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Activities of rumen micro-organisms in water buffalo (*Bos bubalus* L.) and in Zebu cattle

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Summary. Two fistulated adult bulls of the Zebu breed and 2 fistulated adult buffalo steers were given a diet consisting of concentrate mixture (rice bran and cotton-seed cake in equal portions + 2 % calcium hydroxide + 1 % common salt) and straw. This was later changed to an all-roughage diet of first cut berseem (*Trifolium alexandrinum*) Samples of the rumen contents were taken for determination of protozoal differential counts, concentration and relative proportions of volatile fatty acids (VFA), rate of cellulose digestion, fermentation rate, and net growth of rumen micro-organisms. Further data were obtained for the 2 buffalo steers fed sweet Sudan grass. When the feed consisted of concentrate and straw there were significant differences in the numbers of protozoa and in volatile fatty acid concentration in the 2 species of animals. Higher protozoal counts were observed in the rumen of bulls ($7{\cdot}2$–$8{\cdot}1 \times 10^5$ organisms/ml) than in the rumen of buffaloes ($2{\cdot}6$–$3{\cdot}6 \times 10^5$ organisms/ml). Greater concentration of VFA was found in the rumen of buffaloes (5·3–11·2 m-equiv/100 ml rumen liquor) than in bulls (4·8–10·4 m-equiv/100 ml rumen liquor). With berseem, the concentration of VFA in the rumen was generally higher (6·7–11·0 m-equiv/100 ml rumen liquor) and the protozoal counts were lower ($2{\cdot}8$–$5{\cdot}2 \times 10^5$ organisms/ml) but there were no significant differences between bulls and buffaloes.

The rates of cellulose digestion and the maximum fermentation rates were similar in the 2 species indicating a microbial population of the same order. Higher rates of cellulose digestion and of maximum fermentation when the ration consisted of berseem than when it consisted of concentrate + straw indicated a higher concentration of micro-organisms, mostly bacteria.

The water buffalo is a ruminant with many interesting features which are very different from those of other domestic cattle. The growth rate of the buffalo is quite different (Ahmed & Tantawy, 1954). Buffalo calves do not respond to early weaning systems in the same way as do calves of other domestic cattle (Ahmed & el-Shazly, 1960; Khoury, Ahmed & el-Shazly, 1967). The buffalo's milk has a higher fat concentration of the order 6–11 % (Asker, Ragab & Kamal, 1957) and has very little carotene content judging from its white colour. Its vitamin A content is, however, comparatively high (Badr, 1954). Other physiological characteristics of buffalo—heat tolerance (Badreldin, Oloufa & Ghany, 1951; Asker, Ghany & Ragab, 1952; Ragab,

Ghany & Asker, 1953), the magnesium content of the saliva (Sharma, 1936), rumen motility (Bhattacharyya & Mullick, 1965), rates of cellulose digestion in the rumen (Ichhponani, Makkar, Sidhu & Moxon, 1962) and protozoa concentration in the rumen (Mehtiev & Akperov, 1963)—have all been studied and found different from those of other domestic cattle.

The present work is an attempt to compare rumen microbial activities of Zebu cattle and buffaloes under similar conditions of feeding and management.

EXPERIMENTAL

Animals and rations. Two fistulated adult Zebu bulls and 2 fistulated adult buffalo steers were used in the present study. Comparative studies were undertaken using 2 different rations. A period of 3 weeks elapsed between the time the ration was introduced and the beginning of sampling. The rations were given to cover maintenance requirements (Morrison, 1956). The first ration consisted of wheat straw and a concentrate mixture in the ratio of 1:1. The concentrate mixture consisted of rice bran and cottonseed cake in the ratio of 1:1. Sodium chloride (1 %) and calcium hydroxide (2 %) were added to the mixture. The second ration was all roughage, consisting of berseem first cut *Trifolium alexandrinum*. Some data were obtained from the 2 buffalo steers when they were fed sweet Sudan grass (a cross between Sudan grass and *Sorghum saccharatum*). The daily ration was given in 2 equal parts, at 8 a.m. and at 8 p.m. The animals were allowed to drink before each meal.

Rumen sampling. Rumen samples were drawn through the fistula by suction using a rubber tube of $\frac{3}{4}$ in. diam. attached to a filter flask of 500 ml capacity. On each sampling, approximately 300 ml rumen contents were collected. The samples were collected before feeding and at 2, 4, 6 and 8 h after feeding on 2 successive days for each ration.

Counting technique. Two 5-ml samples of rumen contents were diluted 5-fold with 15 ml saline solution and 5 ml Lugol's iodine reagent to make a final volume of 25 ml. After gentle shaking 1-ml portions were taken up immediately in a wide-mouthed 1-ml graduated pipette and 0·1 ml swiftly pipetted to a dry clean slide and spread under a glass cover of 1173 mm^2 (2·3 × 5·1 cm). Differential counting was carried out using a low-power objective (× 10) of field area 1 mm^2. Thirty fields were counted in each slide, and the average count was taken to represent the number of protozoa/mm^2. Three slides were counted for each of the 2 dilutions from each sample. Multiplying the average count by 1173 gives the protozoal count in 0·1 ml of the diluted sample which represents 0·02 ml of the original sample. The coefficient of variation (3 replicates from each of the 2 dilutions) ranged from 0·7 to 14·3 % with an average of 7·38 %.

Methods. Total VFA were determined according to Warner (1956). The individual VFA were separated and determined using silica gel columns after Elsden (1946) as modified by el-Shazly, Khoury & Ahmed (1967).

The rate of cellulose digestion in the rumen was studied using the nylon bag technique (el-Shazly, Dehority & Johnson, 1961).

The fermentation rates, maximum rates of fermentation and net growth were determined following the method of el-Shazly & Hungate (1965).

RESULTS

Protozoal counts. Table 1 presents the total protozoal counts/ml of rumen contents obtained from the bulls and buffaloes fed on different rations. The relative percentage of each species is also given in the same table. The results indicated that *Entodinium* was predominant under all conditions. Feeding sweet Sudan grass to buffaloes caused a marked drop in the total number of protozoa in 1 ml rumen liquor. The relative concentration of *Entodinium* was also reduced in favour of *Isotricha* and *Eudiplodinium.*

Approximately similar numbers of ciliate protozoa were found in the bulls and buffaloes fed on berseem but when the feed consisted of concentrate and straw the numbers in bulls were double those in buffaloes. Table 1 also demonstrates that, on

Table 1. *Differential protozoal counts in rumen contents of adult bulls and buffaloes on different rations, sampled before and at different times after feeding*

Ration	Time of sampling	Total no./ml ($\times 10^5$)	Coefficient of variability*	Population composition, %				
				Entodinium	*Isotricha*	*Dasytricha*	*Diplodinium*	*Eudiplodinium*
			Bulls					
Concentrate +straw	Before feeding	7·2	9·9	97·6	1·6	–	0·8	–
	After feeding, h							
	2	8·4	19·9	97·9	1·6	–	0·5	–
	4	7·7	28·9	98·9	0·8	–	0·3	–
	6	7·3	25·2	98·4	1·1	–	0·5	–
	8	8·1	12·7	98·4	1·0	–	0·6	–
Berseem first cut	Before feeding	3·7	29·7	97·9	1·6	0·5	–	–
	After feeding, h							
	2	3·5	32·8	86·8	8·8	0·4	–	–
	4	2·9	30·9	94·4	4·9	0·7	–	–
	6	3·2	29·2	95·3	4·2	0·5	–	–
	8	5·2	14·3	95·6	4·0	0·3	–	0·1
			Buffaloes					
Concentrate +straw	Before feeding	3·6	23·6	96·3	1·6	–	2·1	–
	After feeding, h							
	2	2·7	20·3	95·8	3·2	–	1·0	–
	4	2·8	20·2	97·7	1·3	–	2·0	–
	6	3·0	23·9	95·7	1·3	–	3·0	–
	8	3·1	16·2	98·7	1·3	–	–	–
Berseem first cut	Before feeding	3·7	10·8	96·9	1·5	0·6	–	1·0
	After feeding, h							
	2	3·3	39·4	89·2	9·5	1·1	–	0·2
	4	3·5	12·7	97·3	2·1	0·6	–	–
	6	3·7	34·6	96·8	3·0	0·2	–	–
	8	4·1	23·5	95·1	4·1	0·2	–	0·6
Sweet Sudan grass	Before feeding	0·6	21·2	89·0	8·0	1·7	–	1·3
	After feeding, h							
	2	0·5	5·4	78·6	16·4	2·2	–	2·8
	4	0·4	1·8	82·8	14·3	0·8	–	2·1
	6	0·5	40·4	77·0	17·8	1·6	–	3·6
	8	0·4	20·1	78·0	15·4	4·1	–	2·5

– Not observed.

* Coefficient of variability = $S/x \times 100$, for the mean of 4 samples, drawn from 2 animals on 2 successive days.

the same ration, both bulls and buffaloes exhibited similar genera of protozoa. However, their relative concentrations were different. Large oligotricha were more abundant in buffaloes. Entodinia in the bulls were mostly *Entodinium minimum* and *simplex*. Larger entodinia, i.e. *E. caudatum* and *longinucleatum*, were observed in the bulls but were more numerous in buffaloes. The higher counts in the rumen of the bulls on the concentrate + straw diet could be ascribed largely to the greater numbers of *E. minimum* and *simplex*.

Variations in size of the same species may also occur in different animals. It was noticed that *Eudiplodinium magii* found in buffaloes had a larger size than in bulls. This was true for adult animals used in the present study and for calves (Naga, Abou Akkada & el-Shazly, 1968).

Table 2. *Total VFA concentration in the rumen contents of bulls and buffaloes on 2 different rations, sampled before and at different times after feeding (m-equiv/100 ml)*

Ration	Before feeding	2 h after feeding	4 h after feeding	6 h after feeding	8 h after feeding
		Bulls			
Concentrate + straw	4·82 ± 1·17	8·65 ± 0·04	10·38 ± 0·51	8·37 ± 0·13	8·90 ± 0·38
Berseem first cut	6·68 ± 2·03	8·49 ± 0·60	10·48 ± 2·92	9·86 ± 1·30	9·59 ± 1·89
		Buffaloes			
Concentrate + straw	5·32 ± 0·71	8·47 ± 0·41	11·16 ± 3·31	9·16 ± 0·95	10·08 ± 2·59
Berseem first cut	8·19 ± 3·01	10·57 ± 1·92	10·73 ± 1·30	11·00 ± 0·91	8·79 ± 1·82

The effect of the diet on the relative concentrations of different types of ciliates was more pronounced. Berseem and sweet Sudan grass caused a marked increase in *Isotricha*, *Dasytricha* and *Eudiplodinium*. It was also observed that while the numbers of entodinia changed only slightly (at any time from feeding) on berseem or concentrate + straw rations, holotricha rose to their highest concentration while large oligotricha decreased 2 h after feeding. On sweet Sudan grass, both holotricha and large oligotricha increased after feeding.

Volatile fatty acid concentration. VFA concentration in the rumen of buffaloes fed on concentrate + straw ration was significantly higher than in the bulls. The concentration of VFA was slightly higher on berseem than on the concentrate + straw ration. However, the differences were not statistically significant (Table 2).

Table 3 presents the relative concentrations of individual VFA in the rumen of the bulls and buffaloes on the 2 rations. There was a tendency for the relative concentration of propionic acid to be lower in the buffaloes than in the bulls both before and 2 h after feeding. On both rations acetic acid concentration was lower and butyric acid concentration higher in samples obtained before feeding from the bulls than from the buffaloes. Acetic acid decreased and butyric acid increased 2 h after feeding in the rumen of buffaloes, whereas in the bulls the trend was not as clear. However, the differences were not statistically significant. The differences in C_4 acids were nearly significant. Day-to-day and between-animal variations were lower 2 h after feeding than before feeding as indicated by the standard deviation values (Table 3).

Cellulose digestion in the rumen. The rate of cellulose digestion in the rumen of the buffaloes was a little lower than in the bulls. The difference was not statistically signifi-

cant. On the berseem ration, cellulose digestion in 48 h was 41 and 25 % greater for bulls and buffaloes, respectively, than on the concentrate + straw ration (Table 4).

Fermentation rates and net growth of rumen micro-organisms. Tables 5 and 6 give the fermentation rates and the maximal fermentation rates (fermentation capacity) of rumen samples from the bulls and the buffaloes on the 2 tested rations. The dry-

Table 3. *The proportions of individual VFA (%) in the rumen contents of bulls and buffaloes on 2 different rations, sampled before and 2 h after feeding*

Ration	Before feeding			2 h after feeding		
	Acetic	Propionic	C_4 and higher	Acetic	Propionic	C_4 and higher
			Bulls			
Concentrate–straw	73·30 ± 0·60	17·40 ± 0·75	9·25 ± 0·07	71·75 ± 0·17	17·05 ± 0·12	11·20 ± 0·90
Berseem first cut	71·59 ± 1·44	17·67 ± 1·39	10·74 ± 0·46	73·32 ± 0·57	17·37 ± 0·50	9·31 ± 0·15
			Buffaloes			
Concentrate–straw	74·82 ± 1·87	16·35 ± 1·50	8·83 ± 1·49	72·30 ± 0·72	16·67 ± 0·62	11·03 ± 0·72
Berseem first cut	74·51 ± 0·94	16·25 ± 0·34 ±	9·25 ± 1·13	69·16 ± 0·73	17·00 ± 0·17	13·84 ± 0·61

Table 4. *Percentage cellulose digested at different periods in the rumen of bulls and buffaloes as studied with the nylon-bag technique*

Ration	Percentage cellulose digested after, h				
	6	15	24	48	72
			Bulls		
Concentrate – straw	0·82 ± 0·30	6·19 ± 0·81	30·40 ± 0·73	56·47 ± 0·50	75·05 ± 0·94
Berseem first cut	0·89 ± 0·05	20·53 ± 0·85	37·43 ± 0·83	79·78 ± 0·29	–
			Buffaloes		
Concentrate – straw	0·43 ± 0·23	4·44 ± 0·92	29·79 ± 0·31	58·10 ± 1·20	77·06 ± 0·75
Berseem first cut	1·51 ± 0·21	11·79 ± 0·26	34·71 ± 0·85	72·94 ± 0·22	–

– Not determined.

Table 5. *The fermentation rate (µl per g per min) of rumen contents from bulls and buffaloes on 2 rations, sampled before and at different times after feeding**

Ration	Before feeding	Time after feeding						
		20 min.	1 h	2 h	4 h	5 h	6 h	8 h	10 h
				Bulls					
Concentrate–straw	6·50	7·15	10·10	15·73	11·96	–	12·74	5·33	–
S.D.	± 0·60	± 0·97	± 2·97	± 2·20	± 3·13	–	± 3·38	± 0·71	–
Berseem first cut	2·97	14·80	–	10·55	15·30	–	9·01	–	5·82
S.D.	± 0·38	± 4·09	–	± 4·34	± 1·31	–	± 2·17	–	± 2·89
				Buffaloes					
Concentrate–straw	5·33	11·70	–	11·96	10·66	8·58	8·45	5·98	–
S.D.	± 0·38	± 0·99	–	± 0·87	± 1·12	± 0·61	± 1·06	± 0·30	–
Berseem first cut	4·28	10·16	–	19·55	15·67	–	7·94	–	4·22
S.D.	± 1·46	± 1·48	–	± 5·01	± 2·44	–	± 0·33	–	± 3·11

* For average and dry-matter percentage see the text.
– Not determined.

matter percentages in the samples from bulls and buffaloes fed on concentrate + straw ranged from 10·61 to 15·30, respectively, and on berseem from 7·02 to 9·33.

The net growths calculated from the maximal fermentation rates before and after 1-h incubation are also shown in Table 6. The mean value of net growth determined at different periods from feeding is the net growth/h, whereas the mean value of maximum fermentation rates before incubation represents the initial concentration of micro-organisms.

Table 6. *Maximal fermentation rate (µl per g per min) and net growth of micro-organisms in rumen contents from bulls and buffaloes on 2 rations, sampled before and at different times after feeding*

Ration	Time of sampling	Maximum fermentation rate		Net growth, %
		Before incubation	After incubation	
		Bulls		
Concentrate + straw	Before feeding	22·60 ± 4·96	23·06 ± 3·71	+ 2·03
	After feeding			
	20 min	28·68 ± 2·17	40·00 ± 3·02	+39·41
	1·5 h	27·80 ± 9·09	56·88 ± 6·81	− 1·59
	2·5 h	55·49 ± 7·32	69·00 ± 2·06	+24·34
	4·0 h	45·40 ± 5·98	51·47 ± 0·61	+13·37
	6·0 h	46·79 ± 6·25	58·82 ± 8·47	+25·71
	8·0 h	36·84 ± 3·42	36·74 ± 7·14	− 0·27
	Average	41·94	47·99	+14·71
Berseem first cut	Before feeding	35·93 ± 13·99	49·93 ± 6·23	+38·90
	After feeding			
	25 min	46·39 ± 7·18	70·76 ± 2·11	+52·53
	2 h	47·25 ± 19·70	64·04 ± 16·08	+35·53
	4 h	83·65 ± 12·61	106·91 ± 5·83	+27·80
	6 h	78·35 ± 1·11	100·35 ± 6·69	+28·10
	10 h	69·97 ± 2·84	99·83 ± 6·26	+42·67
	Average	60·25	81·97	+37·60
		Buffaloes		
Concentrate + straw	Before feeding	50·95 ± 2·88	42·59 ± 4·18	−16·40
	After feeding			
	20 min	35·19 ± 5·18	42·99 ± 6·10	+22·17
	2·5 h	49·06 ± 0·96	50·30 ± 6·52	+ 2·52
	4·0 h	38·53 ± 8·25	50·33 ± 7·00	+30·62
	5·0 h	46·81 ± 6·11	49·88 ± 3·48	+ 6·55
	6·0 h	41·97 ± 5·53	45·19 ± 1·31	+ 7·67
	8·0 h	33·12 ± 2·06	36·50 ± 0·41	+10·20
	Average	42·25	45·39	+ 9·05
Berseem first cut	Before feeding	65·97 ± 12·51	76·71 ± 3·10	+16·28
	After feeding			
	25 min	40·39 ± 11·60	46·93 ± 18·73	+16·19
	2 h	47·80 ± 11·83	72·61 ± 24·15	+51·90
	4 h	43·37 ± 7·13	65·33 ± 7·38	+50·63
	6 h	62·96 ± 9·20	78·26 ± 18·35	+24·30
	10 h	68·74 ± 2·16	104·35 ± 10·73	+51·80
	Average	58·87	74·03	+35·18

There was no significant difference in net growth values of rumen micro-organisms from bulls or buffaloes on either ration. The average microbial net growth values/h were: 14·7 and 9·1 for bulls and buffaloes fed on concentrate + straw, respectively,

and 37·6 and 35·2 when the feed was berseem. The mean values of fermentation rates calculated from Table 5 are 9·9 and 9·7 μl per g per min for bulls and 9·0 and 10·5 μl per g per min for buffaloes on concentrate + straw and berseem, respectively.

DISCUSSION

Although the present study of microbial activities in the rumens of buffaloes and bulls did not reveal striking differences they are wide enough to suggest that differences in the nutritional requirements should be taken into account to exploit fully their potential for production. An important difference is in the total and differential counts of ciliate protozoa. Approximately similar numbers of ciliate protozoa were found in bulls and buffaloes fed on berseem, but the numbers in the bulls were twice those in the buffaloes when the feed consisted of concentrate + straw. Mehtiev & Akperov (1963) found that protozoal counts in buffaloes exceeded those in other cattle; their highest counts were similar to the lowest counts in the present work.

Although the numbers of ciliate protozoa were different in bulls and buffaloes when the feed consisted of concentrate + straw, the cell volume may be more or less similar since *Entodinium minimum* and *E. simplex* were the predominant species in the bulls while *E. caudatum* and *longinucleatum* formed the majority in buffaloes, and these are 2–4 times larger in size. It would be more valid to make the comparison on the basis of both numbers and size. Warner (1962) suggested '. . .it might be more meaningful to measure microbial concentration not as numbers/ml but as volume of its protoplasm/ml. The relative volume of protoplasm contributed by each species reflects more its share in metabolic activity than numbers.' The species *Entodinium* is generally the most abundant in the rumen (Purser & Moir, 1959; Eadie, 1962; Bailey & Clarke, 1963). Variation in size of the same species may also occur in different animals. It was noticed, for instance, that *Eudiplodinium magii*, isolated from the buffaloes, had a larger size than that from the bulls. This was true for both adult animals and calves (M. A. Naga *et al.* unpublished). Warner (1962) observed that considerable size variations may occur in the organisms due to their physiological state.

The effect of the diet was more pronounced on the relative concentrations of different genera of ciliates. Berseem and sweet Sudan grass caused a marked increase in *Isotricha*, *Dasytricha* and *Eudiplodinium*. Similar findings were observed by Oxford (1955) and were attributed to higher concentration of soluble sugars.

Day-to-day variations in protozoal concentration were not small as is indicated from the coefficient of variability in Table 1. However, they were lower than those reported by other investigators (Purser & Moir, 1966; Boyne, Eadie & Raitt, 1957). The animals were fed twice daily. Increasing the frequency of feeding lessens the diurnal variations (Moir & Somers, 1956; Putnam, Gutierrez & Davis, 1961) and probably the day-to-day variations. Such variations could be ascribed to differences in rumen contents which could reach 10 kg (Bath, Ronning, Lofgreen & Meyer, 1966) or more (Whiteman, Loggins, Chambers, Pope & Stephens, 1954).

It was reported by Van der Wath & Myburgh (1941) that acetic and propionic acid concentrations in the rumen affected the protozoal concentration. The peak of acid production was around 4 h after feeding and this coincided mostly with lowest ciliate counts (Tables 1 and 2).

The significantly higher VFA concentrations in the rumen of buffaloes fed on concentrate + straw may account for the highly significant differences in ciliate counts between bulls and buffaloes (Table 1). No such differences in protozoal count or VFA concentration occurred between bulls and buffaloes when the ration was berseem. The higher VFA concentrations in the rumen of buffaloes may reflect either a higher rate of production or a lower rate of absorption. VFAs were never found to leave the omasum in any significant concentration (Elsden, Hitchcock, Marshall & Phillipson, 1946; Gray, Pilgrim & Weller, 1954; Badawy, Campbell, Cuthbertson & Mackie, 1958). The higher values for net growth of micro-organisms from the bull's rumen than from the buffalo's (Tables 5 and 6) reflect a higher turnover rate of rumen contents (el-Shazly & Hungate, 1965) with equal rates of fermentation, and may indicate a higher rate of absorption of VFA from the rumen. Annison, Lewis & Lindsay (1959) concluded that higher rates of outflow of ingesta from the omasum, cause almost total absorption of VFA.

Determination of the rates of production and absorption of individual fatty acids, together with the volume of rumen contents, should throw more light on the function of these acids in domestic cattle and buffaloes and may explain some of the inherent differences of the 2 genera.

Ichhponani *et al.* (1962) found that cellulose digestion in the rumen of the buffalo was double that in the rumen of the Zebu cow. Their finding was not supported in the present study.

On the berseem ration, cellulose digestion was higher than on concentrate + straw both in bulls and in buffaloes. The presence of readily fermentable carbohydrates in the concentrate is responsible for the lower rate of digestion (el-Shazly *et al.* 1961). The presence of stimulating factors in berseem may have also contributed to the difference. Alfalfa ash was shown to improve cellulose digestion *in vitro* (Burroughs, Gerlaugh and Bethke, 1948).

The averages of the rates of fermentation for the rumen contents were of the same order. The mean value (10·16 μl per g per min) found in the present study was comparable to that obtained by el-Shazly & Hungate (1965), namely 10·32 μl per g per min. The differences between the microbial concentrations as detected by the initial maximum fermentation rate (before incubation, Table 6) on the concentrate + straw ration (42 μl per g per min) and that on the concentrate + hay ration (125 μl per g per min) used by el-Shazly & Hungate (1965) reflects a difference in the nutritive value of the 2 rations. The microbial concentration on the berseem ration (59·5 μl per g per min) was almost identical with that of el-Shazly & Hungate (1965) on alfalfa hay (59 μl per g per min). The net growth values (Table 6) were many times higher than those found by el-Shazly & Hungate (1965) especially when the feed consisted of berseem. These authors concluded that net growth is a reflexion of the turnover rate of rumen contents. The turnover rates/day calculated from the present results are 3·36 and 2·16 for bulls and buffaloes, respectively, fed on concentrate + straw and 8·8 and 8·4 for berseem ration. Sperber, Hydén & Ekman (1953) and Castle (1956) reported a turnover rate of 2/day for liquid contents of the rumen. The high turnover rate of berseem (first cut) is due to its high water content. It has a laxative effect on the animals and would therefore be expected to pass quickly through the alimentary tract. This points to the validity

of adding wheat straw in order to reduce the rate of passage of berseem. Another cause may be the ease with which berseem first cut is fermented by rumen micro-organisms (el-Shazly, Abou Akkada & Naga, 1963). Phillipson & Ash (1965) found that with highly fermentable diets the losses of dry matter from the rumen are higher. Furthermore, it is possible that high salivary secretion on the berseem ration may cause a fluid consistency of the rumen contents and a lower dry-matter composition (7·02–9·32). Results pointing to this were reported by Bailey (1961).

Hogan (1964) reported a 2 or 3 times higher rate of outflow for green forages than for dry diets. Balch & Campling (1965) obtained a longer retention time by adding concentrates to a hay ration. The concentrates, when finely ground, sank to the bottom of the rumen, causing a lower rate of passage (Ash, 1962). It has also been suggested that the rumen volume is smaller on a green roughage ration than on a concentrate + dry roughage ration (Balch & Line, 1957; Balch & Campling, 1965).

The net growth of rumen micro-organisms in the bulls was greater than that in the buffaloes (Table 6). It is therefore suggested that the rate of outflow from the bull's rumen is higher. Although the volume of rumen contents in buffaloes has not been determined, it could be concluded from the body size that the rumen size is larger (Warner & Flatt, 1965). Hungate, Phillips, McGregor, Hungate & Buechner (1959) and Purser & Moir (1966) pointed out that animals with small rumens have a faster rate of turnover. Buffaloes exhibit slower rumen movements than do other domestic cattle (Bhattacharyya & Mullick, 1965) which also should result in a slower rate of outflow.

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The action of calf rennet and other proteolytic enzymes on κ-casein

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Summary. The hydrolysis of κ-casein by a number of rennets and other proteolytic enzymes has been followed by measuring the increase in opacity due to the formation of insoluble aggregates of para-κ-caseins. The stability of these precipitates varied markedly, some being solubilized rapidly by the further action of the enzyme. The turbidity obtained with certain enzymes was dependent upon the calcium ion concentration, indicating that the para-κ-caseins produced were not identical for all enzymes.

For high concentrations of calf rennet, the rate of aggregation was linear with respect to time. With low concentrations of enzyme, increase in turbidity was preceded by a lag period which was lengthened by decreasing the enzyme concentration or increasing the κ-casein concentration. This increase in lag is favoured by a high κ-casein/para-κ-casein ratio, suggesting that the aggregation of newly formed para-κ-casein is prevented by the unchanged κ-casein. In addition, small amounts of α_{s1}- or β-caseins present in the κ-casein also markedly affected the aggregation of para-κ-casein, indicating that all 3 major casein components can inhibit the aggregation of para-κ-casein in the absence of calcium ions. In the light of these observations the possible role of protein–protein interactions in casein coagulation by calf rennet is discussed.

When rennet clots milk, the enzyme acts by specifically destroying the micelle stabilizing properties of κ-casein (Waugh & von Hippel, 1956), the time required to convert κ-casein to para-κ-casein being directly comparable to the time required to clot skim-milk (Waugh, 1958). The hydrolysis of soluble κ-casein to insoluble para-κ-casein by rennin is considered to be a limited proteolysis and apparently involves a single link only (Foltmann, 1966). Since Ernstrom (1965) has pointed out that a standard unit of rennet activity is unlikely to be established until a simple substrate is found on which the primary action of rennet can be followed with precision, investigations were carried out on the hydrolysis of κ-casein by calf rennet and other enzymes with rennet-like activity in the hope that the reaction might be developed as an assay. As κ-casein clots when treated with rennin in the absence of calcium ions (Wake, 1959), such a method would have the advantage that all the complications brought about by calcium ions in the clotting of milk would be avoided and might

compensate for the fact that an assay based upon the measurement of turbidity was likely to be complex.

In the course of preliminary studies it became apparent that contamination of the κ-casein by other caseins greatly affected the aggregation of para-κ-casein formed. It therefore seemed appropriate also to examine the action of rennet on κ-casein in the presence of other caseins since this might be expected to throw some light on the role played by protein interactions during gel formation.

MATERIALS AND METHODS

Commercial calf rennet was obtained from the N.Z. Rennet Company. Samples of commercial fungal rennet powders were kindly supplied by the Meito Sangyo Company, Osaka, Japan (U.S. Pat. 3,151,039) and Chas Pfizer and Co. Inc., New York (Br. Pat. 1,035,897), and are designated as Meito and Pfizer rennets in the text. All other proteolytic enzymes were obtained from the Sigma Chemical Company, St Louis, Mo., U.S.A.

Calf rennet was added to the casein solutions to give, except where otherwise stated, a final concentration (v/v) of 1 in 300. All other enzymes were added to give a final concentration (w/v) of 1 in 3000.

Turbidity measurements. Turbidity readings at 550 nm were recorded continuously, using glass cuvettes (1 cm pathlength) at 37 °C in a Zeiss PMQII spectrophotometer. Proportionality between the quantity of para-κ-casein precipitated and the amount of light scattered as measured by absorption would be expected only if the size and shape of the particles were the same in all experiments. Consequently no attempt has been made to compare absolute values obtained in different experiments.

Preparation of casein fractions. A number of investigations into the action of rennin and other proteolytic enzymes on casein fractions have been reported, but the results obtained appear to depend considerably upon the way in which the various caseins were prepared and thus upon their subsequent homogeneity. For this reason the preparation of the caseins used in this study is described in detail.

α_{s1}- and β-caseins. These were extracted from skim-milk obtained from a single cow, essentially by the method of Waugh *et al.* (1962) for the preparation of second cycle casein. The α_{s1}- and β-caseins were separated by a modification of the urea fractionation procedure of Hipp, Groves, Custer & McMeekin (1952). The crude caseins were further purified by elution with 2 salt gradient systems, each of which contained 2–3 drops of 2-mercaptoethanol as suggested by Thompson (1966), on Whatman DE32 ion exchange cellulose prepared according to Thompson (1967). A 2·5 × 25 cm column was coupled to a fraction collector via a flow cell in a Zeiss PMQ II spectrophotometer, the 280 nm absorbance being recorded on a Sargent SRL logarithmic recorder.

Approximately 1·0 g of crude casein dissolved in urea buffer (4·5 M-urea buffered with 0·013 M-imidazole neutralized to pH 7·0 with hydrochloric acid) was placed on the top of the DE-32 cellulose column together with 2 drops of mercaptoethanol. This was washed on to the column with 50 ml urea-buffer solution followed by a 0–0·1M–$MgSO_4$ gradient.

The samples from the fraction collector containing the material eluted at the peak,

as shown on the recorder, were electrophoresed using a polyacrylamide gel apparatus (E.C. Apparatus Corporation, 222, South 40th Street, Philadelphia, Pa., 19104, U.S.A.). The tubes containing the purest fractions were retained, bulked and dialysed against 0·1 M-sodium citrate and then twice against water. Solid urea was added to the sample to bring the urea concentration to 5 M. The sample was then pumped onto the column and rechromatographed as previously described, but using a 0–0·5 M-NaCl gradient. The gradients were run at a rate of 30–40 ml/h by means of a Sigma-motor finger pump (Sigma Motor Inc., North Main Street, Middleport, N.Y., 14105, U.S.A.). Each protein was dialysed, acid precipitated, washed, freeze-dried and stored at −20 °C.

κ-Casein, kindly supplied by Dr W. B. Sanderson of this Institute, was prepared by the sulphuric acid technique of Zittle (1962) as modified by Hartman & Swanson (1965). Gel-electrophoresis showed the κ-casein to be free of all but traces of other caseins.

The casein fractions were dissolved in sufficient dilute sodium hydroxide solution to give a final pH value of 6·3–6·5.

Electrophoresis. Examination of the purity of the caseins, and of their degradation products after enzyme action, was carried out after mercaptoethanol reduction, essentially by the disk electrophoresis method of Davis (1964) using gels containing 6·6 M-urea.

The examination of κ-casein breakdown products was also carried out on blocks of polyacrylamide gel (7 %, w/v) containing 6·6 M-urea, using the tris-citrate-borate buffer system described by Poulik (1957).

After electrophoresis (indicated by the movement of a dye band of Bromophenol blue carried by the borate ion front) the gel was removed and stained with either Amido black (0·1 %) in 5 % aqueous acetic acid or dilute Coomassie fast green GW 150 dye in 10 % aqueous acetic acid. After staining for 4–5 h, the gel was washed free of excess dye by successive washings with 5 % acetic acid.

RESULTS

Aggregation

Effect of rennet strength. In an attempt to obtain quantitative data on the effect of different dilutions of calf rennet on the precipitation of para-κ-casein, the course of aggregation was followed by measuring the increase in opacity of a 0·05 % κ-casein solution during the action of the enzyme. For low dilutions (up to 1/20) the rate of aggregation at pH 6·3 and 37 °C was linear, initially, with respect to time but a lag occurred with higher dilutions (Fig. 1) before turbidity was detected. This increased as the concentration of the rennet was decreased and at very high dilutions (1/1000 or more) several minutes elapsed before any aggregation occurred. The maximum opacity obtained also decreased with increasing dilution of the rennet (Table 1) at any one concentration of κ-casein.

Effect of κ-casein concentration. With any one dilution of rennet, a more linear relationship was obtained between the maximum opacity and the square of the concentration of κ-casein (Fig. 2) than with the concentration alone. As the κ-casein concentration increased, increase in turbidity was preceded by a lag period (Fig. 3).

No simple relationship appeared to exist, however, between the length of the lag period and the concentration of κ-casein.

Effect of different proteolytic enzymes. The rate at which a precipitate was formed differed considerably between the proteolytic enzymes investigated. This was as expected since the purity, pH optima and specificities of the proteinase preparations varied greatly. At the pH value used in this study (between pH 6·3 and 6·5), all the proteinases investigated gave precipitates at suitable concentrations of κ-casein and

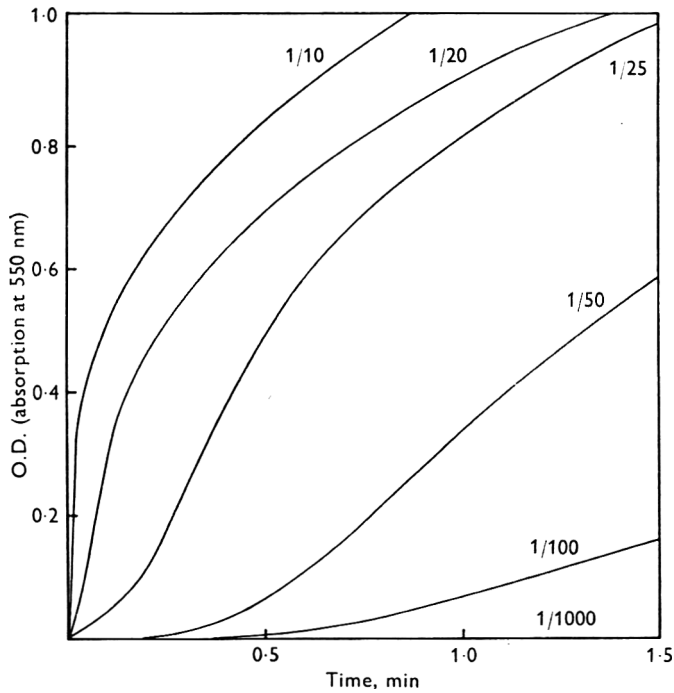

Fig. 1. The effect of different dilutions of calf rennet on the aggregation of para-κ-casein, measured as absorption at 550 nm, during the hydrolysis of κ-casein (0·05 %, w/v) at 37 °C and pH 6·3.

Table 1. *The effect of calf rennet concentration on the maximum opacity (absorbance at 550 nm) obtained in the degradation of κ-casein at pH 6·3 and 37 °C*

Concentration κ-casein, %	Rennet strength	Maximum opacity
0·05	0·10	1·60
	0·05	1·40
	0·01	1·00
0·03	0·001	0·60
	0·05	1·20
	0·01	0·55

enzyme. Even such a highly specific proteinase as thrombin slowly formed a precipitate when incubated with κ-casein at 37 °C. In the absence of calcium ions, the hydrolysis of 0·1 % (w/v) κ-casein resulted in a rapid increase of opacity only with calf

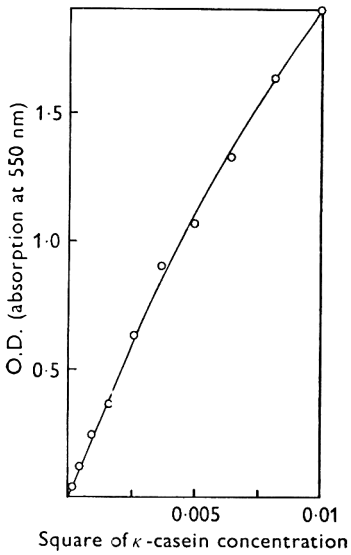

Fig. 2

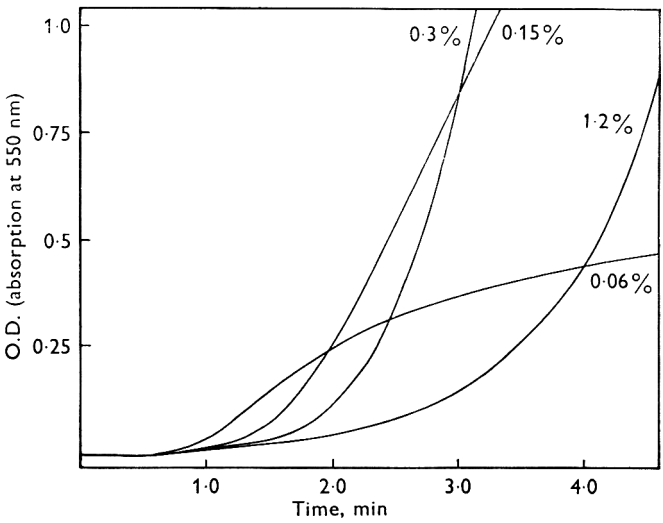

Fig. 3

Fig. 2. The effect of κ-casein concentration on the maximum opacity obtained by calf rennet (final concentration of 1/300, v/v) at 37 °C and pH 6·3.

Fig. 3. The effect of κ-easein concentration on the aggregation of para-κ-casein formed at 37 °C and pH 6·3 by calf rennet (final concentration of 1/3000, v/v).

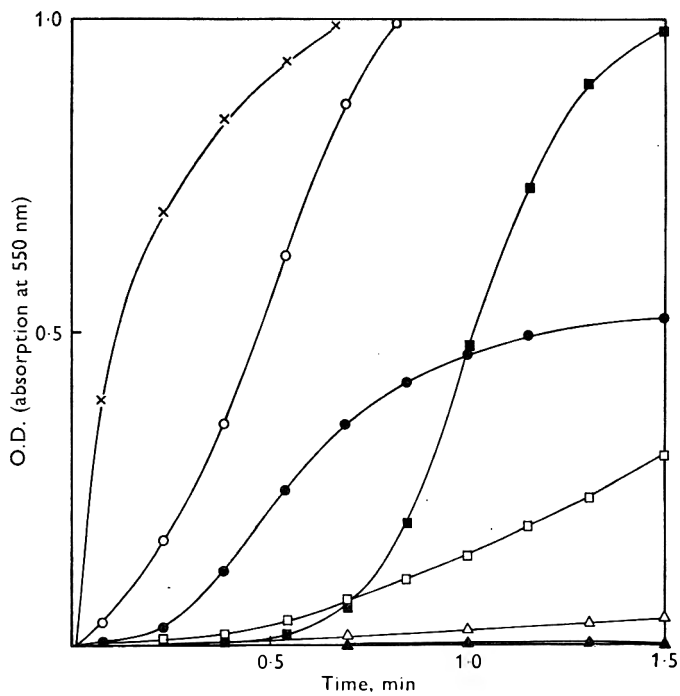

Fig. 4. The rate of aggregation of para-κ-caseins produced at 37 °C and pH 6·3 from 0·1 % (w/v) κ-casein, in the absence of calcium ions, by pepsin, ○—○; chymotrypsin, ●—●: trypsin, □—□; Pfizer rennet, ■—■; subtilisin, △—△; Meito rennet, ▲—▲; all used at a final concentration of 1/3000 (w/v), and by calf rennet, × — ×, at a final concentration of 1/300 (v/v).

rennet, pepsin and, after a short lag, Pfizer rennet. Meito rennet (0·03 %, w/v) gave no precipitate but an increased concentration (0·3 %) did so after a short lag (Fig. 4).

The concentration of κ-casein used was also important, since 0·01 % (w/v) chymotrypsin and subtilisin formed a precipitate with 0·1 % but not with 0·03 % κ-casein. This was believed to be a function of the rate at which the para-κ-casein was further degraded since, of the 9 proteinases investigated, these 2 enzymes most readily further degraded the clot of para-κ-casein formed. The para-κ-caseins obtained in the presence of trypsin, calf rennet, Meito rennet and pepsin were, however, comparatively stable towards solubilization by the enzymes. The para-κ-casein formed in the absence of calcium ions by pepsin was unchanged after a further 24 h.

It is apparent that the increase in opacity is a measurement of the rate of formation of para-κ-casein relative to its further rate of degradation. With any one concentration of κ-casein, it was noted that high concentrations of the less specific proteinases such as subtilisin and chymotrypsin gave less total turbidity than did lower concentrations.

Effect of calcium ion concentration. In the presence of 20 mM-$CaCl_2$, 0·01 % (w/v) chymotrypsin, subtilisin, pepsin and Meito rennets gave precipitates with 0·03 % κ-casein when they had not done so in the absence of calcium. It would appear, therefore, that the para-κ-casein formed by these particular enzymes in the presence of calcium ions is different from that formed by enzymes such as calf rennet which gave a precipitate in the absence of calcium ions. It is also interesting to note that the 4 enzymes which are in commercial use as milk-clotting agents produced aggregates of para-κ-casein the most readily in the presence of calcium.

Effect of α_{s1}- and β-caseins on the aggregation of para-κ-casein. At pH 6·3 and 37 °C, α_{s1}-casein exerted a marked inhibitory effect, in the absence of calcium ions, upon the coagulation of para-κ-casein (Fig. 5). The addition of a low concentration of α_{s1}-casein (5 % of the κ-casein concentration) resulted in a 50 % decrease in opacity and the presence of 10 % α_{s1}-casein totally prevented aggregation of the para-κ-easein formed by calf rennet in the first 2 min. The maximum opacity obtained was also greatly decreased by the presence of α_{s1}-casein (Fig. 6).

The association between para-κ-casein and β-casein was less firm than that with α_{s1}-casein. A ratio of concentrations of κ:β of less than 1:1 was necessary to prevent the normal clotting of κ-casein by calf rennet in the absence of calcium (Fig. 7) and the maximum opacities obtained in the presence of β-casein were much higher than those with the same concentrations of α_{s1}-casein (Fig. 6).

Gel-electrophoresis studies showed that the presence of α_{s1}- and β-caseins did not prevent the formation of para-κ-casein but merely its aggregation. After 2 min all the κ-casein had been degraded under the conditions of the experiment (Fig. 8) and turbidity only increased after the α_{s1}- or β-caseins present had been substantially altered by the enzyme. A small proportion of both the α_{s1}- and β-caseins was apparently broken down even before all the κ-casein has disappeared. The products of α_{s1}-casein were resolved very easily by either block or disk electrophoresis using acrylamide gels but those of β-casein were more difficult to resolve. After 15 min, however, a second band could be clearly seen very close to the original β-casein (Fig. 8). The β-casein appeared to be degraded slightly more readily than the α_{s1}-casein as judged

by the relative rates of disappearance of the original casein and the rate of appearance of the major degradation band in each case. It seems likely, therefore, that the greater inhibitory effect of α_{s1}-casein was the result of its stronger binding capacity with the para-κ-casein formed.

The extent to which α_{s1}- and β-caseins inhibited the aggregation of para-κ-casein varied, however, with the proteinase investigated and was markedly dependent upon the rate at which the α_{s1}- and β-caseins could be degraded. Thus, both caseins were extremely effective, in the absence of calcium ion, in preventing aggregation of para-κ-casein formed by pepsin, calf rennet, Pfizer and Meito rennets and, to a lesser

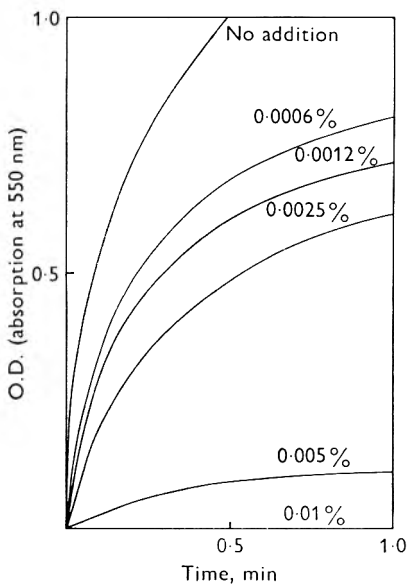

Fig. 5

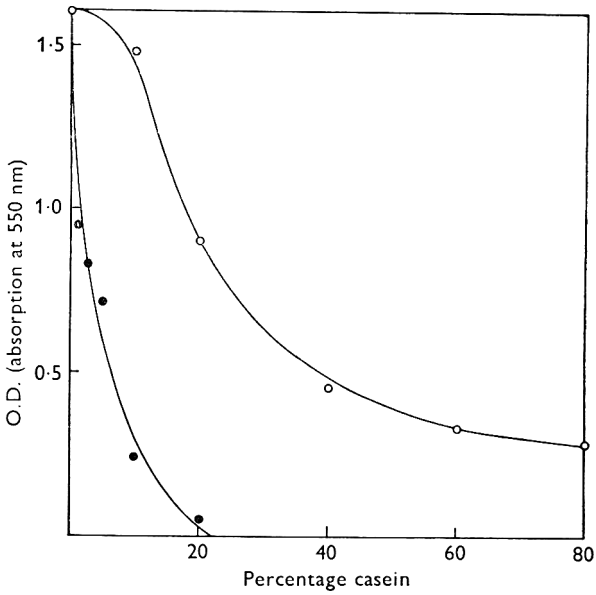

Fig. 6

Fig. 5. The effect of α_{s1}-casein concentration on the inhibition of aggregation of para-κ-casein formed from κ-casein (0·05 %, w/v) at 37 °C and pH 6·5 by calf rennet (final concentration 1/300, v/v).

Fig. 6. The effect of β-casein (○—○) and α_{s1}-casein (●—●) concentrations on the maximum opacity obtained by the hydrolysis of κ-casein (0·05 %, w/v) at pH 6·5 and 37 °C by calf rennet (final concentration 1/300 v/v).

extent, trypsin. The less specific enzymes such as papain, chymotrypsin and subtilisin were able to overcome this inhibitory effect much more readily. Thus, a precipitate was produced by 0·01 % subtilisin within a few seconds with a concentration ratio (κ:α_{s1}) of 1:4, whereas with 0·01 % solutions of trypsin and pepsin no increase in opacity was noted after 8 h.

