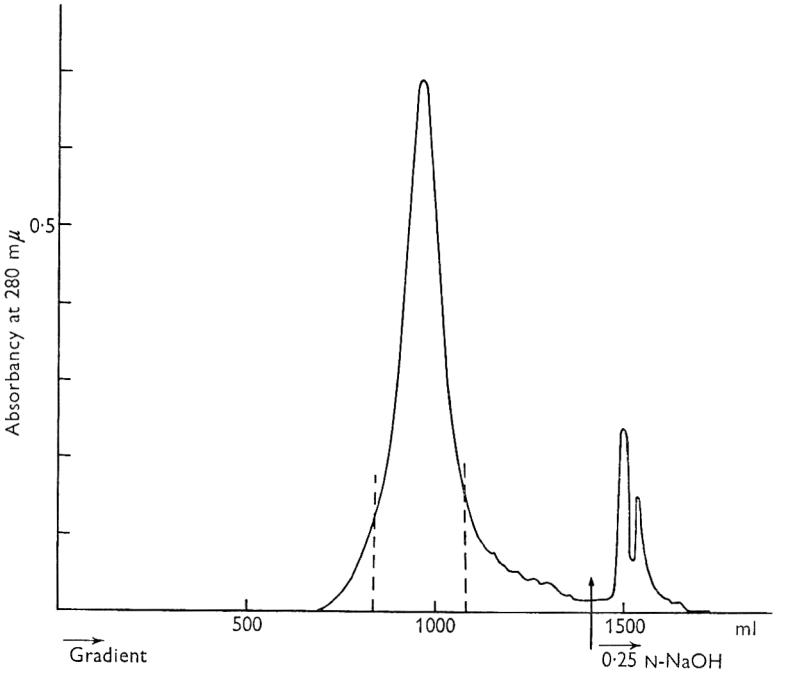

Fig. 2. First chromatography of crude β-casein in Tris buffer at pH 8·2 on DEAE cellulose.

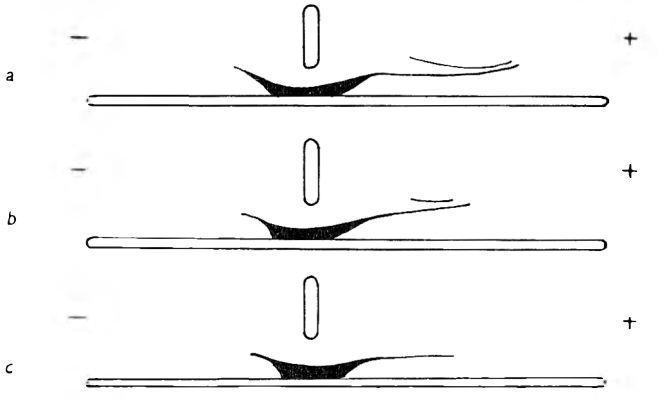

Fig. 3. Immunoelectrophoretic diagrams of: (*a*) crude β-casein after urea-imidazole chromatography; (*b*) β-casein after the first chromatography in Tris buffer at pH 8·2; (*c*) final β-casein preparation after rechromatography in Tris buffer at pH 8·2. Concentration of β-caseins, 0·5 % (w/v).

RESULTS AND DISCUSSION

Chromatography in Tris buffer (pH 8·2) was carried out to remove the fast moving immunoelectrophoretic component which contaminates crude β-casein preparations (Fig. 3*a*). This impurity is an α_s-casein fraction other than α_s-1, 2.

Usually a second chromatography in the same conditions is needed to obtain an immunologically homogeneous β-casein (Fig. 3*c*). It is unlikely that the 'tail' prolonging the main arc of precipitation is due to an impurity since there is a complete immunological cross-reaction with β-casein and only a single band is observed on starch gel electrophoresis (Plate 1*c*).

The homogeneity of the β-casein was also checked by the double diffusion technique (Kaminsky, 1954), with various β-casein concentrations from 0·06 to 2 %.

The purity of the β-casein obtained depends on the quality of the DEAE cellulose used. For instance, with some commercially available samples of DEAE cellulose we failed to get pure β-casein, although using the chromatographic schedule described above. A good test for the suitability of this ion exchanger is its ability to demonstrate the presence of minor constituents appearing between the β-casein peak and the α_s-casein peak during chromatography of whole casein in urea (Fig. 1). All attempts to increase the yield of homogeneous material above 30 % have failed. To ensure the purity of the final preparation it is necessary to collect only those fractions which strictly correspond to the β-casein peak (see dotted lines in Figs. 1 and 2).

There is a small but definite difference in mobility at pH 8·2 in veronal buffer between the A, B and C genetic variants of β-casein. However, the resolving power of chromatography in Tris buffer is still not sufficient to separate the genetic variants although the asymmetry of the peak suggests a slight degree of separation. Consequently, the genetic variants can at present only be obtained by chromatography of the caseins of individual samples of milk from cows homozygous for β-casein.

Starch gel electrophoresis diagrams of samples of β-caseins obtained from individual samples of milk at 2 pH values and at various concentrations are presented in Plate 1. One of the preparations (Plate 1*a*, *b*) comes from a cow which was heterozygous for β-casein, and gives 2 bands corresponding to β-casein A and β-casein B. The other preparation (Plate 1*c*) comes from a cow which was homozygous, and gives a single band. Both preparations are free from contaminants even at a concentration as high as 2 %.