As might be expected, the same order of activity in overcoming the inhibitory effect of α_{s1}- and β-caseins was shown by the above enzymes towards 0·5 % (w/v) solutions of sodium caseinate and of mixtures of α_{s1}-, β- and κ-caseins (in a ratio of 3:2:1) at the same pH value (6·3). Subtilisin caused almost immediate precipitation in both cases and pepsin none at all after 8 h. The order of effectiveness for the other 6 enzymes investigated was as above.

It must be emphasized that in all these experiments the extent to which the α_{s1}- and β-caseins prevented the aggregation of the para-κ-casein formed was greatly decreased by increasing the concentration of the proteinase since the inhibitory caseins were then degraded more rapidly. Gel-electrophoresis studies showed that in these experiments the κ-casein was rapidly degraded to a para-κ-casein by all the enzymes investigated and it would thus appear that degradation of α_{s1}- and β-caseins usually proceeds more rapidly than the further breakdown of the para-κ-caseins formed.

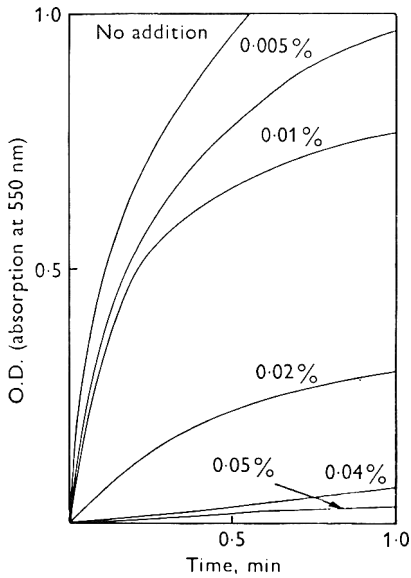

Fig. 7

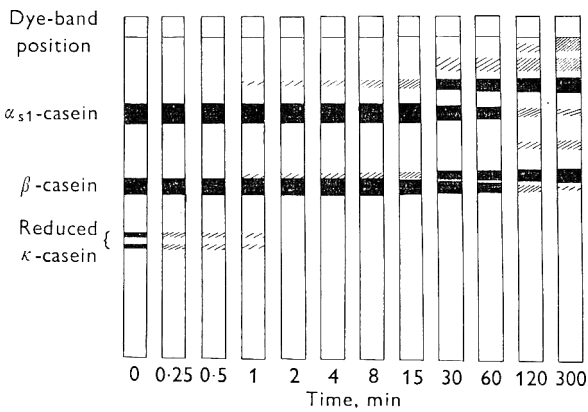

Fig. 8

Fig. 7. The effect of β-casein concentration on the inhibition of aggregation of para-κ-casein formed from κ-easein (0·05 %, w/v) at 37 °C and pH 6·5 by calf rennet (final concentration 1/300, v/v).

Fig. 8. Polyacrylamide-gel analysis of a mixture of α_{s1}-casein (0·45 %, w/v), β-casein (0·30 %, w/v) and κ-casein (0·15 %, w/v) during the action of calf rennet (0·1 ml of 1/10 dilution) at pH 6·5 and 37 °C. Final volume 6 ml. Samples (0·5 ml) were removed at times stated and rennet action stopped by the addition of a urea-mercaptoethanol mixture (final urea concentration, 6 M).

Effect of temperature on inhibitory effect of α_{s1}- and β-caseins. α_{s1}-Casein exerted the same inhibitory effect at 25 and 4 °C as it did at 37 °C, indicating that complex formation between α_{s1} and para-κ-casein still occurred at 4 °C. This contrasts with the conclusion of Noble & Waugh (1965) that an α_{s1}-κ-casein complex did not occur at 5 °C. On the other hand, concentrations of β-casein that were inhibitory at 37 and 25 °C were no longer so at 4 °C.

DISCUSSION

The aggregation of para-κ-casein was found to depend upon pH and temperature, confirming similar observations by Cheeseman (1962). The turbidity obtained with certain enzymes was also dependent upon the calcium ion concentration, indicating that the para-κ-caseins produced by all enzymes were not identical. The para-κ-casein formed was not a stable coagulum but was solubilized by the further action

of the enzyme. In the absence of calcium this took place comparatively slowly with such enzymes as calf rennet, pepsin and trypsin, but was very marked with less specific proteinases such as subtilisin and chymotrypsin. With any one concentration of κ-casein, a quantitative relationship was not found between the concentrations of enzyme used and the maximum opacities obtained, suggesting that the rate of formation of para-κ-casein relative to its further rate of degradation was being measured. In addition, a more linear relationship was found to hold between the maximum opacity obtained and the square of the κ-casein concentration than with concentration alone. Estimation of the formation of para-κ-casein from κ-casein by turbidimetric measurement of its rate of aggregation does not, therefore, appear to be a suitable assay for rennet-like activity.

With low concentrations of calf rennet or high concentrations of κ-casein, increase in turbidity was preceded by a lag period. Increase in this lag period was favoured by a high ratio of κ-casein to para-κ-casein suggesting that the aggregation of newly formed para-κ-casein is prevented by unchanged κ-casein. κ-Casein exists as a series of polymers under the conditions of this investigation, part of the size heterogeneity arising out of intermolecular –S–S– bonding (McKenzie, 1967). Before an increase in opacity is obtained, sufficient κ-casein in each polymer must presumably be converted to para-κ-casein to allow the whole polymer to aggregate with other partially hydrolysed polymers.

The presence of small amounts of α_{s1}- or β-caseins also markedly affected the aggregation of para-κ-casein indicating that the solubilization of para-κ-casein by high concentrations of κ-casein mentioned above is of a more general nature and that all 3 major casein components can inhibit the aggregation of para-κ-casein in the absence of calcium ions. The presence of 10 % α_{s1}-casein completely prevented the precipitation of para-κ-casein formed by calf rennet. β-Casein was less effective in preventing the aggregation of para-κ-casein and a concentration slightly greater than that of the initial κ-casein concentration was needed for total inhibition.

Gel-electrophoresis studies showed that the κ-casein was rapidly degraded to para-κ-casein but that the turbidity only increased after the α_{s1}- or β-caseins had been substantially altered. The ease with which this was achieved varied considerably amongst the enzymes tested but it may be significant that the 4 enzymes which are in use commercially (calf rennet, pepsin, Meito and Pfizer rennets) were those which (*a*) further degraded the para-κ-casein comparatively slowly, and (*b*) did not readily degrade α_{s1}- and β-caseins and were thus unable to overcome readily the inhibitory effect of these caseins towards the aggregation of para-κ-casein. Gel-electrophoresis showed that α_{s1}- and β-caseins were degraded at about the same rate by calf rennet and therefore the greater inhibitory effect of α_{s1}-casein must be a result of a stronger association between α_{s1}-casein and para-κ-casein than between β-casein and para-κ-casein.

These results confirm, in general, similar conclusions put forward by Garnier and his co-workers that the normal clotting of κ-casein is prevented in the presence of α_{s1}-casein (Garnier, Yon & Mocquot, 1964), and of β-casein (Garnier, Dumas & Brignon, 1964). They gave, however, few experimental details and the present findings indicate that α_{s1}-casein exerts a much greater effect than has been previously reported. The apparent inhibition which results from the formation of the complex

α_{s1}-para-κ-casein has been found to be complete for a ratio of concentrations considerably smaller than those postulated for κ-α_s complexes by Garnier, Yon & Mocquot (1964) or Garnier (1967).

The properties of the coagulum formed when rennet clots milk depend to a large extent upon the mineral substances present in the milk, particularly the various forms of calcium, but Mocquot & Garnier (1965) have pointed out that the role played by the proteins themselves is probably far from negligible. It is generally accepted that when κ-casein, as part of the colloidal micelle system in milk, is attacked by rennet, its ability to stabilize the micelle is destroyed. In the presence of calcium ions, the calcium sensitive fractions along with insoluble para-κ-casein form a clot. The findings of this investigation suggest that it may be possible to define this mechanism more closely. In the absence of calcium ions, a mixture of α_{s1}- and β-caseins complexes with para-κ-casein thereby preventing its aggregation. There is no reason to suppose, *a priori*, that calcium ions would interfere with the interactions between α_{s1}-, β- and para-κ-caseins within the complex. The presence of calcium ions may, therefore, modify the complex of associated proteins *as a whole* in such a way that it is no longer stable and can therefore aggregate with other like complexes to form a clot.

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A micro-method for the quantitative estimation of rennets and other proteolytic enzymes

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Summary. An agar diffusion slide assay using a thin layer of calcium caseinate has been developed as a micro-method for the quantitative study of calf rennet and other proteolytic enzymes. The number and width of the precipitation zones formed as a result of proteolytic activity depended upon the enzyme used, the concentration of calcium ion, pH and temperature. The assay was highly reproducible and particularly suitable for the measurement of large numbers of samples. It was considerably more sensitive than the milk clotting technique and, in general, more sensitive than other standard methods for measuring proteolytic activity.

During investigations into the suitability of microbial extracts as substitutes for calf rennet, it was found that the milk clotting activity in the supernatants of growth cultures of a number of micro-organisms tested was too low to be assayed by standard methods, although some of these micro-organisms produced zones of proteolysis when grown on milk agar plates. This suggested that it might be possible to develop a caseinate agar diffusion method that would be more sensitive than existing assays. Since the enzymic phase of milk coagulation is considered to involve a limited proteolysis of κ-casein (Foltmann, 1966), an attempt was made in preliminary studies to use the hydrolysis of κ-casein in agar as an index of rennet-like activity. The degree of proteolysis was measurable as the diameter of the ring of precipitated para-κ-casein at the leading edge of the diffusing enzyme. It was found, however, that whole casein, in the presence of calcium ions, was a more satisfactory substrate than κ-casein. Cheeseman (1963) observed zones of precipitation when proteolytic enzymes diffused through agar gels containing various caseins but considered that the technique did not compare favourably with the milk clotting method (Berridge, 1952) as an assay for calf rennet. We have found that when Cheeseman's method is suitably modified, an agar diffusion assay using calcium caseinate is very sensitive and reproducible and is suitable for both the qualitative and quantitative study of rennets and other proteolytic enzymes.

MATERIALS AND METHODS

Preparation of agar-caseinate slides. The method of Cheeseman (1963) was slightly modified by the use of very thin layers of agar-caseinate. Caseinate solutions were prepared by blending acid precipitated whole casein or individual casein fractions

with sufficient 0·05 N-NaOH to give a final pH value of 6·0, in a Polytron homogenizer (Kinematica GMBH, Lucerne, Switzerland). A 10-ml sample of the caseinate solution (usually 10 % for whole casein) was then added to 90 ml of hot Davis agar (1 %) containing 10–20 mM-Ca^{2+}. One ml of this solution was spread on a microscope slide over an area measuring 2 in. × 1 in., which could be conveniently and exactly outlined by means of cellulose tape. A hole, 2 mm in diam., was bored with a thin steel tube and 0·003 ml of the enzyme solution was added by means of a syringe microburette (Micro-Metric Instrument Co., Cleveland, Ohio). The slides were placed in a plastic box (30 × 26 × 10 cm) containing moist filter paper and incubated at 37 °C for periods up to 40 h. The diameters of the zones of precipitation or of transparent zones were measured with vernier calipers.

The agar was usually autoclaved with water but 0·1 M-acetate buffer, pH 5·2–5·4, was also used in the assay of microbial supernatants in which acid had been produced, since caseinate is precipitated at pH values below 4·8.

Commercial calf rennet was purchased from the N.Z. Rennet Company and samples of fungal rennet powders were kindly supplied by the Meito Sangyo Company, Osaka, Japan, (U.S. Pat. 3,151,039) and Chas. Pfizer and Co. Inc., New York (Br. Pat. 1,035,897). These are designated as Meito and Pfizer rennets, respectively, in the text. All other enzymes were obtained as crystalline solids from the Sigma Chemical Company, St Louis, Mo., U.S.A.

Agarose was purchased from L'Industrie Biologique Française S.A. Gennevilliers (Seine), France, agar from Davis Gelatine (N.Z.) Ltd., Christchurch, New Zealand, and Ionagar No. 2 from Oxoid Ltd, London.

Casein fractions were obtained, and their degradation followed by polyacrylamide gel electrophoresis using methods previously described (Lawrence & Creamer, 1969).

RESULTS

Diffusion of proteolytic enzymes through agar containing purified casein fractions. A study was first made of the diffusion of a number of proteolytic enzymes through agar gels containing purified casein fractions. Although these were found to be unsuitable as substrates for assaying proteinases, the results obtained were, in a number of cases, different from those obtained by Cheeseman (1963) and are therefore presented in some detail. It is apparent that the way in which the different casein fractions are prepared does influence very considerably the type of precipitation pattern obtained.

κ-Casein agar. A previous attempt to use κ-casein as a substrate for rennet activity by measuring the opacity of the para-κ-casein formed was not successful (Lawrence & Creamer, 1969), mainly because the opacity obtained was found to be a function of the rate of formation of para-κ-casein relative to its further degradation by the enzyme. An attempt was therefore made to stabilize the precipitation of para-κ-casein by carrying out the degradation in agar gels. Comparatively faint zones of precipitation were obtained with κ-casein concentrations up to 1 % (w/v), both in the presence and absence of calcium ion, even with enzyme solutions of high activity.

κ-α_{s1}-Casein agar. The addition of α_{s1}-casein to the κ-casein agar to give a concentration ratio of 1:3 (κ:α_{s1}) resulted in a single well-defined white zone in the absence

of calcium, and in 2 clear widely separate zones in the presence of calcium ion at 37 °C, with calf rennet (Plate 1), pepsin and Pfizer fungal rennet. Trypsin and chymotrypsin also gave 2 zones but these were extremely close together. Meito fungal rennet and subtilisin both gave a single zone in the presence of calcium ion. These findings were unexpected as the addition of α_{s1}-casein to κ-casein in the absence of agar inhibited the aggregation of para-κ-casein (Lawrence & Creamer, 1969). It must be assumed that neither of the white zones obtained with calf rennet was due to para-κ-casein alone.

κ-β-Casein agar. The addition of β-casein to κ-casein resulted in a very similar pattern of zones to that with the κ-α_{s1}-casein agars at 37 °C except that the zones obtained were, comparatively, not as sharp.

β-Casein and α_{s1}-casein agars. No white precipitation zones at all were obtained with β- and α_{s1}-caseins either individually or as a mixture.

κ-α_{s1}-β-Casein agars. These caseins in a ratio of 1:3:3 (κ:α_{s1}:β) gave zones very similar to that with whole casein, both in the presence and absence of calcium ions (Plate 1). There appeared, therefore, to be no advantage in using pure casein fractions and, in all subsequent work, whole caseinate was used.

Diffusion of proteinases through calcium caseinate agar. For routine use in screening supernatants of microbial isolates for proteolytic activity, an unbuffered substrate was usually found to be more suitable than the buffered caseinate used by Cheeseman (1963). A number of microbial proteinases were apparently inhibited by the presence of acetate ions. The sensitivity of Cheeseman's method was greatly increased by the use of a thin layer of agar-caseinate on a defined area of a slide (see Methods).

Further increase in sensitivity of the assay could be obtained by decreasing the concentration of the caseinate from 1·0 to 0·5 % but this decrease was at the expense of sharp definition of the zones. For routine use a 1 % caseinate containing 10–15 mM-Ca^{2+} was found to be the most suitable substrate.

Two zones of precipitation were usually produced by calf rennet, pepsin, papain, chymotrypsin and trypsin, in the form of rings separated by circular zones of relatively clear agar (Plate 1). Only one white zone was usually obtained with subtilisin and with the Meito and Pfizer fungal rennets.

The difference in patterns of the white zones obtained with various proteinases appeared to be mainly due to the relative sensitivity to precipitation by calcium ions of the high molecular weight products formed in the initial stages of the caseinate breakdown.

The ratio of the diameters of the outer white zone and the inner transparent zone obtained with calf rennet was very similar to that obtained with Pfizer rennet. The inner transparent zone obtained with Meito rennet was comparatively much smaller. The inner transparent zones did not stain with Amido black, whereas the outer white zones did, indicating that the size of the inner transparent zone could be used as a measure of proteolysis beyond the stage of para-casein formation.

The quantitative validity of the agar diffusion assay has been determined with a wide range of microbial, animal and plant proteinases and appears to have general applicability. The formation of white precipitation zones is apparently a property of the caseinate and not the result of hydrolysis by proteinases with specific properties. The rate of proteolysis, as measured by the area contained by the outermost

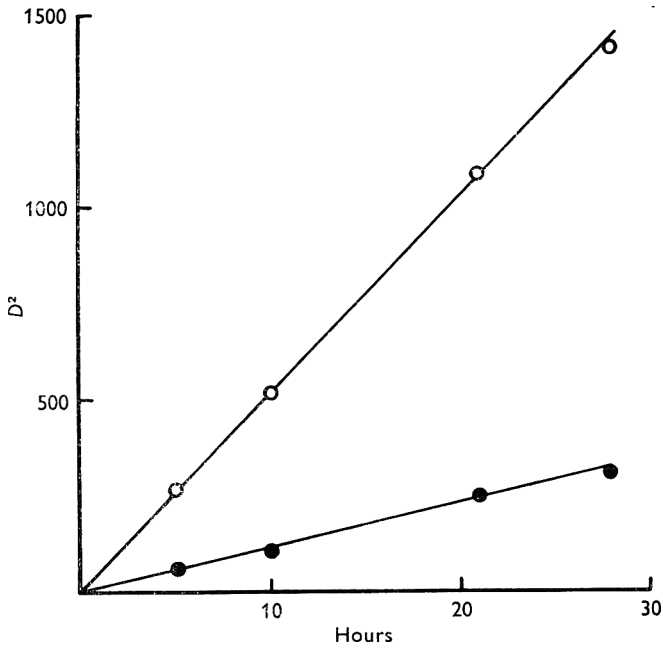


Fig. 1. The extent of hydrolysis of 1 % (w/v) caseinate, buffered with M/10 acetate at pH 5·4, by calf rennet over an extended period of incubation at 37 °C, as shown by areas enclosed by the outer white zone of precipitation (O—O) and inner transparent zone (●—●). *D* is the diameter in mm of the zones of proteolysis.

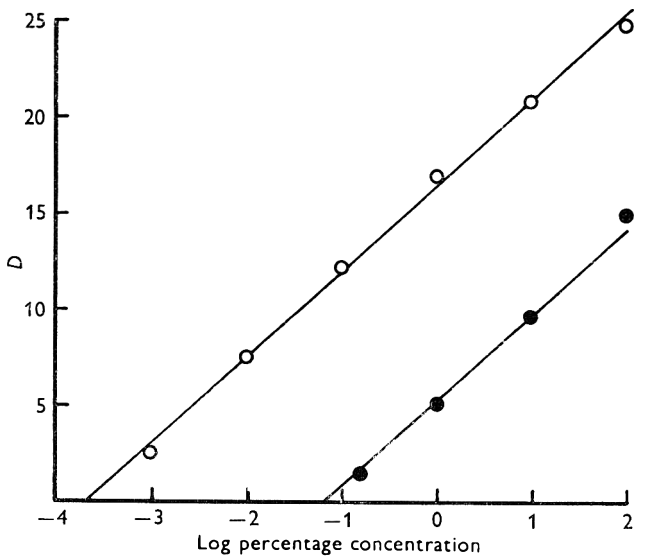


Fig. 2. The extent of hydrolysis of 1 % (w/v) caseinate at pH 6·0 by dilutions of subtilisin after 20 h at 37 °C, as shown by the outer white zone of precipitation (O—O) and by the inner transparent zone (●—●). *D* is the diameter in mm of the zones of hydrolysis.

white zone or of the inner transparent zone, was proportional to the period of incubation (Fig. 1) and remained linear for at least 40 h. A linear relationship was also obtained when the logarithm of the dilution of the proteinase was plotted against the diameter of the outer white zone of precipitation or the inner transparent zone of hydrolysis after incubation at 37 °C (Fig. 2). The points where these straight lines cut the axis were the minimal proteinase concentrations which gave a discernible white zone or transparent zone, respectively. These could be calculated and confirmed by experiment. Since also the ratio of the response for high and low dilutions was not significantly different for the various enzymes (the ratio of high to low dilutions being the same), the assay therefore satisfies the mathematical conditions postulated for a valid agar diffusion assay (Cooper, 1963). The coefficient of variability for a series of determinations of zone diameter obtained with a given enzyme dilution was 0·66 % with a confidence interval of $\pm$ 0·36 mm for a single determination.

Electrophoresis of casein-agar gels. The electrophoresis of samples of agar gel taken successively closer to the central well indicate that one cannot state unequivocally that the appearance and disappearance of the precipitation bands correlate exactly with changes induced by the enzyme, but rather, that when these induced changes have reached a certain level, then the precipitation bands appear. The effect of temperature, protein concentration and ion concentration on the position and extent of the precipitation bands in the agar also supports this view. Thus, although the broader outer white precipitation bands could be correlated with the breakdown of κ-casein to para-κ-casein, slight breakdown of all caseins in this region was detected. In the transparent zone closest to the well, extensive degradation of the major individual caseins had occurred. The relative areas of the outer white and inner transparent zones give an indication of the ability of the proteinase not only to carry out the initial breakdown of the caseins to para-caseins but also to further degrade these products. Solutions containing low amounts of proteinase showed little or no ability to further degrade the white zone formed round the well.

Factors affecting the number of white zones obtained on caseinate-agar

Effect of calcium concentration. In all instances the sensitivity of the assay, as measured by the deposition of an outer white zone, was increased by the addition of calcium (Table 1). The response however varied considerably between enzymes, calf rennet and trypsin being comparatively very sensitive. As the calcium concentration was increased, a point was reached when 2 or more white zones were precipitated by calf rennet. A further increase caused the zones to merge (Table 1). The size of the inner transparent zone was not, however, significantly affected by the calcium concentration except with trypsin and pepsin. With these 2 enzymes the transparent zone also increased with increase in calcium concentration.

The white zones of precipitation became only marginally smaller with increase in Davis agar concentration from 0·5 to 2 % (Table 2) indicating that zone formation is unlikely to involve interaction between the agar and degradation products of the casein. Other gelling agents, such as 'Ionagar', agarose and polyacrylamide, produced a similar pattern of zones to those obtained with Davis agar, despite wide differences in the endogenous calcium content.

Effect of magnesium concentration. An equivalent concentration of magnesium ions

in the medium was found to be almost as effective as calcium. The number of zones obtained was the same but the size of the zones was marginally smaller.

Effect of pH. Distinct white zones were obtained only if the casein was between pH 5·0 and 6·5. For enzymes such as trypsin with an alkaline pH optimum, the inner transparent zone became larger as the pH value was increased above 6·5 but the outer white zone became more narrow and more difficult to read. At pH 8·0 no white zone at all was detected with trypsin. The zone of proteolysis at this pH value could however be readily shown by staining the unhydrolysed caseinate with Amido black (0·01 %, w/v) in dilute acetic acid (7 %, v/v) but such zones were still not as large as the white zones of precipitation obtained between pH 5 and 6. For enzyme preparations with an acid pH optimum, such as calf rennet, both the outer white zones of precipitates and the inner transparent zones decreased with increase in pH value.

Table 1. *The effect of calcium concentration on the diameter and number of precipitation zones produced in* 0·05 % (*w/v*) *caseinate-agar by calf rennet and* 1 % (*w/v*) *Pfizer fungal rennet, at pH* 6·0 *and* 37 °*C*

	Calf rennet		Pfizer rennet	
$CaCl_2$, %	No. of zones	Zone diam., mm	No. of zones	Zone diam., mm
—	1	14·5	1	17·5
0·01	1	15·9	1	17·8
0·02	1	16·2	1	19·3
0·03	1	16·4	2	26·3 (19·6)
0·04	1	16·7	2	26·6 (19·5)
0·05	2	20·8 (13·2)	2	27·1 (19·5)
0·07	3	28·5 (22·3) (16·5)	2	28·8 (19·2)
0·09	2	30·0 (17·0)	1	28·9
0·11	2	30·2 (16·9)	1	29·1

Table 2. *The effect of agar concentration on the diameters of the outer precipitation and inner transparent zones produced in* 1 % (*w/v*) *caseinate-agar, containing* 0·07 % (*w/v*) $CaCl_2$, *by calf rennet and* 1 % (*w/v*) *Pfizer rennet at pH* 6·0 *and* 37 °*C*

	Calf rennet		Pfizer rennet	
Agar, %	Precipitation zones, mm	Transparent zones, mm	Precipitation zones, mm	Transparent zones, mm
2·0	26·6	13·7	28·1	15·3
1·5	26·6	13·6	28·8	15·4
1·2	27·0	13·6	29·4	15·6
1·0	27·3	13·8	29·6	15·4
0·75	27·7	14·1	30·2	15·5
0·5	28·2	14·1	31·1	16·1

Since caseinate is precipitated at pH values below 4·8, proteinases in supernatants of microbial cultures in which acid had been produced were assayed on caseinate buffered with 0·1 M acetate between pH 5·2 and 5·4. White zones were readily formed both on this buffered substrate and also on unbuffered caseinate (initially at pH 6·0) by all proteinases tested, irrespective of whether their pH optima were on the acid or alkaline side.

The pattern of white zones obtained with caseinate buffered below pH 5·4 depended on the buffer used. Thus, calf rennet precipitated one white zone with caseinate

buffered at pH 5·2 by citrate, when one would expect only a low concentration of free calcium ion to be present, whereas acetate at pH 5·2 resulted in the usual 2 white zones. Although agar is known to contain replaceable hydrogen ion (Cooper, 1963) it seems unlikely that the white zones precipitated in caseinate buffered at low pH are identical to those obtained in unbuffered casein containing calcium ions.

Effect of source of enzyme. A number of workers (Ganguli & Bhalerao 1965; De Konig, 1966; Ritter & Schilt, 1967) have suggested that animal rennets can be distinguished from non-animal rennets by the number of white zones precipitated on whole casein agars. In the present investigation, about 90 % of the microbial proteinases, produced from about 100 different micro-organisms taken at random, gave only one white zone of precipitation after 24-h incubation. Many of these, however, gave 2 or 3 distinct zones after incubation for a further 2–3 days. In addition, many microbial proteinases gave 2 or more zones when the enzyme was allowed to diffuse through caseinate-agar containing no added calcium, before flooding the agar surface with calcium chloride solution or buffers at a pH value between 4·5 and 5·0.

Table 3. *Comparison of caseinate-agar diffusion assay against other standard assays for the detection of minimal quantities of proteolytic activity*

	Minimum quantity of enzyme detected, μg/ml		
Enzyme	Milk clotting*	Folin-Ciocalteu†	Caseinate-agar
Pepsin	1	1·0	0·1
Calf rennet‡	10	10·0	0·1
Fungal rennet I	15	2·0	0·1
Subtilisin	20	5×10^{-2}	5×10^{-2}
Chymotrypsin	25	1·0	0·1
Fungal rennet II	50	1·0	0·1
Trypsin	150	0·1	0·1
Papain	700	20·0	10·0

* Minimum quantity of enzyme necessary to clot milk in 1 h using method of Berridge (1952).
† Measurement of TCA soluble phenol and indolyl residues.
‡ Calf rennet protein estimated from Kjeldahl nitrogen determinations.
Fungal rennets I and II were commercial fungal rennet preparations.

Similarly, the ability of animal rennets to give 2 or more zones on calcium caseinate agars was a function of their concentration. Thus, at low concentrations of calf rennet only the one white zone was precipitated instead of 2 and the inner transparent zone vanished completely. In the presence of high concentrations of calcium ion (above 0·1 %) or when the caseinate was buffered below pH 5·4 the white zones also tended to merge so as to appear to be one zone (Table 1).

The only vegetable proteinase tested, papain, also gave between 2 and 5 zones depending upon the conditions of the assay. The assay is, therefore, not universally applicable for distinguishing animal from non-animal rennets.

Effect of temperature. In some instances the temperature at which the slides were incubated affected the number of zones obtained. Thus, Meito rennet gave only one thick zone after incubation for 20 h at 37 °C but 2 easily discernible zones at 45 °C. Similarly, some enzymes, including subtilisin, showed only one faint zone when

allowed to diffuse at 4 °C. On further incubation at 37 °C for 1 h, however, 2 very distinct white zones appeared.

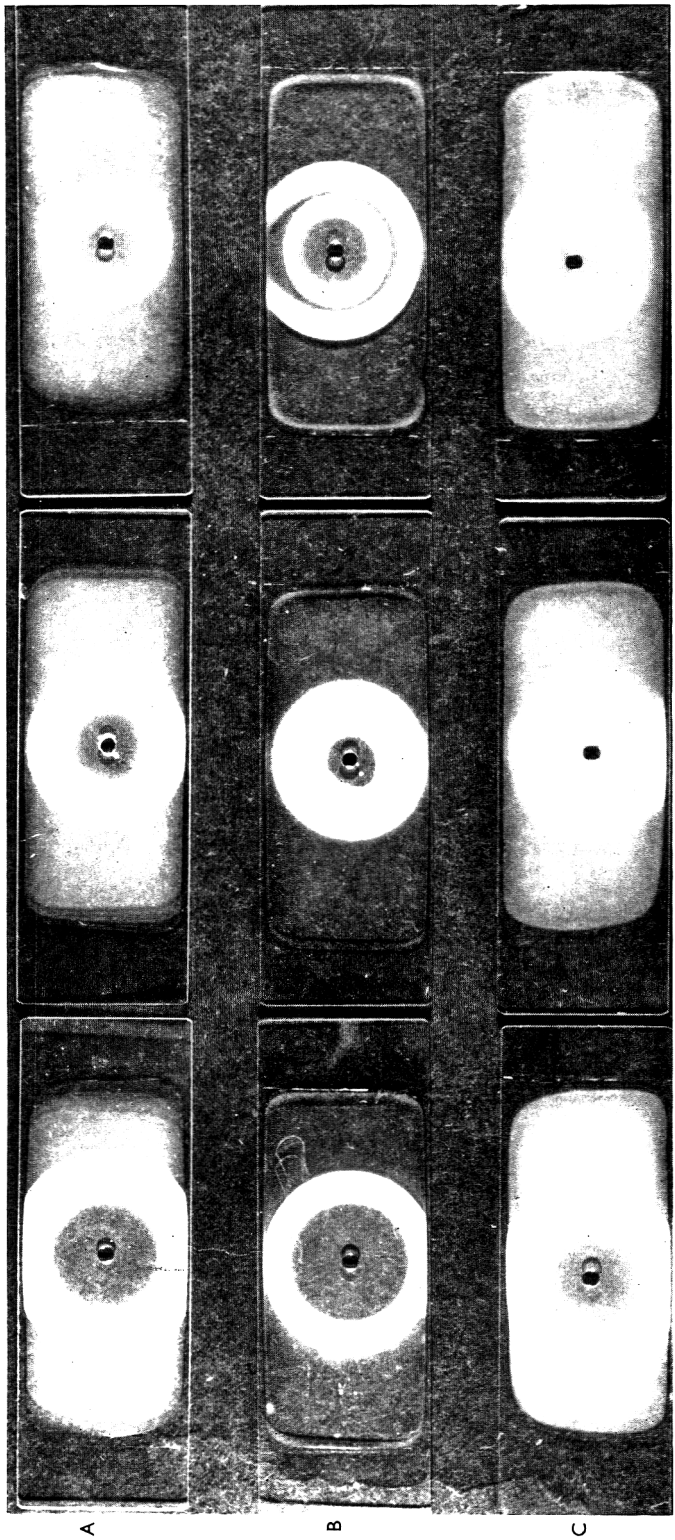
Comparison of the thin-layer assay with other methods. All of the wide range of proteinases tested gave white zones on caseinate-agar slides. Concentrations of proteinases as low as 0·1 μg/ml could be quantitatively estimated by this assay, which was for subtilisin and trypsin at least as sensitive, and for the other proteinases investigated more sensitive, than measurement by the Folin–Ciocalteu method of the extent of release of phenolic hydroxyl groups. For all proteinases the slide assay was considerably more sensitive than the standard milk clotting method of Berridge (1952). The enzyme preparations used in these studies were mostly impure and the minimum quantities of enzymes that could be detected by the 3 methods (Table 3) should be taken only as relative values.

It must be emphasized that when screening micro-organisms for potential milk clotting ability, the culture supernatants being examined normally contained only low concentrations of proteinase, and it was essential to use an assay that would detect these small amounts. The proteinase could then be concentrated by standard methods and partially purified by column chromatography. Here again, the ease with which the agar-caseinate assay could be carried out and the small size of the sample needed (0·003 ml) made it particularly useful for following the purification of proteinases by column procedures.

DISCUSSION

Investigations are being carried out in many countries to find suitable substitutes for calf rennet in cheese-making and the International Dairy Federation, I, Commission for Cheese (1967) have recently concluded (unpublished) that the need still exists for new methods for measuring rennet-like activity. Cheeseman (1963) reported a caseinate agar diffusion method but considered that the sensitivity for calf rennet was poor and did not compare favourably with the milk clotting technique. In the present study, however, it has been shown that Cheeseman's method can be simply modified to give an assay that is both sensitive and reproducible. It appears to be highly suitable for both the qualitative and quantitative study of rennets.

The leading edge of the outer white precipitation zone was shown by gel electrophoresis to be associated with the breakdown of κ-casein to para-κ-casein although some breakdown of α_{s1}- and β-caseins was also observed. In the transparent zone closest to the well, extensive degradation of the major caseins had occurred. The relative areas of the outer white and inner transparent zones give an indication of the ability of the proteinase not only to carry out the initial breakdown of the caseins to para-caseins but also to further degrade these products. Solutions containing low amounts of proteinase showed little or no ability to further degrade the white zone formed around the well. Enzymes producing large inner transparent zones, relative to the outer white zone, would be unlikely to make suitable milk clotting agents in cheese manufacture as they would further degrade the clot too readily. In this laboratory we have found it advantageous, when screening micro-organisms for rennet-like activity, to select proteinases which gave large white precipitates but comparatively small inner transparent zones on the slides.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The sensitivity of the agar-caseinate assay in comparison with others rests not only in the use of a thin layer of substrate but also in that it is the initial stages of casein degradation that are detected. The factors affecting the production of white zones appear to be very similar to those which influence the formation of a clot when proteinases are incubated with milk. In the same way, therefore, that all proteolytic enzymes are said to clot milk (Berridge, 1951), all proteinases of a wide range tested gave white zones on caseinate-agar slides. Concentrations of proteinases as low as 0·1 μg/ml could be quantitatively estimated by this assay which was at least as sensitive as standard proteinase assays and usually considerably more sensitive. One major advantage of the caseinate-agar diffusion assay was that the outer white zones deposited after casein degradation were obtained with equal readiness with proteinases with acid pH optima and with those with alkaline pH optima. This was particularly important when screening microbial culture fluids containing proteinases whose optimum pH values were unknown.

The difference in patterns of the white zones obtained with different proteinases appeared to be mainly due to the relative sensitivity to precipitation by calcium ions of the high molecular weight products formed in the initial stages of the caseinate breakdown. Some proteinases formed 2 or more zones on the unbuffered substrate, but the zones tended to merge when the substrate was buffered below pH 5·4 or the calcium concentration in the substrate was increased. On the other hand, enzymes which produced only one white zone after 24-h incubation were found to give several distinct zones after incubation for 2–3 days. In general, animal proteinases gave 2 white zones of precipitation after 24-h incubation and microbial proteinases only one, but this was not invariably the case. For this reason caution is needed when applying the technique to distinguish animal from non-animal rennets by the number of zones produced on agar-caseinate (Ganguli & Bhalerao, 1965).

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EXPLANATION OF PLATE

The action of 1 % (w/v) subtilisin (1), Meito fungal rennet (2) and commercial calf rennet (3) on 1 % (w/v) κ-casein (A), 1 % (w/v) α_{s1}-casein plus 0·3 % κ-casein (B) and 2 % (w/v) whole casein (C). Slides were incubated for 18 h at 37 °C.

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Weed taints in dairy produce

I. *Lepidium* taint

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SUMMARY. A re-investigation has been made of the nature of the tainting substances present in butterfat possessing the weed taint which arises through the ingestion of *Lepidium* spp. by dairy cattle. Some 0·5 ppm of skatole were isolated from *Lepidium*-tainted butterfat, along with 0·3 ppm indole. Flavour evaluation tests demonstrated that the skatole was principally responsible for the flavour defect, somewhat modifying the conclusions arrived at by earlier investigators.

The presence of weed taints in dairy produce arising from the ingestion of certain annual winter weeds by dairy cattle is of considerable concern in some dairying districts of New Zealand and south-eastern Queensland and to a lesser extent in the Northern Rivers district of New South Wales (McDowall, Morton & McDowell 1947; Conochie, 1950; Major, 1960). In contrast to many fodder taints, these weed taints are not readily removed by the normal milk or cream processing techniques, being actually intensified in some instances (McDowall, McDowell, Morton, Singleton & O'Dea, 1951). The most troublesome of these weeds are the cruciferous plants *Coronopus didymus*, *Lepidium hyssopifolium*, *Lepidium bonariense* and *Rapistrum rugosum* (Conochie, 1950).

Little is known of the nature of the substances in the weeds which are responsible for the development of these weed taints (McDowall *et al.* 1947; Forss, 1951; Park, 1965, 1967) or of the tainting substances present in the affected dairy produce. The isolation and identification of the tainting substances is a task which could not be attempted previously because of the limitations in experimental techniques available to handle materials present in trace amounts only; thus, the concentration of benzyl mercaptan in butter necessary to impart a distinct *Coronopus*-like flavour is of the order of 1 part in 100 million of the butter (Forss, 1951).

The taint arising in milk and butter through the ingestion by dairy cattle of *Lepidium hyssopifolium* and related species, which are collectively known as peppercress or pepperwort, is usually described as possessing a 'faecal' odour (Conochie, 1950). Hussong & Quam (1943), in the U.S.A., and later Conochie (1953), in Australia, investigated the *Lepidium* taint in milk and butter and concluded that milk and butter possessing this taint were characterized by the presence of abnormally large

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amounts of indole and/or skatole. However, the non-specific nature of the spectrophotometric procedure employed by these workers for the identification of the indole and/or skatole (utilizing the reaction of pyrrollic or indolic compounds, unsubstituted at the 2 or 3 position, with para-dimethyl amino benzaldehyde (Feigl, 1960; Manske, 1939) cannot be considered today as constituting an adequate proof for these conclusions. Neither indole nor other indoligenic substances were found in significant amount in *Lepidium hyssopifolium* or related species (Conochie, 1953; Park, 1962) and it was concluded by Conochie (1953) that an abnormal concentration of such substances might arise through a breakdown in the normal indole detoxication mechanism of the liver. Another feature of these species of *Lepidium* is the occurrence of significant amounts of the mustard oil glucoside glucotropaeolin in the seed-bearing portions of *Lepidium hyssopifolium* (Park, 1967), as this same component is held responsible for the development of the *Coronopus* taint in dairy produce, through its enzymically catalysed breakdown to benzyl thiocyanate (Park, 1965).

This present investigation is concerned with the isolation and identification of the *Lepidium*-tainting substances from butterfat possessing this flavour defect.

EXPERIMENTAL

Two 56-lb boxes of butter of Pastry quality (84 points) of Mundubbera, Queensland manufacture (1966) which were graded as possessing a strong *Lepidium* weed taint, were melted at 50–55 °C in a batchwise operation. The resultant butterfat was separated from the serum by decantation, followed by passage through a layer of anhydrous sodium sulphate, to give a clear butterfat graded as possessing a strong weed taint.

High-vacuum distillation

Batches (5 kg) of the weed-tainted butterfat were given a preliminary degassing at 10^{-2} torr and 45–50 °C to remove dissolved gases and traces of water. The butterfat was then distilled at 50–55 °C in a cold finger molecular still as used by Forss & Holloway (1967) for from 18 to 24 h to an ultimate pressure of from 2×10^{-6} to 2×10^{-7} torr (Penning gauge). The cold finger was cooled with liquid nitrogen and the butterfat stirred by means of a magnetic stirrer. The distillate (about 1 g) was evaporated into the first cold trap by warming the cold finger to 35–45 °C and the distillate was then washed out of the trap with 15 ml of re-distilled acetone. The combined acetone solutions of the distillates were concentrated to 2 ml by careful evaporation of the solvent through a 110 × 12 mm column packed with Dixon rings. The concentrated extract was then examined by gas chromatography with 5 ft × $\frac{1}{8}$ in. stainless steel columns containing either 5 % of SE-30 or 20 % of QF 1 stationary phases on silanized acid-washed Chromosorb W (60–80 mesh) support at temperatures of 145 or 180 °C, with flame ionization and electron capture detectors measuring the response from equal quantities of column effluent.

Removal of sulphur compounds

The acetone extract was mixed with 2 ml of distilled ethanol, 3 ml of a 20 % solution of lead acetate were added and the mixture was heated on a steam-bath for 15 min, with intermittent shaking. The cooled mixture was partitioned between 10 ml

of *n*-hexane and 5 ml water and the resultant *n*-hexane layer concentrated to about 2 ml prior to gas chromatographic examination.

The aqueous layer was mixed with 6 ml 6 N-hydrochloric acid, heated on a steam-bath for 10 min and extracted with 15 ml *n*-hexane. The *n*-hexane extract was dried over anhydrous sodium sulphate and concentrated to about 2 ml before gas chromatographic examination.

Chromatography on alumina

The *n*-hexane extract of the sulphur-free distillate material (c. 2 ml) was applied to a 150 × 20 mm column of alumina (B.D.H. Ltd, Chromatography grade) and then developed with *n*-hexane (100 ml), *n*-hexane-benzene (1:1, 100 ml), and benzene (100 ml). Fractions (10 ml) of eluate were collected and carefully concentrated to 1–2 ml volume and then examined in the gas chromatograph.

Fractions 27 and 28 were combined, the solvent removed under reduced pressure and the pale-yellow crystalline residue examined as a 1 % solution in carbon disulphide and carbon tetrachloride in a 1-mm cell in a Unicam SP-100 infrared spectrophotometer. The colourless crystalline material in fraction 30 was likewise examined in the infrared spectrophotometer.

RESULTS AND DISCUSSION

The distillate from the weed-tainted butterfat possessed a distinct weedy aroma, whereas the remaining butterfat was almost free of the weedy flavour, indicating a fairly complete removal of the flavouring materials.

The gas chromatogram of the acetone extract of the distillate (Fig. 1) revealed its complex nature. The electron capture detector was employed in this investigation in addition to the flame ionization detector, since this detector is very sensitive to sulphur-containing compounds while quite insensitive to many of the other likely constituents of the distillate. In this instance the electron-capture detector revealed, by retention time comparison, that the distillate did not contain any significant amounts of the sulphur compounds thought likely to be found, e.g. benzyl mercaptan or dibenzyl disulphide. This was confirmed by the gas chromatographic examination of the lead acetate-soluble material, only traces of the components of the lead acetate-insoluble fraction being present. As shown in Fig. 1, two of the main components of the extract correspond in retention behaviour with indole and skatole.

Alumina chromatography

A pilot experiment with a synthetic mixture of indole and skatole indicated that an almost complete separation of these related components could be obtained by chromatography on alumina, elution being effected with *n*-hexane-benzene mixtures. This technique was successfully applied to the *n*-hexane extract of the sulphur-free material from the butterfat distillate. The progress of the chromatographic separation was monitored by use of the gas chromatograph. Thus, fractions 27–29 were found to contain the component suspected of being skatole and fractions 29 and 30 also contained the indole-like component as the principal constituent.

The infrared spectrum of a 1 % solution of the combined crystalline material from fractions 27 and 28 in both carbon disulphide and carbon tetrachloride was found to be indistinguishable from that of an authentic solution of skatole under the same

conditions. Strong bands appeared at 740, 790, 1015, 1078, 1088, 1230, 1250, 1338, 1358, and 3465 cm^{-1} as well as the characteristic aromatic overtone and combination bands at 1718, 1761, 1792, 1840, 1880 and 1918 cm^{-1}.

Similarly, a 1 % carbon disulphide solution of the crystalline material from fraction 30 showed strong bands at 722, 745, 768, 899, 1092, 1250, 1280, 1336, 1353 and 3470 cm^{-1} with aromatic bands at 1710, 1765, 1798, 1846, 1883 and 1920 cm^{-1}. The spectrum was almost identical with that from authentic indole, the few slight additional absorption bands being attributable to the small amount of skatole shown to be present in the fraction by gas chromatography.

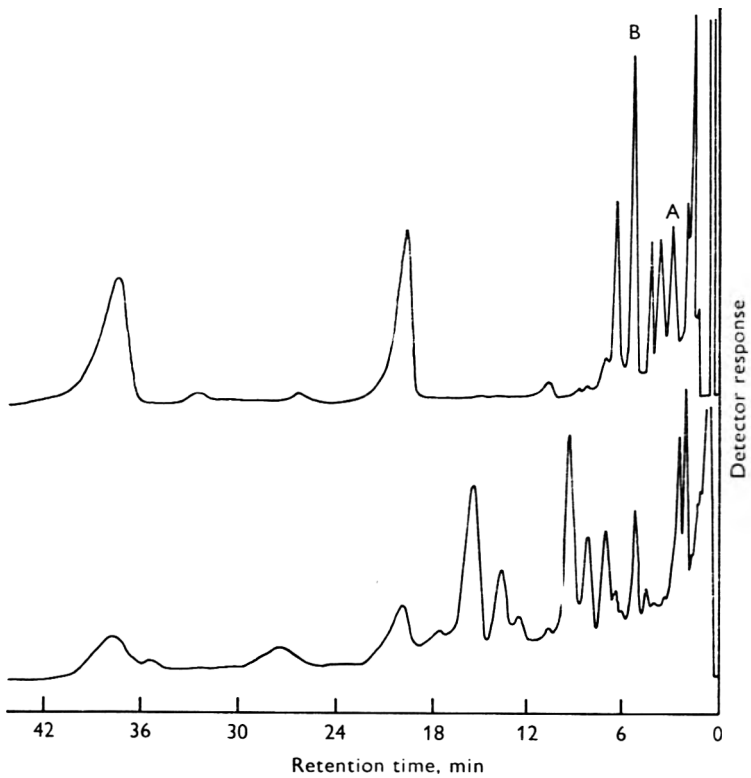

Fig. 1. Gas chromatogram of butter distillate extract on SE-30 stationary phase at 145 °C. The upper trace is the response from the flame ionization detector and the lower trace the equivalent response from the electron-capture detector. Positions marked A and B are, respectively, the retention times of authentic indole and skatole.

The other more volatile components of the distillate extract eluted in fractions 1 and 2 and were presumed to be carbonyl compounds. The remaining 2 major components of the distillate extract, of retention times 19 and 37 min (Fig. 1) did not elute from alumina. However, chromatography on silica gel indicated that these components were lactones, possessing a sweet, peach-like odour and showing strong absorption at 1745 cm^{-1} in the infrared.

These findings provided unambiguous evidence that skatole and indole were present in butterfat possessing the *Lepidium* weed taint. However, Conochie (1953) obtained evidence to indicate that indole and/or skatole were constituents of taint-free butterfat at a level of 0·05 ppm and this has been substantiated in recent

investigations (Urbach, 1966). The *Lepidium*-tainted butter examined here yielded about 18 mg of skatole and 12 mg of indole. This corresponds to 0·5 ppm of skatole and 0·3 ppm of indole in the butterfat, with the actual concentrations in the tainted butterfat being somewhat higher, as losses would inevitably occur during the isolation processes.

These results fall within the limits of tainting concentrations proposed for the *Lepidium* taint by Conochie (1953) of 0·95–5·1 ppm indole and/or skatole. However, it was considered desirable to confirm this by comparison of artificial mixtures of indole and skatole in taint-free butterfat with that of the natural tainted butterfat. It was thus shown that an artificial mixture of 0·5 plus 0·3 or 1·0 plus 0·6 ppm of skatole and indole, respectively, in taint-free choice quality butterfat was indistinguishable in flavour character from the natural taint. The intensity of the taint in the natural butterfat was very similar to that containing 1 and 0·6 ppm added skatole and indole, respectively. Furthermore, when 1 ppm skatole or 0·6 ppm indole in taint-free butterfat were compared with the natural taint it was found that 1 ppm of added skatole imparted a *Lepidium*-like taint to the butterfat indistinguishable from that of the mixture of the 2 components. In contrast, 0·6 ppm added indole in butterfat was described as having no distinctive flavour.

This investigation has confirmed but somewhat modified the findings of earlier investigators by the isolation of abnormally high amounts of skatole and indole from butterfat characterized as possessing the *Lepidium* weed taint and the demonstration that a high concentration of skatole is apparently responsible for the flavour defect, with indole playing a minor role at the most.

This investigation was part of a study of the chemistry of weed taints in dairy produce financed by the Australian Dairy Industry Research Funds administered by the Australian Dairy Produce Board. The author thanks Dr D. A. Forss, late of the Dairy Research Division, C.S.I.R.O., for the provision of details of the cold-finger molecular still; the dairy produce graders of the Queensland Department of Primary Industries for their expert opinions; Mr T. J. Beckmann of the Agricultural Chemical Laboratory of the Queensland Department of Primary Industries for the measurement of infrared spectra; and Mr Y. Varabioff for technical assistance.

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Weed taints in dairy produce

II. *Coronopus* or land cress taint in milk

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Summary. Unheated *Coronopus*-tainted milk yielded butterfat from which benzyl methyl sulphide, benzyl isothiocyanate, benzyl cyanide, indole and skatole were isolated by high-vacuum distillation. The first 3 of these compounds were also isolated from the corresponding skim-milk, buttermilk and butter serum by vacuum steam distillation: identification was by gas chromatography and mass spectrometry.

The flavour threshold of benzyl methyl sulphide in milk and butter oil was found to be respectively, 1 part in 10^8 and 1 part in 10^7, whereas the amount isolated from butterfat was approximately 1 part in 10^6.

This evidence, together with the similarity in flavour of the *Coronopus* taint in milk and butter oil with that of untainted milk and butter oil to which had been added respectively, 1 part in 10^7 and 1 part in 10^6 of benzyl methyl sulphide, demonstrates that benzyl methyl sulphide is a principal contributor to the flavour defect. A hypothesis is proposed to explain the origin of the sulphide and related components in the milk. The possibility remains that in commercial weed-tainted butter, which is made from heated cream, other compounds may be present.

The *Coronopus* or land cress taint in dairy produce, characterized by a biting taste and sharp odour in milk and a burnt flavour in butter, arises through the ingestion by dairy cattle of the cruciferous annual weed *Coronopus didymus* Sm. (Conochie, 1950; McDowall, Morton & McDowell, 1947). This taint is of considerable economic concern to the dairying industries of Australia and New Zealand, particularly in southern Queensland where the weed is widespread in dairy pastures during late winter and spring (Armitt, 1968).

Unlike many fodder or weed taints, the *Coronopus* taint cannot be removed from milk during pasteurization or from cream for buttermaking by vacuum steam-pasteurization in a vacreator or similar cream-treatment unit (McDowall, McDowell, Morton, Singleton & O'Dea, 1951), although some success has been achieved by the

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use of higher temperatures in another cream-treatment unit (Major, 1966). The *Coronopus* taint in cream is actually accentuated by the heat treatment applied during vacreation (McDowall *et al.* 1951).

Forss (1951) reported that when benzyl mercaptan, isolated from steam-distilled *C. didymus* plants, was added to taint-free butter at concentrations as low as 1 part in 10^9 the resultant flavour was very similar to that of the *Coronopus* taint in butter. It was assumed that the benzyl mercaptan originated as a product of the breakdown of glucotropaeolin, the constituent mustard oil glucoside of the weed. Forss was, however, unable to isolate benzyl mercaptan from tainted butter oil or to show what caused the taint to develop. Park (1965), in a study of the enzymically catalysed breakdown of glucotropaeolin in *C. didymus*, found that moistened, crushed seeds of *C. didymus* gave rise to benzyl isothiocyanate as a primary product of the breakdown of the glucoside, which isomerized to benzyl thiocyanate within a few minutes, presumably through enzymic action. Furthermore, it was found that when a benzyl thiocyanate preparation was administered to a cow by oral dosing a strong *Coronopus*-like taint developed in the milk within 2 h. The addition of 1 part of benzyl thiocyanate in 10^7 of milk produced a taint similar to the odour associated with the weed.

This paper describes the isolation and identification of several substances from unheated *Coronopus*-tainted milk by vacuum distillation, gas chromatography and mass spectrometry, and evaluation of their contribution to the weed taint, followed by a brief speculation as to their origin.

EXPERIMENTAL

An 8 acre pasture of young lucerne (*Medicago sativa* L.) at Boonah, South-East Queensland, was found to be very heavily infested with *C. didymus* (about one half of the total foliage). A herd of 40 dairy cows of mixed Jersey and Australian Illawarra Shorthorn breeding strip-grazed this pasture for 6-8 h/day for 10 days, the grazing mainly being carried out between the a.m. and p.m. milkings. During this period the milk possessed a very strong *Coronopus* taint. The milk obtained from the p.m. milkings on the fifth, sixth and eighth days of grazing was separated and part of the skim-milk (90 gal) was used for vacuum steam distillation. The resultant cream was stored at 5 °C and conditioned overnight at 13 °C prior to churning, 10 days after feeding began. The buttermilk (4·5 gal) was used for vacuum steam-distillation, while the butter was stored at 0 °C for 10 days, melted at 50–55 °C bath temperature and filtered through a bed of anhydrous sodium sulphate, to give a clear butter oil which was stored at −10 °C until required for high-vacuum distillation. The butter serum also obtained was stored at 0 °C overnight prior to vacuum steam-distillation.

The skim-milk was distilled in the Luwa turbulent thin film evaporator described by Leverington & Morgan (1964, 1967) operating at a pressure of 15 torr and a feed temperature of about 10 °C. The distillate (18 gal) was recycled through the evaporator under the same conditions and this distillate (10 l) cycled twice through a cyclone evaporator similar in design to that described by Lindsay, Day & Sandine (1965) at a pressure of about 15 torr to give a final distillate volume of 200 ml. This distillate

was then further concentrated by re-distillation at 5 torr through a vertical condenser held at 0 °C until 50 ml of distillate was collected in CO_2-ethanol traps and the 50 ml re-distilled until about 10 ml distillate was collected. The final distillate was extracted with a small volume (2–3 ml) of re-distilled hexane, after the addition of 10 ml of saturated ammonium sulphate solution. Control experiments demonstrated that the solvent did not yield any detectable contaminants.

The buttermilk was passed through the cyclone evaporator in the manner described above, the distillate recycled, followed by further concentration of the distillate through the 0 °C vertical condenser at 5 torr, to give a final distillate volume of 10 ml. This distillate was extracted with 2–3 ml re-distilled hexane.

The butter serum was likewise passed through the cyclone evaporator and the distillate through the 0 °C vertical condenser at 5 torr, to give a final distillate volume of about 10 ml. This distillate was also extracted with 2–3 ml hexane.

The butter oil distillation was carried out in a cold finger, high-vacuum distillation apparatus similar to that described by Forss, Stark & Urbach (1967). The butter oil (3-kg batches) was degassed initially in a feed flask fitted with a degassing tube and a Springham high-vacuum diaphragm valve. The distillate was allowed to flow down through 2 Davies condensers at a controlled rate to allow optimal degassing and collected in a 2-l reservoir. Two liquid nitrogen cooled traps were used to collect condensible material from these preliminary degassing stages, but the traps were found to contain water as the principal component with no evidence for the presence of components related to the flavour defect under investigation (Forss *et al.* 1967). The final pressure in the reservoir was about 10^{-2} torr (Pirani gauge). The butter oil was then allowed to flow into a 10-l flask at a slow rate, so that the pressure in the flask did not rise above 5×10^{-3} torr (Penning gauge). The flask was heated at 50 °C in a bath while the charge was magnetically stirred. Degassing was continued for three 8-h periods and the cold finger was filled with liquid nitrogen after the pressure had dropped below 5×10^{-4} torr, while 2 additional liquid nitrogen traps were employed to condense any material not collected on the cold finger. A final pressure of less than 2×10^{-5} torr was obtained. The cold finger was removed at the end of each 8-h period, the liquid nitrogen removed and the distillate washed from the finger with re-distilled acetone. The contents of the liquid nitrogen traps were also washed out with acetone and combined with the washings from the cold finger. The solvent was carefully removed from the combined acetone washings by distillation at reduced pressure through a short column ($12 \times 0{\cdot}8$ cm) filled with Fenske glass helices. The residue was then partitioned between redistilled hexane (10 ml) and saturated ammonium sulphate solution (5 ml). The components of interest passed into the hexane layer. The acids were removed from the hexane extract by washing with sodium bicarbonate solution.

The hexane extracts of the vacuum distillates of the skim-milk, buttermilk and butter serum and the acid-free butter oil distillate were examined in a gas chromatograph equipped with 1:1 exit stream splitter and a dual channel detector system (Oaks, Hartmann & Dimick, 1964). The detectors employed were a flame ionization and a concentric tritium-foil electron capture detector, the response from each detector being amplified in separate channels of a dual channel electrometer and recorded in a side by side, dual-pen, 1 mV recorder. Three $\frac{1}{8}$-in. columns were

employed; one of 5 ft, containing 20 % QF-1 on 60–80 mesh Chromosorb W-acid washed, was used isothermally at 125, 150 and 185 °C; another of 5 ft, containing 5 % SE-30 on 60–80 mesh Chromosorb W-acid washed, was operated isothermally at 100 °C and the third of 10 ft, containing 10 % Apiezon L on 60–80 mesh Chromosorb W-acid washed, operated isothermally at 150 °C. The detector oven temperature was standardized at 175 °C and flow rates of 15–20 ml/min of oxygen-free nitrogen were employed.

A portion of the hexane extract of the butter oil distillate was chromatographed on chromatographic grade alumina in a column $8 \times \frac{3}{4}$ in., 10 ml-fractions being collected by use of a fraction collector. Hexane, hexane-benzene (4:1) and benzene (200 ml each) were used as eluants. The solvent was evaporated from the fractions and the volume made up to $\frac{1}{2}$ ml with hexane prior to examination in the gas chromatograph.

The distillate extracts and the alumina chromatography fractions of interest were analysed by combined gas chromatography and mass spectrometry (Stark, Smith & Forss, 1967) on a 4·5 ft $\times \frac{1}{8}$ in. column containing 20 % QF-1 on 100–140 mesh Gas Chrom A, programmed from 100 to 150 °C at 4 °C/min, held at 150 ° for 7·5 min and the programme continued to 180 °C at 4 °C/min, with a flow rate of 15 ml/min, of which 4 ml went to the mass spectrometer. Authentic compounds were treated in the same way to obtain gas chromatographic retention data and reference mass spectra and the components of the extracts were taken to be identified when retention data and mass spectral data of the known and isolated compounds agreed.

Fresh taint-free raw milk or choice quality butter oil were used as vehicles for flavour evaluation experiments and assessments were carried out by dairy produce graders of the Queensland Department of Primary Industries, or for the determination of average flavour threshold, by non-expert laboratory personnel.

RESULTS

Characterization of distillate components

The skim-milk distillate extract possessed a strong odour which, in small amounts, was strongly reminiscent of that of a strong *Coronopus* taint in milk. Figure 1 shows a typical gas chromatogram with the dual detector system of flame ionization and electron-capture detectors obtained from the skim-milk distillate. The 3 major components of the distillate were, in order of elution from QF-1, benzyl methyl sulphide, phenyl acetonitrile (benzyl cyanide) and benzyl isothiocyanate, as established by mass spectral and retention data. Approximate concentrations of the components in the skim-milk, assuming 100 % recovery, were respectively, 1, 8 and 0·5 parts in 10^8, by quantitation from gas chromatographic data. The mass spectrum of benzyl methyl sulphide differed significantly from the published spectra of its isomers the 3 toluyl methyl sulphides and phenyl ethyl sulphide (Bowie, Lawesson, Madson, Schroll & Williams, 1966).

The ratio of the response from the electron-capture detector to that obtained from the flame ionization detector, as described by Oaks *et al.* (1964) (for different classes of compounds) has proved quite a useful parameter in this investigation, due to the wide range of responses of the components in the electron-capture detector. The

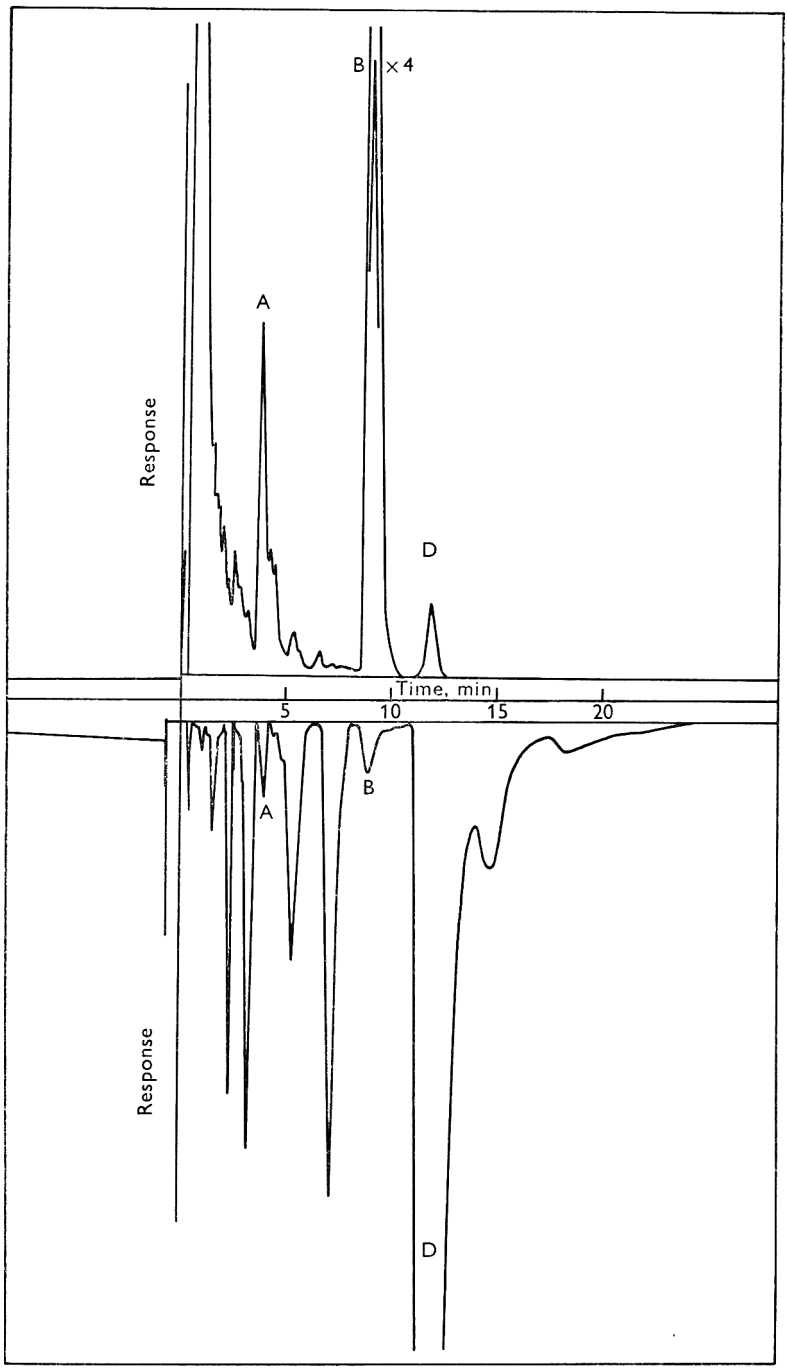

Fig. 1. Dual channel gas chromatogram of skim-milk distillate extract from *Coronopus*-tainted milk on QF-1 stationary phase at 150 °C. Top trace is the response from a flame ionization detector and the lower trace is from an electron-capture detector. A, benzyl methyl sulphide; B, benzyl cyanide; D, benzyl isothiocyanate.

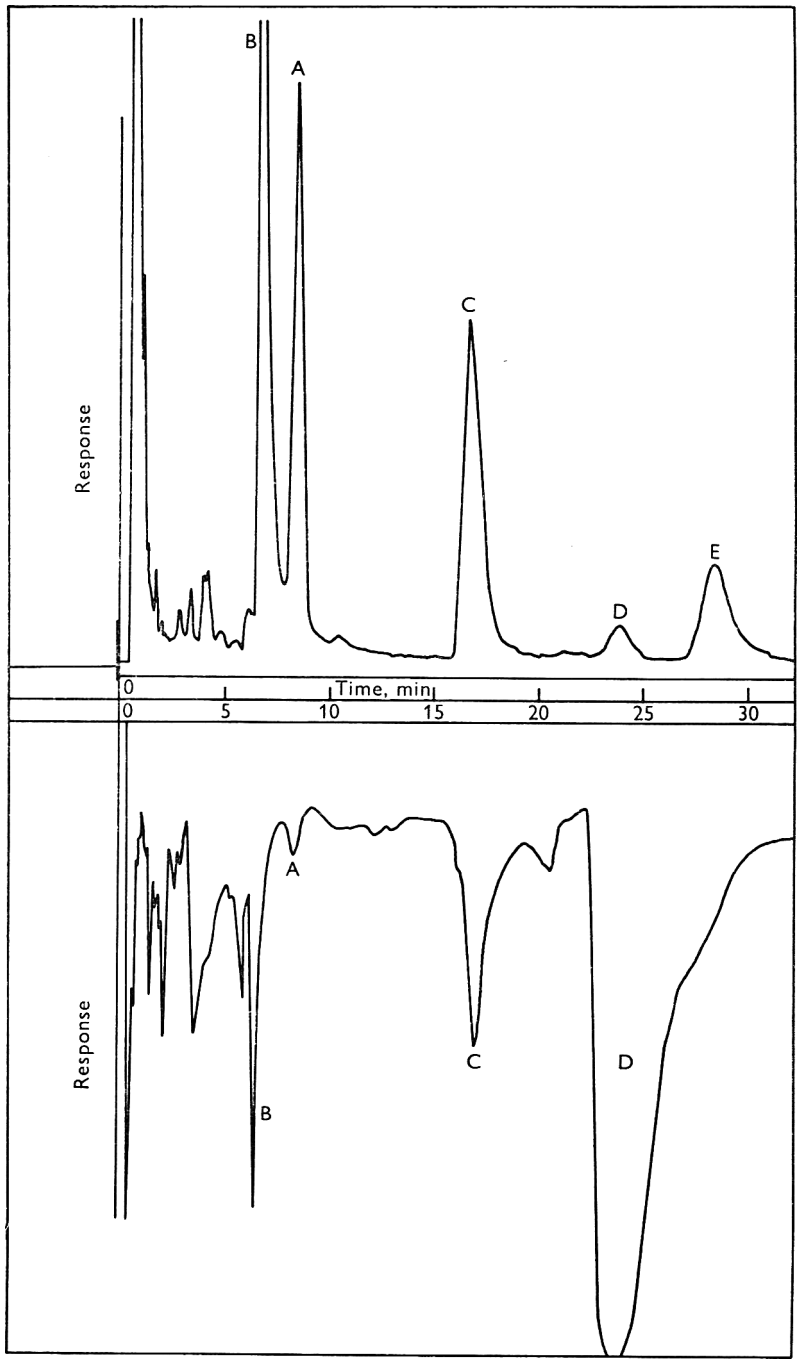

Fig. 2. Dual channel gas chromatogram of butter oil distillate, non-acidic extract from *Coronopus*-tainted milk on SE-30 stationary phase at 100 °C. Top trace is from a flame ionization detector at a signal sensitivity level 10 times higher than in the lower trace from an electron-capture detector: A, benzyl methyl sulphide; B, benzyl cyanide; C, indole; D, benzyl isothiocyanate; E, skatole.

electron-capture detector response of benzyl isothiocyanate is high, being sensitive to less than 10^{-9} g, with a response ratio of 1000 in favour of the E.C. detector, whereas the response ratios of benzyl methyl sulphide (14), benzyl cyanide (3), indole (0·5) and skatole (1·0) were considerably lower.

The hexane extracts of the buttermilk and butter serum distillates possessed odours similar to that of the skim-milk distillate extract and contained the same 3 components, as shown by gas chromatography.

The butter oil distillate obtained on the cold finger had a strong 'weedy' odour which was equally evident in the hexane extract of the non-acidic material. Figure 2 shows the relevant part of a dual channel gas chromatogram of the butter oil distillate non-acidic extract on SE-30 stationary phase. The components were identified as benzyl methyl sulphide (A), benzyl cyanide (B), indole (C), benzyl isothiocyanate (D), and skatole (E). The relative concentrations in the oil, calculated by quantitation of the gas chromatographic data, were respectively, 1, 3, 0·6, 0·2 and 0·25 parts in 10^6.

Chromatography of the butter oil distillate extract on alumina effected a partial fractionation of the distillate components, as shown by gas chromatographic and mass spectral examination of the fractions obtained. Fractions 1–6 were found to contain hydrocarbons, principally C15–C18 and containing up to two double bonds; fractions 9 and 10 contained the benzyl methyl sulphide and benzyl isothiocyanate and fractions 35–38 contained benzyl cyanide, indole and skatole.

The odour and flavour of the distilled butter oil showed a considerable diminution in intensity of the off-flavour, but the flavour defect was still detectable, indicating incomplete distillation. However, the added recovery obtained by distillation for twice the normal period of 24 h was considered insufficient to justify the additional time for isolation.

Flavour evaluation experiments

Solutions of authentic benzyl methyl sulphide, benzyl cyanide and benzyl isothiocyanate were prepared in milk at levels of 1 part in 10^5, 10^6, 10^7 and 10^8 and were evaluated by the graders. Benzyl cyanide was readily detectable at 1 part in 10^5, but barely in 1 in 10^6 and not at lower concentrations. The taint was described as being 'nutty' or 'marzipan-like' in character and in no way resembling the *Coronopus* taint. Benzyl isothiocyanate gave a distinct flavour at 1 part in 10^6 and a 'very slight' taint at 1 part in 10^7 which was described as 'artificial' or 'burning' and quite unlike *Coronopus* taint.

Benzyl methyl sulphide gave a slight but definite taint at 1 part in 10^8 described as 'very similar' to a slight *Coronopus* taint and at 1 part in 10^7 described as 'a strong Coronopus-like taint' by all graders. The determination of average flavour threshold value for benzyl methyl sulphide in milk was based on the method employed by Patton & Josephson (1957). The average flavour threshold found was 1 part in 10^7 of milk.

Solutions of the above compounds in butter oil were evaluated by the graders. Benzyl cyanide and benzyl isothiocyanate gave slight taints at 1 part in 10^5, described as quite unlike the *Coronopus* taint in butter oil, although reminiscent of the odour associated with the weed. Benzyl methyl sulphide gave a definite but slight taint 'indistinguishable' from a slight *Coronopus* taint at 2 parts in 10^7 in the graders'

opinions and at 1 in 10^6 was 'very like' a strong *Coronopus* taint in butter oil, such as the oil used for the isolation of the tainting components described above.

DISCUSSION

In the present investigation, we have isolated benzyl methyl sulphide, benzyl cyanide and benzyl isothiocyanate from skim-milk, buttermilk, butter serum and butter oil derived from a common bulk of milk possessing a strong *Coronopus* weed taint. These components have not been reported to occur in dairy products examined previously. The indole and skatole, on the other hand, which were isolated from the tainted butter oil, have been found to occur as normal constituents of Australian butters (Urbach, 1964–5, 1966) as well as being associated with the *Lepidium* weed taint in butter (Conochie, 1953; Park, 1969).

The flavour evaluation tests carried out on the novel constituents isolated here have demonstrated that benzyl methyl sulphide is present at a level above its average flavour threshold in milk and that it contributes most significantly to the *Coronopus* taint in both the unheated milk and the unheated butter oil studied in these experiments. On the other hand, these tests have shown that benzyl isothiocyanate and benzyl cyanide occur near or below their flavour threshold values in the milk and are unlikely to contribute directly to the taint to any significant extent. However, the possibility cannot be entirely excluded that one or other of these components might add some character to the over-all flavour by synergistic or other action.

The total concentration of indole and skatole isolated here at 0·85 parts in 10^6 butter oil is slightly below that proposed by Conochie (1953) as the lower limit for the production of a taint similar to that associated with the *Lepidium* weed taint. However, the level is well above those which Conochie found to be normal levels of these components in untainted butter at 0·05–0·3 ppm. It is also interesting to note that the relative concentration of indole to skatole found here favours indole, whereas skatole was found to be the major component in *Lepidium*-tainted butter examined by Park (1969). In the latter investigation, skatole was found to contribute much more to the taint than indole, the flavour threshold of skatole in butter oil being below 0·5 parts in 10^6. It therefore appears likely that the level of skatole found here, at 0·25 parts in 10^6, would be sufficient to contribute to the over-all taint to a slight extent only.

No evidence was found to indicate the presence of either benzyl thiocyanate or benzyl mercaptan in the *Coronopus*-tainted raw milk, even at concentrations below their respective flavour thresholds. However, it should be pointed out that quantities of 1–2 μg of benzyl mercaptan could not be recovered in the gas chromatography–mass spectrometry system. The present findings contrast with the findings in an earlier investigation (Park, 1965) that benzyl thiocyanate, a constituent of crushed moist *C. didymus* seeds, when administered to dairy cattle in a drenching preparation, imparts a taint to milk indistinguishable in flavour from the natural weed taint. Benzyl thiocyanate, when added to untainted milk at concentrations of 1 part in 10^7 produces a taint similar to the natural weed taint (Park, 1965) but, as has been shown here, the taint is more closely associated with the actual plant odour than the taint produced by benzyl methyl sulphide.

Benzyl isothiocyanate can break down chemically under certain conditions (for example, at about 100 °C in the presence of metal catalysts, as has been found in our laboratory in previous attempts at distillation through stainless steel fractionating column packing) to produce benzyl mercaptan or benzyl thiocyanate. However, laboratory experiments have indicated here that this does not occur in the presence of cysteine at temperatures of about 78 °C.

Benzyl cyanide and benzyl isothiocyanate are constituents of crushed, moistened *C. didymus* plants and their presence in milk could be attributed to absorption from the rumen into the blood and subsequent transfer into the milk. Benzyl isothiocyanate can also be derived from benzyl thiocyanate by isomerization (Bacon, 1961), such isomerization reactions being quite common among allylic thiocyanates. These can proceed as thermal isomerizations even without enzymic or chemical catalytic action, e.g. ^{1}-methyl-^{1}-phenyl ethyl thiocyanate is completely isomerized at 72 °C (Bacon, 1961). A completely thermal isomerization of benzyl thiocyanate is not likely to occur below 100 °C as benzyl thiocyanate can be steam-distilled in the laboratory without detectable change. On the other hand, it is conceivable that an isomerase could be available to convert the thiocyanate absorbed from the rumen to the isothiocyanate.

The presence of benzyl methyl sulphide in the tainted milk is harder to explain. Earlier investigators found no evidence to indicate the presence of the sulphide in *C. didymus* plants, nor has it been found to occur in extracts of crushed, moistened and incubated *C. didymus* seeds examined in this laboratory. We now propose that benzyl methyl sulphide may be a metabolite of benzyl thiocyanate, formed through a methylation of the thiocyanate or an intermediate metabolite such as benzyl mercaptan and taking place at an appropriate site in the body of the cow, such as the liver. Laboratory experiments have shown that benzyl thiocyanate reacts with such chemical methylating agents as dimethyl sulphate in alkali to produce benzyl methyl sulphide in fair yield. Alternatively, an enzyme such as S-adenosylmethionine:thiol S-methyltransferase (Enzyme Commission no. 2,1,1,d, Dixon & Webb, 1964) which acts on a wide range of methyl accepting substrates, including non-physiological sulphydryl compounds (Bremer & Greenberg, 1961) and which occurs in bovine liver, could catalyse the methylation reaction.

Another pathway for the production of benzyl methyl sulphide in the animal body could be the reduction of benzyl thiocyanate to benzyl mercaptan and the subsequent methylation of the mercaptan. Laboratory experiments have shown benzyl thiocyanate to be readily reduced to benzyl mercaptan by such reducing agents as cysteine.

For the results described here to be consistent with the earlier work of Forss (1951) regarding the likely role of benzyl mercaptan in *Coronopus*-tainted butter and with the reports by McDowall *et al.* (1947, 1951) of the effect of heat treatment on *Coronopus*-tainted cream, it is necessary to postulate that the nature of the tainting substances is altered during such heat treatment. In our work no evidence was obtained to indicate the presence of benzyl mercaptan in unheated milk even at levels well below that of the components isolated here. However, if for instance the benzyl isothiocyanate was converted to benzyl mercaptan by a reaction such as described above, the mercaptan would then be expected to contribute significantly to the total

flavour since its flavour threshold is of the order of 1 part in 10^9 of butter (Forss, 1951). It therefore remains to determine the nature of the tainting substances in heated dairy products. Such an investigation is planned.

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The induction by exogenous hormones of enzymes metabolising glucose 6-phosphate in the mammary gland of the pseudopregnant rabbit

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Summary. A study was made of the effects of the hormones, prolactin and cortisol acetate, on the activities in the mammary gland of the pseudopregnant rabbit of the enzymes concerned with lactose biosynthesis and glucose 6-phosphate metabolism, namely: UDP-glucose pyrophosphorylase, UDP-glucose-4′-epimerase, phosphofructokinase, phosphoglucomutase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Significant increases in enzyme activities were observed in rabbits examined 3 or 4 days after receiving a mammary intraductal injection of prolactin. The activities were not significantly increased at examination 2 days after injection of prolactin. Rabbits receiving cortisol acetate each day for 3 days also showed no significant increases in mammary enzyme activity. However, when prolactin and cortisol acetate were given simultaneously over a period of 3 days, increased enzyme activities were found suggesting synergistic action of the hormones.

INTRODUCTION

The activities of several enzymes in the mammary glands of many species increase with the onset of lactation (Baldwin, 1966). It is thought that these increases are hormonally controlled. *In vitro* studies have shown that various hormones when administered to cultured ex-plants of the mammary glands of late pregnant mice (e.g. Lockwood, Turkington & Topper, 1966) and dogs previously treated with oestrogen and progesterone (Barnawell, 1967) induce lactogenesis in the gland. Similar mammary gland development has been observed with *in vivo* studies and many mammalian species have been brought into lactation by suitable hormone treatment. (For review see Cowie, 1961.)

To date, however, little information is available on the effects of hormones on enzyme systems in the mammary gland, although Thibodeau & Thayer (1967) observed an increase in mammary gland aspartate carbamoyl-transferase (E.C. 2.1.3.2) when growth hormone was administered to female rats previously treated with oestrogen and progesterone. On the other hand, Shatton, Gruenstein, Shay & Weinhouse (1965) were unable to detect any rise in the activities of UDP-glucose pyrophosphorylase (UDPG-PPase) and UDP-glucose-4′-epimerase (UDPG-

epimerase) when they treated rats with lactogenic hormones even though they found marked proliferative changes in the gland. Heitzman (1967, 1968*a*) reported that the subcutaneous administration of prolactin together with cortisol acetate for several days brought about increases in the activities of the enzymes UDPG-PPase and UDPG-epimerase in the mammary glands of pseudopregnant rabbits. The present communication describes a detailed study of the effects of prolactin and cortisol acetate on these 2 enzymes and also on the enzymes phosphofructokinase (PFK), phosphoglucomutase (PGM), glucose 6-phosphate dehydrogenase (G6PDH), and 6-phosphogluconate dehydrogenase (6PGlucDH).

The enzymes were chosen because of their importance in the biosynthesis of lactose and the metabolism of glucose 6-phosphate in the mammary gland. Glucose 6-phosphate is the starting-point of lactose synthesis and of the Embden–Meyerhof and pentose phosphate pathways. Jones (1967) reported that the activity of several of these enzymes in rat mammary tissue decreased after hypophysectomy or weaning. He suggested that these decreases were caused by a lack of prolactin in the target organ, the mammary gland.

A preliminary report of some of this work has already been published (Heitzman, 1968*b*).

MATERIALS AND METHODS

Animals and hormone treatment. Virgin New Zealand white rabbits were made pseudopregnant by an intravenous injection of human chorionic gonadotrophin (125 i.u.). After 14 days the rabbits were treated in 4 groups as follows:

Group 1. Anaesthetized with Nembutal and injected intraductally in the mammary gland with prolactin (5 i.u. in 0·3 ml of 0·85 % NaCl solution). Killed 2, 3 and 4 days after injection.

Group 2. As group 1 but also received cortisol acetate (5 mg in 0·4 ml of 0·85 % NaCl solution) subcutaneously daily for 3 days. Killed 3 days after first injection.

Group 3. Received cortisol acetate (5 mg in 0·4 ml of 0·85 % NaCl solution) subcutaneously daily for 3 days. Killed 3 days after first injection.

Group 4. Control group. Anaesthetized with Nembutal and injected intraductally with 0·3 ml of 0·85 % NaCl solution. Killed 2, 3 and 4 days after injection.

Enzyme assay procedure. The rabbits were killed by cervical dislocation. The mammary glands in the prolactin-treated rabbits showed local responses similar to that described by Bradley & Clarke (1956). Regions with obvious local mammary development were observed 3 and 4 days after the administration of prolactin. Two days after the administration of prolactin this development was not so well defined. These regions and the glands of animals treated with cortisol acetate and saline were carefully excised. All subsequent procedures were carried out at 0–4 °C. After removal of most of the fibrous tissue the glands were washed to remove excess milk in mannitol–sucrose–EDTA medium (0·25 M-mannitol, 0·027 M-sucrose, 1 mM-EDTA, pH 7·5). Homogenization for 90 s using 6 vols medium was carried out using a Potter–Elvehjem homogenizer. The homogenate was strained through 4 layers of sterilized gauze and a portion retained for DNA estimation. The remainder was centrifuged at 55 000 × *g* for 30 min in a Spinco Model L centrifuge. The clear supernatant was used within 4 h for all the enzyme estimations. Three replicate determinations were made for each enzyme.

Enzyme assays. All the enzymes are believed to occur wholly in the particle-free fraction of the cell homogenate.

UDPG-PPase and UDPG-epimerase were assayed essentially by the method of Shatton *et al.* (1965). PGM, G6PDH, 6PGlucDH and PFK were assayed as described by Jones (1967). All readings were made at 340 nm on a Unicam SP500 spectrophotometer with a Hilger–Gilford attachment for automatic recording. The assays were carried out at 25 °C. The incubation media were as follows:

Assay of UDPG-PPase (*UTP*: α-D-*glucose* 1-*phosphate uridylyltransferase E.C.* 2.7.7.9). Tris buffer of pH 8·9 (30 μmoles), UDP-glucose dehydrogenase (166 i.u.), NAD (0·5 μmole), UTP (1 μmole), $MgCl_2$ (2μmoles), glucose 1-phosphate (1 μmole) and tissue extract. Final volume 0·5 ml.

Assay of UDPG-4'-epimerase (*E.C.* 5.1.3.2). Tris buffer of pH 8·9 (30 μmoles), UDP-glucose dehydrogenase (166 i.u.), NAD (0·5 μmole), UDP-galactose (0·015 μmole) and tissue extract. Final volume 0·4 ml.

Assay of phosphoglucomutase (α-D-*glucose*-1, 6-*diphosphate*: α-D-*glucose*-1-*phosphate phosphotransferase E.C.* 2.7.5.1). Tris buffer of pH 7·4 (70 μmoles), NADP (0·2 μmole), glucose 1-phosphate (0·5 μmole), $MgCl_2$ (2 μmole) and tissue extract. Final volume 1 ml.

Assay of glucose 6-*phosphate dehydrogenase* (D-*glucose*-6-*phosphate*: *NADP oxidoreductase E.C.* 1.1.1.49). Tris buffer of pH 7·4 (75 μmoles), $NADP^+$ (0·2 μmole), glucose 6-phosphate (0·5 μmole) and tissue extract. Final volume 1 ml.

Assay of 6-*phosphogluconate dehydrogenase* (6-*phospho*-D-*gluconate*: *NADP oxidoreductase* (*decarboxylating*) *E.C.* 1.1.1.44). Tris buffer of 8·9 (65 μmoles), NADP (0·2 μmole), $MgCl_2$ (5 μmoles) 6-phosphogluconic acid (1 μmole) and tissue extract. Final volume 1 ml.

Assay of phosphofructokinase (*ATP*: D-*fructose*-6-*phosphate* 1-*phosphotransferase E.C.* 2.7.1.11). Tris buffer of pH 8·2 (40 μmoles), NADH (0·1 μmole), fructose 6-phosphate (4 μmoles), NH_4Cl (10 μmoles), $MgCl_2$ (1 μmole), ATP (0·2 μmole) and tissue extract. Final volume 1 ml. The rate was measured before and after addition of ATP.

DNA was estimated by the method of Schneider (1945) using the diphenylamine colour reaction of Dische (1955). Calf thymus DNA was used as the reference standard.

The probability values (*P*) were obtained by Student's '*t*' test. Hormones and substrates were obtained from Sigma Chemical Co. Ltd., or Boehringer Corporation Ltd.

RESULTS

The mammary enzyme activities expressed against unit wet weight of tissue are shown in Table 1, and against unit weight of DNA in Table 2. The amounts of DNA per unit weight of tissue are also given in Table 2.

The results show that when measured against wet weight of tissue all the enzyme activities were significantly increased 3 and 4 days after prolactin administration. A similar pattern was observed on a DNA basis, the only exceptions being 6PGlucDH 3 days after prolactin administration and PFK 3 and 4 days after administration. In these instances the observed mean activities were higher than the controls but not significantly so. The activities were not significantly increased (except G6PDH

and PGM which increased on the basis of wet weight) 2 days after prolactin administration, nor after treatment with cortisol acetate alone. When prolactin and cortisol acetate were administered together most of the activities increased compared with those for the rabbits which received only prolactin for 3 days. The activities of UDPG-PPase, PGM, PFK per unit wet weight of tissue and PFK per unit weight of DNA were significantly increased ($P < 0.05$). G6PDH and 6PGlucDH activities per unit weight of DNA were the same in both groups of animals. For the last-named enzyme this was probably because the 3 measurements made for the prolactin-treated rabbits were insufficient.

The glands of the saline-treated animals showed no visible difference at 2, 3 or 4 days after injection. The results for the individual enzymes were always similar and therefore the saline results have been pooled to give one mean value of activity per enzyme.

Table 1. *The activities of mammary gland enzymes in normal and hormonally stimulated pseudopregnant rabbits*

(Enzyme activities are expressed as mμmoles substrate utilized per min per g wet tissue ± S.E.M. The number of observations are given in parentheses. *P* values for the hormonally treated animals are given over saline controls.)

	Saline controls	Prolactin			Cortisol acetate for 3 days	Cortisol acetate + prolactin for 3 days
		For 2 days	For 3 days	For 4 days		
UDPG-PPase	61 ± 7 (15)	73 ± 8 (12)	124 ± 4 (6)	187 ± 18 (6)	83 ± 13 (6)	265 ± 36 (6)
P	—	N.S.	< 0·001	< 0·001	N.S.	< 0·001
UDPG-Epimerase	56 ± 9 (14)	69 ± 12 (12)	101 ± 15 (6)	138 ± 16 (6)	67 ± 10 (6)	257 ± 84 (6)
P	—	N.S.	< 0·02	< 0·001	N.S.	< 0·001
G6PDH	228 ± 43 (7)	488 ± 108 (11)	548 ± 82 (7)	980 ± 183 (9)	209 ± 35 (6)	771 ± 83 (8)
P	—	< 0·05	< 0·01	< 0·01	N.S.	< 0·01
6PGlucDH	156 ± 2 (3)	—	335 ± 50 (3)	418 ± 57 (6)	—	357 ± 47 (7)
P	—	—	< 0·05	< 0·02	—	< 0·05
PGM	43 ± 9 (9)	77 ± 13 (12)	104 ± 16 (8)	121 ± 22 (11)	58 ± 8 (6)	194 ± 22 (8)
P	—	< 0·05	< 0·01	< 0·001	N.S.	< 0·001
PFK	100 ± 18 (8)	125 ± 22 (11)	140 ± 18 (9)	234 ± 57 (8)	87 ± 10 (6)	395 ± 104 (8)
P	—	N.S.	< 0·05	< 0·05	N.S.	< 0·02

DISCUSSION

The results showed quite clearly that the administration of prolactin alone to the intact pseudopregnant rabbit was capable of inducing the synthesis of several enzymes concerned in the metabolism of glucose 6-phosphate and in the biosynthesis of lactose. In general, the increases in activities were not significant until 3 days after an intraductal injection of prolactin. Cortisol acetate has no measurable inductive effect. However, when administered with prolactin there was an increase in enzyme activities greater than that observed when prolactin was given alone. This suggests a synergistic mode of action for these 2 hormones as found by Meites, Hopkins & Talwalker (1963) in mammary glands of 15-day pregnant rabbits.

The animals in the present study had an intact endocrine system. Thus, the observed increases in activities for the rabbits treated with prolactin would result from a synergistic action with one or several of the endogenous hormones present in the mammary gland. It is also possible that cellular proliferation is an essential

Table 2. *Enzyme activities and DNA levels in normal and hormonally stimulated rabbit mammary tissue*

zyme activities are expressed as mμmoles substrate utilized per min per mg DNA ± S.E.M. The number of observations are given in parentheses. Details assays and treatment of rabbits are given in the Materials and Methods section. *P* values are given for the hormonally treated animals compared with line controls.)

		Prolactin				
	Saline controls	For 2 days	For 3 days	For 4 days	Cortisol acetate for 3 days	Cortisol acetate + prolactin for 3 days
UDPG-PPase	135 ± 41 (15)	129 ± 36 (12)	314 ± 50 (6)	543 ± 109 (6)	154 ± 45 (4)	385 ± 47 (6)
P	—	N.S.	< 0·05	< 0·001	N.S.	< 0·01
UDPG-Epimerase	90 ± 24 (13)	105 ± 24 (12)	255 ± 49 (6)	388 ± 74 (6)	118 ± 26 (4)	348 ± 95 (6)
P	—	N.S.	< 0·01	< 0·001	N.S.	< 0·01
G6PDH	495 ± 82 (7)	583 ± 84 (10)	1070 ± 227 (7)	1424 ± 300 (8)	320 ± 63 (4)	1062 ± 139 (8)
P	—	N.S.	< 0·05	< 0·02	N.S.	< 0·01
6PGlucDH	276 ± 69 (3)	—	845 ± 272 (3)	1222 ± 259 (6)	—	732 ± 97 (7)
P	—	—	N.S.	< 0·01	—	< 0·02
PGM	87 ± 16 (6)	149 ± 51 (12)	213 ± 56 (7)	217 ± 39 (8)	137 ± 25 (5)	289 ± 43 (6)
P	—	N.S.	< 0·05	< 0·02	N.S.	< 0·01
PFK	164 ± 45 (7)	150 ± 19 (10)	184 ± 30 (6)	259 ± 43 (6)	165 ± 33 (4)	660 ± 178 (8)
P	—	N.S.	N.S.	N.S.	N.S.	< 0·05
DNA (mg/g wet tissue)	0 67 ± 0 10 (10)	0 79 ± 0 10 (10)	0 70 ± 0 16 (8)	0 76 ± 0 09 (9)	0 59 ± 0 16 (5)	0·73 ± 0·07 (11)

prerequisite for prolactin to extend its action on mammary enzyme synthesis, but the current study provides no evidence on this point.

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With 1 *plate*

The use of high-temperature short-time scalding in continuous curd-making

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Summary. Experiments with continuously made curd leading to the use of a 4-min scalding period at 48 °C for Cheddar curd-making are described. The short time at 48 °C had only a slightly adverse effect on the starter, and the moisture content of the drained curd was within the range usually found in conventionally made curd at the same stage. The starter was allowed to grow in the drained curd during cheddaring. After 3–6 h the pH and moisture content were the same as those of normal cheddared curd.

The usual method of making Cheddar cheese includes a long period of scalding while the curd is stirred in the whey at an elevated temperature. A process for the continuous production of curd on the laboratory scale has been demonstrated (Berridge, 1968), but if it were to be scaled up a long period of scalding would diminish the advantages of continuous production. Experiments to determine the feasibility of shortening the scalding period by raising the temperature were therefore undertaken.

The defects arising from a too rapid increase of temperature are well known and were described in detail by Schulz (1951). Some years ago it was found to be possible to overcome these defects by acidifying the milk before renneting and then periodically neutralizing the whey (Berridge, 1963). The resulting curd, however, was too soft and moist for the manufacture of typical hard-pressed cheese. Curd made by the continuous process first described by Berridge (1968) is normally already at a high temperature when it leaves the apparatus and therefore problems such as those encountered in conventional curd making when an attempt is made to raise the temperature too rapidly need not be considered; there remains merely the problem of deciding when the curd should be cooled and to what temperature. The method permits the production of curd at high temperatures which would be lethal to starter organisms if maintained for more than a minute or two. Such temperatures, however, cause rapid syneresis and so it might be advantageous to produce curd at a high temperature and to cool it quickly. If too high a temperature were used, difficulties might arise from the exact time control that would be required to preserve the starter as well as from the differences between the heat resistance of different starters; and for this reason the experimental temperatures have not exceeded 50 °C.

METHODS AND MATERIALS

Curd-making

The design and operation of the curd-maker have already been described (Berridge, 1968), but it is convenient to repeat here a brief summary of the method. Heat-treated milk was adjusted to pH 6·2 and cooled to < 10 °C and 1 ml of rennet/gal of milk was added. The temperature was maintained at between 10 and 3 °C overnight and next day the milk was pumped through a 3-ft length of $\frac{1}{4}$-in. diam. Visking tubing surrounded with flowing 0·1 % phosphoric acid at 60 °C. The milk clotted as it passed along the tube. The resulting curd and the whey were led via a glass tube to plastic tubing which then facilitated a variety of further operations. Plate 1*a* shows 2 units of the apparatus. Plates 1*b* and *c* show curd emerging from the plastic tube into a beaker of clear water. (Normally the curd is collected in whey, see below.) The curd took the form of a cylinder about 5 mm diam. and of indefinite length and it was produced, together with the whey, at the rate of 80 ml total volume/min. In some experiments the temperature of the emerging curd was controlled between 36 and 50 °C, but normally the temperature was 44–46 °C. The curd was usually received into at least an equal volume of whey to facilitate the control of temperature. Thus, when curd from 400 ml of milk was required, 400 ml or more of whey was separated from recently formed curd and fresh curd was caused to flow into this whey for 5 min. Gentle stirring by hand was used to prevent the curd matting at the bottom of the receiver. A little experience was necessary as the curd was fragile at this stage; too vigorous mixing broke the curd into small pieces and produced turbidity in the whey. The whey for receiving the curd was normally obtained during the same run with the same milk during a short period just before the experiment itself began. The curds and whey were then subjected to the selected schedule of temperatures and times indicated in the section below on Experiments and Results.