Preliminary experiments performed on β-casein A, B and C showed a complete cross reaction between them by the techniques of double diffusion in agar and immunolectrophoresis. The same result was found by Gough & Jenness (1962) for β-lactoglobulins A and B.

A small amount of sialic acid was found in β-casein A or AB, i.e. 0·018–0·03 %. This could be explained by a trace of κ-casein tightly bound to β-casein, and not detectable by our techniques, or by a possible interference of the protein itself in the determination of sialic acid since an excess of β-casein had to be used to allow the small amount of sialic acid to be measured. Such a small amount has also been found in purified α_s-casein by Schmidt & Payens (1963).

The ultracentrifugation diagram of the purified β-casein showed a single symmetrical peak which according to Payens & van Markwijk (1963) corresponds to the monomer of β-casein. The sedimentation coefficient $s_{20,w}$ obtained was $1{\cdot}22 \times 10^{-13}$ sec.

The phosphorus content of 0·52 % for β-casein A agrees with 4 atoms of phosphorus for a molecular weight of 25,000 determined by Payens & van Markwijk (1963) and the nitrogen content was 14·4 %.

The extinction coefficient at 278 mμ in phosphate buffer, pH 7 (μ = 0·1, for a 1 % solution in a 1 cm path cell) has been found to be $E^{1\%}_{1\,cm} = 4{\cdot}6$ for β-casein A.

Chromatographic separations can be easily mechanized and in the near future automatic protein analysers will be available. The preparation of large amounts of purified casein fractions by chromatography will then become possible due to the high resolving power of this technique.

We sincerely thank V. Moreno, Ghislaine Brignon and Monique Veaux for their technical assistance.

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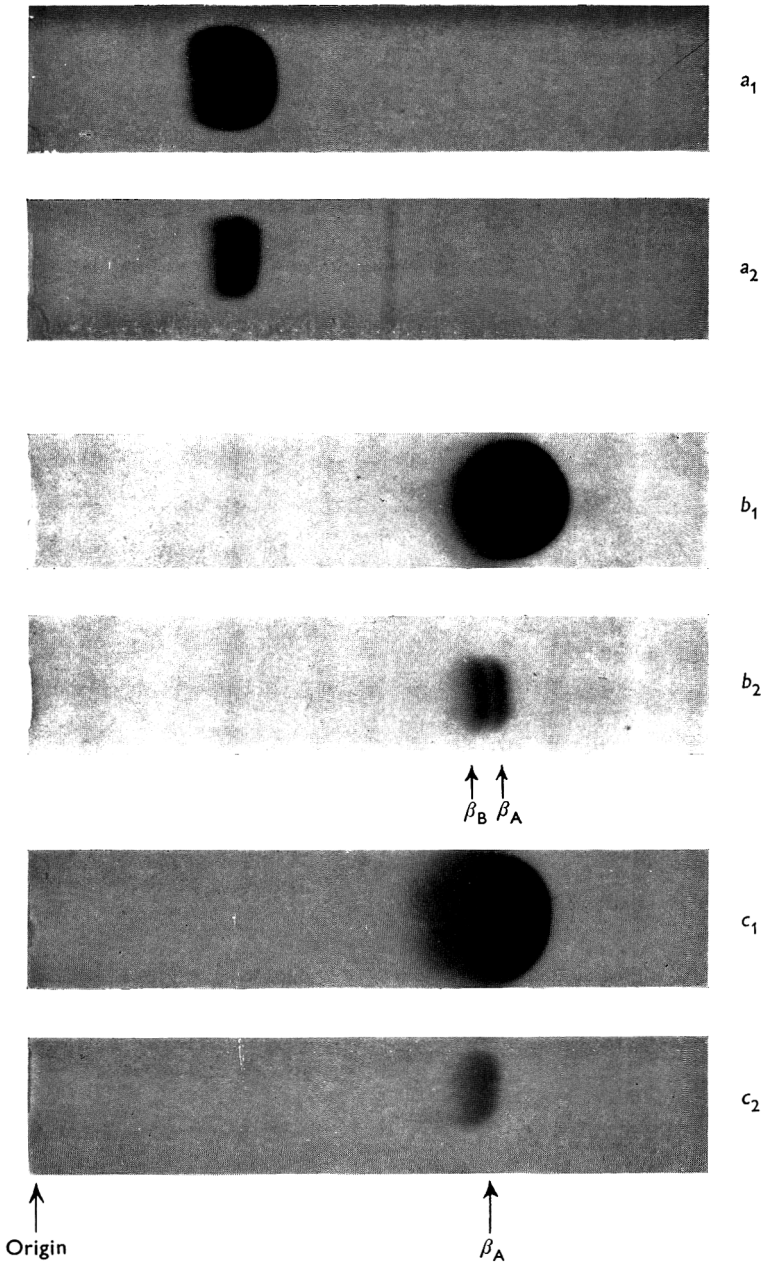
EXPLANATION OF PLATE

PLATE 1

Starch gel electrophoresis of β-casein in urea. (*a*) β-casein AB, pH 3·8, in 5 M urea at concentrations of 2 % (a_1) and 0·2 % (a_2); (*b*) β-caseins AB, pH 8·6, in 7 M urea at concentrations of 2 % (b_1) and 0·1 % (b_2); (*c*) β-casein A, pH 8·6 in 7 M urea at concentrations of 2 % (c_1) and 0·1 % (c_2).



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