Cold-renneted milk

Bulked single-herd milk, heat-treated at 71·6 °C (161 °F) for 17 sec and cooled to below 10 °C (< 50 °F) was normally used. It was adjusted to pH 6·25 ± 0·05 with dilute HCl, and 1 ml rennet/gal was added. Starter also was added at this stage. The mixture was then stored overnight in a cold room at 4 °C.

Starter

The starters were obtained from the National Collection of Dairy Organisms at the National Institute for Research in Dairying, Shinfield, Reading. The starter normally used was NCDO 1200, but occasionally NCDO 712 had to be used owing to the presence of 'phage' for NCDO 1200. The 'phage' was later controlled by more stringent hygienic measures, such as the use of alkaline detergents and hypochlorite rinse. On some occasions (indicated below) the starter was neutralized from time to time during its growth and when a diminution in the rate of acid production was observed, the neutralized culture was cooled and kept at 0 °C until it could be added to the milk. Experiments not reported here suggested that a more vigorous culture was obtained in this way. Lack of vigour, as shown by the rate of acid formation in

curd, was noticed occasionally in cultures not treated by neutralization, but as it was not possible to determine the vigour of the cultures accurately at the time, the value of neutralization awaits assessment.

Cheddaring technique

All cheddaring took place in an incubator at 30 °C. The curd was pressed with increasing weights in vertical tubes of increasing diameter, namely 3·4, 5·0 and 7·3 cm, with suitable bases and disks. The curd was transferred from one tube to the next after 30 and 60 min with a sufficient increase in weight to bring the pressure to approximately the same value after the curd had spread to the larger diameter. The schedule of times, diameters and pressures in Table 1 will make this clear.

The first tube would hold 100 g of curd, but it was more conveniently operated with 50–80 g. This amount of curd formed only a thin layer in the last tube, and so on some occasions the first tube was duplicated. Cheddaring was terminated either after 90 min or when the required pH had been reached (see Results). The curd was left in the largest tube under the heaviest weight during the extra period required to reach the designated pH.

Table 1. *Schedule of times and pressures for cheddaring*

Time from beginning of cheddaring, min ...	0–15	15–30	30–45	45–60	60–75	> 75
Tube diam., cm	3·4	3·4	5·0	5·0	7·3	7·3
Pressure, g/cm²	4·4	6·0	6·0	9·0	9·0	11·0

Determination of curd pH

A sample of the curd (4 g) was ground and mixed with 6 ml distilled water using a flat-ended glass rod in a small beaker. A combined glass and calomel dip type electrode (Radiometer) was used to determine the pH of the slurry. This technique does not give the true pH of the curd since a different dilution will give a different result, but providing the dilution is kept constant comparable figures can be obtained.

Determination of curd moisture

Curd samples were refrigerated in moisture-proof containers. A small amount of whey always drained out of the curd before refrigeration was complete and some condensation occurred on the walls of the containers. All the moisture was incorporated into the curd as thoroughly as possible by chopping and mixing before smaller samples were taken for moisture determination.

A metal dish containing about 10 g sand and a short piece of glass rod was dried for several hours at 98 °C. The minimum time needed for constant weight to be attained was 30 min, but the longer period was often convenient. About 5 g curd was weighed accurately into the cooled dish and ground up with the sand. The loss in weight at 98 °C was determined. The minimum time of heating to obtain a constant weight of dried curd was 4 h. However, it was often convenient to leave the curd in the oven overnight, and as no significant further loss in weight occurred this was often done (cf. British Standards Institution, 1963).

EXPERIMENTS AND RESULTS

The effect of the pH and temperature of the whey in the receiver on starter activity and curd quality

As pointed out on p. 53 curd can be produced at temperatures which are quickly lethal for the starter but high temperatures have the advantage of shortening the time for curd formation and syneresis. Under such conditions a rapid cooling of the curd might be significant in maintaining starter vigour. Therefore, curd was produced at 50 °C and cooled rapidly by receiving the curd into a large volume of whey at 3 °C. The whey for this experiment was produced at the Institute's Experimental Dairy during normal cheese-making. It was heated at 80 °C for 5 min. Curd-making was as already described except that 5·6 % of starter was used to obtain rapid acidification in those samples in which the lower temperatures allowed the starter bacteria to remain fully active.

About 5-min operation of the apparatus gave sufficient material for the test, and at the end of this time the mixture of curds and whey was warmed quickly to 30 °C. It was maintained at this temperature for 90 min with occasional stirring, sufficient to prevent the threads of curd from matting together. The curd was then drained and left in an incubator at 30 °C. Samples of whey for pH determination were obtained by manual pressure 3 and 4 h after curd formation.

As soon as the first sample of curd had begun its incubation in the whey at 30 °C a second sample was collected in whey at 10 °C and this followed the first sample, receiving identical treatment. Similarly, a third sample was collected in whey at 20 °C and treated in the same way. Thus, the effect of different cooling treatments could be assessed.

In conventional curd treatment much time is spent in achieving the correct acidity. Previous work had shown that when curd is in very thin strips its pH can readily be modified by the pH of the whey in which it is suspended. It might therefore save time if curd could be acidified by being collected in an acid whey. On the other hand, it is known that a soft curd is formed in normal cheese-making if the milk is too acid at the time of renneting, and some advantages were obtained when acid curds were scalded in neutralized whey as mentioned above (Berridge, 1963). It was therefore felt worthwhile to try both an acid and an alkaline whey. With this object in view the experiment was continued by repeating the making of 3 samples using different cooling temperatures but with whey at pH 4·0 and again with whey at pH 8·0. The first set of samples had been cooled in whey at the same pH as that of the milk, namely 6·2. As far as can be judged from the results (see Table 2) there was no advantage in cooling the whey below 20 °C nor in adjusting its pH value.

The effect of lower temperatures of curd-making on starter activity

In the previous experiment the curd was at 50 °C at the outlet from the apparatus, and there was a possibility that even the most rapid cooling had not sufficed to protect the starter from some damage due to heat. The curd production of the previous experiment was therefore continued without interruption but at lower temperatures, including the lowest at which curd could easily be made with this apparatus. Only small samples of curd were collected, with no whey other than that

which accompanied them. Each sample was incubated at 30 °C and its pH value was determined in the well-stirred mixture after 3 h. Curds and whey which had been produced at 42 °C gave a pH of 5·57, at 39 °C pH 5·60 and at 36 °C pH 5·62. This latter curd was soft but the other 2 were firm. An attempt was made to use the cold-renneted milk as a control by incubating a sample at 30 °C for 3 h, but reliable pH readings could not be obtained. Good curd was not, of course, produced. It was mushy and the whey was cloudy and greasy. The pH value of the curd seemed to be about 5·5 and of the whey about 5·6, but the difference was almost certainly due to the difficulty of using the glass electrode in such a mixture. All the pH values obtained in this experiment were slightly lower than the corresponding values in Table 2 (5·70) indicating that the possibility of a slightly deleterious effect at 50 °C remains.

Table 2. *Effect of pH and temperature of whey in the receiver on starter activity*

Cooling whey		pH Values of whey pressed from curd		
Temp. °C	pH	After 3 h	After 4 h	Remarks
3·5	6·2	5·70	5·35	Curd fused normally
10	6·2	5·70	5·35	
20	6·2	5·70	5·36	
3·5	4·0	5·35	5·20	Milky whey, soft curd did not fuse
10	4·0	5·37	5·25	
20	4·0	5·37	5·25	
3·0	8·0	5·92	5·45	Clear whey, curd fused normally
10	8·0	5·88	5·48	
20	8·0	5·88	5·48	

The effect of incubation of curds and whey before scalding

During conventional cheese-making the starter grows for a long time at temperatures not too far from the optimum, i.e. before the scald temperature reaches its maximum. It seemed possible therefore that a period at 30 °C before scalding might be beneficial.

Curd was made in the normal way. Starter was added to the cooled milk (the quantity was not recorded, but it was probably about 3 %). Three batches of curd were collected in cool whey (< 30 °C) for 10 min at intervals of 30 min, and then stored in an incubator at 30 °C for 90, 60 and 30 min, respectively, with gentle stirring every 10 min. Thus, 3 batches of curd which had been incubated for different lengths of time were ready simultaneously. A fourth batch was collected in whey at 38–40 °C and this received no incubation at 30 °C but was maintained at the scalding temperature of 38 °C. The first 3 batches were brought to 38 °C by the addition of whey at 50 °C. Two workers processed these curds so that the scalding of all 4 batches could be begun, and ended, almost simultaneously. The error in the timing of the incubation period was negligible. After 30 min at 38 °C with occasional stirring, cold whey was added to all 4 batches to bring the temperature rapidly to 30 °C. After 1 min the whey was decanted from each batch and the curd piled separately on a sloping tray in the incubator at 30 °C. The pH values of the curd were determined from time to time. By plotting the pH values against the total time that had elapsed at 30 °C

since the beginning of the collection of each batch of curd it could be seen (Fig. 1) that all the points fell near a straight line irrespective of the proportion of the incubation time that elapsed before the scald, and therefore that the incubation of curd in whey before scalding is not necessary. Each curd was sampled 292 min after curd formation and the moisture content determined. The values all fell between 44 and 46 %.

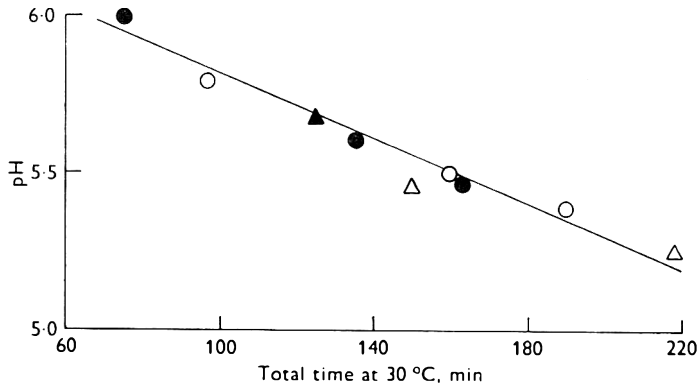


Fig. 1. The effect of different periods of incubation before scalding on the acid development in curd at 30 °C. △, Pre-scald incubation for 90 min; ▲, for 60 min; ○, for 30 min; ●, no pre-scald incubation.

The effect of different scalding temperatures and times on starter activity

In order to avoid uncertainties arising from differences in pH between curds and whey it was decided to blend the 2 immediately the scalding period was over and to determine the pH value in the relatively homogeneous mixture after incubation for 3 and 5 h.

Fresh whey was prepared with the curd-making apparatus using cold-renneted-milk with no starter, the curd being received as described on p. 54. For the preparation of the curd the starter culture was NCDO 1200. This was in the state of a soft clot. It was shaken vigorously with 2 vol autoclaved milk and incubated for 1 h at 30 °C. Two per cent of this mixture was then added to the cold-renneted milk just before use. Our normal procedure is to add starter to the cold milk on the previous evening at the same time as rennet is added, as described under Methods on p. 54). Each sample of curd was received for 2 min into 160 ml of the whey already prepared. This period of 2 min was just sufficient for 160 ml of curd to be produced.

Care was taken to keep the temperature of production well below 50 °C (about 44–46 °C) and to keep the temperature in the whey in the receiver at about 30 °C. The mixture of curds and whey was then heated to the chosen scalding temperature over a bunsen flame (with no gauze, the time required to attain this temperature was less than 2 min). The mixture was then kept at that temperature for the required time (see Tables 3 and 4). After that it was immediately cooled in running water to about 34 °C. This also took no longer than 2 min. The timing of the incubation period which was to follow was begun as soon as 34 °C was reached. The mixtures were stirred gently by hand during the heating and cooling. Blending (with the MSE 'Atomix') followed at once and this required 40 sec. The temperature was then 30 °C.

After incubation for 3 h each mixture was stirred and its pH value determined. This operation was repeated after a further 2 h. The curd settled during the incubation but was readily dispersed again by stirring with a glass rod in spite of a slight tendency for parts of it to stick together.

The experiment was repeated to extend the observations to lower scalding temperatures and slightly more starter was used. The pH values were recorded after 4 h.

Table 3. *Effect of certain scalding temperatures and times on starter activity as indicated by pH*

Scalding temperature, °C ...	pH after 3 h at 30 °C			pH after 5 h at 30 °C		
Scalding period, min	50	48	46	50	48	46
1	6·00	—	—	5·71	—	—
2	6·02	—	—	5·83	—	—
4	6·08	5·97	—	6·00	5·69	—
7	—	—	5·95	—	—	5·65*
8	6·06	6·01	—	6·01	5·79	—
14	—	—	6·00	—	—	5·82
18	—	6·01	—	—	5·98	—
30	—	—	6·01	—	—	5·97*
32	—	6·02	—	—	6·01	—
60	—	—	6·01	—	—	6·00*

* By extrapolation from 2 readings between 4 and 5 h.

Table 4. *Effect of certain scalding temperatures and times on starter vigour; pH values after subsequent incubation for 4 h at 30 °C*

Scalding temperature, °C ...	By interpolation from Table 3			Measured		
Scalding period, min	50	48	46	44	42	40
1	5·85	—	—	—	—	—
2	5·93	—	—	—	—	—
4	6·04	5·83	—	—	—	—
7	—	—	5·80	5·80	5·70	5·65
8	6·04	5·94	—	—	—	—
14	—	—	5·91	5·82	5·70	5·68
18	—	6·00	—	—	—	—
30	—	—	5·99	5·93	5·76	5·67
32	—	6·02	—	—	—	—
60	—	—	6·00	6·02	5·71	5·61

Controls: (*a*) curd, no scald, blended and incubated, pH 5·81; (*b*) cold-renneted milk and equal volume of whey, heated to 30 °C and incubated, pH 5·89 (*c*) inoculated cold-renneted milk alone, heated to 30 °C and incubated, pH 5·50.

The results for the higher temperatures are given in Table 3. Table 4 shows the results for the lower temperatures together with 4-h values for the higher temperatures as determined by straight line interpolation from the results in Table 3. It is to be

emphasized, however, that the results are not strictly comparable since more starter culture was used for the second part of the experiment. Nevertheless, an adequate guide to the temperature sensitivity of NCDO 1200 in curds and whey was obtained. It is clear, for example, that only slight damage, if any, was done to the starter by heating at 46 °C for 7 min, 48 °C for 4 min or 50 °C for 1 min. Table 4 suggests a slightly stimulating effect at lower temperatures.

The effect of scalding treatment on curd moisture (a) at end of scald, (b) after cheddaring

Uninoculated raw milk at pH 6·2 and 5 °C was treated with 8 ml rennet/gal and was ready for use in 45 min. (This procedure was used occasionally whenever cold-renneted milk was needed quickly. There was no evidence that milk prepared in this way was different from that treated with a smaller amount of rennet for a longer period.) Curds and whey were made for 30 min and then the curd produced over a period of 5 min (400 ml) was received into 400 ml of whey just separated from the curd made during the previous 30 min. The mixture was warmed rapidly to 40 °C over a bunsen with gentle stirring and maintained at this temperature. A sample of curd was removed from the whey after 10 min, allowed to drain for 5 min on a sloping tray in an incubator at 37 °C, and then immediately refrigerated. Another sample of the curd was similarly treated after being kept in the whey at 40 °C for 20 min. The procedure was repeated using scalding temperatures in the whey of 43, 46 and 49 °C with the sole difference that at 49 °C curd samples were removed after 5 and 10 min instead of 10 and 20 min.

This experiment was also part of a series leading to the design of a larger apparatus and for this reason a mechanically strengthened membrane was used. This gave the tubular membrane a smaller cross-section and the faster linear flow which was necessary to maintain the constant output resulted in a slightly disturbed curd which is known to be faster-draining than normal curd (Cheeseman & Chapman, 1966). Two of the tests were therefore repeated at a normal linear flow rate. The results are shown in Table 5. Clearly, any of the time-temperature combinations should produce curd suitable for Cheddar making. It was confirmed that faster drainage occurred with disturbed curd.

An extension of the results for scalding at 46 °C was obtained in a further experiment using milk renneted overnight with the normal amount of enzyme. Starter was also added but it failed to take the curd below pH 6·0 during a cheddaring-period of 2 h. In spite of the starter failure a good deal of moisture was removed during the cheddaring, (samples nos. 5, 6, 9, 12 of Table 5).

The effect of different times and temperatures of scalding on moisture and pH values of curd after cheddaring

Previous experiments indicated that moisture retained as a result of too little scalding treatment would not easily be lost during cheddaring (Table 5). It was therefore necessary to give adequate scalding. The shorter the scalding time the more convenient will a process be, but the use of a short time would require a high temperature. As there was still some doubt about the advisability of using temperatures as high as 50 °C it seemed safer to begin experiments at 48 °C. Consequently, experi-

ments were carried out with scalding for 4 min at 48, 10 min at 46 and 10 min at 44 °C. Curd production was carried out as already described, the curd being received into cooled whey so that the final temperature did not exceed 33 °C. Neutralized cooled starter NCDO 712 (500 ml) had been added to the milk (4 gal) with the rennet the previous evening.

Scalding for 4 min at 48 °C was not precise since 9 min were required to raise the temperature of the mixture to this value. After scalding the curd was drained and cheddared as usual until the pH values were between 5·45 and 5·56. Scalding for 4 min at 48 °C gave a moisture content of 42 % and 10 min at 44 °C gave 41 %. Surprisingly, 10 min at 46 °C gave 44 %. Thus, it seemed that 4 min at 48 °C would be satisfactory but confirmation was needed.

Table 5. *Effect of scalding temperature and time on curd moisture content*

	Scalding		Moisture, % after drainage for 5 min	Moisture, % after cheddaring for 2 h
Sample no.	Time, min	Temp., °C		
1	10*	40	52	—
2	20*	40	51	—
3	10*	43	50	—
4	20*	43	48	—
5	5	46	55	45
6	10	46	51	43
7	10	46–47	50	—
8	10*	46	48	—
9	20	46	47	41
10	20*	46	45	—
11	20	46–47	46	—
12	30	46	48	39
13	5*	49	46	—
14	10*	49	45	—

* Disturbed curd (see text).

A comparison of different techniques of scalding

This experiment was used to see whether the value of high-temperature short-time scalding could be confirmed using NCDO 1200. The curd was produced by the usual technique; 500 ml of neutralized cooled starter was used in 5 gal milk. Curd no. 1 was produced at 44 °C and kept at this temperature for 20 min. Two batches were used each taking 6 min to collect, so that the average time at 44 °C was 23 min. The 2 batches were then drained and cheddared together. Curd no. 2 was produced at 44 °C and collected for 12 min in whey at about 30 °C. The temperature was then raised very quickly to 48 °C by adding enough whey at 60 °C. After 4 min the curd was drained and placed in an incubator at 30 °C. After a few minutes it was loaded into a cheddaring tube. Curd no. 3 was produced at 48 °C and passed through a coil of plastic tubing, of such length (10 m) that about 4 min were required for the passage. A fall in temperature of about 3 °C occurred during passage through the coil. The treatment was thus less severe than had been intended. This curd was drained and cheddared as for nos. 1 and 2.

The pH value fell during cheddaring almost linearly with time between 2·5 and 6 h,

the curd scalded at 48 °C requiring 5 h 6 min to reach pH 5·40 and that scalded at 44 °C requiring 5 h 59 min. The curd which had been passed through the coil of plastic tubing (48–45 °C) needed 5 h 40 min to reach pH 5·40. When the pH values had fallen below 5·4 the curd was torn into pieces, 2% salt added and the whole pressed in miniature cheese moulds for 20 h. The moisture contents of the resulting cheese were determined but the results were improbably low (33–34% moisture). However, the cheeses were very firm and it is possible that over pressing occurred as the pressure indicators on the miniature presses were not working properly. The pH of cheese no. 3 after 6 days was 5·05–5·10, confirming that treatment for 4 min at 46–48 °C is satisfactory for scalding this type of curd.

A further trial of scalding for 4 min at 48 °C combined with a test of the effect of the addition of different quantities of starter

Cooled neutralized starter prepared as described under Methods (p. 54) was added to 3 batches of the cold renneted milk the previous evening, in the proportions of 2, 4 and 6%, respectively. Curd was made in the usual way at 44–46 °C and collected into cool whey. The temperature was then raised to 48 °C by adding hot whey as described on p. 61, and after 4 min at this temperature the curd was drained for about 5 min at 30 °C and put into the first cheddaring tube. Cheddaring was continued as described under Methods until a pH value below 5·4 was reached. The results for pH during cheddaring and for moisture content at the end of cheddaring are shown in Table 6.

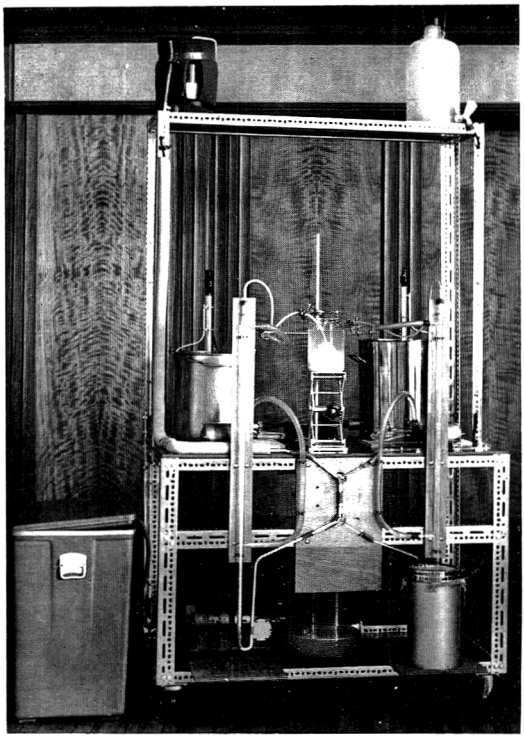
Table 6. *pH values during cheddaring and final moisture contents of curd prepared from milk with different proportions of starter*

Curd no.	Proportion of starter, % (v/v)	Duration of cheddaring, h	pH	Moisture, %
1	2	3	5·89	—
		4	5·52	—
		5	5·51 (!)	—
		6	5·37	38·4
2	4	2·5	5·73	—
		3·5	5·44	—
		4·5	5·34	39·8
3	6	2	5·68	—
		3	5·46	—
		4	5·30	42·0

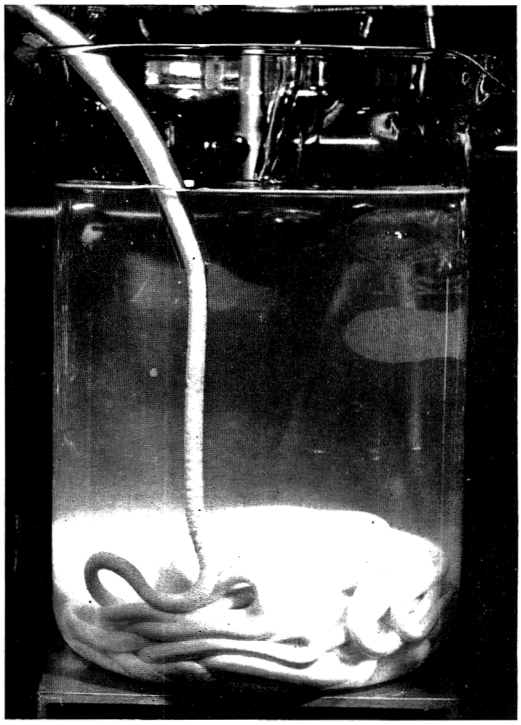
DISCUSSION

These experiments show the steps which led to the selection of the preferred scalding time and temperature of 4 min at 48 °C.

Whenever a more complex procedure was tried, for example the use of cooled whey, it was found to be unnecessary. It is possible that the heat treatment may be slightly damaging to the starter, but the results suggest that any such damage is insignificant. The different pH values reached by curd and inoculated milk in the same time are shown at the bottom of Table 4. This was the result of the planned dilution of curd samples with whey.



(*a*)



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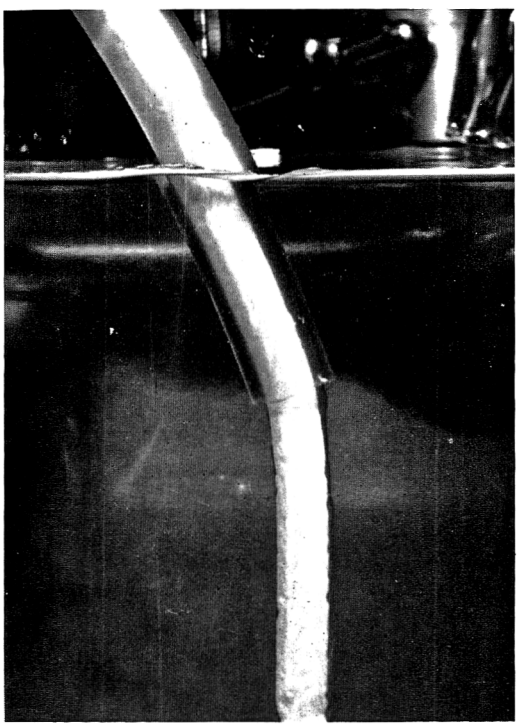

(*c*)

N. J. BERRIDGE

(*Facing p.* 63)

The effect of incubation before scalding was compared with that after scalding because a period of active growth might have affected the behaviour of the starter during the scalding period. Figure 1 shows that the points obtained by plotting the pH against time at 30 °C fall on or near a straight line. It was, therefore, immaterial whether the curd was kept at 30 °C before or after the scald. However, the scalding treatment was not severe and a severe scalding might have shown some differences.

Table 6 shows the time that can be saved by the use of increased doses of starter. It is interesting to note that the increased rate of acid production was not paralleled by an increased rate of drainage.

Pimblett (1962) gives the average and the maximum and minimum values for the moisture content of good curd at various stages during commercial production. These values were used as a guide in the present work. Thus, curds with a moisture content of less than 45 % at the end of cheddaring (Table 6) were regarded as satisfactory.

The results as a whole suggest that it would be possible to use the continuous process to make curd with an acidity and a moisture content suitable for Cheddar cheese production. The process would involve a curd-making time of about 20 sec followed by a scalding period of 4 min. The curd would then be stored and cheddared in a suitable container until the required acidity had been developed. It is hoped to carry out work on this part of the process in due course.

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EXPLANATION OF PLATE

(*a*) General view of a laboratory apparatus for continuous curd-making. Cold-renneted milk flows from the container (top left) vertically downwards and across the bench to the taps (centre) whence it can be directed either to the right or left to the bottom of the membrane in one or other of the vertical hot water jackets, in this case the left-hand one. The curd flows from the top of the membrane through a plastic tube into the beaker (centre). Similarly, water from the vessel (top right) can be used for rinsing either membrane. The lower end of the membrane and jacket is normally immersed in ice in a 'Thermos' flask (as on the right) to ensure against premature coagulation of the milk. The rest of the apparatus is for pumping milk from the insulated container (left) to the top reservoir, for the independent circulation of hot water to each jacket, and for the collection of whey.

(*b*) Curd flowing from the plastic tube into a beaker of water. The crinkled surface of the emerging curd can be seen and is in contrast with the smooth surface of the major part of curd already in the beaker. The crinkled surface is characteristic of satisfactory performance. It is possibly produced following the reversal of momentary adhesion between the curd and the membrane (Berridge, 1968). It appears on perhaps 5 % of the curd.

(c) An enlarged view of the same curd. The high refractive index of the whey causes it to appear as a darker layer surrounding the emerging curd below the end of the plastic tube.

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Hydrolysis of fat and protein in small cheeses made under aseptic conditions

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Summary. Aseptically drawn milk was made into small cheeses (100 g) under aseptic conditions. The influence of the cow's feeding régime on the composition of the cheese fat, and the hydrolysis of the cheese fat and protein by bacterial and native milk enzymes, was studied.

In the absence of a strongly lipolytic bacterial cheese flora, the free fatty acid (FFA) content of cheese appeared to depend on several variables:

1. The initial FFA content of the milk varied according to the mode of milking (the lowest was obtained by cannulation of the udder) and the feeding régime of the cow. When one of a pair of monozygotic twin cows was maintained on pasture and the other on winter feed (hay and concentrates), the cheese made from the milk of the latter contained less FFA than did that from the former.

2. The major changes in the composition of the milk fat of the cows on winter régime were an increase of myristic and palmitic acids from 8 to 11 % and 21 to 28 %, respectively, and a decrease in stearic and oleic acid from 11 to 7·5 % and 38 to 28 %; minor changes in the other fatty acids were also observed.

3. The milk lipase in raw milk cheese liberated substantially more FFA than did the weakly lipolytic lactic acid streptococci. Milk lipase was active at the low pH value of cheese, but was inactivated when the milk was pasteurized at 63 °C for 30 min.

4. Although rennet did not produce any amino acids in the mature cheeses it hydrolysed some of the cheese protein to nitrogen soluble in water (WS), trichloroacetic acid (TCA-S) or phosphotungstic acid (PTA-S). Some of the WS-N appeared to be utilized by the lactic acid streptococci. The native milk protease resisted the heat treatment used and liberated amino acids at the low pH value of cheese, but its contribution to the proteolysis was relatively less important than the contribution of the milk lipase to the fat breakdown in cheese.

5. The free amino acids of the milk, which appeared in the whey, were not recovered in the cheese. The cheese made from unheated milk with δ-gluconic acid lactone contained low amounts of free amino acids apparently due to the action of milk protease. The proteolytic activity of the lactic acid bacteria increased the amino acid content appreciably.

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The value of aseptic cheese-making techniques (Mabbitt, Chapman & Sharpe, 1959; Chapman, Mabbitt & Sharpe, 1966) in flavour research is now established (Perry & McGillivray, 1964; Reiter, Fryer, Sharpe & Lawrence, 1966; Reiter *et al.* 1967; McGugan *et al.* 1968). However, such methods used for making 40-lb cheeses under aseptic conditions do not allow the study of the native milk enzymes and their effects because the milk has to be heat treated to eliminate the contaminating flora. Mabbitt & Douglas (1960) described a small aseptic cheese-making vat of 1l capacity, small enough to use raw milk aseptically withdrawn by cannula from bacteria-free udders. It can be operated by one person and the cheeses are suitable for bacteriological and chemical analysis, although they are too small for the assessment of flavour by a tasting panel. Results obtained with such small aseptic cheeses supplement in many respects those obtained with the large aseptically made cheese (Reiter *et al.* 1967; McGugan *et al.* 1968), particularly in the study of the effect of enzymes of raw milk on cheese.

The present paper is concerned with the effects of the method of milking and the diet of the cow, and with the activity of milk lipase and lactic acid bacteria, on the hydrolysis of the cheese fat, and also with the effects of rennet, milk protease and lactic acid bacteria on the hydrolysis of the cheese protein.

METHODS

Cheese-making. Milk was withdrawn aseptically by cannula from bacteria-free udder quarters and was used either raw or after heating at 63 °C for 30 min. Machine-drawn milk was also obtained from a nearby commercial herd (S-herd) and from the Institute herd. Cheddar and Edam cheese was made aseptically using commercial cheese practices. Bacteria-free cheese was made using δ-gluconic acid lactone (GAL) to replace starter, as described by Mabbitt, Chapman & Berridge (1955).

Each cheese weighed about 100 g and was made aseptically from 1 l of milk in an aseptic vat. The moisture content of the Cheddar cheese was below 40 % and that of the Edam below 45 %. The cheese was stored under medicinal paraffin to prevent loss of moisture during accelerated maturation at room temperature.

Starter. Streptococcus cremoris 924 (NCDO 924) and *Str. cremoris* TR (NCDO 1200) were used. They were obtained from the National Collection of Dairy Organisms.

CHEMICAL ANALYSES

Determination of free fatty acids (FFA). FFA (C_2 (acetic), C_3 (propionic), C_4 (butyric) acids and $> C_4$ (free fatty acids higher than butyric)) were determined by chromatography on silicic acid columns, the fractions being titrated with 0·01 N-KOH according to the method described by Harper (1953).

Fatty acid composition of milk fat. Representative samples from 4 milkings were refrigerated and combined during each period of the experiment for the analysis of the milk fat; morning milk only was used for cheese-making on the same day. The milk fat was extracted from the milk in chloroform–methanol 2:1, v/v), converted to methyl esters of the fatty acids in sealed tubes and analysed by gas–liquid chromatography as described by Storry & Millard (1965) and Storry, Rook & Hall (1967).

Determination of protein fractions. The stage of protein breakdown was measured by

estimation of nitrogenous fractions soluble in water (WS), in 12% trichloroacetic acid (TCA-S) and in 5% phosphotungstic acid (PTA-S). The nitrogen in each fraction was determined by the Kjeldahl method, and the results expressed as a percentage of total N, WS-N and TCAS-N, respectively.

Amino acid determination. Cheese (20 g) was homogenized with 100 ml distilled water at room temperature and filtered. One ml of the filtrate, equivalent to 0·2 g cheese, was deproteinized by the addition of 5 ml 3% sulphosalicilic acid. The free amino acids were determined by ion-exchange chromatography using an automatic amino acid analyser (Evans Electroselenium Ltd., Halstead, Essex).

RESULTS

Lipolysis

With the continuous improvement of the bacterial quality and the almost universal heat-treatment of milk used for cheese-making it is to be expected that strongly lipolytic contaminants such as micrococci and Gram-negative bacteria should play an ever decreasing part in the hydrolysis of cheese fat. The FFA concentration will depend, therefore, mainly on the metabolism of the majority flora of the cheese—the lactic acid bacteria (Reiter *et al.* 1967; Fryer, Reiter & Lawrence, 1967).

Table 1. *A comparison of the levels of FFA in 3-month-old Edam cheeses made with and without lactic acid bacteria*

	FFA, μmoles/5 g cheese*		
Cheeses made from heated milk with	C_2	C_4	$> C_4$
δ-Gluconic acid lactone (GAL)	4·7	0·16	0·7
Str. cremoris 924	16·7	0·38	1·4

* Average values for 4 cheeses.

Preliminary data from the analysis of the small cheeses confirmed the lipolytic activity of the lactic acid bacteria. The absolute concentrations of FFA were, however, very low (Table 1) compared with the values obtained for the 40-lb aseptic cheese, which averaged 4·7 μmoles C_4 acid and 49·0 μmoles $> C_4$ acid/5 g cheese during summer and 0·2 μmoles C_4 and 14·0 μmoles $> C_4$ during winter (Reiter *et al.* 1967). As the low FFA levels in the cheese could be a feature of the cheese-making in the small vat or of the use of the milk of one cow only, this finding was further investigated.

The effect of the mode of milking on the FFA content of cheese made with δ-gluconic acid lactone (GAL cheese)

Bacteria-free cheese made from heated milk with δ-gluconic acid lactone was known to contain the lowest FFA concentrations and it was thought that the low FFA of these cheeses reflected the FFA content of the milk (Reiter *et al.* 1967). Milk from a single cow was, therefore, obtained by different methods of milking and compared with mixed herd milk (Table 2). Milk from a single cow was withdrawn by cannula from 2 quarters of the udder, while the other quarters were milked by hand. Twenty-four hours later, the cow was machine-milked and a sample withdrawn from

the milk jar. The cheese made from cannulated milk contained the lowest concentration of FFA and that made from milk from the hand-milked quarters the highest, indicating a possible correlation between the level of FFA and the amount of agitation of the milk. The FFA concentration of milk obtained by machine milking was dependent on the point of sampling; thus, milk samples taken from the milk jar always contained less FFA than did milk from the bulk tank, presumably because the former had not been pumped through a lengthy pipe into the bulk tank. The differences in the FFA content of the cheese made from the milk of the 2 herds cannot

Table 2. *Effect of mode of milking on the content of fatty acids higher than butyric (FFA > C_4) of Cheddar cheese*

Source	Milked by	FFA > C_4 μmoles/5 g cheese
Cow 209	Cannula	1·6
	Machine	2·7
	Hand	4·4
Herd N.I.R.D.	Machine	
Composite sample from	(*a*) Sampled from milk jar	15·0
6 cows	(*b*) Sampled from bulk tank	26·0
Herd N.I.R.D.*	Machine–sampled from bulk tank	42·0
Herd S*	Machine–sampled from bulk tank	19·0

* Viable count, 10^2/ml; lipolytic count, 10/ml.
† Viable count, 10^5/ml; lipolytic count, 10^3/ml.

Table 3. *Concentration of FFA > C_4 in Cheddar cheese (ex press made) made with δ-gluconic acid lactone (GAL) from milks of monozygotic twins (cows 265 and 266) on either summer or winter feed*

	Cow 265 FFA > C_4, μmoles/5 g cheese		Cow 266 FFA > C_4, μmoles/5 g cheese	
Cheese made	Feed	> C_4	Feed	> C_4
6. vii. 66	Pasture	8·5	Pasture	6·5
15. vii. 66	Hay and concentrates	7·9	Pasture	6·6
22. vii. 66	Hay and concentrates	5·4	Pasture	12·1*
29. vii. 66	Hay and concentrates	3·4	Pasture	6·0

* Unexplained discrepancy

	Cow 265	Cow 266
Calved	19. ix. 65	14. ix. 65
Dried-off	2. x. 66	21. viii. 66
Calved	26. xi. 66	5. x. 66

be explained at present. The milk of the S herd was transported for 10 miles and always contained more lipolytic organisms than that of the other herd, but the cheese made from it contained consistently less FFA. The difference between the FFA content of the cheese made from the milk of one cow and the composite sample from 6 cows, both machine-milked, indicated that there must exist a great individual variation, which was indeed found when the milk of individual cows was examined for FFA content.

The effect of giving different feeds to monozygotic twins on the pattern of FFA in cheese made from their milk

The consistent differences in the concentration of FFA in winter and summer cheese (Reiter *et al.* 1967) were considered to be due to differences in feeding. A pair of monozygotic twin cows, at nearly the same stage of lactation, was kept on pasture and one twin was later changed to winter feed of old hay and concentrates. The cows were milked by machine, and part of the morning milk made into GAL cheese. The winter feed régime (cow 265) gradually depressed the content of FFA > C_4 (Table 3), while the level of FFA > C_4 in cheese made from the milk of the cow 266 kept on pasture remained nearly constant: moreover, this latter animal became dry much earlier, and the stage of lactation is known to influence the amount and the relative proportions of the fatty acids in milk (Stull, Brown, Valdez & Tucker, 1966).

The changeover from pasture to a winter feed régime resulted not only in decreased total FFA > C_4 but also in major changes in the composition of the fat (Table 4). Myristic acid increased from 8 to 11 % and palmitic acid to 21 to 28 %, whilst the stearic acid decreased from 11·2 to 7·5 % and the oleic acid from 38 to 28 %. On changing back again from pasture to winter feed the proportions of these fatty acids returned to their original levels. The composition of the milk fat of the control twin which stayed on pasture remained constant and was almost identical to that of the

Table 4. *Fatty acid composition of the milk fat of monozygotic twin cows kept on pasture and on a winter feed régime of hay and concentrates*

			Fatty acids, % of total					
	Fat, %	SNF, %	4:0	6:0	8:0	10:0	12:0	14:0
			Cow 265 (experimental)					
Precontrol	3·00	8·47	2·2	1·0	0·6	1·3	1·9	8·0
Experimental	3·35 ± 0·33	8·60 ± 0·04	2·7 ± 0·30	1·3 ± 0·10	1·2 ± 0·17	2·6 ± 0·50	3·6 ± 0·90	11·2 ± 0·66
Postcontrol	3·52	8·69	1·9	0·9	0·6	1·3	2·0	7·9
			Cow 266 (control)					
Control	3·47 ± 0·71	8·65 ± 0·37	2·0 ± 0·60	0·9 ± 0·02	0·6 ± 0·15	1·2 ± 0·10	1·8 ± 0·18	7·4 ± 0·42

			Fatty acids, % of total					
	Fat, %	SNF, %	14:1	16:0	16:1	18:0	18:1	18:2
			Cow 265 (experimental)					
Precontrol	3·00	8·47	1·3	21·0	3·1	11·2	38·6	1·7
Experimental	3·35 ± 0·33	8·60 ± 0·04	1·7 ± 0·20	28·3 ± 0·81	3·3 ± 0·95	7·5 ± 2·72	28·4 ± 2·66	2·7 ± 0·36
Postcontrol	3·52	8·69	1·2	20·8	3·2	12·1	37·7	2·8
			Cow 266 (control)					
Control	3·47 ± 0·71	8·65 ± 0·37	1·2 ± 0·14	21·5 ± 0·93	3·4 ± 0·22	11·6 ± 1·40	38·4 ± 1·59	2·4 ± 0·76

Precontrol and control, animals on pasture. Experimental, change over to winter feed régime for 3 weeks. Postcontrol, return to pasture.

milk fat of the experimental animal while on pasture. Besides these major changes, smaller changes in the proportions of nearly all the other fatty acids occurred during the experimental winter feed régime.

Effect of milk lipase and lipolytic activity of starters on FFA content of cheese

It has been previously shown that cheese made with starters contained more FFA than did cheese made with δ-gluconic acid lactone (Reiter *et al.* 1967); the lipolytic activity of starters was confirmed, and that of the mill lipase investigated (Table 5). The GAL-cheese made with raw milk had an appreciably higher content of FFA > C_4 than had the cheese made with heated milk, and the FFA < C_4 content

Table 5. *Effects of milk lipase and starter on the concentration of FFA > C_4 in unmatured Cheddar cheese (ex press)*

	Cheese made with			
	δ-Gluconic acid lactone		*Str. cremoris* 924	
	Raw milk	Heated milk	Raw milk	Heated milk
Origin of milk	FFA > C_4 expressed in μmoles/5 g of cheese*			
Herd S	23·6	17·7	25·8	18·8
Herd N.I.R.D.	41·6	33·3	51·1	40·1

* Average values for 2 cheeses.

Table 6. *Concentrations of FFA in 6-month-old Cheddar cheese made from raw or unheated milk taken from the udder by cannula*

		*FFA expressed as μmoles/5 g cheese	
	Milk	C_4	> C_4
Cheese made with:			
δ-Gluconic acid lactone	Raw	1·9	8·9
δ-Gluconic acid lactone	Heated	0·1	2·4
Str. cremoris TR	Raw	2·9	18·6
Str. cremoris TR	Heated	0·8	5·8

* Average values for 4 cheeses of each kind.

in starter cheese made from raw milk was still higher. The differences in the content of FFA > C_4 of cheese made from the milks of the 2 herds (herd S and the N.I.R.D. herd) were very marked and the differences between the cheese made from the raw and heat-treated milk of the N.I.R.D. herd, with a higher initial FFA level, appeared to be greater than those between the cheese made from the milk of the herd S which had a lower initial level.

The cheese, made from milk taken by cannula and heated, contained very low concentrations of FFA, even after 6 months maturation, compared with the cheese made from raw milk; thus, the effect of the milk lipase was quite marked (Table 6). The bacteria-free cheese made from raw milk contained appreciably higher concentrations of FFA than did the cheese made with starter from heated milk, and the cheese made with starter from raw milk contained the highest concentration.

Proteolysis

At 1 and 3 months, the GAL cheeses contained more WS-N than the starter cheese (Table 7). Most of the breakdown to WS-N in the GAL cheese occurred in the first month and continued at a slower rate up to 3 months. The starter appeared to utilize some of the WS-N hydrolysed by the rennet or by the native milk protease or by both, because the starter cheese contained considerably less WS-N than did the GAL cheese at 1 month. Thereafter, accumulation of the WS-N continued at a much higher rate, the total concentration at 3 months approaching the values in the GAL cheese of the same age. The TCA and PTA-soluble contents were higher in the starter cheese than in the GAL cheese and represented a higher proportion of the WS and TCA-S N, respectively; the differences in the TCA- and PTA-S fractions of the starter cheese were more pronounced than those in the WS fractions. Thus, in the starter cheese a greater proportion of the protein was hydrolysed to smaller peptides and eventually to amino acids (Table 8). Three-month-old GAL cheese made from heated and raw milks contained the same concentration of WS nitrogen, but the raw milk cheese contained slightly more TCA-S and PTA-S nitrogen.

Table 7. *Protein breakdown in Edam cheese made without starter (GAL) and with different starters*

	Water-soluble N, % of total N		Trichloroacetic acid-soluble N, % of total N		Trichloroacetic acid-soluble N, % of water-soluble N	
Months ...	1	3	1	3	1	3
Cheese made with						
GAL (heated milk)	16·1	23·4	2·3	3·8	14·3	16·2
GAL (raw milk)	—	23·8	—	4·2	—	17·7
Str. cremoris 924	6·6	16·7	3·3	7·9	50·0	47·3

	Phosphotungstic acid-soluble N, % of total N		Phosphotungstic acid-soluble N, % of water-soluble N		Phosphotungstic acid-soluble N, % of trichloroacetic acid-soluble N	
Months ...	1	3	1	3	1	3
Cheese made with						
GAL (heated milk)	0·75	0·78	4·7	3·3	32·6	20·5
GAL (raw milk)	—	1·2	—	5·0	—	28·5
Str. cremoris 924	1·5	3·0	22·8	18·0	45·5	38·0

Concentration of amino acids

Milk contains an appreciable amount of free amino acids, about 70–80 mg/l (Deutsch & Samuelsson, 1959; Ghadimi & Pecora, 1963) which appear in the whey during cheese-making (Professor D. M. Irvine, University of Guelph, Canada, personal communication). The whey of the GAL cheese contained about 80 mg/l (Table 8) (the accuracy of the amino acid determination by the automatic amino acid analyser must be low at these minute concentrations), but the curd contained only traces of glutamic acid, aspartic acid and glycine, although hard cheese contains about 1/3 of whey. Even after 1 month maturation the GAL-cheese made from heated milk con-

tained only 8 mg/100 g amino acids (the content of the raw milk cheese was not determined). After 3 months the amino acid content increased about 10-fold (Table 8). As no 100 g duplicate cheese had been made at the time, the amino acid concentration in a large 40-lb cheese made under aseptic conditions is cited for comparison in Table 8. At these low concentrations of amino acids, it is difficult to interpret whether the higher amino acid content in the GAL cheese made from raw milk is significant. It is, however, remarkable that in each of the cheeses the same amino acids are either high, low or absent. The same holds true for the contribution to the amino acid content by the starter organisms. The total amount is appreciably increased and with 2 exceptions (proline and methionine, both present in relatively low concentrations) the concentrations of the individual amino acids are of the same order of magnitude.

Table 8. *Free amino acids in 3-month-old cheese (mg/100g) and in whey (mg/l)*

		Cheese made with:				
		GAL, from milk:			*Str. cremoris* 924, from milk:	
	Whey	Heated,*	Heated†	Raw*	Heated*	Heated†
Aspartic acid	18	1	Trace	Trace	20	15
Threonine	7	Trace	Trace	—	19	12
Serine	—	1	3	—	67	58
Glutamic acid	24	5	7	4	164	127
Proline	—	—	—	—	13 (?)	Trace
Glycine	20	2	2	1	10	11
Alanine	1	1	1	2	15	17
Valine	3	10	22	12	56	56
Methionine	—	Trace	Trace	—	19 (?)	Trace
Isoleucine	1	3	Trace	11 (?)	7	9
Leucine	1	17	28	20	157	112
Tyrosine	—	3	4	4	26	25
Phenylalanine	—	20,	21	45	74	71
Lysine	—	15	15	28	45	45
Histidine	—	Trace	Trace	Trace	Trace	Trace
Arginine	1	10	15	44	74	51
Ammonia	4	1	5	1	2	11
Total	76	88	118	171	766	609

* 100 g aseptic Edam cheese.
† 40-lb aseptic cheese, milk heated 161 °F 15–20 sec (A.P.V. HX type, plate heat exchanger).

DISCUSSION

The present investigations are part of a larger project on cheese flavour (Reiter *et al.* 1966, 1967; McGugan *et al.* 1968). As long as the identity of the flavour compound or compounds is not established, the progress of lipolysis and proteolysis remains the best guide to the maturation process of cheese, although the activities of lipolytic and proteolytic enzymes may not produce flavour products *per se* (see reviews by Sjøstrom, 1959; Mabbitt, 1961; Marth, 1963; Reiter *et al.* 1966). The advantage of the aseptic cheese-making technique lies in the opportunity it affords to separate the effects of the native milk enzymes from those of rennet and bacteria. The results indicate that this separation has been largely achieved.

The mode of milking, and the degree of agitation to which milk is submitted, is known to influence the degree of fat hydrolysis, but the extremely low level of FFA in milk withdrawn by cannulation of the udder was nevertheless unexpected. It appears, therefore, that problems connected with the activity of lipase in milk could be investigated with advantage by using milk obtained by cannulation.

The elimination of the raw milk flora by what is an unnecessarily severe heat-treatment of the cheese milk (Franklin & Sharpe, 1963; Reiter, Fewins, Fryer & Sharpe, 1964), now generally practised in the cheese industry, results in inactivation of the milk lipase, and the FFA content of cheese, therefore, depends largely on the mode of milking, the feeding régime and the weak lipolytic activity of the lactic acid bacteria, which are the majority flora in cheese.

Every cheese-maker is aware that summer and winter milk differ in their cheese-making qualities, and flavour studies show that winter cheeses made under aseptic conditions and using the same starter develop the characteristic Cheddar flavour much slower than do summer cheeses (B. Reiter, T. F. Fryer & M. E. Sharpe, unpublished). Although no detailed analysis of the cheese fat has been made so far, the gross differences in total FFA $>C_4$ in the milk (Table 3) indicate that studies on cheese made from 'summer' and 'winter' milk obtained from identical twin cows on different feeding régimes may yield valuable results. The major changes in the composition of the fat, although based on only one experiment, are supported by the findings of several large surveys, made in different countries, on the fat composition of summer and winter milk (Guyot & Piraux, 1965; Svensen & Ystgaard, 1966; Artamentova & Martynushkina, 1966; Hall, 1967). It is, however, not possible to assess the significance of the minor changes in the fat composition. The increased content of stearic and oleic acids during pasture feeding can be expected, because of the increased intake of C_{18} acids from young herbage, mainly in the form of linolenic and linoleic acids. The lack of any appreciable increase of linoleic acid in the milk fat is due to its hydrogenation in the rumen, partially to oleic acid or fully to stearic acid.

Wolf (1941) was probably the first to detect weak lipolytic activity in lactic acid cultures and this has recently been unequivocally demonstrated with various substrates and in cheese (Fryer *et al.* 1967; Reiter *et al.* 1967). The present work confirms the lipolytic activity of starter streptococci in cheese.

It is now possible to answer some of the questions raised by Bills & Day (1964) regarding the lipolytic activity of milk lipase and bacterial lipase in cheese: (1) Milk lipase and bacterial lipase do possess activity at the low pH value of cheese. (2) The milk lipase is inactivated during pasteurization at 63 °C for 30 min (Table 6); in the absence of a strongly lipolytic flora (e.g. micrococci, coliforms), the FFA produced by the milk lipase are relatively important in cheese. (3) The initial FFA content of the milk depends on the mode of milking (e.g. milk obtained by single-bucket milking machines would contain less FFA than milk obtained by parlour machine milking) and on the feeding régime of the cows. (4) C_4 acid is hydrolysed from the cheese fat when single-strain starters are used, but whether C_4 acid or other FFAs are derived from amino acids in cheese containing a mixed, complex bacterial flora is not clear.

The results for the breakdown of the protein illustrate the roles of the native milk protease, rennet and lactic acid streptococci. The significance of the protein break-

down for cheese flavour has been somewhat over-emphasized in the past, but recent reviewers agree that the products of proteolysis provide a background to the cheese flavour (Mabbitt, 1961; Marth, 1963; Reiter *et al.* 1966). The GAL-cheese made from heated milk contained about the same WS-N as did the cheese made from raw milk, but less TCA-S and PTA-SN and smaller amounts of amino acids. During the clotting action of rennet NPN is produced but the pure enzyme rennin is not generally thought to be capable of breaking down protein to amino acids even during prolonged maturation of the cheese.

Unfortunately, the commercial rennet preparation may have contained proteolytic enzymes as impurities, which could have produced at least some of the amino acids present in the mature GAL cheeses. This, however, seems to be unlikely, if we accept that the differences in the amino acid contents in the GAL cheeses (both of which of course contained rennet) made from raw and heated milk are significant, and the differences in the amino acid contents of this cheese could be rather ascribed to the partial inactivation of the milk protease in the heated milks (Warner & Polis, 1945; Kiermeier & Semper, 1960). It appears, therefore, that it is the milk protease which is responsible for the low concentration of free amino acids present in the GAL cheese. Aseptic GAL cheeses made from raw milk are now being matured for up to one year and it is expected that they will contain sufficiently high concentrations of amino acids to enable a comparison to be made of the distribution of individual amino acids in the bacteria-free GAL cheese and in the cheese made with starter. Thus, it will be possible to compare the specificity of the proteolytic enzymes of the milk and of the starter bacteria. Even at the low amino acid levels in the GAL-cheese the results are remarkably constant and indicate that different amino acids are liberated in the GAL cheese than in the starter cheese. The much higher concentrations of amino acids in the cheese made with starter indicate that the accumulation of the amino acids follows a certain pattern, which seems to be very similar in the 2 cheeses analysed. We have also data on cheeses made with different strains of starters (B. Reiter *et al.*, unpublished) which contained higher concentrations of amino acids but of the same pattern as was obtained with the less proteolytic starter used in these experiments. It appears, therefore, that the aseptic cheese-making technique is suitable for the study of proteolytic activity of the native milk protease (using crystalline rennin), starter bacteria and non-starter bacteria. GAL cheese would also be suitable for comparing the proteolytic effect of the various rennet substitutes with that of rennet.

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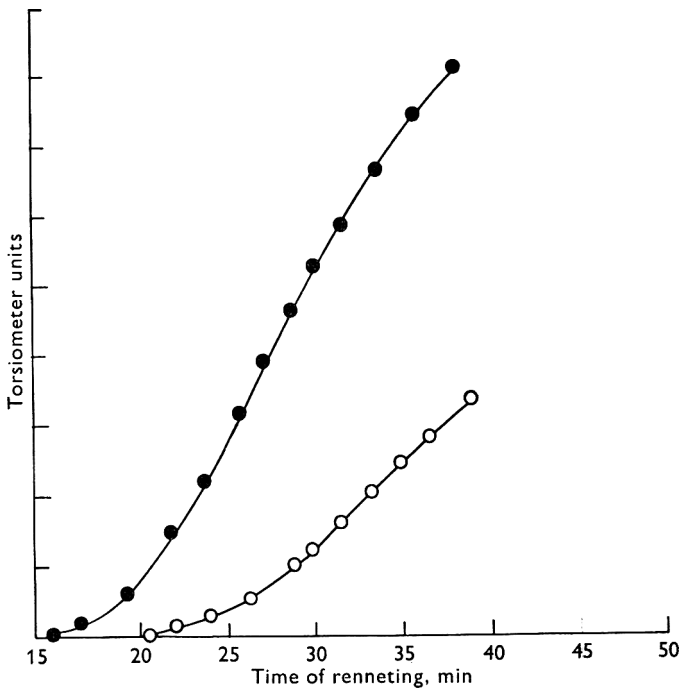

Fig. 1. Torsiometer measurements of the rigidity of rennet coagulum of the milks of the monozygotic twins when one was kept on pasture and the other on winter feed régime (old hay and concentrate). ●——●, Cow 266, pasture; ○——○, cow 265, hay and concentrate.

ADDENDUM

Burnett & Scott Blair (1963) described a speed-compensated torsiometer for measuring the setting of milk by rennet. A bench type of this instrument has now

been developed by the same authors. Mr J. Burnett measured the firmness of the curd-setting from the milk of monozygotic twin cows, one of which was kept on pasture and the other on a winter feed régime. Figure 1 shows that the milk of the twin cow on pasture clotted appreciably sooner than the milk of the cow on winter feed and was also appreciably firmer at 40 min, the usual time when the milk coagulum is about to be cut for Cheddar cheese-making. Chapman, Harrison & Burnett (1967) measured the rigidity of renneted cheesemilk obtained from 2 herds over 2 years and came to the same conclusions, namely that winter milk formed a rather weaker gel than summer milk.

Cheese-makers know that they have to use more rennet for cheese-making in winter than in summer because the same amount of rennet does not set the milk sufficiently firmly to permit cutting at the same time in winter. The increased fat content of winter milk was generally given as the reason for the required increase in rennet. Table 4 shows, however, that the 2 milks from the monozygotic twins were similar in fat content, and it seems probable, therefore, that some other factor was involved.

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The isolation and identification of 4,8,12-trimethyltridecanoic acid from butterfat

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SUMMARY. The isoprenoid fatty acid 4,8,12-trimethyltridecanoic acid has been isolated from butterfat and identified. This acid was found to be a DD diastereoisomer, and was thought to have been derived from the phytol moiety of chlorophyll. It was estimated that in the sample of butterfat investigated, 4,8,12-trimethyltridecanoic acid constituted about 0·005 % of the total weight of fatty acids.

The polymethyl branched-chain fatty acid 4,8,12-trimethyltridecanoic acid has recently been isolated and identified from marine fish oils (Sen Gupta & Peters, 1966), from whale oil (Sano, 1967), and from sheep perinephric fat (Hansen, 1968). It has also been detected in cod liver oil (Ackman, Sipos & Tocker, 1967), herring oil (Ackman & Hooper, 1968) and finwhale milk (Ackman, Eaton & Hooper, 1968). The isolation of 4,8,12-trimethyltridecanoic acid from butterfat, has not previously been reported as far as the author is aware, although gas-chromatographic evidence supporting the presence of this and other polybranched fatty acids in butterfat and beef tallow, has been furnished by Peters & Wieske (1966), who examined by gas–liquid chromatography (GLC) concentrates of branched-chain fatty acids which they separated from a number of animal fats and plant seed oils by means of urea adduction.

Of particular metabolic interest has been the identification of this acid as a degradation product in the livers of mice and rats dosed with phytanic acid (Mize, Steinberg, Avigan & Fales, 1966; Steinberg *et al.* 1967; Hansen, Shorland & Prior, 1968). The work reported in this communication was a continuation of studies on the identification and biosynthesis of trace fatty acid constituents in ruminant fats.

EXPERIMENTAL

The acid (B 101) reported in this communication was isolated from a large sample (B/36, 37; *c.* 20 kg) of butterfat, and the techniques employed in its separation included fractional distillation *in vacuo* of the methyl esters, repeated low-temperature crystallization from solvents, hydrogenation of selected and bulked 'liquid' fractions in which 4,8,12-trimethyltridecanoic acid was concentrated, and preparative GLC (cf. Hansen, 1968). Final purification involved its separation from associated urea adduct-forming components.

The preparative GLC used in this investigation was a model A-700 Aerograph (Wilkens Instrument and Research, Inc., California, U.S.A.) accommodating a thermal conductivity detector and aluminium columns 2·4 m × 7 mm int. diam. packed with acid-washed celite (30–80 mesh) silanized and impregnated with (*a*) 20 % (w/w) Apiezon L (ApL), (*b*) 20 % (w/w) ethylene glycol adipate polyester (EGA). Hydrogen was used as carrier gas and the operating temperatures were 174 and 180 °C, respectively.

Analytical GLC analyses were carried out with an instrument constructed in this laboratory and fitted with a ^{90}Sr detector (Lovelock, James & Piper, 1959). Aluminium columns 2·4 m × 7 mm int. diam. were used and were packed with acid-washed celite (30–80 mesh) which had been silanized and impregnated with (*a*) 20 % (w/w) EGA, and (*b*) 5 % (w/w) ApL. The carrier gas was argon and the operating temperatures were 207 and 170 °C.

Retention volumes (V_R) were determined on the methyl esters of fatty acids and were calculated relative to methyl stearate which was added as a marker to fractions after they had been scanned. Carbon numbers were determined by the method of Woodford & van Gent (1960).

Infrared examination of fraction B 101 was made on thin films of the methyl ester between KBr disks using a Perkin–Elmer model 137E spectrophotometer.

An A.E.I. MS 9 mass spectrometer was employed for the analysis of B 101 methyl ester.

Melting points were determined in capillaries open at one end with a Hoover Unimelt instrument.

Optical rotatory dispersion curves were measured on a 1967 model of the Bellingham and Stanley/Bendix–Ericcson automatic recording spectropolarimeter 'Polarmatic 62'. Concentration: 8 mg/ml. Solvent: methanol. Cell: 0·2 cm.

Optical activity was determined on a Hilger standard polarimeter MK III model M 412 using a 5-cm micropolarimeter tube.

RESULTS

Fraction B 101

Saponification equivalent of fatty acid: 257·0 (calc. for $C_{16}H_{32}O_2$: 256·4). Iodine value: 0·0, determined on methyl ester (Wijs solution, 1 h).

Melting point: no definite melting point was observed for either the acid or the methyl ester. B 101 acid was solid at −65 °C and melted between −42 and −10 °C. The methyl ester of B 101 appeared cloudy at −70 °C and cloudiness disappeared over the range −35 to 25 °C.

A critical solution temperature of 175–178 °C was determined on the fatty acid using ethylene glycol, by the method of Schmid, Mangold & Lundberg (1965) as adapted from the micro method of Fischer (1962).

Optical rotatory dispersion measurements made on B 101 fatty acid were as follows:

[Ø]	+85 at 222 nm;	[Ø]	+50 at 250 nm;
[Ø]	+30 at 300 nm;	[Ø]	+25 at 400 nm.

Optical activity: $[\alpha]_D^{17°}$ +4·4. (Concentration: 0·0541 g acid made up to 1 ml in chloroform.)

GLC analyses. Examination of the methyl ester of B 101 on both EGA and ApL analytical GLC columns showed one peak only, indicating a high degree of purity. However, when B 101 was applied by Dr Ackman (Fisheries Research Board of Canada) to a highly efficient open-tubular capillary column coated with butanediol succinate polyester (BDS) as described formerly (Ackman & Hansen, 1967), one major peak and 3 minor peaks were revealed, the main component occupying 95·9 % of the total peak areas and the accompanying peaks representing 2·7, 0·4 and 1·0 %, respectively. These minor peaks have not been identified, but it was found that they corresponded in equivalent chain length with peaks of components separated from B 101 in the final stages of purification, and which prior to separation had constituted a posterior shoulder on B 101 when examined on the analytical GLC using an EGA column.

On the EGA analytical column at 207 °C the V_R was 0·34 (carbon number 14·1) and at 170 °C it was 0·27 (carbon number 14·2). With the ApL column the V_R was 0·25 (carbon number 14·5) at 207 °C and 0·18 (carbon number 14·4) at 170 °C. These retention volumes are in close agreement with those earlier observed in this laboratory (Hansen, 1968) for 4,8,12-trimethyltridecanoic acid isolated from sheep fat namely V_R 0·32 at 207 °C and 0·26 at 170 °C on the EGA column, and V_R 0·24 at 207 °C and 0·18 at 170 °C on the ApL column. (For 4,8,12-trimethyltridecanoic acid Ackman *et al.* (1967) reported equivalent chain length (ECL) 14·14 using an open-tubular column coated with butanediol-succinate (BDS) polyester liquid phase at 170 °C. Sano (1967) reported an ECL value of 14·3 for this acid on a polyester column at 210 °C.) When equal volumes of the methyl esters of B 101 and 4,8,12-trimethyltridecanoic acid from sheep fat (sample H 155; Hansen, 1968) were mixed together and applied to the polyester and ApL GLC columns, respectively, the 2 substances co-chromatographed at 207 and at 170 °C.

Diastereoisomerism. When fraction B 101 methyl ester was examined on open-tubular GLC columns (0·01 in. int. diam.) coated with BDS and ApL, respectively, (Ackman & Hansen, 1967), the major component registered as one symmetrical peak. With the BDS column, this peak matched the second partially resolved peak obtained when 4,8,12-trimethyltridecanoic acid synthesized from farnesol was chromatographed under corresponding conditions, as did 4,8,12-trimethyltridecanoic acid isolated from sheep fat (see Fig. 2, Ackman, 1968). Therefore it was presumed to be DD in structure.

Mass spectrum. The mass spectrum of the methyl ester of fatty acid fraction B 101 (Fig. 1) is similar to that obtained for the methyl ester of 4,8,12-trimethyltridecanoic acid (fraction H 155) earlier isolated from sheep perinephric fat (Hansen, 1968).

Salient features of the spectrum are as follows:

(1) The parent ion at *m/e* 270. An appreciable peak at mass 256 suggests the presence of C 15 impurity.

(2) A small M-31 (270–31 = 239) peak characteristic of fatty acid methyl esters (Ryhage & Stenhagen, 1960*a*, *b*).

(3) A prominent re-arrangement peak at *m/e* 74 (73+1) which indicates the grouping

$$CH_3—O—\overset{\overset{O}{\|}}{C}—CH_2$$

(cf. Ryhage & Stenhagen, 1960*a*, *b*; Hansen, Shorland & Morrison, 1965). Sen Gupta & Peters (1966) also observed this strong *m/e* 74 peak in 4,8,12-trimethyltridecanoic acid from marine fish oil, and reported that it indicated there was no α-branching in the molecule.

(4) The base peak at *m/e* 87 (M-183 = 87) together with the medium-strength peak at *m/e* 115 which, according to Sen Gupta & Peters (1966), indicates a methyl side chain on the fourth carbon.

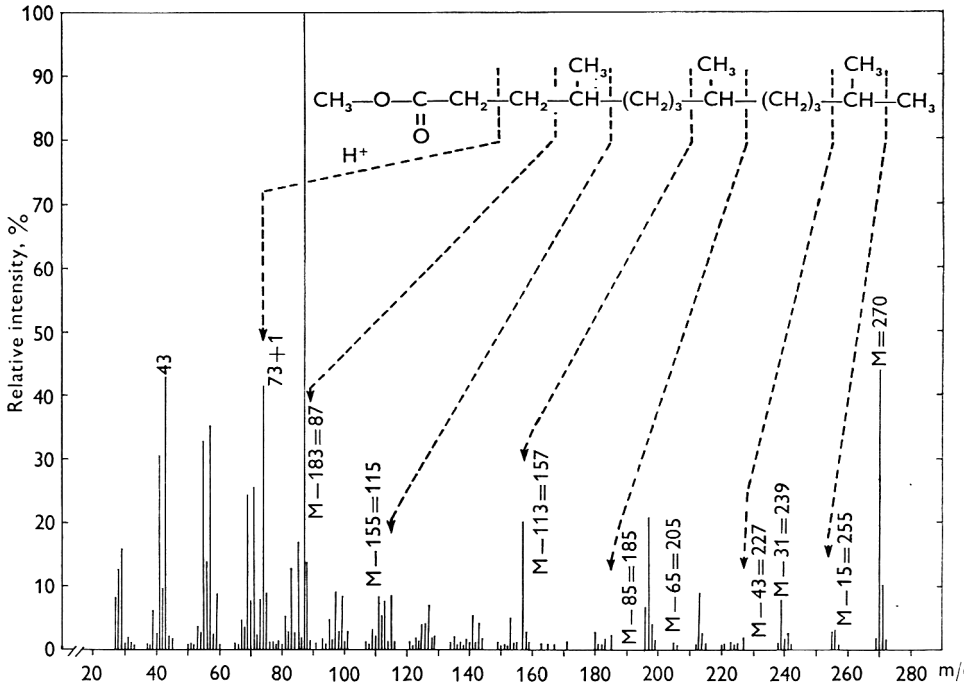


Fig. 1. Mass spectrum of the methyl ester of fatty acid fraction B 101.

(5) A small peak at *m/e* 205 (M-65 = 205) provides supporting evidence for the presence of an isopropyl group (Ryhage & Stenhagen, 1960*a*, *b*) as was earlier observed for 2,6,10,14-tetramethylpentadecanoic acid (cf. Hansen & Morrison, 1964).

(6) Peaks at *m/e* 87, 115, 157, 185, 227 and 255 indicate a regular triple methylene sequence in accord with the isoprenoid structure of 4,8,12-trimethyltridecanoic acid (cf. Sen Gupta & Peters, 1966).

Infrared spectrum. The methyl ester of B 101 exhibited an infrared spectrum (Fig. 2) very similar to that of 4,8,12-trimethyltridecanoic acid (H 155) from sheep fat (Hansen, 1968).

An isopropyl grouping was indicated by a strong band at about 1173 cm^{-1} accompanied by a weak shoulder at about 1155 cm^{-1}. 4,8,12-Trimethyltridecanoic acid from sheep fat (Hansen, 1968) displayed corresponding absorptions, but the spectra of this acid isolated by Sen Gupta & Peters (1966) from marine fish oil and by Sano (1967) from whale oil did not reveal a shoulder in the vicinity of 1150 cm^{-1}. Simpson & Sutherland (1949) and Pliva & Sörensen (1950) reported that a strong band at 1170 m^{-1} with a shoulder at about 1150 cm^{-1} is due to skeletal vibrations of the

isopropyl group $(CH_3)_2CH$. Further evidence for an isopropyl group was provided by the presence of a doublet at approximately 1368 and 1380 cm^{-1}. 4,8,12-Trimethyltridecanoic acid isolated from sheep fat (Hansen, 1968) showed a doublet at 1368 and 1381 cm^{-1}, while Sen Gupta & Peters (1966) observed a doublet at 1363 and 1374 cm^{-1} for the methyl esters of 4,8,12-trimethyltridecanoic acid, pristanic and phytanic acids. Pristanic and phytanic acids earlier isolated in this laboratory showed doublets at 1370 and 1380 cm^{-1} (Hansen & Morrison, 1964) and at 1364 and 1376 cm^{-1} (Hansen, Shorland & Morrison, 1965), respectively. Two closely spaced bands at 1368 and 1381 cm^{-1} have been attributed by Thompson & Torkington (1945) to the C—H deformation modes of isopropyl groups.

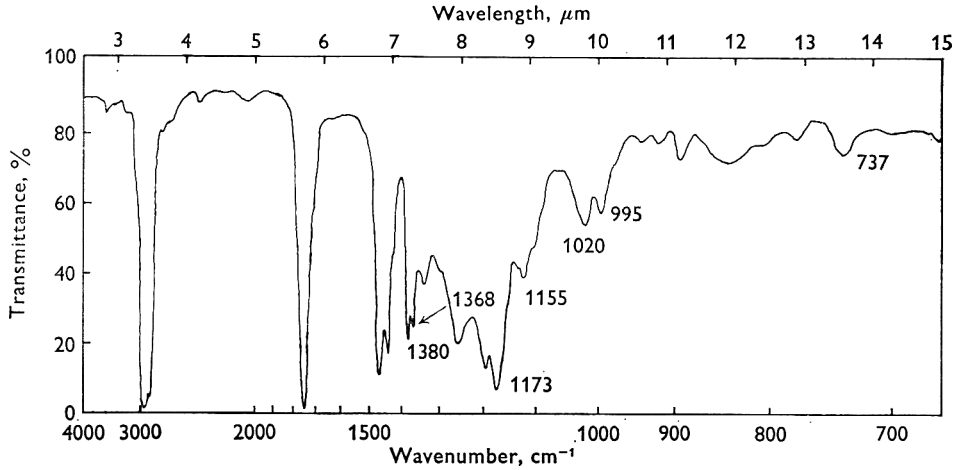


Fig. 2. Infrared spectrum of the methyl ester of fatty acid fraction B 101.

A regular isoprene chain sequence was indicated by a band at approximately 737 cm^{-1}, without an accompanying distinct shoulder at about 727 cm^{-1}. The corresponding band at 733 cm^{-1} was observed with 4,8,12-trimethyltridecanoic acid from sheep fat (Hansen, 1968). Sen Gupta & Peters (1966) reported a band at 733 cm^{-1} and the absence of absorption at 720–725 cm^{-1} for this acid as well as for phytanic and pristanic acids. Pliva & Sörensen (1950) and Bendoraitis, Brown & Hepner (1962) reported that a strong band at 735 cm^{-1} resulting from the methylene rocking vibration of the R—$(CH_2)_3$—R group was significant in proving the regular isoprene chain sequence.

A prominent doublet was observed for the methyl ester of B 101 at 995 and 1020 cm^{-1}: identical absorption was recorded for the methyl ester (but not the acid) of 4,8,12-trimethyltridecanoic acid from sheep fat (Hansen, 1968) Sen Gupta & Peters (1966) reported a doublet at 988 and 1010 cm^{-1} in the methyl esters of 4-methyl valeric acid, 4,8,12,16-tetramethylheptadecanoic acid and 4,8,12-trimethyltridecanoic acid, and accredited it with being characteristic of a methyl side chain on the fourth carbon.

DISCUSSION

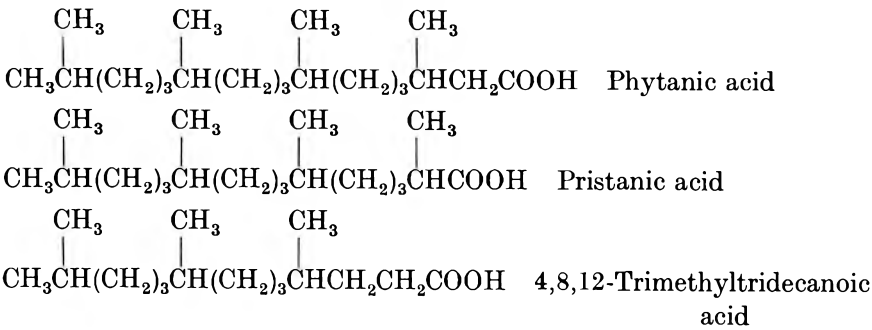
Based on mass and infrared spectrometry, saponification equivalent, iodine value and GLC, fraction B 101 isolated in this investigation is identified as 4,8,12-trimethyl-

tridecanoic acid. Corroboratory evidence was provided by the methyl ester co-chromatographing with authentic 4,8,12-trimethyltridecanoic acid from sheep fat (Hansen, 1968) when equal quantities were mixed and applied to both polyester (EGA) and ApL GLC columns at 207 and 170 °C, respectively. Independent examination of fraction B 101 with highly efficient open-tubular capillary columns coated with both polar and non-polar liquid phases, afforded further confirmation of the identity of this acid. The open-tubular column coated with BDS also indicated that the major component in B 101 was comprised of one peak only and by comparison with 4,8,12-trimethyltridecanoic acid synthesized from farnesol (see Ackman, 1968), was presumed to be the DD diastereoisomer. This GLC analysis further showed that the fraction contained approximately 4·1 % of impurity which was observed in the mass spectrum and which appeared to correspond with a shoulder on the rear of its peak prior to final purification, when chromatographed on the analytical EGA column.

Although hydrogenation was employed in this work, it was used only after the presence of 4,8,12-trimethyltridecanoic acid had already been detected by GLC in a concentrate of methyl esters separated from the unhydrogenated material.

In the sample of butterfat investigated, it is estimated that 4,8,12-trimethyltridecanoic acid constituted approximately 0·005 % of the total weight of fatty acids.

The route of biosynthesis of the polybranched fatty acid 4,8,12-trimethyltridecanoic acid from butterfat has not been established. Although some of the fatty acid constituents of butterfat are known to be synthesized in the mammary gland, there is no evidence to suggest that the isoprenoid acids originated from this source. The structure of 4,8,12-trimethyltridecanoic acid, however, like that of phytanic acid (3,7,11,15-tetramethylhexadecanoic acid), pristanic acid (2,6,10,14-tetramethylpentadecanoic acid), and the C_{19} and C_{20} hydrocarbons pristane (2,6,10,14-tetramethylpentadecane), and phytane (2,6,10,14-tetramethylhexadecane), suggests a relationship with the isoprenoid alcohol phytol (3,D-7,D-11,15-tetramethylhexadec-*trans*-2-en-1-ol (Burrell, Jackman & Weedon, 1959), which constitutes about 30 % of the chlorophyll molecule (cf. Bendoraitis *et al.* 1962; Hansen *et al.* 1965; Patton & Benson, 1966). The following chemical formulae of these 3 isoprenoid fatty acids illustrate their similarity of chemical structure:



It has been demonstrated by Steinberg and colleagues (Steinberg, Avigan, Mize & Baxter, 1965; cf. also Steinberg *et al.* 1967) and by Klenk & Kramer (1965), that when phytol is fed to rats some of it is converted to phytanic acid which is rapidly metabolized. In experiments with mice, Mize *et al.* (1966) established that administered phytanic acid is α-oxidized to pristanic acid which is further degraded by

successive β-oxidation steps to 4,8,12-trimethyltridecanoic acid, 2,6,10-trimethylundecanoic acid, and 4,8-dimethylnonanoic acid (cf. also Steinberg *et al.* 1967; Shorland, Hansen & Prior, 1966; Hansen *et al.* 1968). It is thought that in lactating ruminants the free phytol derived from ingested chlorophyll is converted to phytanic acid by rumen bacteria, and that after the bacterial lipids have been assimilated, part of the phytanic acid in the blood is degraded to pristanic acid and to 4,8,12-trimethyltridecanoic acid prior to redistribution in the body and milk fats of the animal. β-Oxidation of pristanic acid would appear to occur in ruminants as in mice, and available evidence indicates that the site of this enzymic or bacterial activity lies elsewhere than in the rumen (cf. Akashi & Saito, 1960). In support of this theory, phytanic acid has been isolated in minor proportions from rumen bacteria (Hansen, 1966) and from rumen contents (Patton & Benson, 1966), but an examination of the lipids from these sources did not reveal the presence of either pristanic acid or 4,8,12-trimethyltridecanoic acid. These 3 isoprenoid acids have, however, been found in ruminant body and milk fats.

The occurrence of diastereoisomers in phytanic and pristanic acids from terrestrial and marine sources was recently reported by Ackman & colleagues (Ackman & Hansen, 1967; Ackman *et al.* 1967; Ackman & Hooper, 1968). It was found that when using highly efficient open-tubular GLC columns coated with BDS, both phytanic and pristanic acids from a number of fats including sheep fat, butterfat, herring oil and finwhale blubber, were resolved into 2 peaks. By comparison with samples of authentic DDD phytanic and pristanic acids isolated from the lipids of bacterium *Halobacter cutirubrum* (Kates, Joo, Palameta & Shier, 1967), these peaks were identified as diastereoisomers. Of the 2 peaks exhibited by both phytanic and pristanic acids from butterfat, the larger was shown to be the DDD isomer and the smaller the LDD. With phytanic acid the mean ratio of LDD to DDD was 0·50, while with pristanic acid this proportion increased to 0·96. In marine animals, however, the LDD diastereoisomer predominated. The fraction of 4,8,12-trimethyltridecanoic acid isolated in the present investigation was found to exhibit the DD diastereoisomer only, and this is consistent with the derivation of 4D,8D,12-trimethyltridecanoic acid from phytol (3D,7D,11,15-tetramethylhexadec-*trans*-2-en-1-ol (Burrell *et al.* 1959), or from predominantly 2D,6D,10D,14-tetramethylpentadecanoic (pristanic) acid by stereospecific oxidation. The presence of one diastereoisomeric peak (DD) for the fraction of 4,8,12-trimethyltridecanoic acid from butterfat reported in this communication, corresponds with that observed for the same acid isolated from sheep fat (Hansen, 1968), and it accords in sign and magnitude with the small positive optical rotatory dispersion values measured on these samples.

It is probably of biological significance that small amounts of the isoprenoid hydrocarbons pristane and phytane (the latter generally in lesser proportions) have been found in crude petroleum (Dean & Whitehead, 1961; Bendoraitis *et al.* 1962); ancient geological sediments (Cummins & Robinson, 1964; Eglinton *et al.* 1966), recent marine sediments (Blumer & Snyder, 1965), human serum and tissues, rat liver, bovine serum, bovine tissues and milk, shark liver (Avigan, Milne & Highet, 1967), and algal mats (Oró & Nooner, 1966). Pristane has for a long time been known as a constituent of shark liver oil (Tsujimoto, 1917); it is also present in whale oil (Tsuchiya & Kaneko, 1951), herring oil (Hallgren & Larsson, 1963), ambergris (Lederer & Pliva, 1951), coal

tar (Kochloefl *et al.* 1963), and wool grease (Mold, Means, Stevens & Ruth, 1964). One species of higher plant (*Pimpinella anisum* L.) has also been reported to contain pristane (Brieskorn & Zimmermann, 1965). Appreciable proportions of this hydrocarbon have been found by Blumer, Mullin & Thomas (1963) in copepod planktonic crustaceans of the genus *Calanus*, and these investigators considered that pristane entered the food cycle of marine fish and marine mammals through dietary zooplankton (cf. also Sörensen & Sörensen, 1949).

The common occurrence of pristane in marine animal life, and of phytol in plant life, and the presence of isoprenoid hydrocarbons and corresponding isoprenoid fatty acids in ruminant fats, human fats, marine fish oils, whale oils, crude petroleum and geological sediments, indicate the probable existence of more than one pathway in the biosynthesis of 4,8,12-trimethyltridecanoic acid. It appears probable, however, that the phytol moiety of chlorophyll is the precursor of this and other isoprenoid fatty acids, whether they are of terrestrial or marine origin.

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With 2 plates

An inducible antibacterial agent produced by a strain of *Streptococcus cremoris*

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Summary. An antibacterial agent is produced by ultraviolet (UV) irradiation of *Streptococcus cremoris* strain C 11–56. Its production is accompanied by cell lysis and it attacks the strain C 11–56 which produces it as well as a number of other *Str. cremoris* strains. A correlation between sensitivity to the antibacterial agent and sensitivity to trypsin has been demonstrated. Electron micrographs have shown the presence of particles resembling phage heads, most of which are empty, and of only very few intact phage-like particles in the lysate. The active agent in the lysate is associated with a particle of $M > 100000$. The evidence suggests that the activity of the lysate may be due to the presence of a defective phage or of a lethal component of it.

Various species of bacteria are known to produce antibacterial substances, which are grouped under the name of bacteriocins. They may be produced in a culture spontaneously, or induced by exposure to UV irradiation or other inducing agents. They have the ability to kill sensitive cells but do not cause them to produce more bacteriocin. Recently, the association of phage-like particles, and of particles which resemble parts of phages, with various bacteriocins has been demonstrated (Bradley & Dewar, 1966; Ishii, Nishi & Egami, 1965; Fredericq, 1963) and the subject has been reviewed by Bradley (1967).

Like lysogenic strains of bacteria which are immune to the phages they produce, bacteriocinogenic strains are immune to their bacteriocins. Colicin 15, a bacteriocin produced after UV irradiation of *Escherichia coli* strain 15, is unusual in that it is able to attack the strain which produces it. Its production is accompanied by cell lysis (Ryan, Fried & Mukai, 1955). Recently, electron microscope studies of colicin 15 have revealed the presence of phages and parts of phages (Endo, Ayabe, Amako & Takeya, 1965; Sandoval, Reilly & Tandler, 1965; Mennigmann, 1965). It has been suggested that the action of colicin 15 is related to the presence of defective phage.

The production of bacteriocins of enterococci has been described by Brock, Peacher & Pierson (1963) and of group A streptococci by Kuttner (1966). The present communication describes an antibacterial substance produced after UV irradiation of a *Str. cremoris* strain C 11–56. Like colicin 15 its production is accompanied by cell lysis and the strain which produces it is also sensitive to it.

METHODS

Cultures. The original *Str. cremoris* culture strain C 11 was stored for many years by 2 methods. The culture C 11–56 used in the present study was stored in freeze-dried form, and a culture C 11-S was stored in litmus milk containing 3% calcium carbonate. The 2 cultures were found to differ from each other in sensitivity to the phage c 11, which produced a titre on C 11–56 which was 1000-fold lower than that on C 11–S.

Source of ultraviolet radiation. An Oliphant type lamp with a 24-in. tube of low-pressure mercury vapour and maximum emission at 2537 Å was used at a distance of 36 cm for 30 sec.

Irradiation of culture. Str. cremoris C 11–56 was grown for 6 h in 9 ml of tryptone yeast extract broth (TYB) of pH 6·8, containing tryptone, 3·0%; yeast extract, 1·0 %; lactose, 0·5%; meat extract, 0·2%; NaCl, 0·3%; and K_2HPO_4, 0·5%. The culture was centrifuged and the deposit resuspended in 9 ml of $\frac{1}{4}$ strength Ringer's solution. Three-millilitre quantities of this suspension were transferred to Petri dishes and exposed to UV radiation. The irradiated suspension was then inoculated into TYB at the rate of 4 ml of suspension to 18 ml of broth, incubated at 30 °C, for 1·5 h, allowed to stand at room temperature and examined at intervals for lysis by measuring the percentage transmittance at 700 nm with a Beckman DK 2 spectrophotometer. This wavelength was selected because it minimized interference from components of the medium.

Tests for antibacterial activity. Twofold serial dilutions were made of the lysates produced by the UV irradiation of the cultures and spotted, using drops from a Pasteur pipette, on plates of tryptone yeast extract agar (TYA; of composition similar to that of the broth but with the addition of 1·5% agar) which had been surface-seeded with indicator cultures. Plates were examined for clear zones produced by the lysate after incubation at 30 °C overnight. The indicator cultures used were the strains C 11–56 and C 11–S and other group N streptococcal strains.

Test for heat sensitivity. The heat sensitivity of the antibacterial substance was determined at 60 °C by immersing the lysate in sealed phials in a water-bath and testing for activity at intervals of from 2 to 30 min by spotting on agar seeded with C 11–56 and *Str. cremoris* strain BK 5.

Tests for transfer of activity. The transferability of the activity of the antibacterial substance was investigated by taking sections of clear zones from the plates and transferring them to broth and litmus milk inoculated with strains C 11–56 and C 11–S and other strains known to be sensitive to the lysate. After incubation at 30 °C overnight, the broths were centrifuged and the supernatant fluids spotted on to seeded plates which were then incubated for 24 h and examined for clear zones and plaques. Litmus milks were centrifuged after incubation at 30 °C overnight and the whey tested for bacteriophage as described below.

Test for effect of filtration. The effect of filtration of the lysate was determined by passing it through Seitz filters and membrane filters (Hemming filters, Oxoid Ltd). Both types of filtrate were titrated on strain C 11–56 and *Str. cremoris* strain BK 5.

Tests for bacteriophage. Tests for bacteriophage were made on 1 ml quantities of lysate by the double-layer plate technique (Gratia, 1936), using TYA without

phosphate as the base layer and a medium containing 0·17 % calcium chloride and 0·9 % agar as the seed layer. The strains C 11–S and C 11–56 and 19 other strains of *Str. lactis*, *Str. cremoris* and *Str. diacetilactis* were used to seed the plates. Lysates were also titrated to extinction by spotting on seeded plates and examining the areas where spots had been placed for phage plaque.

Electron microscope studies. Lysates and the ammonium acetate washings of agar seeded with strain C 11–56 which had been cleared by the action of the lysate were examined under the electron microscope. They were centrifuged at 120000 ***g*** and the deposits were negatively stained with neutral potassium phosphotungstate and examined with a Siemen's electron microscope at a magnification of × 40000.

Separation of active fraction. The lysate was separated into fractions by passage through a Sephadex column 5 cm diam. and containing 25 g of Sephadex G 100. A 0·2 M phosphate buffer of pH 6·6 was used for elution. The column was calibrated by passage of casein micelles, β-lactoglobulin (M.W. 35000) and α-lactalbumin (M.W. 17000). The various fractions of the lysate were titrated on strains C 11–56 and BK 5.

RESULTS

Spotting of the UV irradiated suspension of C 11–56 on a lawn of C 11–56 resulted in a clear zone on incubation of the plate. The irradiated suspension itself did not lyse on storage at room temperature.

On incubation, the broth (TYB) inoculated with the UV irradiated suspension increased in turbidity for at least 4·5 h and then gradually cleared. After standing overnight at room temperature, the clearance was almost complete and proceeded to completion even after the culture was transferred to a refrigerator. Spotting of the lysate on a lawn of C 11–56 resulted in a marked clear zone which increased in clarity but not in area when plates were held for several days at room temperature. Supernatants of non-irradiated cultures showed no activity when spotted on seeded agar.

Nine out of 12 strains of *Str. cremoris* were sensitive to the lysate and none of the 2 strains of *Str. lactis* or of the 3 of *Str. diacetilactis* was sensitive. Titres on the various sensitive strains ranged from 1/16 on strain C 11–56 to 1/256 on strain BK 5.

Resistant cultures isolated from the lysate of C 11–56, and from plates seeded with C 11–56 which had been directly irradiated, varied in their sensitivity to the lysate and in their ability to lyse and produce the active substance on re-irradiation. Culture C 11–56 therefore contained mutants.

The culture C 11–S did not lyse completely after irradiation but produced some of the antibacterial substance. Two isolates from it, selected because of their lower sensitivity to phage, did not lyse when irradiated. Both produced the active substance to a very slight extent but one was sensitive to the lysate of C 11–56, whereas the other showed only a delayed reaction. The parent C 11–S culture was of lower sensitivity to the lysate than C 11–56 and showed only a delayed reaction. Zones sometimes appeared only after holding plates for several days at room temperature. The culture C 11–S apparently also contained mutants.

Exposure of the lysate to a temperature of 60 °C gradually destroyed the active substance. The active substance did not diffuse in agar, since the activity was restricted to the drop area. Seitz and membrane filtration reduced the activity of the lysate 8-fold.

In attempting to demonstrate the effect of trypsin on the lysate it was found that the culture C 11–56 was sensitive to trypsin, which caused a complete clearing of the seeded agar. The culture C 11–S was insensitive to it. Other strains which were very sensitive to lysate were also sensitive to trypsin. It was therefore difficult to interpret what effect trypsin had on lysate action.

The activity of the lysate was destroyed by 0·02 N-hydrochloric acid at pH 3·9 so the effect of pepsin could not be determined.

All attempts to transfer activity from clear zones on seeded plates failed, as did attempts to detect the presence of bacteriophage by cultural techniques.

The sensitivity to lysozyme of the 9 group N streptococcal strains which were sensitive and of the 8 strains resistant to the action of the lysate was determined by the same method as was used for determining the sensitivity to lysate, except that pure egg-white lysozyme (100 mg/ml, in phosphate buffer of pH 6·6) was used instead of lysate. A correlation between the lysate sensitivity and lysozyme sensitivity was shown, with 2 exceptions. Strain C 3 of *Str. cremoris* was sensitive to lysate and resistant to lysozyme and *Str. cremoris* EB 7 was resistant to lysate and sensitive to lysozyme.

Examination under the electron microscope of the lysate and of the ammonium acetate washings from plates cleared by the action of the lysate revealed the presence of hexagonal bodies 500 Å in diam. These bodies resembled phage heads in their morphology. The majority of the heads were empty (Plate 1, Fig. 2 and Plate 2, Fig. 3). Only 3 particles resembling intact phages were seen in the many fields examined (Plate 1, Fig. 1). Their presence was demonstrated in the lysate preparation only.

The intact phages of strain C 11 are shown in Plate 2, Fig. 4 which was prepared as a control to show that the technique employed for preparation and staining was capable of demonstrating and preserving intact a virulent phage particle.

By filtration of the lysate through a calibrated column of Sephadex G 100 it was demonstrated that its activity occurred only in the fraction containing solute of molecular weight > 100000.

DISCUSSION

The behaviour of C 11–56 after UV irradiation closely resembles that of *Escherichia coli* strain 15 (Ryan *et al.* 1955) and the lysates of both have closely corresponding properties and activity. The presence in the electron micrographs of particles resembling phage heads most of which were empty and of only very few intact phage-like particles, together with failure of cultural techniques to demonstrate the presence of phage, suggests that the UV irradiation of C 11–56 resulted in the liberation of a defective phage. The possibility remains that the apparent inability of the particles to reproduce could be due to the absence of a suitable host. The fraction from the Sephadex column containing solute of M.W. > 100000, which was found to contain the activity, would include the phage-like particles and suggests that the phage-like particles or particles resembling phage parts are responsible for the observed activity of the lysate. There was no activity in the Sephadex fractions which would contain low molecular weight enzymes, but the possibility must be kept in mind that such enzymes could be attached to larger particles. It is possible that lethal protein of defective phage (Fredericq, 1963) is responsible for the action of the lysate.

The activity of the antibacterial substance occurs over a wide range of *Str. cremoris* strains. It seems likely that the active agent is adsorbed on the cell wall and that the difference between the 2 strains C 11–56 and C 11–S and between the sensitive and resistant strains of other group N streptococci lies in a difference in cell-wall composition. This suggestion is supported by evidence that those strains which are strongly sensitive to lysate action are also strongly sensitive to trypsin, while those strains which are not sensitive to lysate are not sensitive to trypsin. It has been reported (N.I.R.D. 1965, 1966) that trypsin acts directly on the cell wall of some group N streptococci and that the material removed contains some of the phage receptor material.

Two lines of evidence suggest that lysozyme is not the active substance in the lysate. The first is the absence of activity in the Sephadex fraction that would contain it. The second is the lack of complete correlation between egg-white lysozyme and lysate sensitivity.

The present communication provides further evidence of an association of defective phage with bacteriocin-like activity, and of the ability of yet another bacterial species to produce, on exposure to an inducing agent, phage-like particles which are incapable of self reproduction in a recipient host.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Electron micrograph showing 2 intact phage-like particles. Preparation made from the lysate produced by the UV irradiation of *Str. cremoris* strain C 11–56. Magnification, × 200 000; 1 cm = 500 Å.

Fig. 2. Electron micrograph showing hexagonal bodies resembling phage heads, some of which are empty. Preparation made from the lysate produced by UV irradiation of *Str. cremoris* strain C 11–56. Magnification, × 200 000; 1 cm = 500 Å.

PLATE 2

Fig. 3. Electron micrograph showing hexagonal bodies resembling phage heads, the majority of which are empty. Preparation made from the ammonium acetate washings of agar plates seeded with *Str. cremoris* strain C 11–56 and cleared by the action of the lysate produced by the UV irradiation of that strain. Magnification, × 200 000; 1 cm = 500 Å.

Fig. 4. Electron micrograph of virulent phages of *Str. cremoris* strain C 11. Magnification, × 200 000; 1 cm = 500 Å.

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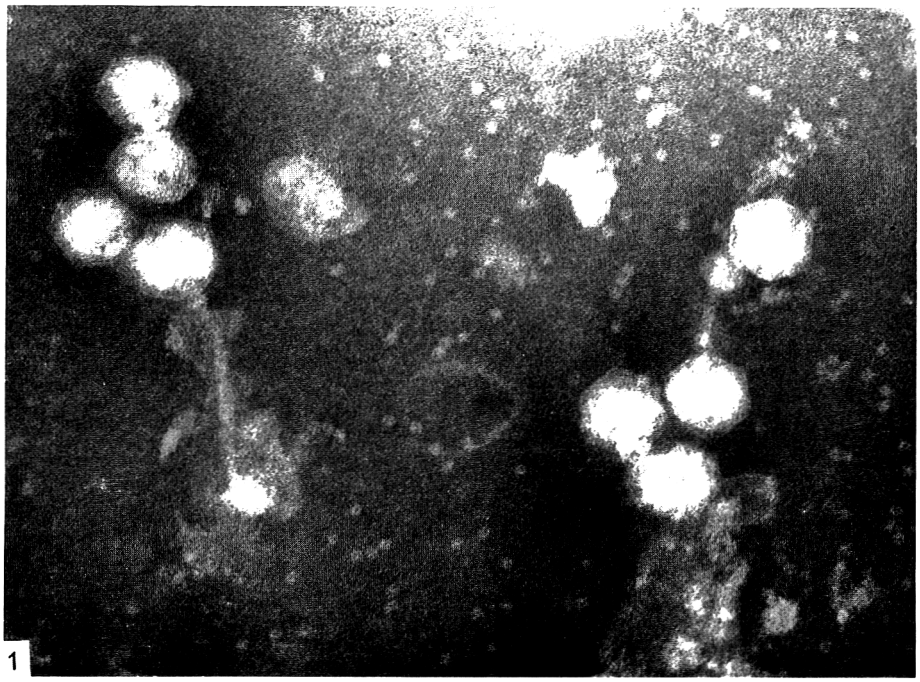

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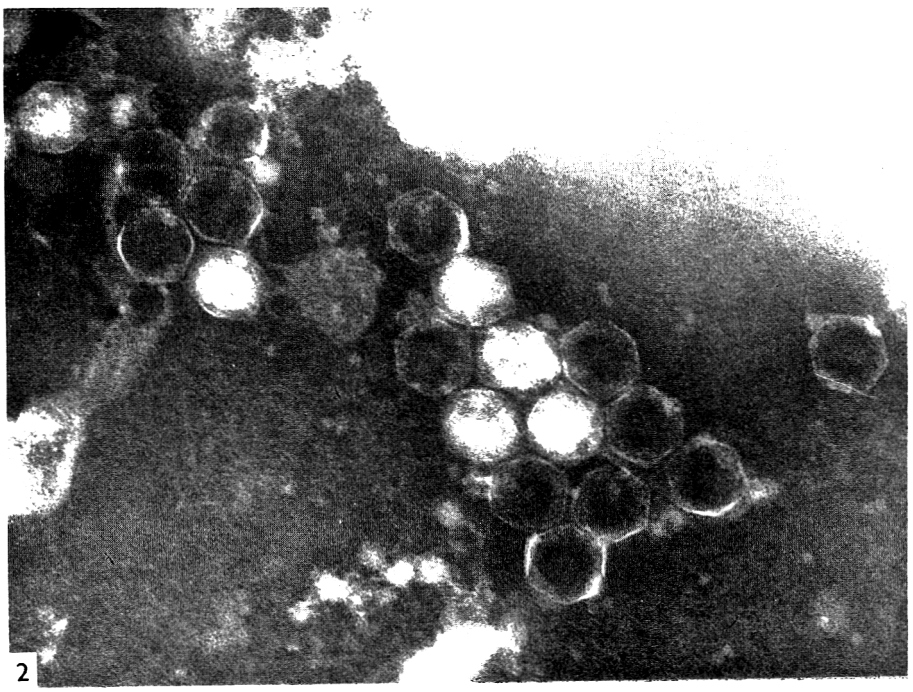

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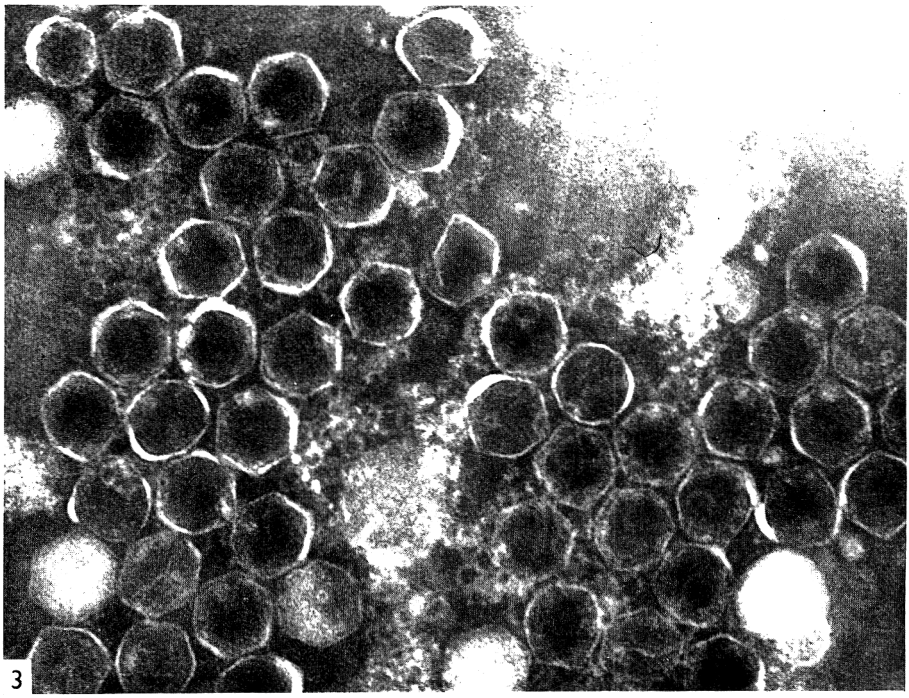

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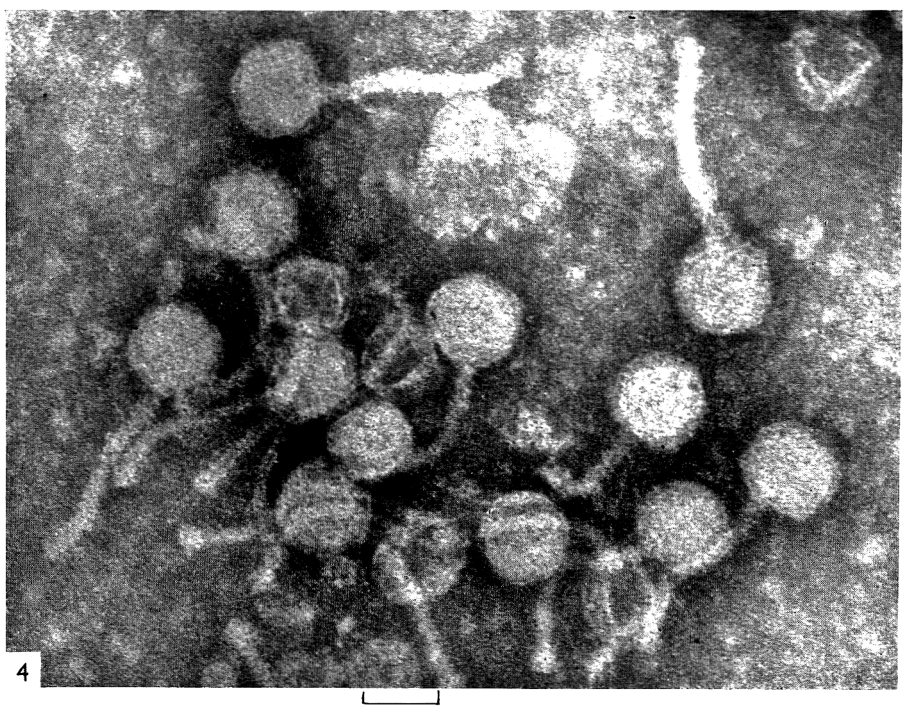

4

BARBARA P. KEOGH AND P. D. SHIMMIN

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With 1 *plate*

Lactose, lactic acid and mineral equilibria in Cheddar cheese manufacture

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Summary. In experimental small-scale manufacture of Cheddar cheese a study was made of the movement of lactose, lactic acid, calcium and phosphorus between curd and whey, and of the effect of high acidity developed in the curd before whey separation, on the pH and physical properties of the cheese. It was found that while the curd particles remain in contact with the whey, the lactose fermented in the curd is replaced by lactose diffusing from the whey. Lactic acid produced in the curd diffuses into the whey rather slowly.

When a high lactic acid level is attained rapidly the curd does not cheddar well, the pH of the cheese is low, the flavour is sour and bitter, the body is crumbly and the cheese is bleached. When a high lactic acid level is reached slowly, the curd also does not cheddar properly but the pH of the cheese may be within normal limits up to 3 weeks, at which age its body is coherent and its colour is normal. However, on maturing, the cheese becomes acid, the body crumbly and liquid separation occurs even at 2 months of age. This points to a risk in grading cheese at an early age.

It is suggested that the abnormal behaviour in cheddaring of 'high-acid' curds is connected with an excessive loss of calcium. When high acidity is developed over an extended time in the whey, the curd retains more lactose but loses more phosphorus so that its buffering capacity is reduced. Cheese made from such curd becomes highly acid at maturity and this leads to the development of a bitter flavour.

It is, therefore, concluded that both the rate of development of acidity, and the time the curd is in the whey, are critical factors in controlling the quality of Cheddar cheese.

In their extensive studies on the chemistry of Cheddar cheese, Dolby, McDowall & McDowell (1937*a*, *b*) have shown a close relationship between the acidity or pH of the whey at 'running' and both the pH and the mineral content of the cheese. Later Dolby (1941) confirmed that the lower the pH value of the whey at 'running', the lower was the pH value of the cheese. He also observed that curd from whey with the higher acidity at 'running' had in the later stages of the process a higher lactose content for the same pH value, and thought that this was probably caused by replacement of lactose used up in the curd particles by lactose from the whey. Brown & Price (1934) had earlier observed a correlation between the pH at 'running' and the mineral

content of the cheese. Mattick (1938) suggested that differences in the mineral content of Cheddar, Cheshire, Leicester, Lancashire and Stilton cheese probably play a part in determining their characteristics.

Whilst these observations leave little doubt of the existence of these relationships, the mechanisms responsible have not been adequately defined. Further, it is known from practical experience that high acidity of the whey at running does not always result in a low pH of the cheese. To throw more light on these phenomena, we have studied the lactose–lactic acid equilibria between the curd and the whey and their effect on the following properties of the cheese: (*a*) pH; (*b*) calcium and phosphorus content; (*c*) physical and flavour characteristics. An additional purpose of this study was to gain a better understanding of the efficiency of traditional control measures in the manufacture of cheese, as a basis for the design of automatic control systems for producing cheese of uniformly high quality.

EXPERIMENTAL

Cheddar cheese was made in 3 series of experiments in vats of 50 gal (Imp.) capacity, using either C_1 or C_2 single-strain starter cultures in amounts ranging from 2·4 to 3·0 % for the control and extended time vats and double the amount for the rapid vats. The milk was evening bulk milk held overnight at 38–40 °F and pasteurized at 162 °F for 15 sec. The purpose of series I was to assess the effect of higher whey acidities at 'running' and also of extended time of contact between the curd and the whey. It consisted of 4 experiments in which both a control vat and a test vat were inoculated with the same amount of the same starter. Rennet was added immediately thereafter and the curd was cut about 30 min later and stirring commenced. The titratable acidity of the whey 5 min after cutting was determined and the curd was 'cooked' in 40–45 min to 100 °F. When the titratable acidity of the whey rose by 0·025 % above that at cutting, half of the whey was drained off. The vat contents were then held at 100 °F and stirred until the acidity of the whey reached 0·165–0·185 at which stage all the whey was drained from the control vat. In the test vats, stirring in the whey and development of acidity was continued for further periods of 50, 95, 135 and 240 min, after which all the whey was run off. The curd was banked, cheddared, milled, salted, hooped and pressed in the normal manner. The cheese was then matured at about 55 °F, and sampled and examined at intervals.

Series II comprised 4 experiments in which the curd in both the control and the test vats remained in contact with the whey for the same time, on average 1 h 50 min, but the rate of acid development in the test vat was much faster. This was achieved by inoculating the milk in the test vat with double the amount of starter. At the time of running off all the whey, the titratable acidity in the test vat was 0·05–0·07 % higher than in the control vat. From this point the curd was handled as in series I.

In series III, one control vat and 3 test vats were used. In the first of the test vats the curd was held in the whey for an additional 135 min and in the second test vat for an additional 240 min. In the third test vat the curd was held in the whey for the same time as in the control vat but to obtain fast acid development twice as much starter was added.

Samples of whey, curd and cheese were taken at the following points: (*a*) 20 min

after cutting; (*b*) when half of the whey was run off; (*c*) when all of the whey was run off; (*d*) at 'dry' (series I only); (*e*) at milling; (*f*) on completion of pressing; (*g*) at 7 days and in some cases at 14 days after manufacture.

The samples from series I and II were analysed for pH and for moisture, lactose, lactic acid, calcium, and the whey also for phosphorus. Salt content was determined on samples from the cheese. Reflectance measurements using 'Agtron' equipment were made on the cheese from series II. Samples from series III were analysed for titratable acidity of the whey, pH of the curd, calcium content of the whey and curd and phosphorus content of the whey at the time of complete whey separation. In addition, pH of the cheese ex press, after 1, 2, 3 and 9 weeks, calcium content ex press, buffering capacity and moisture and salt content of the cheese were determined.

Analytical methods

For the measurement of pH a glass electrode was used and a 1:1 (w/w) slurry of grated cheese and distilled water. Moisture content was determined by difference, drying overnight at 102 °C. Lactose in curd and whey was measured by a modification of the phenol–sulphuric acid method of Barnett & Tawab (1957), and in cheese by the method of Sutherland & Van Leeuwen (1967). Lactic acid was determined by chromatographic separation according to Velasco & Noll (1957) and the colorimetric procedure of Davidson (1949). Calcium was measured by the indirect method of Sawyer & Hayes (1961) and phosphorus by the colorimetric method of Allen (1940). Buffer capacity was measured as follows: 10 g of cheese was macerated for 5 min with 50 ml distilled water at room temperature and centrifuged. The macerate was then chilled in iced water and the fat removed with a spatula. The aqueous portion was filtered through a no. 41 Whatman paper and the curd washed with distilled water until the filtrate had a volume of 100 ml; 50-ml aliquots were then titrated with 0·05 N-NaOH and the volume (in ml) required to increase the pH value from 5·50 to 7·40 was recorded as the buffer capacity.

RESULTS AND DISCUSSION

A major change which occurs in the conversion of milk into cheese is the removal of water from the coagulated milk solids. In the manufacture of Cheddar cheese this removal of water from the gel and the establishment of desirable levels of lactose, lactic acid and minerals in the cheese must largely be achieved while the curd is in the whey. The process of syneresis is accompanied by the development of lactic acid with the consequent solubilization of mineral constituents of the casein complex and by the diffusion of substances such as lactose and lactic acid into or out of the curd. The extent to which these constituents remain in the curd is affected not only by the movement of water but also by the rate of acid development, the nature of the casein, and the membrane formed on the surface of the curd granule in the cooking process.

The cheesemaker must manipulate the variables such as the amount of starter added, the cooking temperature, the length of time the curd is in the whey and the level of salt, to achieve the desired cheese quality. The better his understanding of the complex interactions in the cheese-making process, the better will be his control. The discussion which follows is an attempt to explain the results as shown in the accompanying figures and tables and to increase this understanding.

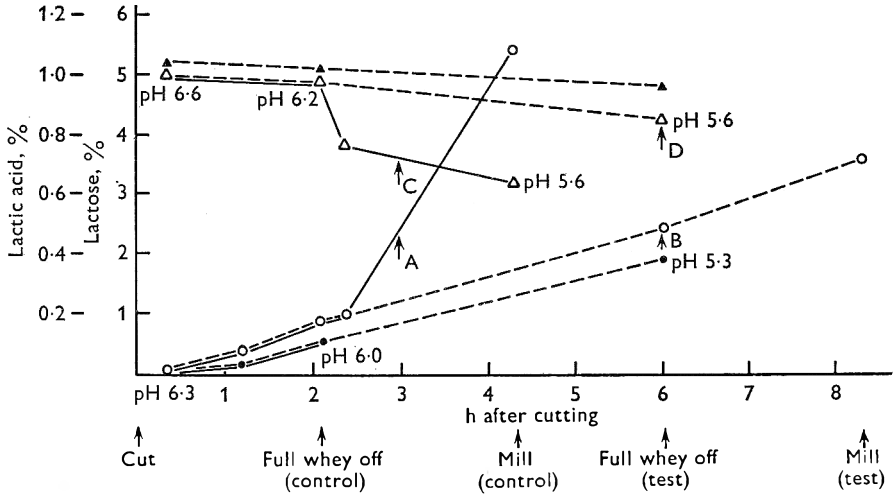


Fig. 1. Lactose, lactic acid and pH in curd and whey in a control vat and a test vat with extended time of contact of curd with the whey. ——, Control vat; – – –, test vat; ○, lactic acid in curd water; ●, lactic acid in whey; △, lactose in curd water; ▲, lactose in whey.

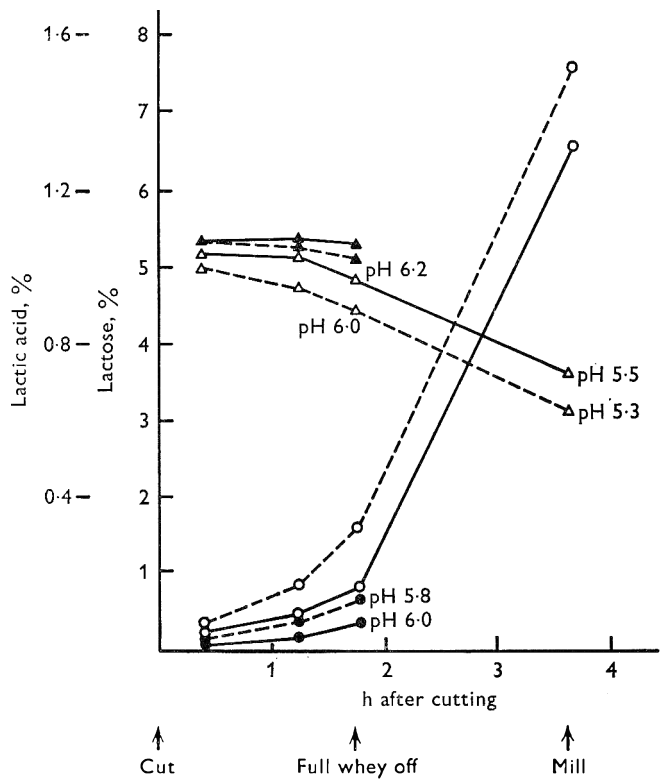


Fig. 2. Lactose, lactic acid and pH in curd and whey in a control vat and test vat with rapid development of acidity. ——, Control vat; – – –, test vat; ○, lactic acid in curd water; ●, lactic acid in whey; △, lactose in curd water; ▲, lactose in whey.

Lactose and lactic acid equilibria

As can be seen in Figs. 1 and 2, and Tables 1 and 2, the concentration of lactose is always higher in the whey than in the water of the curd. The concentration of lactic acid, on the other hand, is always lower in the whey than in the water of the curd although, because of the greater buffering capacity, the pH of the curd is higher than that of whey. These effects are due to the much greater fermentative activity in the curd where most of the bacterial cells are to be found. As the lactic acid is produced and diffuses into the whey the lactose level in the curd declines. But as Dolby (1941) thought, some of the lactose used up in the curd is replaced by lactose diffusing from the whey. This replacement is clearly demonstrated in Fig. 1 where it can be seen that for the same lactic acid contents in the 2 curds (points A and B), the lactose content of the control curd is 3·75 % (point C) while for the curd held longer in the whey it is 4·32 % (point D).

Table 1. *Experiments on extended time in whey (series I): pH value and lactose and lactic acid content of curd and whey at the time of whey separation*

Expt no.	Extension time in whey, mins	pH value		Lactose, %		Lactic acid, %	
		Whey	Curd	Whey	Curd water	Whey	Curd water
1	50	5·96	6·11	5·24	4·38	0·12	0·169
2	95	5·70	5·75	5·20	4·56	0·237	0·420
3	135	5·63	5·70	5·17	4·46	0·270	0·430
4	240	5·32	5·62	4·90	4·32	0·352	0·487
Controls*	0	6·00	6·16	5·16	4·68	0·106	0·148

* Average results from 4 expts.

Table 2. *Experiments on fast acid development (series II): pH value and lactose and lactic acid content of curd and whey at the time of separation, and pH value of cheese ex press and at one week*

Expt no.	pH value		Lactose, %		Lactic acid, %		pH value of cheese		Salt in cheese water, %
	Whey	Curd	Whey	Curd water	Whey	Curd water	Ex press	At 1 week	
1	5·70	5·81	4·86	4·59	0·195	0·326	5·03	4·96	4·54
2	5·67	5·92	5·20	4·35	0·200	—	5·06	5·00	4·72
3	5·70	5·76	5·17	4·54	0·191	0·336	5·20	5·20	4·96
4	5·72	5·92	5·13	4·53	0·201	0·337	5·30	5·35	5·10
Controls*	6·00	6·15	5·18	4·67	0·121	0·180	5·16–5·54	5·15–5·61	4·86

* Average results from 4 expts.

The rate at which lactose diffuses back into the curd depends on 2 factors—the rate at which the lactose in the curd is converted to acid and the permeability of the curd particle and its membrane. With rapid acid development the concentration of lactose in the curd-water falls more sharply than in the controls or in the 'extended' vats (Figs. 1, 2). This suggests that when acid development is fast, lactose is not replaced as rapidly as it is used up. However, at normal rates of acid production the

levels of lactose in the curd-water and in the whey are relatively little affected by the level or rate of acid development in the curd (Table 5).

The use of a uniform cooking temperature throughout should have reduced to a minimum any variations in the nature of the curd particle membrane.

It was initially intended to follow the fermentation of lactose into lactic acid in the early stages of maturing of the cheese from these experiments. However, due to the non-specificity of the modified Barnett and Tawab method for lactose determination in the presence of other reducing compounds this was not possible for series I and II. As the interference was of little significance in curd samples taken during manufacture the analytical data obtained using this method were considered acceptable. In the later experiments with extended-time and fast-acid (series III) the analyses of cheese for lactose were made by the method of Sutherland & van Leeuwen (1967) which is specific for lactose.

Table 3. *Experiments on extended time and fast acid development (series III): pH value of curd at the time of whey separation and of cheese at various ages, and percentage moisture and salt in the cheese*

Vat	Expt no.	pH value of curd	pH value of cheese						Moisture, %	Salt in cheese water, %
			Ex press	1 week	2 weeks	3 weeks	9 weeks	18 weeks		
Controls	1	6·10	5·24	5·12	5·10	5·13	—	5·20	36·9	4·40
	2	6·14	5·50	5·30	—	5·48	5·32	—	36·0	5·30
Extension 135 min	1	5·38	5·17	5·05	5·12	5·03	—	4·80	35·2	5·48
	2	5·17	5·12	5·03	—	5·14	4·80	—	34·0	5·35
Extension 240 min	1	5·17	5·12	5·02	5·05	5·05	—	4·73	34·1	5·86
	2	5·14	5·11	5·02	—	5·12	4·80	—	35·8	4·95
Fast acid	1	5·66	5·06	4·90	4·85	4·91	—	4·90	36·5	4·44
	2	5·75	5·22	5·15	—	5·17	5·12	—	36·9	5·34

Table 4. *Calcium contents in curd and whey and phosphorus levels in whey at the time of whey separation, and pH values of cheese ex press (averages for each series)*

Expt	Ca in whey, mg/ml	Ca in curd, mg/g solids	Phosphorus in whey, mg/ml	pH value of cheese, ex press
Controls	0·80	15·0	0·41	5·16–5·60
Fast acid (series II)	1·08	12·5	0·48	5·03–5·30
Fast acid (series III)	1·05	10·7	0·55	5·06
Extension 135 min (series III)	1·55	8·6	0·74	5·17
Extension 240 min (series III)	1·89	6·0	0·88	5·12

Mineral equilibria—calcium and phosphorus

Before calcium and phosphorus can be lost from the curd they must no longer be bound. At the pH of milk, colloidal calcium phosphate is only sparingly soluble, but when the pH drops as in normal cheese manufacture some of this goes into solution and is lost into the whey. Under abnormal conditions of manufacture, such as when high acidity develops either rapidly or over an extended time, more of the calcium phosphate is solubilized and greater loss occurs.

When high acidity is reached quickly sufficient calcium is removed to alter the physical properties, but insufficient phosphate is lost to seriously affect the buffer capacity of the cheese (Tables 4 and 7). This would indicate that in this system the calcium ion has greater mobility than the phosphate ion.

When high acidity is reached over extended time a very significant loss of both calcium and phosphorus occurs (Table 4). The loss of phosphorus in this case is of much more consequence than when the acid is developed rapidly because it is sufficient to cause a substantial lowering of the buffer capacity of the cheese (Table 7).

Table 5. *Experiments on extended time and fast acid development (series I and II combined): pH value and lactose and lactic acid content of curd and whey at the time of whey separation*

Expt	pH value		Lactose, %		Lactic acid, %		Lactic acid difference, % curd-water and whey
	Whey	Curd	Whey	Curd water	Whey	Curd water	
Extension 135 min (series I)	5·63	5·70	5·17	4·46	0·270	0·430	0·160
Extension 240 min (series I)	5·32	5 62	4·90	4·32	0·352	0·487	0·135
Fast acid* (series II)	5·70	5·84	5·09	4·50	0·197	0·333	0·136
Controls†	6·00	6·16	5·17	4·68	0·114	0·164	0·050

* Average results from 4 expts.
† Average of series I and II.

Table 6. *Lactic acid and lactose content of cheese from 2 experiments on extended time and fast acid development (series III)*

	Expt A. 3 weeks		Expt B 1 week		Expt B 3 weeks	
	Lactic acid, % in cheese	Lactose, % in cheese	Lactic acid, % in cheese	Lactose, % in cheese	Lactic acid, % in cheese	Lactose, % in cheese
Control	0·80	0·024	0·54	0·43	0·80	0·27
Extension 135 min	0·60	0·43	0·36	0·83	0·41	0·64
Extension 240 min	0·35	0·79	0·36	0·79	0·52	0·64
Fast acid	0·98	0·016	0·74	0·44	1·13	0·26

pH

At 2 weeks the pH of cheese from the control and the 'extended-time' vats had dropped to normal values whilst that of the 'fast-acid' vat was considerably lower (Table 3). However, at 9 weeks the pH of the cheese of the 'extended-time' vats became abnormally low. Thus, if a high acidity is reached over a long period in the whey, the pH of the cheese at 3 weeks, the usual time for grading Australian cheese for export, is not greatly affected, but the cheese ultimately becomes highly acid. An extended time in the whey results in a high lactose content at 3 weeks (Table 6) and a loss of phosphorus (Table 4) which lowers the buffer capacity of the cheese (Table 7). Because of the low buffering capacity the lactic acid eventually produced from the additional lactose causes an abnormally large fall in pH.

The pH of the cheese from 'fast-acid' vats at 2–3 weeks was lower than in the controls (Tables 2 and 3) due to the higher level of lactic acid (Table 2). The normal

buffer capacity of cheese from the 'fast-acid' vats (Table 7), in spite of the high acidity, is attributed to there having been insufficient time for a substantial loss of phosphorus from the curd particles into the whey (Table 4).

Table 7. *Experiments on extended time and fast acid development (series III): the buffering capacity and phosphorus content of extracts of cheese, and the pH value of cheese at* $3\frac{1}{2}$ *weeks of age*

Expt	Buffering capacity*	Extracted phosphorus, mg/g cheese	pH of cheese
Control	10·0	1·67	5·13
Extension 135 min	8·2	1·40	5·03
Extension 240 min	5·5	0·98	5·05
Fast-acid	11·0	1·97	4·91

* See p. 95.

Table 8. *Experiments on fast acid development (series II): reflectance of cheese from control and test vats*

Expt no.	Agtron reflectance reading, % 0–97 scale	
	Control	Test
1	52·0	58·8
2	53·8	59·4
3	58·8	66·4
4	61·8	72·0

Physical properties

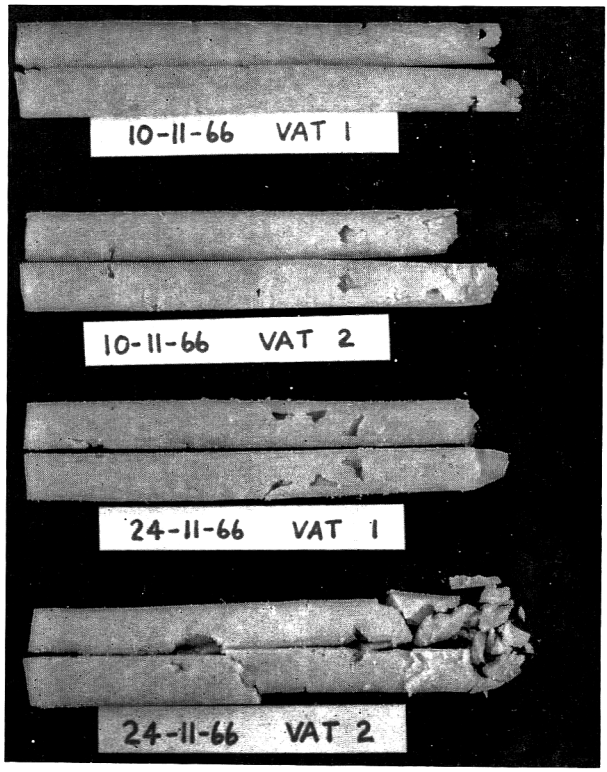
(*a*) *Body and texture*

In comparison with the curd in the control vats the curd in all the more acid vats did not fuse readily during cheddaring, but tended to remain granular. The most obvious explanation is that this failure was due to loss of calcium (cf. Mattick, 1938).

On maturing, the cheese from acid vats was crumbly and mealy so that on sampling the plugs tended to disintegrate as illustrated in Plate 1. The poor body and texture may be explained by the effect of low pH on the colloidal phosphate in association with a low calcium content. It is known that at low pH more of the colloidal phosphate becomes soluble and no longer contributes to curd structure (McGann & Pyne, 1960; Pyne & McGann, 1962). The precise effect of the level of phosphate on the physical properties of the cheese is not known. Shehata, Iyer, Olson & Richardson (1967) have shown, however, that cheese made with phosphoric acid and therefore containing a high level of phosphate is firmer in body than is that made with lactic or hydrochloric acid.

(*b*) *Colour*

Cheese from vats in which there was rapid acid development while the curd was in the whey was consistently whiter than that from control vats (Table 8). Extension of time in the whey did not affect the colour of the cheese until after prolonged storage. In the preliminary study of this phenomenon (J. Czulak *et al.*, unpublished) it was shown that the whiter colour was not due to bleaching of the carotene in the fat. Some evidence was obtained that it may have been due to structural changes in the casein complex which occur at lower pH. This will be the subject of a further study.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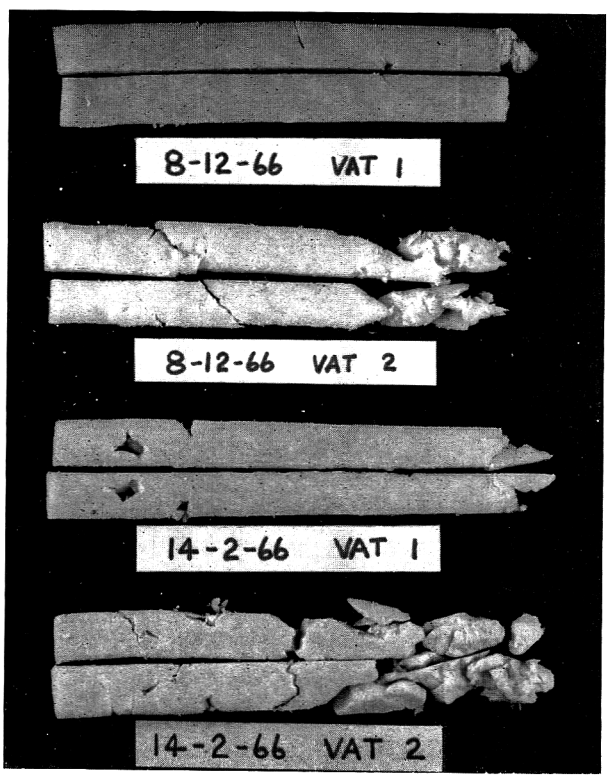
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(*Facing p.* 101)

Flavour

The cheeses from the 'fast-acid' vats had low pH and as a consequence were sour and became bitter in flavour. A similar relationship between the pH and the flavour of the cheese was observed in cheese from 'extended-time' vats. At 3 weeks the cheese had an acceptable flavour, but after a longer period of storage when all the lactose had been fermented the cheese became sour and bitter. It is possible that the accumulation of lactate ions in a reduced buffering system in cheese from 'extended-time' vats is responsible for the slower rate of fermentation of the residual lactose. This further emphasizes the importance of the rate of acid development in Cheddar cheese manufacture.

CONCLUSIONS

Whilst further studies are needed to explore the complex reactions of calcium and phosphate with the casein, one practical conclusion is justified from these experiments—that in Cheddar cheese manufacture it is not sufficient to standardize on the pH or acidity of either the whey or the curd alone, it is also necessary to control the rate of acid development and the time the curd is held in the whey. The observation that cheese made with extended-time in the whey may be of acceptable quality when graded at an early age, and yet develop serious defects on maturing, highlights a deficiency of the grading system.

Because, for the purpose of clarity, extreme limits for both the rate of acid development and the extension of time were allowed, we plan to make a further study to determine the effects of less severe deviations.

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EXPLANATION OF PLATE

1. Experiments on fast acid development (series II): longitudinally bisected plugs of matured cheese. Vat 1, controls; vat 2, test cheeses.

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The variation throughout a year in the fatty acid composition of milk fat from 2 dairy herds

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Summary. Differences in milk-fat composition in summer and winter have been demonstrated and related to feeding for an autumn calving Friesian herd and an Ayrshire herd in which calving was equally divided between spring and autumn. The observations are in agreement with previous results obtained for milk fat in the Northern Hemisphere.

Summer milk fat contained more C_{18} acids and less C_4–C_{16} acids than winter milk fat, the effect being particularly marked for the autumn calving herd.

Changes in the pattern of feeding altered the fatty acid composition of milk fat from both herds, and abrupt changes in feeding were clearly reflected even at times when stage of lactation effects were greatest.

Gas chromatography has been applied to studies of seasonal variations in butterfat composition (Patton, McCarthy, Evans & Lynn, 1960; Jensen, Gander & Sampugna, 1962). Patton, McCarthy, Evans & Lynn looked at the composition of early March and June samples of fat from pooled herd milk and showed that the percentage of C_{18} acids was considerably higher in the summer fat sample. Jensen, Gander & Sampugna examined variation throughout the year in milk-fat composition and showed that when cows had access to pasture the C_{18} acids were present in much higher concentration than when the animals were housed. Jensen *et al.* did not discuss the cause of these variations but referred to the work of Patton, *et al.* who attributed the higher C_{18} fatty acid content in the summer milk fat to a 'greater availability' of C_{18} dienoic acid from the pasture lipids and partial hydrogenation of this acid in the rumen. Presumably Patton *et al.* believed that pasture has a greater content of C_{18} dienoic acid than other feeds.

It was felt that the subject of variation in milk-fat composition throughout the year had not to date been adequately studied. Therefore, a survey was carried out for 12 months commencing on 10 March, 1966. Two dairy herds were used and milk was sampled once weekly from the bulk tanks used on each farm to store the daily milk.

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EXPERIMENTAL

Animals and their management. The Friesian herd consisted of approximately 120 cows, the calvings of which were concentrated in autumn; a few calved in July. Milking was carried out 3 times daily at 8-h intervals.

The Ayrshire herd consisted of approximately 60 cows, the calvings of which were concentrated equally in the periods March–May and October–November. This calving system resulted in the production of approximately equal amounts of milk in summer and in winter. Cows were milked twice daily.

Feeding. From May to June the Friesian herd grazed large, irrigated paddocks of 3-year leys based on perennial ryegrass and, in addition, each cow received 1 lb of mineral-rich concentrates (2 parts moist barley: 1 part mineral balancer pencils) at each milking to offset any mineral deficiencies of the herbage. From late June to the end of September the cows grazed haylage aftermaths and from mid-July onwards cows yielding more than 3 gal milk/day also received 4 lb concentrates (2 parts moist barley: 1 part high-fat concentrate: 1 part protein balancer) for every extra gallon of milk produced. The high-fat concentrate contained 6 % fat which analysis showed to comprise approximately 60 % C_{18} acids, 35 % C_{16} acids and small amounts of branched-chain acids.

It should be noted that in the first 3 weeks of June the herbage tended to become very stemmy while in late June leafy haylage aftermaths were grazed. From the beginning of July to the end of September the Friesian cows were drawing the major part of their energy intake from grass.

The Friesian herd was loose housed in winter (November–March) and the cows received chopped haylage (self-fed) plus a concentrate mix (2 parts moist barley: 1 part high-fat concentrate as above: 1 part protein balancer). Barley straw was always available in wall racks and as bedding. Winter feeding for this herd was effectively terminated at the end of April.

The Ayrshire herd strip-grazed leys and pastures from May to September inclusive and throughout May the cows continued to receive some hay. The system of grassland management was based on a rotational principle. On any one field the herbage was rapidly removed either by grazing animals or by machine, the field was then rested for 4–6 weeks and the herbage again removed. With this system almost every field was defoliated 4–5 times in the year. Where possible each field was alternately grazed and cut to avoid accumulation of dung patches. No irrigation of pastures or leys was carried out. Throughout the grazing period the minimum of concentrates was fed; the total amount averaged about 0·6 lb concentrate/gal of milk yielded, of which over 90 % was provided in April and late September. The composition of the concentrate was 28 parts bruised barley: 7 parts groundnut cake: 4 parts flaked maize: 1 part minerals, and this was fed when required at 3·5 lb/gal of milk. In mid-September, the herd was introduced to winter feed and by mid-October was permanently housed and under the complete winter-feeding régime in which silage and hay were the 2 main feeds. These were supplemented with the concentrate mix referred to above, fed at 3·5 lb/gal of milk yielded.

Milk analyses. The weekly milk yield was recorded and the milk samples were analysed for fat content by the Gerber method; total solids content was also deter-

mined. The composition of the fats was determined as described by Hutton, Prescott, Seeley & Armstrong, in a paper submitted for publication to *Animal Production*.

RESULTS

Observations on feeding. For the Friesian herd the period from the beginning of July to the end of September will be referred to as the summer period. Changeover to housing and winter feeding took place over a period of 1 month commencing on 27 September and the period from mid-November to the end of March will be referred to as the winter period.

For the Ayrshire herd, the summer period has been taken from July to September inclusive, during which time the cows received only grass except at the end of September when small amounts of winter feeds were introduced. The changeover to housing and winter feeding for the Ayrshires took place over a period of about 1 month commencing on 13 September and the winter period is taken as December–February inclusive.

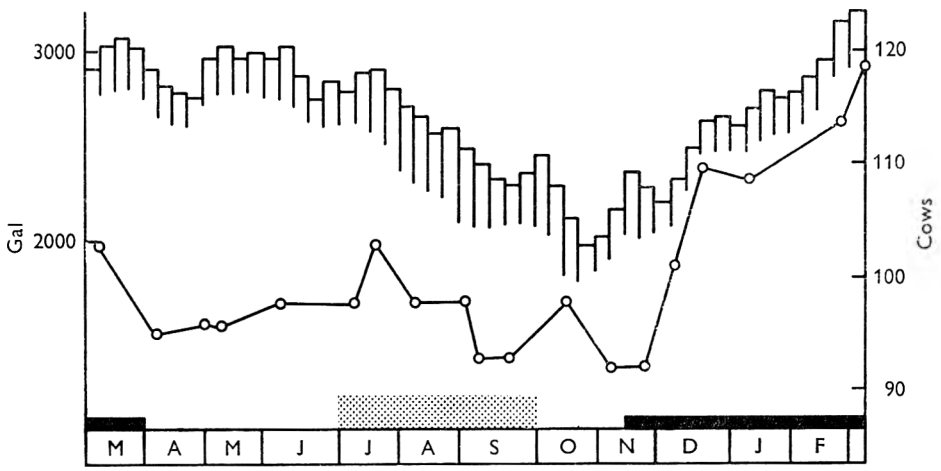

Fig. 1. The number of cows in milk (○—○) and the total weekly milk yield of the Friesian herd. Winter period, ■; summer period, ▨.

In choosing the summer and winter periods for the Ayrshire herd consideration was given to stage of lactation effects as well as feeding pattern. It can be seen from Fig. 2 that in the Ayrshire herd spring calving reached a maximum in the first week in June and autumn calving was almost complete by early November. The marked changes occurring in fatty acid composition of milk fat in early lactation have been shown (Stull & Brown, 1965; Stull, Brown, Valdez & Tucker, 1966; Decaen & Adda, 1966); the full effects of these changes would be expected to have taken place before the intervals chosen to represent summer and winter periods in this herd.

The weekly milk yield. It can be seen from Figs. 1 and 2 that the weekly milk yields for both herds closely followed cow numbers. A marked increase in milk yields was noted for both herds at the beginning of May when grazing commenced and when the number of animals did not change.

The content of solids-not-fat. Figure 3 shows that for the Friesian milk the SNF was

highest in May and June, and in September. In the Ayrshire herd the highest SNF was recorded in October (Fig. 4). From April to mid-August SNF levels in this herd were, with the exception of those in the second half of June, high. The fall in SNF at the end of June was also seen in the Friesian milk.

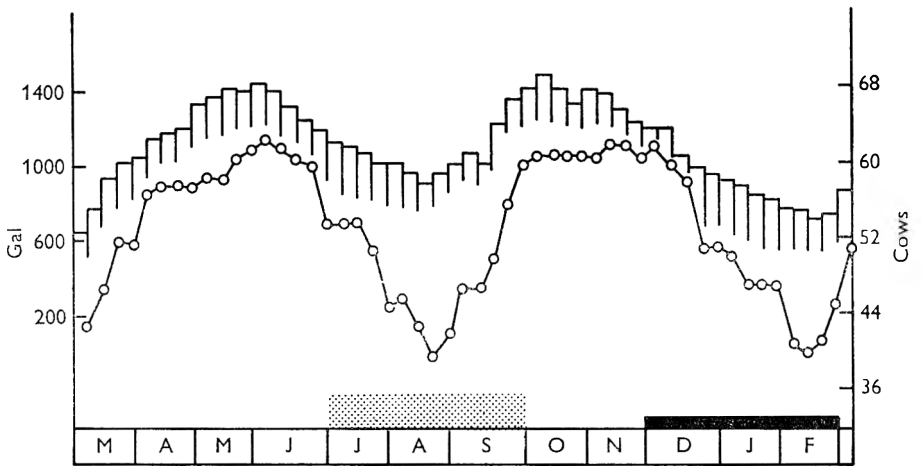

Fig. 2. The number of cows in milk (○——○) and the total weekly milk yield of the Ayrshire herd. Winter period, ■; summer period, ▦.

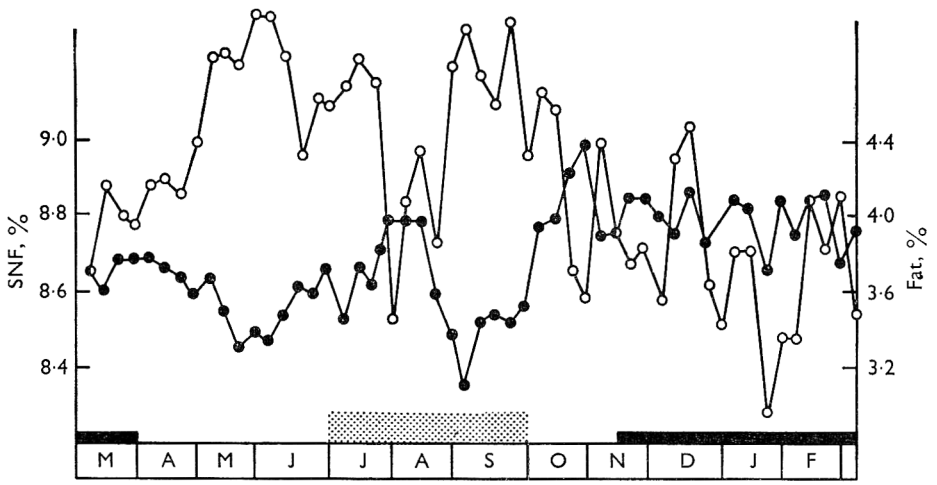

Fig. 3. The weekly milk SNF percentages (○—○) and fat percentages (●—●) for the Friesian herd. Winter period, ■; summer period, ▦.

The fat content. The fat content of the milk from the Friesian herd (see Fig. 3) dropped with the onset of grazing at the beginning of May, but gradually recovered through June and July. Towards the end of August a drastic depression in fat percentage occurred and a minimum of 3·1 % was recorded. This was followed by an increase throughout September and October at the end of which time the maximum fat percentage for the year was attained. The peak value occurred at the time when cows had been grazing during the day, housed at night and fed winter rations. When

access to grass during the day was stopped and the herd was permanently housed a drop in the fat content of the milk occurred, and from November through to February the fat content remained reasonably steady around 4%. A low value in January corresponded to the feeding of haylage from the bottom of a silo. Recovery of fat percentage followed the feeding of haylage from a fresh tower.

The over-all result (see Table 1) was that for the Friesian herd the milk-fat content in the summer period (beginning of July to the end of September) was significantly ($P < 0{\cdot}001$) lower than in the winter period (mid-November to the end of March). Furthermore, while the fat content in winter remained relatively constant,

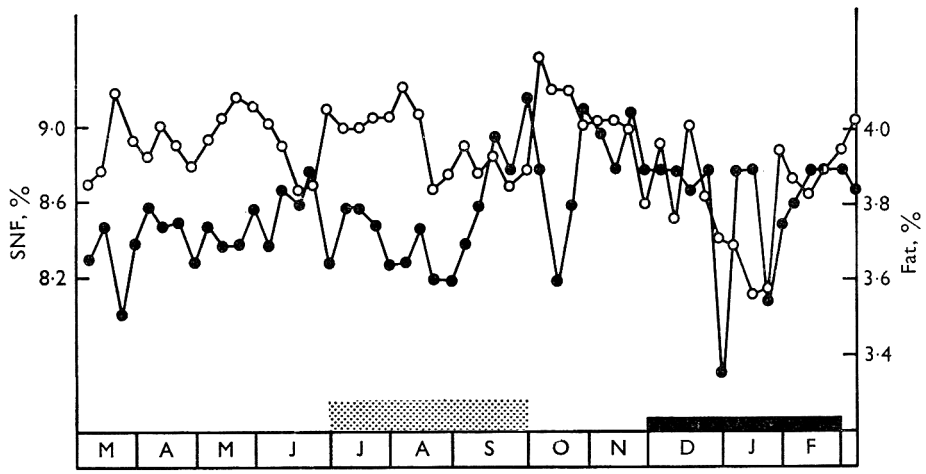

Fig. 4. The weekly milk SNF percentages (○—○) and fat percentages (●—●) for the Ayrshire herd. Winter period, ■; summer period, ▦.

Table 1. *Differences in the yield and composition of milk fat in summer (July, August and September) and winter (mid-November to the end of March) for the Friesian herd*

	Summer		Winter		Difference between means	Percentage change in winter	Statistical significance
Component	Mean	S.D.	Mean	S.D.			
Fat, %	3·61	±0·26	3·99	±0·14	±0·38	+10	$P < 0{\cdot}001$
Acids as % of total fatty acids							
C_4–C_{14}	24·03	±1·24	26·32	±1·65	+2·29	+10	$P < 0{\cdot}001$
C_{16}	23·23	±1·02	30·91	±1·36	+7·68	+39	$P < 0{\cdot}001$
C_{18} (total)	52·76	±1·45	42·76	±1·64	−10·00	−19	$P < 0{\cdot}001$
$C_{18:0}$	13·87	±0·95	12·51	±0·52	−1·36	−10	$P < 0{\cdot}001$
$C_{18:1}$	36·23	±1·65	27·67	±1·10	−8·59	−24	$P < 0{\cdot}001$
$C_{18:2}$	2·63	±0·46	2·58	±0·65	−0·05	−2	N.S.
Acids, yield in g/kg of milk							
C_4–C_{14}	8·18	±0·69	9·92	±0·51	+1·74	+21	$P < 0{\cdot}001$
C_{16}	7·93	±0·67	11·69	±0·76	+3·74	+47	$P < 0{\cdot}001$
C_{18} (total)	17·98	±1·35	16·14	±0·93	−1·84	−10	$P < 0{\cdot}001$

that during the summer showed great variation rising through July to a peak in mid-August and then falling markedly towards the beginning of September. Thereafter it showed a marked rise.

The fat content of the milk from the Ayrshire herd was about 3·5–3·7 % in March

and remained remarkably constant at about 3·7–3·8 % through to June (see Fig. 4). There was little consistent effect of spring grazing, but a depression in milk-fat content was noted for the week when day-grazing commenced. In June, the fat content rose slightly to about 3·9 % and then decreased to about 3·6 % by the beginning of September. Throughout September milk-fat content rose and by the end of September was 4·1 %. When winter feeding commenced in October the fat content showed a sharp decline, but rose again to about 3·9 % by November. It remained constant until March except for a sharp depression in late December and another depression in late January.

Table 2. *Differences in the yield and composition of milk fat in summer (July, August and September) and winter (December, January and February) for the Ayrshire herd*

	Summer		Winter		Difference between means	Percentage change in winter	Statistical significance
Component	Mean	S.D.	Mean	S.D.			
Fat, %	3·78	±0·15	3·81	±0·17	+0·03	+0·71	N.S.
Acids as % of total fatty acids							
C_4–C_{14}	24·13	±1·08	27·10	±1·06	+2·97	+11·23	$P < 0·001$
C_{16}	29·48	±1·29	34·20	±0·93	+4·72	+16·01	$P < 0·001$
C_{18} (total)	46·39	±2·08	38·70	±1·68	−7·69	−16·58	$P < 0·001$
$C_{18:0}$	13·40	±0·66	10·48	±0·46	−2·92	−28·79	$P < 0·001$
$C_{18:1}$	30·59	±1·69	25·56	±1·36	−5·03	−16·44	$P < 0·001$
C_{18} (total)	2·40	±0·32	2·66	±0·19	+0·26	+10·83	$P < 0·02$
Acids, yield in g/kg of milk							
C_4–C_{14}	8·64	±0·39	9·79	±0·47	+1·15	+13·31	$P < 0·001$
C_{16}	10·54	±0·35	12·35	±0·55	+1·81	+11·72	$P < 0·001$
C_{18} (total)	16·63	±1·32	13·99	±0·95	−2·64	−15·87	$P < 0·001$

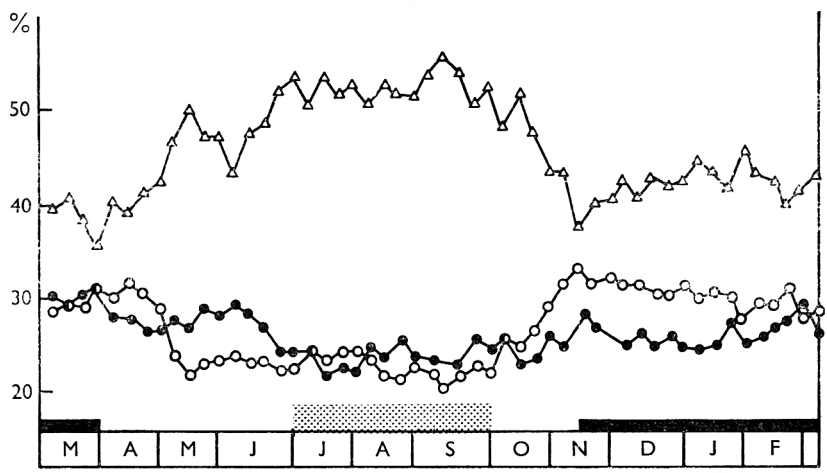

Fig. 5. The weekly fatty acid composition of the milk fat of the Friesian herd expressed on a percentage by weight basis. Acids: C_{18}, △—△; C_{16}, ○—○; C_4–C_{14}, ●—●. Winter period, ■; summer period, ▦.

The over-all result (Table 2) was that for the Ayrshire herd the milk-fat content in the summer period was slightly lower than that in the winter period, but the difference between the mean values was not statistically significant.

The fatty acid composition of the fat. The variations in the fatty acid composition of the milk fat of the Friesian herd are shown in Fig. 5. Table 1 shows the mean values for the fatty acids of the milk fat obtained for the summer period (July, August and September) and the winter period (mid-November to the end of March). From this table it can be seen that the content of C_{18} acids in the milk fat was very much greater ($P < 0{\cdot}001$) in the summer than in the winter period. The contents of palmitic acid and of the C_4–C_{14} acids were significantly lower in summer than in the winter period (C_4–C_{14}, $P < 0{\cdot}001$; C_{16}, $P < 0{\cdot}001$).

Figure 7 shows the weekly proportions of $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$ acids in the fatty acids of milk fat from the Friesian herd, and from this figure it can be seen that the component principally responsible for the over-all increase in the content of C_{18} acids in milk fat during the summer period was the $C_{18:1}$ acid (see also Table 1).

During March and April when the herd was housed there was little change in the fatty acid composition of the milk fat except at the end of March, when the feeding of haylage with markedly depressed ether extract and fibre contents was reflected in a lowered content of C_{18} acids in the milk fat and an increased content of C_4–C_{14} acids (Fig. 5).

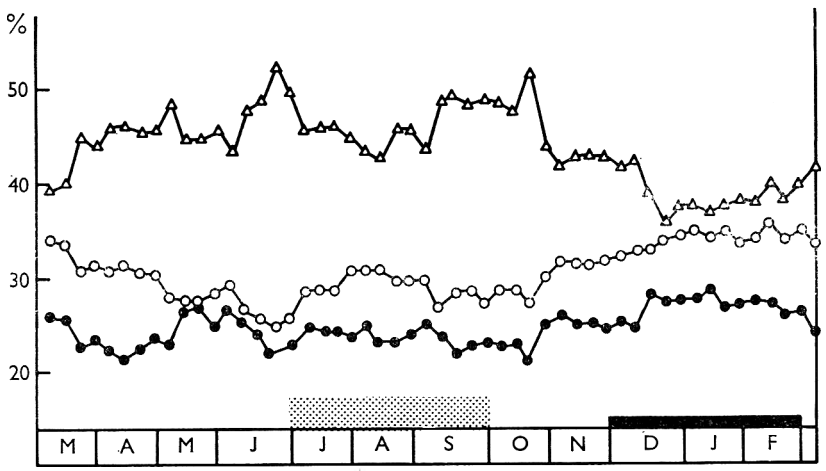

Fig. 6. The weekly fatty acid composition of the milk fat of the Ayrshire herd expressed on a percentage by weight basis. Acids: C_{18}, △—△; C_{16}, ○—○; C_4–C_{14}, ●—●. Winter period, ■; summer period, ▦.

Following the introduction of the Friesian cows to grass at the beginning of May marked changes occurred in the composition of the milk fat. There was a sharp increase in the percentage of C_{18} acids as a whole (Fig. 5) due to a marked increase in both $C_{18:1}$ and $C_{18:2}$ acids (Fig. 7), there being no change in the stearic acid content. This increase in the content of total C_{18} acids in the milk fat was accompanied by a marked fall in palmitic acid content, but no apparent change occurred in the content of C_4–C_{14} acids (see Fig. 5). After 4 weeks on grass the content of $C_{18:2}$ in the fat had dropped almost to its winter level and the $C_{18:1}$ content had also dropped slightly, but was still higher than the winter value. The content of stearic acid remained unchanged. The resulting drop in total C_{18} acid content of milk fat was accompanied

by a corresponding increase in the content of C_{16} and a slight increase in C_4–C_{14} acids.

With the onset of winter feeding a drastic depression in the content of total C_{18} acids in the fat occurred which was due almost entirely to a marked depression in the $C_{18:1}$ acid. This depression in the content of total C_{18} acids was accompanied by a sharp increase in palmitic acid content and a slight increase in the content of C_4–C_{14} acids in the milk fat. By mid-November a steady state appeared to have been reached.

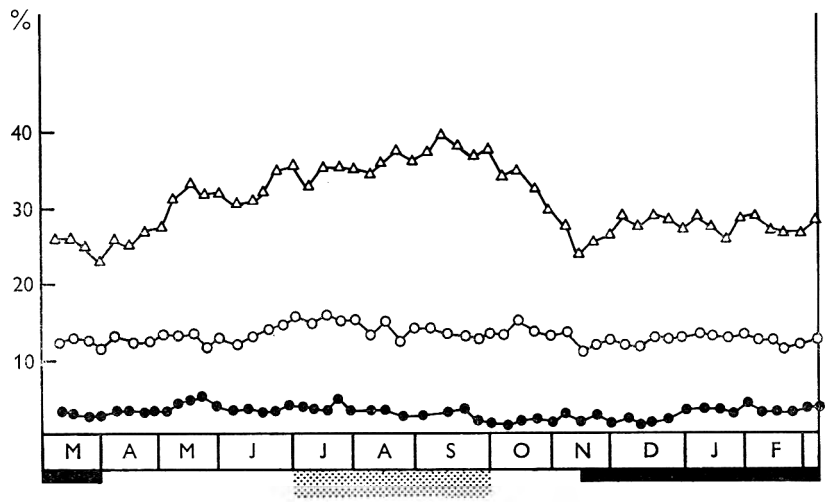

Fig. 7. The weekly proportions of $C_{18:0}$ (○—○), $C_{18:1}$ (△—△) and $C_{18:2}$ (●—●) acids in the fatty acids of the milk fat of the Friesian herd expressed on a percentage by weight basis. Winter period, ■; summer period, ▨.

With reference to the Ayrshire herd the changes in fatty acid composition of the milk fat throughout the year are shown in Figs. 6 and 8. The mean values for the contents of fatty acids in the milk fat are given in Table 2 for the summer (July, August and September) and winter (December, January and February) periods. From this table it can be seen that the content of C_{18} acids in the milk fat was very much greater ($P < 0{\cdot}001$) in the summer than in the winter period. The contents of palmitic acid in the C_4–C_{14} acids were significantly lower in summer than in the winter period (C_4–C_{14}, $P < 0{\cdot}001$; C_{16}, $P < 0{\cdot}001$).

From Fig. 8 and Table 2 it can be seen that the components responsible for the over-all increase in the content of C_{18} acids in milk fat during the summer period were the $C_{18:0}$ and $C_{18:1}$ acids. The content of the $C_{18:2}$ acid in milk fat was significantly decreased in the summer period ($P < 0{\cdot}02$).

During March and April, when the herd was housed, the content of C_{18} acids in the milk fat showed a steady rise, and palmitic acid content decreased. Following the introduction of cows to grass at the end of April no marked changes occurred in the composition of the milk fat. There was an increase in the content of C_{18} acids (Fig. 6), but this was only for the first week on grass. A small drop in palmitic acid was observed, and C_4–C_{14} acids showed a rise. In early June a marked rise in the content of C_{18} acids occurred, accompanied by a marked decrease in the contents of both pal-

mitic acid and the C_4–C_{14} acids. However, at the end of June and throughout July the content of C_{18} acids in milk fat declined and that of palmitic acid increased. In early September, the content of C_{18} acids in the milk fat showed a rise again and palmitic acid and C_4–C_{14} acids both showed a corresponding decrease.

Towards the end of October, when cows had been housed and fed winter rations for about 10 days, a marked drop in the content of C_{18} acids in milk fat occurred which was due to a drop in both $C_{18:0}$ and $C_{18:1}$ acids (Fig. 8). This depression in the content of total C_{18} acids was accompanied by a rise in the contents of palmitic acid and C_4–C_{14} acids in the Ayrshire milk fat.

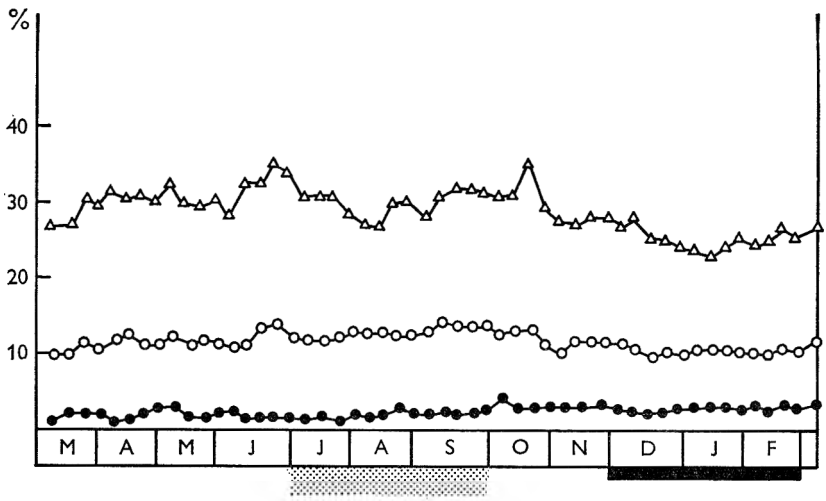

Fig. 8. The weekly proportions of $C_{18:0}$ (○—○), $C_{18:1}$ (△—△) and $C_{18:2}$ (●—●) acids in the fatty acids of the milk fat of the Ayrshire herd expressed on a percentage by weight basis. Winter period, ■; summer period, ▦.

Thus, for both the Friesian and Ayrshire herds, the percentage of C_{18} acids in the milk fat and also the yield of C_{18} acids in the milk were significantly higher in summer than in winter. Also the percentages of C_4–C_{14} acids and of palmitic acid in the milk fat were lower in summer than in winter and so were the yields of these acids in the milk. The mean milk fat content was lower in summer than in winter, but this depression was not significant for the Ayrshire herd.

DISCUSSION

Summer milk fat from both herds contained more C_{18} acids than winter milk fat, the effect being particularly marked with the Friesian herd. The pattern of calving may have caused the partial masking of this effect in the Ayrshire herd, since the influence of stage of lactation on milk composition is likely to be greater than the influence of diet at peak calving times.

It is clear that the higher fat content of winter milk was largely due to the increased yield of C_4–C_{14} acids and of palmitic acid/kg of milk. The effect of this increased yield of C_4–C_{14} acids on the fat content of the milk was only partly offset by a decreased output of C_{18} acids/kg of milk in the winter period.

Milk fatty acids C_4–C_{14} are derived mainly from plasma acetate, and palmitate arises partly from acetate and partly from plasma triglycerides (Annison, Linzell, Fazakerley & Nichols, 1967). In the present experiments, the increased yield of C_4–C_{14} acids/kg of milk in winter must thus reflect an increased incorporation of acetate (and/or β-hydroxybutyrate) into C_4–C_{14} acids by the mammary gland during this period; the same trend for palmitic acid suggests that the same is also true for this acid. Such an increased incorporation of C_2 and C_4 fragments into the C_4–C_{16} acids of milk in winter may possibly be due to an increase in the proportion of energy absorbed from the alimentary tract in the form of acetate and butyrate, or to a preferential utilization of these fragments by other tissues during the summer period (see Armstrong, 1968).

The major sources of C_{18} acids for milk fat synthesis in the mammary gland are the chylomicra and low density lipoproteins of the blood plasma (Barry, Bartley, Linzell & Robinson, 1963). Without knowledge of the levels of these components in the blood reaching the mammary gland during the different periods, it is difficult to account for the increased level of C_{18} acids in summer milk.

Several workers in the Northern Hemisphere, where cows are housed in winter, have reported seasonal variations in the iodine values of milk fat (Stadhouders & Mulder, 1956; Wood & Haab, 1957; Jensen *et al.* 1962). The iodine values of the milk fat of such cows were lower in winter than in summer; the results obtained in the present study are consistent with this finding and show that the acid responsible for the greater part of the change in iodine value was the mono-unsaturated C_{18} acid.

In the North Island of New Zealand, where cows are maintained on pasture throughout the year, the seasonal variation is somewhat different (see McDowall, 1962). There is a fall in iodine number of milk fat in late spring and early summer, which may be an effect of stage of lactation since calving in New Zealand's North Island is primarily in late winter and early spring. It is known that the contents of $C_{18:0}$ and unsaturated acids are high in colostrum and in early lactation, falling rapidly in the first few weeks of lactation (Stull & Brown, 1965; Stull *et al.* 1966; Decaen & Adda, 1966). Again, in contrast to the findings obtained in the Northern Hemisphere, there is a rise in iodine number of milk fat in late summer and early winter accompanied by a depressed Reichert–Meissl value. These observations, which are typical of undernutrition and consistent with mobilization of body fat (Smith & Dastur, 1938), have been considered as reflecting the extent to which milking cows draw on body reserves of fat when pasture is of poor nutritional quality (Hansen & Shorland, 1952; McDowall, 1962).

In the present study the value of feeding hay during the period of introduction to spring grazing is clearly demonstrated. Where the changeover from winter to summer feeding occurred with no hay being fed during the changeover period, as with the Friesian herd, there was a marked decline in milk-fat content due entirely to a depression in the output of palmitic acid. However, when hay was fed to the Ayrshire herd during the early grazing period little change occurred in milk-fat content and composition.

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Sulphydryl and disulphide groups in casein

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Summary. The method of Gaitonde (1967) was adapted for the measurement of cysteine and cystine in acid hydrolysates. Analysis of 3 different casein preparations indicated the presence of both cysteine and cystine in whole casein in the proportions of 1:1 by weight and in the molecular ratio of 2:1. If all the cysteine and cystine is associated with κ-casein then it appears that one mole of the latter contains 2 moles of cysteine and 1 mole of cystine. The sulphydryl group in whole casein appears not to be highly labile.

The question of whether cysteine or cystine, or both occur in casein has been a subject of investigation for many years and is still a subject of controversy (McKenzie, 1967). Early work suggested the presence of cystine only, e.g. Kassel & Brand (1938); Gordon, Semelt, Cable & Morris (1949); Zweig & Block (1953); Hipp, Basch & Gordon (1961); Yoshino, Wilson & Herreid (1962); Waugh (1961). Christ (1956) was unable to detect any sulphydryls in casein but postulated that -SH groups were involved in the syneresis of rennet-casein gels. However, Beeby (1964) showed the presence of sulphydryl groups in freshly prepared κ-casein, the estimation being done at a high pH value(9·0) in strongly disaggregating conditions and in the absence of calcium ions. It appeared from his work that calcium and/or hydrophobic bonding masked the sulphydryl groups from -SH reagents. Hill (1964), working on whole casein and using more drastic methods for its disaggregation (e.g. proteolysis with Pronase), observed the presence of cysteine only. Subsequently, MacKinlay & Wake (1964) submitted evidence that contradicted the findings of Beeby (1964) and Hill (1964) and, later, Nakai, Wilson & Herreid, (1965) were also unable to substantiate the findings. The present paper describes a study of the cysteine/cystine balance in whole, acid precipitated casein. The work was undertaken as part of an investigation into the factors contributing to the syneresis of rennet–milk gels.

EXPERIMENTAL

All chemicals used were of A.R. grade and only glass-distilled water was used.

Preparation of acid casein

Three different casein preparations were made: (*a*), from freshly collected raw milk; (*b*) from industrially separated skim-milk; (*c*) from industrial acid casein. The methods of preparation were as follows:

(*a*) Bulk raw milk of good bacteriological quality was obtained from the Massey University dairy herd. It was cooled to 10 °C and the fat was removed by centrifugation in polyethylene containers. The skim-milk was bulked in a polyethylene bucket, the temperature having risen to 14 °C. Acid precipitation of the casein at pH 4·5 was carried out with a Radiometer Automatic Titration apparatus using N-HCl, care being taken to ensure adequate mixing. The fine precipitate obtained on centrifuging the slurry was washed twice with distilled water, using centrifugation to recover the precipitate. The washed precipitate was then dissolved in distilled water by addition of N-NaOH to pH 8·0 and reprecipitated by addition of N-HCl to pH 4·6. The Automatic Titrator was used for all pH adjustments. After thorough washing with distilled water, the final slurry was frozen at − 15 °C and freeze-dried in an accelerated freeze-drying apparatus. The freeze-dried casein was stored at − 15 °C. During the additions of acid and alkali the skim-milk was agitated by means of an electrically driven stirrer and to avoid aeration and minimize possible oxidation of sulphydryl groups in the casein, oxygen-free nitrogen was passed through 2 diffuser heads placed in the bottom of the bucket. The rate of nitrogen diffusion was adjusted to give a gentle bubbling over the entire liquid surface.

(*b*) Skim-milk was obtained from the Palmerston North Milk Treatment Station. The milk had been heated to about 48–50 °C for the separation in a conventional separator. Casein precipitation was done at 25 °C using the Automatic Titrator, following the same procedure as for casein (*a*) except that nitrogen was not bubbled through during the precipitation and solubilization processes. There was, therefore, ample opportunity for oxidation of the sulphydryls to occur, both at the Milk Treatment Station and during the isolation of the casein in the laboratory. This casein (casein (*b*)) was freeze-dried and stored at room temperature.

(*c*) Wet lactic casein was obtained from a commercial casein factory. Although the detailed history of its preparation is not known a standard commercial process was followed. The pressed curd was freeze-dried and stored at room temperature.

The 3 casein preparations (*a*), (*b*), (*c*) had, therefore, widely differing pretreatments, the most drastic treatment being suffered by casein (*c*).

Hydrolysis of casein and estimation of sulphydryls and disulphides in the hydrolysate

Hydrolysis. 2·5 g casein was hydrolysed under reflux with a 1:1 (v/v) mixture of 98% formic acid and concentrated HCl for 7 h at 105 °C. During the digestion oxygen-free nitrogen was bubbled through the digest. The mixture was approximately 6 N in HCl. It is important to have this high concentration of HCl in the mixture to reduce the hydrolysis time. Other investigators (e.g. Block & Bolling, 1940) have recommended a 1:1 (v/v) mixture of 6 N-HCl and 90% formic acid giving a final HCl normality of 3 N in the mixture. However, the digestion time has to be prolonged (16 h) and some destruction of cystine and cysteine takes place. Thus, when the mixture was 3 N in HCl acid, total SH + SS content was between 260 and 300 mg/100 g casein whereas with 6 N-HCl the digestion time was reduced to 7 h and total SH + SS content was significantly higher (see Results). A preliminary experiment using 6 N-HCl in the HCOOH-HCl mixture showed (Fig. 1) that about 80% of each of these amino acids was liberated in 2 h. (cf. Leach, 1966).

Before heating, oxygen-free nitrogen was passed through the digestion mixture for 10 min for deaeration. The hydrolysate was transferred to a 200-ml volumetric flask and cooled immediately with running water. Twenty ml of 5 N-NaOH was then added, drop by drop, to the flask which was swirled in a bath containing water at room temperature. This procedure was essential for the removal of heat generated by the addition of the alkali and to avoid local concentrations of the alkali, which otherwise tended to destroy the sulphur amino acids. The mixture was then made up to 200 ml. The addition of the alkali raised the pH to 3·7–4·0, which allowed the precipitation of humin material and its removal by filtration using Whatman no. 540 filter paper. The clear filtrate was used for measuring cysteine and cystine levels.

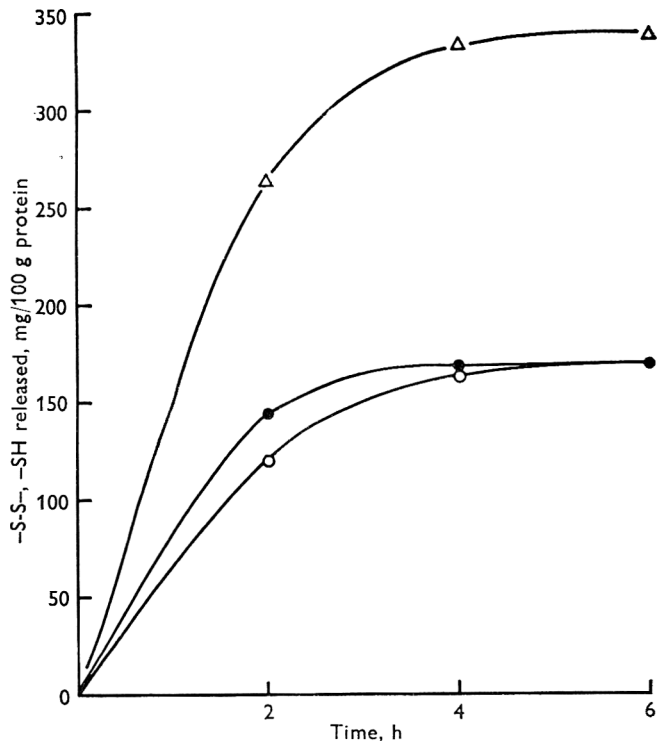

Fig. 1. Rate of release of cystine and cysteine during acid hydrolysis of whole casein, O—O, cystine; ●—●, cysteine; △—△, cystine + cysteine.

Determination of cysteine. The spectrotophometric method of Gaitonde (1967), with some minor modifications, was used to determine the cysteine quantitatively. To 1 ml of the digest filtrate in a test tube were added in turn 1 ml glacial acetic acid and 1 ml ninhydrin reagent (250 mg ninhydrin dissolved in 6 ml glacial acetic acid and 4 ml concentrated HCl). The mixture was heated for 8 min in a boiling water bath and cooled immediately and 10 ml ethanol was added. The pink colour was measured at 575 nm, the absorption maximum, in a Beckman DU Spectrophotometer using as a blank a similarly treated sample to which no ninhydrin had been added.

Determination of cystine. To 20 ml of the digest filtrate were added, drop by drop, 5 N-NaOH (4–6 ml) to adjust the pH value to 5·0. During the addition the mixture was agitated and cooled in a water bath to avoid local concentration of the alkali and to dissipate the heat quickly. Excess sodium sulphite (about 500 mg) was added as a solid and the mixture made to 40 ml with distilled water. The final pH value of the solution lay between 6·5 and 7·0.

The mixture was left at room temperature until reduction was complete (1 h) and then 1 ml was measured as described above for cysteine.

Calibration curve

To 39·4 mg chromatographically pure cysteine hydrochloride (B.D.H. Ltd) were added 50 ml of a 1:1 (v/v) mixture of 98% formic acid and 6 N-HCl. The resulting solution was diluted to 250 ml in a volumetric flask. The cysteine content of this solution was taken as 1 μmole/ml. Further dilutions of this cysteine solution were made using 1:5 dilution of the 1:1 HCl:HCOOH as diluent. Solutions prepared in this way contained: 1·0, 0·75, 0·5, 0·25 and 0·125 μmole/ml of cysteine. The plot of optical density against concentration was linear and passed through the origin.

Because of the protective effect of formic acid against metal catalysed oxidative destruction of cysteine (Haurowitz, 1963), the solutions of pure cysteine used for calibration, were made up in HCl–formic acid mixtures. These solutions were stable for at least 48 h.

Table 1. *Cysteine and cystine content of 3 different casein preparations*

(All values in mg/100 g dry protein.)

Sample		Cysteine	Cysteine after reduction	Cystine by difference
Casein (*a*)	1	165	338	173
	2	165	335	170
	3	155	328	173
	4	165	338	173
	Mean	163	335	172
Casein (*b*)	1	165	347	182
	2	174	337	163
	3	165	328	163
	4	165	335	170
	Mean	167	337	170
Casein (*c*)	1	165	338	173
	2	165	328	163
	Mean	165	333	168

RESULTS

Cysteine and cystine contents of 3 different casein preparations

The cysteine and cystine content of the 3 casein preparations described earlier are given in Table 1.

Table 1 demonstrates remarkably similar cysteine values obtained both before and

after reduction for the 3 casein preparations, despite the different methods of preparation. Accepting the claim of Gaitonde (1967) that the method in the form used is specific for cysteine, then the increased value after the reduction process could presumably only arise as a result of the reduction of cystine to cysteine. It is thus possible, indirectly, to measure the cystine content of the system.

Effects of variables on the recovery and estimation of cysteine and cystine

A check was made to see whether the pure amino acid cystine was being converted to cysteine under the conditions of hydrolysis used for casein.

About 25 mg cystine (chromatographically homogeneous, B.D.H. Ltd) was dissolved in a 1:1 mixture of 98% formic acid and concentrated HCl; the volume was made to 25 ml in a volumetric flask. Ten ml of this solution, containing about 10 mg cystine, was digested with 25 ml of the 1:1 (v/v) HCOOH:Conc. HCl mixture and refluxed for 7 h at 105 °C under a stream of oxygen-free nitrogen, as for casein. Cysteine, and cysteine-after-reduction, were then determined as described above, the digest having been diluted to 100 ml with distilled water. A second portion of 10 ml of the cystine solution was mixed with 25 ml of the 1:1 acid mixture and diluted to 100 ml with distilled water. It was tested immediately for cysteine and cysteine-after-reduction, omitting the heating at 105 °C. Results are given in Table 2. The results show that the conditions of hydrolysis did not convert cystine to cysteine. The approximately 9% cysteine equivalent was probably cysteine contamination present in the original sample as digestion for 7 h with HCOOH:HCl acid mixture at 105 °C caused no further conversion of cystine to cysteine.

Table 2. *Recovery and estimation of cystine*

(All values in mg/10 ml of original solution.)

Treatment	Cysteine	Cysteine after reduction	Cysteine in sample, %	Cystine, %
Unheated	0·99	9·60	9·3	90·7
Heated	0·97	9·84	9·0	91·0

A further check was made to establish whether cystine was reduced to cysteine in the presence of casein under the hydrolysis conditions employed and in the presence of humins. To 1·5 g casein were added 25 μmoles of cystine, and the mixture was hydrolysed under the standard conditions. The values for cysteine and cysteine-after-reduction were determined on the hydrolysate as described above. The results are shown in Table 3. These results indicate that there was (100+)% recovery of the added cystine. An increase of 4 μmoles of cysteine probably represents the cysteine impurity in the cystine mentioned earlier (Table 2), the level being of the same order.

The reduction of cystine to cysteine under the conditions used, therefore, does not appear to be a significant source of error. It is apparent also that oxidation of cysteine to cystine did not occur during the digestion as the level of cysteine contamination was similar in both heated and unheated cystine solutions (Tables 2 and 3).

To establish whether the cysteine and cystine levels found in the caseins were invariant and hence possibly the consequence of some equilibrium state, casein was

digested in the presence of thioglycollic acid. To 2·5 g of casein (*a*) were added 100 μmoles thioglycollic acid and the mixture was hydrolysed under the standard conditions. The values for cysteine and cysteine-after-reduction were determined on the hydrolysate as before and are given in Table 4. In the presence of 100 μmoles thioglycollic acid the mean value for cysteine-after-reduction was the same as that reported in Table 1, but the value for cysteine at 204 mg/100 g dry protein showed a 25 % increase. The thioglycollic acid thus appears to have caused some reduction of the cystine during the digestion.

Table 3. *Recovery of added cystine after digestion in the presence of casein*

(All values in μM/1·5 g casein.)

No.	Sample	Cysteine	Cysteine after reduction	Cysteine in cystine, %
1	1·5 g casein after digestion	20	40	—
2	1·5 g. casein plus 25 μM cystine Before digestion	4	40	10
3	After digestion	24	92	—
4	Difference (3 − 1) representing the recovery of 25 μM added cystine	4	52	7·7

Table 4. *Casein hydrolysis in presence of* 100 μM *thioglycollic acid*

(All values in mg/100 g dry protein.)

Sample		Cysteine	Cysteine after reduction	Cystine by difference
Casein (*a*)	1	204	329	125
	2	204	329	125
	3	204	348	144
	Mean	204	335	131

It has been reported (Benesch & Benesch, 1958) that disulphide exchange can take place in strong HCl (7 N–12 N) through an electrophilic attack of a sulphenium cation (RS^+) on the disulphide bond, the sulphenium ion being produced by the acid acting on the disulphide, thus:

$$R—S—S—R + HCl \rightleftharpoons RSCl + RSH, \quad (1)$$

$$RS^{\oplus}\ Cl^{\ominus} + R'—S—S—R' \rightleftharpoons R—S—S—R' + R'SCl. \quad (2)$$

Thus, there seemed a possibility that cystine values could have been reduced by the hydrolysis system used. This acid catalysed exchange reaction should be inhibited by the addition of mercaptans as reaction (1) is reversed and the concentration of sulphenyl chloride reduced. Theoretically, therefore, if the hydrolyses were to be carried out in the presence of thioglycollic acid or any other mercaptan, the concentration of cystine should either remain the same or increase if in fact cystine was being converted to its sulphenium chloride in previous experiments.

The (100+)% recovery of added cystine recorded in Table 2 and the drop in cystine due to the reducing action of the added thioglycollic acid reported in Table 4, clearly eliminate any possible interference by the sulphenium chloride reaction in the cysteine–cystine balance in casein during digestion.

From all the foregoing it is reasonable to infer that during casein hydrolysis no artifact or equilibrium shift of significance was produced from the cysteine or cystine or through cysteine–cystine interconversion.

DISCUSSION

Acid hydrolysis of proteins is generally believed to cause a certain amount of destruction of the sulphur amino acids cysteine and cystine (Haurowitz, 1963). The destruction is particularly severe in the digestion of glycoproteins, and is apparently caused by the carbohydrate breakdown products (Block & Bolling, 1945). The destruction can be reduced by the use of formic acid and by exclusion of oxygen during the digestion (Haurowitz, 1963). Furthermore, acid conditions below pH 2·0 inhibit air oxidation of cysteine as well as cystine reduction through the presence of thiols or mercaptans. (Leach, 1966).

As indicated on p. 120, the conditions used in the present study did not appear to affect the total recovery of added cysteine and cystine or the balance of the two.

In this study, cystine was determined as cysteine-after-reduction in solution or in the hydrolysate with sodium sulphite. One mole cystine was found to be equivalent to 2 moles cysteine when measured by the method used. It would appear that the colour reaction with ninhydrin below pH 1·0 does not involve the sulphydryl group, because other sulphydryl-containing compounds do not respond similarly, e.g. thioglycollic acid. Gaitonde (1967) tested a whole range of compounds, including —SH compounds and amino acids, and showed that the colour reaction was specific for cysteine under the conditions of the test and that the presence or absence of other amino acids had no effect on the specificity of the test. The increase in cysteine found after the reduction with sodium sulphite in near neutral solution could thus only have arisen through reduction of cystine.

The cysteine plus cystine content of 335 mg/100 g protein found for the 3 caseins is of the same order as figures reported for either cysteine alone (300+mg, Hill, 1964) or cystine alone (340 mg, Gordon & Whittier, 1965). Our results indicate the presence of both cysteine and cystine in a 1 : 1 ratio by weight and so differ from results reported by Nakai *et al.* (1965), Beeby (1964), and Hill (1964).

Hill (1964) used Pronase to open up the structure of whole casein but he gave no indication whether Pronase affected the disulphide bond in any way at all. However, Davidson & Hird (1967) report the possibility of SH–SS exchange reactions during limited proteolysis. Hill found that when the casein was digested with Pronase alone he obtained a much lower sulphydryl content than when the Pronase digest had been reduced with sulphite or $NaBH_4$ or treated with cadmium hydroxide. He ascribed this to the sulphydryl being so strongly protected that even Pronase digestion does not fully expose the sulphydryls. However, study of table 2 of Hill (1964) shows the following: 'The average of the moles cysteine/100000 g protein figures in column 2 (Pronase digest, pH 8·0) works out to 1·27. The average of the values in columns 3, 4 and 5 (sulphite, $NaBH_4$ and $Cd(OH)_2$ treatment) works out to 2·51 moles cysteine/100000 g. We would argue that this difference ($2{\cdot}51 - 1{\cdot}27 = 1{\cdot}24$ moles) is due to conversion of the disulphide to the sulphydryl as a consequence of the reductive treatments given and not due to simple disaggregation and exposure of more —SH groups.'

We have shown that sulphite reduces disulphide quantitatively to –SH. This difference of 1·24 mole of cysteine could thus be equivalent to 0·62 mole of cystine giving 1·27 moles cysteine and 0·62 mole of cystine/100 000 g casein. From the figures reported in this investigation (165 mg cysteine and 170 mg cystine/100 g casein) the moles/100 000 g casein work out to 1·36 moles cysteine and 0·71 mole cystine, respectively.

This re-calculation of Hill's data provides valuable confirmation of our findings.

If it is assumed that the cysteine and cystine are mostly, if not all, in the κ-casein fraction of whole casein (Waugh *et al.* 1960; Hill, 1964) then 0·71 mole cystine/100 000 g whole casein may be written as 0·71 mole/15 000 g κ-casein, assuming that κ-casein comprises 15% of whole casein (Waugh & von Hippel, 1956). This is roughly equivalent to 1 mole cystine/21 000 g κ-casein. Swaisgood, Brunner & Lillevik (1964) report the presence of 1–1$\frac{1}{2}$ mole of cystine/28 000 g κ-casein and Nakai *et al.* (1965) report 1 mole of cystine/20 000 g κ-casein, which is in reasonable agreement with cystine figures reported in this work. Neither reported the presence of cysteine in casein. From a similar calculation, it appears that the concentration of cysteine is equivalent to 2 moles/22 000 g κ-casein. As the molecular weight of κ-casein is about 20 000 (see Jolles, 1966) it appears that one mole of κ-casein contains 2 moles of cysteine and 1 mole of cystine. Hill (1964) was not able to detect sulphydryls of whole casein, even under strongly dissociative conditions and did so only after partially digesting with Pronase. Thus, it appears that the sulphydryl groups are well protected in whole casein. Evidence for the structural protection of sulphydryls in other proteins is also available (Cecil, 1963). Several sulphydryl dependent aggregations, as distinct from SH– SS– interchange reactions, have been reported in the literature (Hughes, 1947; Madsen & Cori, 1956; Deutsch & Morton, 1958; Jensen, 1959) and a similar type of sulphydryl dependent aggregation in whole casein cannot be ruled out.

The fact that 3 different casein preparations used in this study gave similar results indicates that ordinary processing does not affect the sulphydryl-disulphide balance of whole casein. The sulphydryl group may not be as labile as is commonly believed.

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With 1 plate

Dextran sulphate–Toluidine blue method for the histochemical identification of lipoproteins in cheese

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Summary. Details are given of a histochemical technique suitable for the identification of lipoproteins in cheese based on their property of forming insoluble complexes with sulphated polysaccharides.

The use of a histochemical stain for the detection of lipids-containing choline in sections of Parmesan and Pecorino cheese was reported by Bolcato, Pallavicini & de Felice (1964); Bolcato, Pallavicini, de Rosa & de Felice (1964). The phosphomolybdic acid reagent (Pearse, 1960, p. 851) used gave a blue coloration with lipid complexes which at low magnification (50 ×) appeared as small blue granules. In a subsequent paper, Pallavicini & Bolcato (1965) demonstrated that these lipids were not free but were in combination with a protein to form a lipoprotein. The presence of the protein moiety was confirmed by 3 different staining techniques: (1) the method of van Duijn (1961); (2) the acid solochrome cyanine R method (Pearse, 1960, p. 793), and (3) the mercury–bromophenol blue method (Pearse, 1960, p. 792). The lipoprotein was also stained by the method of Carmichael (1963), but the specificity of this method is uncertain (cf. Carmichael & Mander, 1967). The lipid component of the lipoprotein was also stained by the method of Böttcher & Boelsma-van Houte (1964) which is very specific for lipids containing choline. The granules of lipoproteins contain free or weakly bound phosphates (Bolcato & Spettoli, 1969). Pallavicini (1965) has reported very similar granules in 31 ripened cheeses examined, but none in 5 soft cheeses.

It is known that sulphated polysaccharides (heparin, synthetic heparinoids, dextran sulphate, and others) react with lipoproteins of human and animal sera in the presence of metal ions (Ca^{2+}, Mg^{2+}, Cd^{2+}, and others) in the pH range from 6·5 to 8·5. Electrostatic attraction between cationic groups on the protein and anionic groups (sulphate and carboxyl) of sulphated polysaccharides probably plays a major stabilizing role since the complexes are disrupted by the addition of alkali or by increasing the ionic strength of the medium. The literature on the use and action of sulphated polysaccharides in the precipitation of the lipoproteins has been reviewed by Cornwell & Kruger (1961).

The method now to be described for the histochemical identification of lipoproteins in cheese is based upon the fact that sulphated polysaccharides form

insoluble complexes with lipoproteins. The complex so formed was stained with the dyes Alcian blue and metachromatic Toluidine blue used for the histochemical identification of the sulphated polysaccharides.

Sections of cheese, 15 μm in thickness, were cut in a Terzano–Leitz cryostat at -20 °C and defatted for several hours in ethanol. The sections were then exposed to formalin vapour for 30 min, washed with water and immersed for 30 min in a 5% aqueous solution of trichloroacetic acid. This latter treatment, by which the phosphates were removed from the granules, was optional but gave clearer preparations. After careful washing of the sections with water, they were immersed for 40–45 min in a Choplin's jar containing 40 ml of a 1% aqueous solution of dextran sulphate (Schuchardt, Chemische Fabrik, München, Germany) (M about 500000) to which 5 ml of a 5% solution of $CaCl_2$ was added, the pH value being maintained at 7–7·2. The sections, after washing in water to remove the excess of dextran, were stained for 3–4 min with a 0·06% (w/v) solution of Toluidine blue in 0·15 M-citrate-phosphate buffer of pH 4·8, rinsed in water, air-dried and mounted in glycerin jelly. The lipoproteins appeared violet coloured (see Plate 1).

The sections could also be stained with Alcian blue using the procedure of Mowry & Winkler (1956). A 2% (w/v) aqueous solution of heparin (80,000 i.u./g, B.D.H. Ltd, Poole, England) at pH 7–7·2, containing 0·250 mg $CaCl_2$ could be used in place of dextran sulphate.

We have reported (Bolcato & Spettoli, 1969) that the protein moiety could be separated from the phosphates and from the lipid by immersing the sections for 2 h in 0·15 M-citrate buffer of pH 4·7 to solubilize the phosphates, and then for 48 h in chloroform–methanol (2:1, v/v) to solubilize the lipids. After this treatment the sections of cheese did not give histochemical reactions with stains specific for these 2 substances. As the sections still stained for the presence of sulphated polysaccharides, it was apparent that the protein moiety of the lipoprotein reacted with heparin or dextran sulphate and was distinguishable from the other proteins of the cheese. Nevertheless, it was also noted that the sulphated polysaccharides will react with α-lecithin, sphingomyelin and cephalin (Koch–Light Laboratory, Colnbrook, England). If these 3 compounds are incorporated into a section of cheese they stain violet according to the method outlined above. Thus, it seems possible that the dextran sulphate may bind to the lipoprotein at both the lipid and protein fractions of the molecule.

The regions in a section of cheese stained individually for phospholipids containing choline, lipoproteins and proteins were similar to those obtained with the dextran sulphate–Toluidine blue method.

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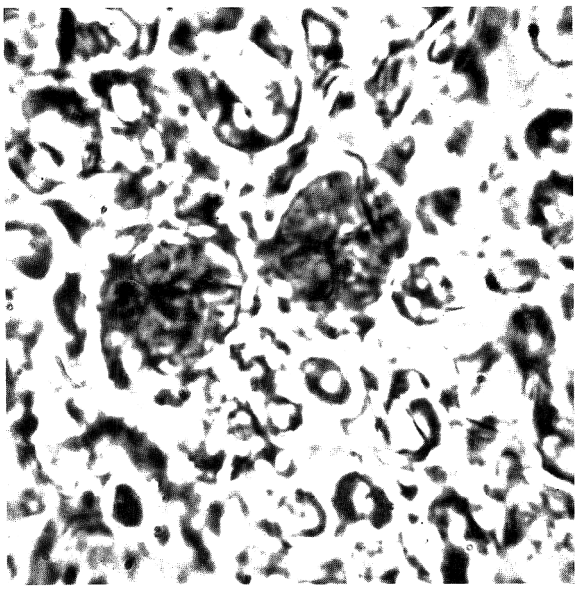

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EXPLANATION OF PLATE

Spots of lipoproteins in a section of Provolone cheese (× 840).

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A pilot plant for the removal of cationic fission products from milk

III. Nutritional evaluation of the product

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Summary. As previously described, the process consists essentially of the passage of milk at pH 5·2–5·3 through an ion exchange resin charged with a suitable mixture of the ions of Ca, K, Na and Mg.

The nutritive quality of plant-treated milk was assessed in feeding experiments with weanling rats and 1- to 2-day-old piglets.

No deleterious effect was found with rats, but tests with piglets indicated that the nutritive quality of the milk was impaired as evidenced by a higher mortality.

Post mortem examinations revealed no specific cause of death.

The use of hydrochloric acid instead of citric acid resulted in a lower mortality, though not as low as that found in piglets receiving untreated milk.

The difference in mortality between animals receiving treated and untreated milk was not consistent and was not found in some experiments.

It is concluded that the milk is unsuitable for piglets and that its suitability for human infants remains in question.

A pilot plant erected in this Institute for the removal of cationic fission products from milk has been described by Glascock, Hall, Suffolk & Bryant (1968). The milk is acidified with citric acid to pH 5·2–5·3, held for 1 h and applied to a cation exchange column charged with a suitable mixture of the ions of Ca, K, Na and Mg. After passage through the column the pH value is adjusted to 6·8 with KOH. It was shown (Glascock & Bryant, 1968) that the process reduced the concentration of radio-strontium added *in vitro* or *in vivo* to 2–4 % of its original value. The treatment had no unexpected effects on the chemical composition of the milk. The milk contained about twice the normal level of potassium citrate, and a loss was found of about 65 % of the thiamine and small losses occurred of vitamin B_6 and nicotinic acid.

The results are now reported of tests of the nutritive quality of milk processed in this way. No deleterious effect was found on weanling rats fed only on treated milk supplemented with trace minerals, but tests with 1- to 2-day-old piglets fed with treated milk supplemented with thiamine and antibiotics indicated that the nutritive quality of the milk had been adversely affected.

Since it seemed possible that the results with piglets might have been caused by the presence in the milk of levels of potassium citrate that had been found to inhibit the enzymic clotting of milk (Glascock & Bryant, 1968), the preliminary acidification was carried out with hydrochloric acid instead of with citric acid. This change led to some loss of radiochemical efficiency and the concentration of residual radio-strontium was then 11 % of its original value (Glascock & Bryant, 1968). However, the results of tests with piglets indicated that this modification reduced the loss of nutritive quality but did not give a completely satisfactory product.

In a further test with piglets an attempt was made to compare the nutritive value of milk treated by the usual procedure with citric or hydrochloric acid with that of milk treated after another modification of the process in which the delay period, during which the milk was held at pH 5·2 before application to the column, was reduced from 1 h to 4 min. In this test no difference in performance was found between the piglets receiving untreated and either kind of treated milk.

The results of our tests with piglets are generally not in accord with the findings of Isaacs *et al.* (1967).

EXPERIMENTAL

Tests with rats

Hooded Norwegian rats were taken at 25 days of age from 12 litters, each comprising 3 female and 3 male rats. The stock colony rats were maintained on a commercial pelleted diet. One male and one female weanling rat from each litter was randomly allocated to each of the 3 experimental diets. Male and female rats were separately housed with 3 animals in each cage. The diets were offered *ad lib.* for 9 weeks and records were kept of the daily consumption of milk by each group of 3 animals. The rats were weighed twice weekly.

Tests with piglets

Large White piglets from the N.I.R.D. herd or purchased from a neighbouring farm (Culham Farms, Hurley, Berks) were removed from the sow when 36 to 48-h old and placed individually in stainless steel cages. Four such cages were kept in each of a series of small rooms in which the temperature was maintained at 21 °C and the relative humidity at about 75 %. On all except one occasion all 4 piglets in each room received the same diet. The details of the number of piglets given each diet and the date when the experiment was carried out are shown in Table 2. Each experiment included several replicates, usually with 4 piglets on each diet. Litter-mate pigs were randomly allocated to the different diets.

The experimental diets were offered *ad lib.* from small troughs, which were automatically supplied from individual containers. The milk was placed in the containers twice daily, at 9.30 a.m. and 4.0 p.m., from a stock kept in a refrigerator at 4 °C. The level of the milk in the trough was kept low by a special automatic device, thus ensuring that very little was wasted. The cages and the feeding equipment have been described in detail by Braude, Mitchell & Suffolk (1968). The amount of milk consumed by each piglet was recorded. The stock of milk was replaced twice weekly by freshly processed milk.

All the cages were thoroughly cleaned and sterilized in a steam chest twice weekly. The troughs and milk containers were sterilized and replaced twice daily.

The piglets remained on test for 26 days, except in one replicate of the first experiment, which was terminated after 21 days. When the piglets were 4 days old, they were given an injection of 2 ml Imposil 200 (Fisons Pharmaceuticals Ltd, Loughborough), as a protection against anaemia. As the experiments progressed the piglets on all the treatments scoured intermittently. As soon as scouring was observed they were given orally, on 2 consecutive days, the prescribed dose of Framomycin (Crookes Laboratories Ltd, Basingstoke).

Diets

Fresh cow's milk, and milk from the same bulk treated in the plant after acidification with citric acid or hydrochloric acid, was pasteurized, and homogenized. The treated milks were supplemented with 3·3 mg thiamine/l. Fresh batches of milk were treated and prepared twice-weekly. Each litre of milk for rats was further supplemented with vitamin D_3 (45 i.u.) and minerals (80 mg, ferric citrate; 5·9 mg, $CuSO_4.5H_2O$ and 4·1 mg, $MnSO_4.4H_2O$) and stored at 4 °C until required. Each litre of milk for piglets was further supplemented with penicillin (4·5 mg) and chlortetracycline (4·5 mg) and stored at 4 °C until required.

RESULTS

Tests with rats

In Table 1 are shown the mean body weights of the groups of male and female rats at the start of the test and after receiving the experimental diets for 3, 6 and 9 weeks and also the mean total food consumption of the rats in each group. As expected, the male rats drank more milk and grew faster than did the females, but it is evident from the results that plant treatment of the milk did not affect feed consumption or growth rate.

Table 1. *Mean body weights and mean total food consumption of rats given untreated milk or milk treated after acidification with hydrochloric or citric acid*

Rats	Treatment of milk	Week of test: Mean rat body weight, g				Total milk consumption, g/rat
		0	3	6	9	
12♂	None	49	124	201	246	696
12♂	Hydrochloric acid	49	116	191	239	679
12♂	Citric acid	50	119	197	248	698
12♀	None	47	106	152	181	551
12♀	Hydrochloric acid	47	90	149	174	548
12♀	Citric acid	46	112	152	176	563

Tests with piglets

Performance

Table 2 summarizes the results of 5 experiments in which the growth performance of piglets given untreated milk was compared with that of litter-mates given milk processed after acidification with either citric or hydrochloric acid. A few piglets did not take to the artificial rearing routine and all the piglets that refused to feed within

and the live-weight gain and efficiency of feed conversion of the survivors.

Treatment of milk	Expt no.	Date	Source of pigs	No. of animals	Weight, kg	No. dead	% dead	Age at death, days	Live-weight gain, g/day	Efficiency of feed conversion, g milk/ g gain
Citric acid	1	20. iv–30. v. 66	N.I.R.D.	14	1·56	11		10, 10, 11, 12, 12, 13, 13, 14, 14, 15, 15	—	—
Citric acid	4	15. x–26. xii. 66	N.I.R.D.	15	1·60	4		8, 10, 12, 18	—	—
			Total	29		15	51·7	—	295·8	8·60
None	1	20. iv–30. v. 66	N.I.R.D.	13	1·54	4		16, 16, 4, 15	—	—
None	4*	15. x–26. xii. 66	N.I.R.D.	15	1·61	1		10	—	—
			Total	28		5	17·8	—	273·9	8·40
Hydrochloric acid	2	10. vi–28. vii. 66	N.I.R.D.	11	1·71	7		15, 17, 19, 19, 9, 11, 16	—	—
Hydrochloric acid	3	14. vii–31. viii. 66	N.I.R.D.	8	1·64	0		—	—	—
Hydrochloric acid	4	15. x–26. xii. 66	N.I.R.D.	13	1·60	1		9	—	—
			Total	32		8	25·0	—	285·8	8·44
None	2	10. vi–28. vii. 66	N.I.R.D.	10	1·72	2		22, 9	—	—
None	3	14. vii–31. viii. 66	N.I.R.D.	7	1·64	1		—	—	—
None	4*	15. x–26. xii. 66	N.I.R.D.	15	1·61	1		—	—	—
			Total	32		4	12·5	—	289·0	8·32
Citric acid	5	17. i–31. iii. 67	Culham Farms	15	1·37	1	6·6	13	304·7	8·57
Citric acid, no delay†	5	17. i–31. iii. 67	Culham Farms	15	1·38	1	6·6	21	294·6	8·48
None	5	17. i–31. iii. 67	Culham Farms	15	1·40	1	6·6	15	292·8	8·66

2 days of the start of the experiment, or that died subsequently from causes not connected with the experiments, have been excluded from the results. The animals placed on experiment each drank 1–1·5 l milk/day and their consumption increased gradually until by the end of their third week of life they were each drinking about 3 l/day. Most of the piglets on all the treatments had one or more periods of scouring; animals that recovered continued to grow normally, whereas those that did not recover spontaneously or did not respond to treatment usually died within 1–3 days of gastro-enteritis with persistent scouring and dehydration. All the piglets that died were given a detailed post mortem examination at the Ministry of Agriculture, Fisheries and Food Veterinary Investigation Centre, Coley Park, Reading.

It is apparent from Table 2 that the number of piglets surviving to the end of the experiment varied markedly in the different trials. Thus, in expt 1, with milk acidified with citric acid, 11 piglets out of 14 died, whereas in expt 4, carried out under similar conditions 9 months later, only 4 out of 15 died. In expt 2, with milk acidified with hydrochloric acid, 7 piglets out of 11 died, whereas in expts 3 and 4 only 1 pig died out of the 21 given milk treated in the same way. Relatively few piglets given untreated milk died, though it is noteworthy that the casualty death rate was highest in expt 1 in which 4 piglets out of 13 died. Almost all the deaths occurred when the piglets were between 10 and 17 days of age.

Post mortem findings

No significant differences were observed in the post mortem examination of piglets receiving the untreated and treated milks; haemolytic strains of *Escherichia coli* were isolated from the intestinal contents and viscera of all the piglets that died. The infective organism was identified as strain OKE4 in expt 1 and as strain G1253 in expt 2. Histological preparations of thyroid, thymus and adrenal glands, and of liver and kidney, and in some piglets of lung, failed to reveal significant differences between piglets that had received untreated or treated milk.

Table 3. *Concentration of potassium (mg/100 ml) in blood plasma from pigs receiving untreated milk or milk processed after acidification with citric acid*

		Age, days			
		3	5	8	10
		Plasma potassium, mg/100 ml			
Pig no.	Treatment of milk				
1A	None	37·2	41·1	40·1	31·3
1B		30·6	44·9	41·3	29·8
2A	Citric acid	41·3	40·2	52·5	55·5
2B		41·5	66·7	50·4	38·3

The presence of kidney lesions and urates in the bladder in piglets from the first 2 experiments suggested that the treated milk might be interfering in some way with urinary secretion. Urine samples were therefore examined qualitatively whenever possible for the presence of abnormal constituents. About half the samples tested were positive for albumin and about one-third were positive for glucose but these were equally distributed between control pigs and those on treated milk, and the degree of abnormality was not considered to be significant.

Kidney abnormalities were present in both groups and the haemopoietic foci normally present in the livers of piglets up to 3 weeks of age were reduced in number and extent in all the piglets examined. It is possible that these histological features may be characteristic of artificially reared pigs.

There was evidence in some piglets of excess deposition of iron pigment in the sinusoids and Kupfer cells of the liver and also in the kidney glomeruli of piglets on the treated milk diets. It is difficult to find any explanation for this phenomenon, but such excess might possibly have interfered with reticulo-endothelial function and rendered the piglets more susceptible to pathogenic bacteria.

In all the piglets normal clotting of the milk in the stomach was found to have taken place. However, the pH values of fresh stomach contents were found to be slightly higher in piglets receiving treated milk than in the controls.

Concentration of potassium in blood plasma

The potassium content of samples of blood plasma taken during the first 10 days of expt 1 from 2 piglets receiving milk acidified with citric acid before processing, and from 2 piglets receiving untreated milk, was measured by atomic absorption spectrophotometry, and the results are shown in Table 3. Pigs no. 1A and 2B subsequently died when 15 days old.

It is evident that the values for the piglets receiving the citric-acid treated milk were somewhat higher than those for the piglets receiving untreated milk. However, all the values were within the rather wide range reported for young piglets by Widdowson & McCance (1956).

DISCUSSION

Cationic fission products are present in milk as a result of the contamination of pasture and fodder by fall-out from nuclear weapons. The principal aim in developing the ion-exchange process was to provide a means of removing them without detriment to the nutritional quality of the milk. To avoid possible danger to health it is particularly necessary to ensure a supply of uncontaminated milk for infants and young children. Thus, in assessing the nutritive value of the plant-treated product it was of paramount importance to determine whether the product was likely to be a satisfactory alternative to ordinary milk for this purpose. The results of tests with weanling rats showed that plant treatment of the milk did not affect its nutritive quality for weaned animals but provided no information about its suitability for young sucking animals. Since the state of physiological maturity at birth of the piglet is generally agreed to be similar to that of the human infant, it seemed likely that the pig would be the most appropriate species with which to carry out further tests.

During the last few years a large number of experiments have been carried out at this Institute in which 2-day-old piglets have been given cow's milk or diets prepared from reconstituted cow's whole milk. The growth rate and over-all performance of these pigs has been good and the mean mortality about 10%. The rate of growth of young piglets depends to a very large extent on their intake of food, and it was found that although the incidence and severity of scouring was lessened when food intake was reduced, the rate of growth was substantially less than that normally found for

suckling pigs. Conversely, when food was unrestricted the incidence of scouring tended to increase, but so did the rate of growth.

Thus, it was considered that the most exacting test of the nutritive value of the plant-treated milk would be accomplished by allowing pigs unlimited access to the diet, and it was hoped that in this way it would be possible to detect any differences that might exist between the quality of the untreated and treated milks. A preliminary experiment with growing rats showed clearly that the nutritive value of milk for rats was not impaired by passage through a cation-exchange column after acidification with citric or hydrochloric acid.

However, the tests with baby pigs gave conflicting results. In expts 1, 2 and 4 a markedly higher mortality was found among piglets receiving the treated milk, whereas in expts 3 and 5 no such difference was observed. Mortality was greatly reduced when hydrochloric acid was used in place of citric acid in expts 3 and 4, but the effect in expt 2 was less definite.

Post mortem examinations of piglets that died gave no indication that the treated milk contained a toxic factor or that it was deficient in an essential nutrient, but it seems possible that piglets receiving the treated milk were more susceptible to the effects of coliform infection than were those receiving the untreated milk.

Chemical analysis of the treated milk (Glascock & Bryant, 1968) indicated that the main differences likely to be of nutritional import were the increase of about 50 % in the level of potassium and the decrease of about 65 % in the level of thiamine. Thiamine had been added to the milks used in the feeding tests, and the higher concentration of potassium was not found to cause an appreciable elevation of blood potassium levels.

Glascock & Bryant (1968) found that acidification of milk with citric acid nearly doubled the level of this acid in the milk used in their experiments, whereas it was essentially unchanged after acidification with hydrochloric acid. The enzymic clotting time (with rennin) of the milk prepared with citric acid was prolonged from 2–5 min to over 24 h, whereas that of hydrochloric acid-treated milk was less than normal. However, normal clotting of the milk was found in the stomach at post mortem examination. It is not yet possible to say whether such alterations in clotting time would be likely appreciably to affect the performance of baby pigs, but it is of interest that Hallgren (1940) and Weikl & Emig (1957) found that the addition of citric acid to cow's milk given to young pigs was beneficial.

It is noteworthy that only 3 pigs died out of the 45 used in expt 5 which was carried out with animals of a different strain. This result, and the varying mortality found in our other experiments, may have arisen from differences in susceptibility to infection of pigs from different sows. Early-weaned piglets kept in individual cages are undoubtedly exposed to physiological stress which we have found to be exacerbated by feeding the diets *ad lib.* Thus, it is probable that an additional stress, such as might be caused by a slightly unsatisfactory diet, will allow them to be overwhelmed.

Whilst our experiments were in progress a report was published of similar studies carried out in the U.S.A. by Isaacs *et al.* (1967) who found that ion-exchange treatment of milk for the removal of cationic radionuclides caused changes in composition similar to those found by us, but that it appeared not appreciably to affect the

nutritive value of the milk for rats and pigs. In their experiments, groups of 3-day-old crossbred pigs were given reconstituted whole milk powders prepared by spray-drying untreated milk, and milk processed after acidification with citric acid; a third group was given a diet of reconstituted whole milk powder from untreated milk to which potassium citrate was added. All the diets were supplemented with salts of iron, copper, manganese and zinc. The diets were reconstituted at the start of the experiment to contain 19% of solids and after 3–4 weeks this was increased to 28–30%. Apart from their content of solids, the only apparent difference between the diets used by Isaacs *et al.* (1967) and those used by us was that the former added trace minerals. In our experience, such additions are unnecessary for very young pigs during the first weeks of artificial rearing. Furthermore, analyses of our untreated and plant-treated milks showed that the treatment had little effect on the concentrations of ions of Cu, Zn, and Mn. Unfortunately, no details were given by Isaacs *et al.* (1967) of their scale of feeding or of the amounts of food given to their piglets, but these must have been small since it is noteworthy that the initial weights of the piglets were 1·10–2·05 kg, and that after 4 weeks the mean weights of the groups given the various diets were between 2·31 and 3·30 kg. Thus, the mean liveweight gain per piglet per day was around 55 g, which may be contrasted with the much greater live-weight gain per day of around 290 g achieved in the 26-day period of our experiments. This markedly slower rate of growth may well have allowed any effect resulting from treatment of the milk in the plant to be masked. No mention was made of scouring and no piglets were reported as having died. Pathological observations on 4 piglets killed from each group at 5 weeks of age indicated no abnormalities in the kidneys, livers or hearts of the animals.

Nevertheless, we conclude from the results of our own tests that the nutritive value of milk for the piglet was adversely affected by treatment in the plant. Furthermore, since no complete explanation could be given of the cause of death in the test animals and since in some tests no difference could be found between treated and untreated milk, it is unlikely that further experiments with piglets would provide decisive information. It is impossible from our findings to draw any firm conclusions about the suitability for human infants of milk treated by this process.

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Carotenoid and tocopherol levels in the serum of apparently healthy dairy cattle

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Total carotenoids, β-carotene and α-tocopherol concentrations were determined in serum from healthy dairy cattle of various ages, grazing good quality pasture, in different areas of New South Wales. The relationships of these components with each other and with age of the animal were examined.

Mean serum β-carotene concentrations for heifers and cows were 0·68 and 1·20 mg/100 ml, respectively. These concentrations were somewhat higher than those found for calves (0·20 mg/100 ml) and weaners (0·37 mg/100 ml). Total corresponding carotenoid values were generally about 20% higher than those for β-carotene for herds.

Mean serum α-tocopherol concentrations for heifers and cows were 0·54 and 0·90 mg/100 ml, respectively. Again, these values were higher than those found for calves (0·22 mg/100 ml) and weaners (0·37 mg/100 ml).

There was a close relationship between serum α-tocopherol and β-carotene ($r = 0·90$), between serum α-tocopherol and total carotenoids ($r = 0·92$), and between serum β-carotene and total carotenoids ($r = 0·99$).

The presence of adequate dietary levels of carotenoids and tocopherols is an important consideration in the nutrition of man and of domestic animals. Deficiencies or imbalances of the carotene derivative, vitamin A, can give rise to well-known disorders, such as defective dark adaptation and impaired growth rate (Moore, 1965), and lack of vitamin E has been associated with dystrophic muscular conditions in several species of animals (Hartley & Dodd, 1957; Blaxter & Brown, 1952). The interrelationships of these vitamins in biological systems have been investigated but apparently they are not well understood (Green, 1962).

The nutritional aspects of vitamin A in cattle have been studied extensively but there are few reports on the concentrations of carotenoids in bovine blood (Payne & Kingman, 1947; Watkins & Knox, 1954). Similarly, there are few reports on serum tocopherol levels (Latschar, Wise, Parrish & Hughes, 1949; Blaxter, Brown & MacDonald, 1953).

In the present investigation an attempt was made to determine the concentrations of total carotenoids, β-carotene and α-tocopherol, and the relationship between these components, in the serum of apparently healthy dairy cattle.

EXPERIMENTAL

Serum samples were collected from cattle in 3 well-managed dairy herds in different districts of New South Wales. Within each herd, samples were collected from 5 calves aged between 2 weeks and $5\frac{1}{2}$ months, either weaned or unweaned; from 5 weaners aged from 6 months to about 18 months; from 5 pregnant, uncalved heifers, aged from 20 months to 3 years and from 5 adult cows at different stages of pregnancy and lactation. All the animals were in good condition and apparently in good health. Table 1 gives details of the farms and animals sampled.

Table 1. *Details of breed, age, location, pasture and soil type for each herd examined*

Herd	M	G	O
District	Sydney	Moss Vale	Orange
Altitude, ft	140	2200	3100
Date of sampling	20. vi. 67	2. v. 67	14. vi. 67
Breed	Mixed crossbred	Australian Illawarra Shorthorn	Friesian
Soil type	Clay over shale	Alluvial creek flats	Basaltic
Pasture species	Kikuyu and paspalum	Ryegrass, clover and phalaris	Ryegrass, clover, phalaris and oats
Mean age, and range:			
Calves, months	1·8 (0·2–3·5)	2·4 (0·5–5·0)	2·7 (0·7–5·5)
Weaners, months	11 (6–18)	13 (8–18)	16 (6–24)
Pregnant heifers, years	2·6 (1·7–3·2)	— (1·4–2·0 approx.)	2·7 (2·3–2·9)
Cows, years	5·4 (2–10)	— (3–7 approx.)	3·3 (2–4)

Collection of serum

Approximately 200-ml samples of blood were collected from the jugular vein and protected from light, by putting them immediately into cool, insulated boxes during clotting and transport of the samples from the field to the laboratory. Serum was separated in a refrigerated centrifuge, dispensed in 10–15 ml quantities and stored at −20 °C pending analysis. Elapsed time from bleeding to storage of serum varied from 3 h (herd M) to 9 h (herd O) depending on the distance the samples were transported.

Chemical analyses

Determinations of β-carotene and α-tocopherol were carried out on light petroleum extracts of serum from all 60 animals. The extracts were examined by 2-dimensional paper chromatography, and the separated constituents were determined spectrophotometrically, as described previously (Bayfield, Falk & Barrett, 1968).

Total carotenoids were determined directly on the excess light petroleum extract, by measuring the optical density at 450 nm and calculating the result as for β-carotene (Bayfield *et al.* 1968). Because of a shortage of serum these determinations were restricted to 16 samples from herd M and 20 from herd O.

All samples were analysed in duplicate and the precision of the duplicates was ascertained by use of the formula

$$s = \sqrt{\frac{\Sigma (d^2)}{2N}},$$

where d = difference between duplicates and N = number of samples analysed.

RESULTS

The serum concentrations of β-carotene are given in Table 2. For the 3 herds there was a wide variation in values from zero to 3·20 mg/100 ml, with an over-all mean of 0·61 ± 0·55 mg/100 ml. Highest values were found in adult animals and lowest in calves (Fig. 1) and, generally, values for herd O were lower than those for herds M and G.

Table 3 gives the concentrations of α-tocopherol in the same series of samples. The values fell within a wide range from zero to 1·53 mg/100 ml with an over-all mean of 0·51 ± 0·36 mg/100 ml. As with β-carotene, adult cattle had the highest values (Fig. 1), and the values for herd O were lower than those for herds M and G.

Results for total carotenoids are given in Table 4. There was a trend for concentration to rise with increasing age.

Table 2. *Concentrations of β-carotene (mg/100 ml) in serum of normal dairy cattle*

(Within each herd the animals are arranged in order of increasing age.)

	Herd						
	M		G		O		
Class	Serum level	$\bar{x}$ ± S.D.	Serum level	$\bar{x}$ ± S.D.	Serum level	$\bar{x}$ ± S.D.	Class $\bar{x}$ ± S.D.
Calves	0·04	0·14 ±	0·08	0·43 ±	0·05	0·03 ±	0·20 ± 0·28
	0·10	0·21	0·23	0·34	0·01	0·02	
	0·50		0·35		0·01		
	0·01		0·95		0·05		
	0·04		0·55		0·01		
Weaners	0·19	0·05 ±	0·63	0·73 ±	0·25	0·32 ±	0·37 ± 0·30
	0·04	0·08	0·66	0·08	0·22	0·15	
	0·01		0·78		0·52		
	0·03		0·73		0·43		
	0·00		0·83		0·19		
Heifers	0·87	0·96 ±	0·74	0·62 ±	0·50	0·44 ±	0·68 ± 0·25
	0·78	0·17	0·62	0·09	0·42	0·04	
	0·93		0·66		0·43		
	1·23		0·60		0·39		
	1·00		0·50		0·47		
Cows	1·63	1·73 ±	1·23	1·17 ±	1·10	0·68 ±	1·20 ± 0·66
	1·14	0·86	1·03	0·19	0·46	0·26	
	3·20		1·28		0·51		
	1·63		0·93		0·59		
	1·06		1·39		0·75		
Herd		0·72 ± 0·82		0·74 ± 0·34		0·37 ± 0·28	0·61 ± 0·55

The ratio of β-carotene to total carotenoids was reasonably constant for each class. Thus, the mean ratio was for calves, 70 % (50–88 %); for weaners, 80 % (75–87 %); for heifers, 86 % (83–89 %) and for cows 82 % (70–91 %).

There were close relationships between the concentrations of β-carotene and α-tocopherol (Fig. 2(*a*)), between total carotenoids and α-tocopherol (Fig. 2(*b*)), and between β-carotene and total carotenoids (Fig. 2(*c*)). Regression lines are shown and the relationships between these and other parameters are given in Table 5.

Table 3. *Concentrations of α-tocopherol (mg/100 ml) in serum of normal dairy cattle*

(Within each herd the animals are arranged in order of increasing age.)

	Herd						
	M		G		O		
Class	Serum level	$\bar{x} \pm$ S.D.	Serum level	$\bar{x} \pm$ S.D.	Serum level	$\bar{x} \pm$ S.D.	Class $\bar{x} \pm$ S.D.
Calves	0·15	0·22 ±	0·17	0·36 ±	0·10	0·09 ±	0·22 ± 0·21
	0·20	0·24	0·24	0·21	0·10	0·04	
	0·63		0·20		0·08		
	0·07		0·53		0·13		
	0·04		0·64		0·03		
Weaners	0·18	0·05 ±	0·61	0·57 ±	0·51	0·49 ±	0·37 ± 0·25
	0·04	0·07	0·56	0·03	0·48	0·16	
	0·01		0·54		0·73		
	0·04		0·54		0·44		
	0·00		0·60		0·28		
Heifers	0·63	0·72 ±	0·45	0·52 ±	0·40	0·38 ±	0·54 ± 0·16
	0·80	0·07	0·58	0·07	0·39	0·10	
	0·77		0·60		0·34		
	0·73		0·47		0·25		
	0·68		0·50		0·52		
Cows	0·88	1·08 ±	0·95	1·07 ±	0·90	0·55 ±	0·90 ± 0·36
	0·73	0·32	0·75	0·28	0·57	0·21	
	1·53		1·33		0·47		
	1·28		0·90		0·38		
	1·00		1·40		0·45		
Herd		0·52 ±		0·63 ±		0·38 ±	0·51 ± 0·36
		0·46		0·32		0·22	

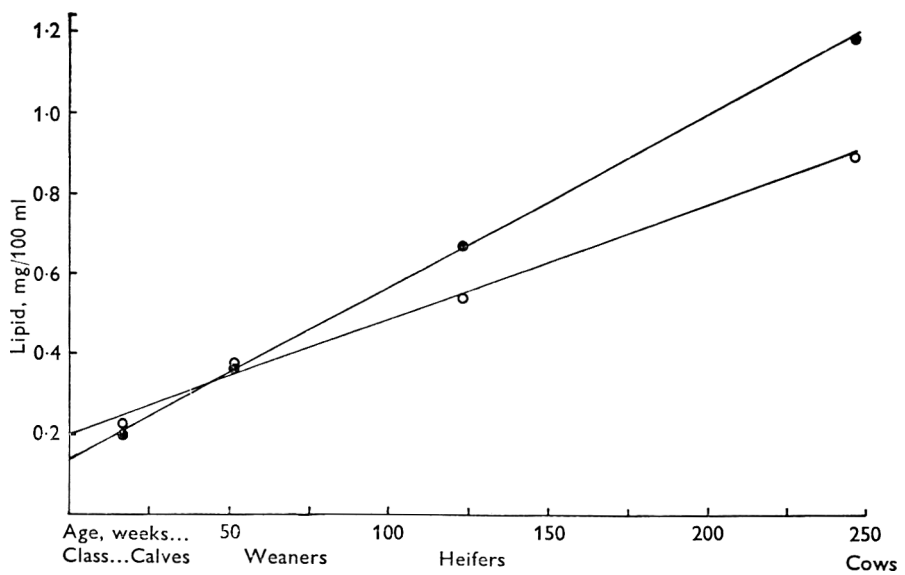

Fig. 1. Relationship of serum α-tocopherol (○) and β-carotene (●) to age. The points represent the mean concentrations for the different classes of animals.

The precision of the duplicates for β-carotene determinations was 0·02 mg/100 ml (over-all mean concentration 0·61 mg/100 ml), and for α-tocopherol determinations, 0·03 mg/100 ml (over-all mean concentration 0·51 mg/100 ml).

Table 4. *Concentrations of total carotenoids (mg/100 ml) in serum of normal dairy cattle.*

(Within each herd the animals are arranged in order of increasing age.)

	Herd				
	M		O		
Class	Serum level	$\bar{x} \pm$ S.D.	Serum level	$\bar{x} \pm$ S.D.	Class $\bar{x} \pm$ S.D.
Calves	0·05	0·20 ± 0·25	0·08	0·04 ± 0·03	0·11 ± 0·18
	0·13		0·02		
	0·57		0·02		
	0·05		0·06		
	—		0·02		
Weaners	0·21	0·13 ± 0·12	0·33	0·37 ± 0·19	0·30 ± 0·20
	0·04		0·29		
	—		0·60		
	—		0·51		
	—		0·13		
Heifers	1·00	1·11 ± 0·20	0·58	0·49 ± 0·09	0·80 ± 0·36
	0·89		0·48		
	1·06		0·38		
	1·41		0·44		
	1·19		0·57		
Cows	1·92	2·02 ± 0·88	1·42	0·88 ± 0·36	1·45 ± 0·87
	1·34		0·55		
	3·50		0·59		
	2·00		0·79		
	1·34		1·07		
Herd		1·04 ± 0·92		0·45 ± 0·36	0·71 ± 0·73

Table 5. *Statistical data on relationships of serum constituents*

Relationship	No. of samples	Correlation coefficient	Regression equation
β-Carotene and age	60	0·68	$y = 0{\cdot}00346\,x + 0{\cdot}236$
α-Tocopherol and age	60	0·75	$y = 0{\cdot}00245\,x + 0{\cdot}244$
β-Carotene and α-tocopherol	60	0·90	$y = 0{\cdot}581\,x + 0{\cdot}155$
α-Tocopherol and total carotenoids	36	0·92	$y = 0{\cdot}470\,x + 0{\cdot}168$
β-Carotene and total carotenoids	36	0·99	$y = 0{\cdot}874\,x - 0{\cdot}024$

For all correlation coefficients, $P < 0{\cdot}001$.

DISCUSSION

Many values reported for carotene in biological materials have been measures of total carotenoids (Watkins & Knox, 1954; Leitner, Moore & Sharman, 1960*a*), whereas others have been expressed as β-carotene (Hartmann & Lascelles, 1965). Therefore, it is difficult to compare published results on carotene because of the different components measured. In the present report, total carotenoids were measured and these were compared with values for β-carotene, separated from other carotenoids by means of paper chromatography.

From Tables 2 and 4 it can be seen that, as a general rule, total carotenoid values were about 20% higher than those for β-carotene and this should be taken into account when comparing these results with carotene values from other reports.

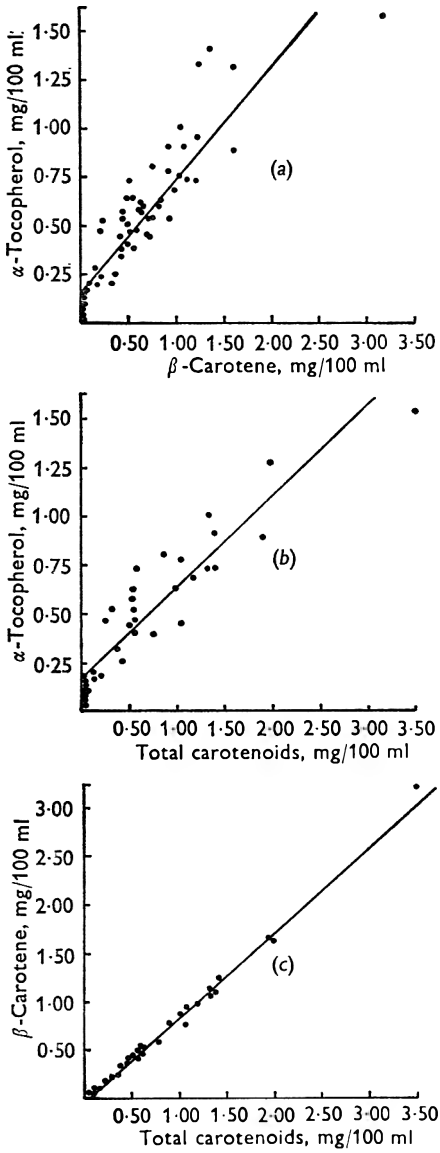

Fig. 2. Relationship between α-tocopherol and β-carotene (*a*); α-tocopherol and total carotenoids (*b*); β-carotene and total carotenoids (*c*).

In the current investigation, the mean values for β-carotene concentrations in the serum of heifers and cows were 0·68 and 1·20 mg/100 ml, respectively. These values are higher than those for American range cattle of 0·45 mg/100 ml (Watkins & Knox, 1954), 0·60 mg/100 ml (Repp & Watkins, 1958) and 0·63 mg/100 ml (Ralston & Dyer, 1959). They are similar to the levels of 0·50–0·90 mg/100 ml reported for American

cattle on good quality pasture (Watkins & Knox, 1954) and also to the values of about 1 mg/100 ml for lactating cows on medium quality pasture (Hartmann & Lascelles, 1965).

There are fewer published values for β-carotene in the serum of young cattle than for adults. In the present study, the mean value for calves under 6 months of age was 0·20 mg/100 ml, and for weaners and yearlings, 6–18 months of age, 0·37 mg/100 ml. As would be expected, these values are higher than the concentration of 0·009 mg/100 ml found for calves on low carotene diets (Church, MacVicar, Bieri, Baker & Pope, 1954), and the level of 0·006 mg/100 ml for calves on dry feed (Bayfield *et al.* 1968).

The difficulty of comparing serum tocopherol levels for different areas, and the apparent unreliability of some of the results, have been discussed (Roels, 1967). In the work now reported, α-tocopherol was separated from other reducing constituents, simultaneously with β-carotene, by paper chromatography, so that a reliable estimate was possible.

The mean concentrations of α-tocopherol for heifers and cows in the present study were 0·54 and 0·90 mg/100 ml, respectively. These values were generally similar to those of 0·72–1·47 mg/100 ml for pasture-fed cows and 0·45 mg/100 ml for barn-fed animals reported from America (Latschar *et al.* 1949), and were also comparable with values of 0·04–1·20 mg/100 ml reported from Scotland (Blaxter *et al.* 1953). For calves, the mean value of 0·22 mg/100 ml was higher than that of 0·15 mg/100 ml found for healthy calves in Scotland (Blaxter, 1953), and higher than the levels of 0·10 mg/100 ml (Rousseau *et al.* 1957) and 0·06 mg/100 ml (Bayfield *et al.* 1968).

For all 3 components studied there appeared to be an age effect upon serum concentration. Generally, the concentrations were lowest in calves and weaners and rose to highest values in adult animals (Fig. 1). Increasing serum levels of carotene and tocopherol with age has also been reported in man (Leitner *et al.* 1960*b*).

There appeared to be a difference between herds for the different components. Serum values in herds M and G were of the same order, but were about twice those found in herd O. The herds were in different environments and were of different breeds so that there are many possible reasons for the differences observed. For example, the influence of soil type on pasture carotene has been shown (Payne & Kingman, 1947), and a relationship between pasture growth and serum carotene has been reported (Gillam & El Ridi, 1935).

The correlation of plasma carotene and tocopherol with various factors has been studied by several workers (Ralston & Dyer, 1959; Rousseau *et al.* 1957). In the present work, the plot of serum α-tocopherol against serum β-carotene for all animals showed a good correlation ($r = 0{\cdot}90$). A similar result was obtained when serum tocopherol was plotted against serum total carotenoids ($r = 0{\cdot}92$). It was apparent that serum β-carotene increased at a faster rate than serum α-tocopherol, which agrees with findings for serum carotenoids and tocopherol in humans (Leitner *et al.* 1960*b*). The existence of this correlation may be associated with the fact that the levels of vitamin E and carotenoids in the blood are apparently controlled by similar mechanisms (Leitner *et al.* 1960*b*).

Some information has been reported on the proportion of β-carotene to total carotenoids in the serum of man (Leitner *et al.* 1964). It is apparent from Fig. 2(*c*)

that β-carotene levels were approximately 80 % of those for total carotenoids, and that there was a close correlation ($r = 0{\cdot}99$). In view of this, it may be satisfactory for some purposes to estimate the concentration of β-carotene from the level of total carotenoids, which are determined more simply. Similarly, total carotenoid concentrations may give some indication of serum tocopherol levels in cattle.

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Reviews of the progress of dairy science

Section A. Recent developments in the biochemistry of the mammary gland

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CONTENTS

INTRODUCTION

This review will attempt to describe recent developments in the biochemistry of the mammary gland and some related topics in a selective rather than exhaustive way. For earlier work the reader is referred to the general reviews by Hansen & Carlson (1961) and Folley (1961) and, in addition, for more detailed information on protein metabolism, lactose synthesis and milk fat synthesis to the respective reviews of Barry (1961), Leloir & Cardini (1961) and Folley & McNaught (1961). For the most part, references given in these earlier reviews are not repeated here and, in addition, the well-established facts of general biochemistry are unsupported by references.

On considering the large volume of published work on mammary gland biochemistry one is struck by the dichotomy between research on ruminants and non-ruminants. Most of the work on ruminants has concentrated on the relationship between the uptake of metabolites from the blood and the output of products in the milk while in the bulk of studies on intermediary mammary gland metabolism at the enzymic level non-ruminants have been used. The need for economy directs most workers to small mammals, i.e. non-ruminants, for biochemical studies while technical difficulties discourage their use in the balance type of experiments. This is unfortunate as there are important differences between the metabolism of ruminants and non-ruminants which make correlation of the 2 types of result difficult at times. However, a complete separation of data from ruminants and non-ruminants would

be too restrictive, so they are here considered together in the various sections, though the possibility of errors of interpretation arising from this must be borne in mind.

There are 2 major aspects of mammary gland biochemistry to be dealt with: the metabolic pathways in the actively lactating gland and the changes in the pattern of this activity through the lactation cycle and especially at lactogenesis, the commencement of milk synthesis. In this review, the first sections will be concerned with the peak lactation picture and the changes will be considered subsequently.

LIPID SYNTHESIS

Since the review of Folley & McNaught (1961) most of the research on this topic has been directed at determining which components of ruminant milk lipids are synthesized in the gland and which originate from the blood, and in investigating the mechanism by which fatty acids of different chain lengths are synthesized.

The origin of milk fat

As reviewed by Barry (1964) it appears that, at least in the ruminant, the longer chain fatty acids originate from the blood whereas the shorter chain acids are synthesized *de novo* in the mammary gland. Experiments carried out using the intact goat (Barry, Bartley, Linzell & Robinson, 1963), and perfused goat udder (Lascelles, Hardwick, Linzell & Mepham, 1964) and the intact cow (Glascock *et al.* 1966) have all confirmed that plasma triglycerides are the source of the long chain fatty acids of milk fats. Lascelles *et al.* (1964) studied the fate of labelled chylomicra, obtained by cannulating the lymph duct of a goat given [^{3}H]stearate, when injected into a lactating animal and showed that they were rapidly taken up by both mammary gland and liver and had a half life in the blood circulation of approximately only 10 min. Delay in the appearance of this activity in the milk was attributed to the presence of a large pool of unlabelled material in the gland. Barry *et al.* (1963) showed that low density lipoproteins contribute to milk fat in the goat and in the cow. Glascock *et al.* (1966) concluded that the β-lipoprotein triglycerides are the serum lipid fraction making the major contribution to milk fat though they did not rule out a minor contribution from phospholipids. Though there is no net uptake of free fatty acids by the goat mammary gland there is an exchange between plasma free fatty acids and those of the gland (Linzell, Annison, Fazakerley & Leng, 1967).

As the goat gland takes up more stearic acid than oleic acid while milk contains 3–4 times more oleic than stearic a large conversion must take place. This conversion was demonstrated in the cow by Lauryssens, Verbeke & Peeters (1961) and goat milk has been shown to contain an enzyme capable of converting stearic to oleic acid (McCarthy, Ghiardi & Patton, 1965).

The question of whether palmitate is synthesized in the gland or taken up from the blood was studied by Linzell *et al.* (1967) who perfused the isolated goat gland with [1-^{14}C]acetate and examined the activity of the milk fatty acids. It was shown that fatty acids up to and including C_{14} were wholly synthesized *de novo*, palmitate was partially synthesized, and oleate came entirely from the plasma. A similar pattern was found when arterio-venous differences were measured across the mammary gland in the intact goat (Annison, Linzell, Fazakerley & Nichols, 1967). In this connexion,

Storry, Rook & Hall (1967) observed that the level of palmitate in cow's milk is relatively independent of dietary palmitate, which is not the case with stearate and oleate. On the other hand, milk palmitate can be increased by the intravenous infusion of acetate (Storry & Rook, 1965). From this it seems clear that in ruminants milk fatty acids up to and including C_{14} are synthesized in the gland, the C_{18} acids originate from plasma triglycerides, and palmitate occupies an intermediate position. The proportion of the fatty acids of non-ruminant milk which is derived from the plasma remains unknown due to the difficulties of applying the technique of perfusion and arteriovenous differences to these smaller animals.

The mechanism of the uptake of the chylomica which contain triglycerides is not known though lipoprotein lipase increases greatly in the guinea-pig mammary gland at the time of parturition (McBride & Korn, 1963; Robinson, 1963) and this enzyme is believed to be concerned with the uptake of lipoproteins. However, according to McBride & Korn (1963) the lipoprotein lipase content of the gland can be accounted for by the level of retained milk so its role in lipid uptake remains unproven.

Fatty acid synthesis

Precursors. The original observation of Balmain, Folley & Glascock (1954) that, whereas rat mammary gland slices utilized both glucose and acetate for fatty acid synthesis, sheep gland slices used only acetate in significant amounts remains unchallenged. Indeed, glucose infusion in the cow actually reduced milk fat yields (Storry & Rook, 1965). The importance of β-hydroxybutyrate in addition to acetate as a fatty acid precursor has been confirmed (Linzell *et al.* 1967) though it is not certain to what extent it must be split to 2 carbon units before being utilized. Linzell *et al.* (1967) concluded that only a small amount of direct incorporation of 4 carbon units would occur, while Kumar, Singh & Keren-Paz (1965), using soluble enzyme systems from goat and rabbit mammary glands, found that β-hydroxybutyrate and acetoacetate were incorporated into butyrate without prior cleavage. This discrepancy remains unresolved but may reflect a difference between intact and fractionated tissue.

Acetyl-CoA synthesis. As acetyl-CoA is a key intermediate in fatty acid synthesis its formation is important and embodies one of the most striking differences between the metabolism of ruminant and non-ruminant glands. In the non-ruminant, where glucose is an effective fatty acid precursor, it is converted to pyruvate in the cytosol (the soluble fraction of the cell) and the pyruvate is oxidized to acetyl-CoA in the mitochondria by a well-established sequence of reactions. However, as the weight of evidence suggests that fatty acid synthesis takes place in the cytosol and mitochondria are impermeable to acetyl-CoA a transportation problem exists (see review by Srere, 1965). Figure 1 shows the mechanism for the transport of 2 carbon units (mainly based on evidence from tissues other than mammary gland) which has been suggested by Srere and others. This involves the alternate formation and cleavage of citrate and the cycling of a 4 carbon unit between the mitochondria and the cytosol.

The evidence that this scheme is important in the non-ruminant mammary gland is considerable though circumstantial. The cytosol enzyme, ATP-citrate lyase, is highly active in the lactating mammary gland of the rat (Howanitz & Levy, 1965)

and mouse (Spencer & Lowenstein, 1966), increases rapidly at the onset of lactation and declines rapidly in the involuting gland (Jones, 1967). Citrate is a more effective precursor of fatty acids than glucose in a soluble enzyme system from rat mammary gland (Spencer, Corman & Lowenstein, 1964). Bartley, Abraham & Chaikoff (1965) investigated the conversion of doubly labelled ([^{14}C] and [^{3}H]) glucose, lactate and acetate into fatty acids by rat mammary gland slices. Their results indicated that the hydrogens of the methyl group of acetate were labilized during their conversion to fatty acids and of various hypothetical schemes they considered that only the citrate pathway should give this result.

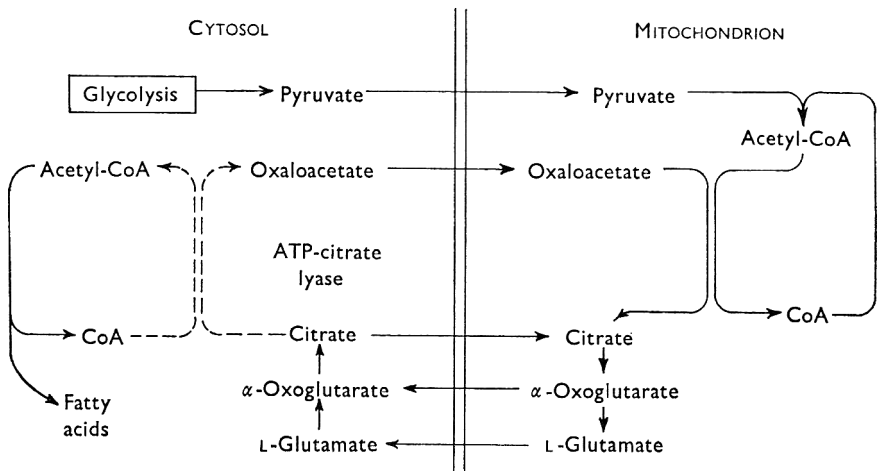

Fig. 1. The formation of acetyl-CoA in the cytosol. The pathway marked --- is absent in the ruminant mammary gland.

The possibility that α-oxoglutarate is involved in the above scheme is suggested by the work of Madsen, Abraham & Chaikoff (1964) who showed that rat mammary gland slices incorporated [2 and 5]^{14}Cglutamate into fatty acids in a glucose-dependent reaction. These particular carbon atoms would not be incorporated if the glutamate were oxidized via the Krebs cycle but would be if glutamate were converted to citrate with α-oxoglutarate as an intermediate. It was calculated that in the presence of glucose 20–30 % of glutamate utilized was converted to fatty acid. The importance of this pathway *in vivo* remains to be determined though it may be valuable because of the relative impermeability of some types of mitochondria to citrate. The conversion of α-oxoglutarate to citrate has also been noted in the perfused goat udder (Hardwick, 1964).

Assuming the citrate pathway to be important in non-ruminants there remains the question of the non-utilization of glucose for fatty acid synthesis in the ruminant. As Hardwick (1966) pointed out, the oxidation of glucose by the goat udder does yield acetyl-CoA as judged by the labelling of milk citrate and casein glutamate but the fatty acids remain unlabelled. This is explained by the absence of ATP-citrate lyase in the goat (Hardwick, 1966), sheep and cow (Hanson & Ballard, 1967) which prevents the extra-mitochondrial utilization of acetyl-CoA synthesized within the mitochondrion. On the other hand, ruminant glands contain an active acetyl-CoA synthetase and are thus capable of forming acetyl-CoA directly from acetate.

Fatty acid synthesis from acetyl-CoA. The mechanism of fatty acid synthesis in a variety of tissues is now well established and can be outlined as a 2-step reaction:

$$\text{Acetyl-CoA} + CO_2 + \text{ATP} \rightarrow \text{malonyl-CoA} + \text{ADP} + P_i, \quad (1)$$

$$\text{n-malonyl-CoA} + \text{acetyl-CoA} + 2\text{n-NADPH} \rightarrow CH_3.CH_2.(CH_2.CH_2)_{n-1}.CH_2.\text{COCoA} + \text{n-CoA} + 2\text{n-NADP} + \text{n-}CO_2 \quad (2)$$

Reaction (1) is catalysed by a single enzyme, acetyl-CoA carboxylase, while equation (2) describes a complex cyclic sequence of reactions which have not been studied separately in the mammary gland and which will not be considered individually here. Acetyl-CoA carboxylase is active in the lactating rat mammary gland (Howanitz & Levy, 1965) but declines rapidly in the involuting gland (Jones, 1967). In most tissues studied, including rat mammary gland, acetyl-CoA carboxylase is found solely in the cytosol, but Smith, Easter & Dils (1966) reported that the rabbit mammary gland activity is divided almost equally between the soluble and microsomal fractions.

Reaction sequence (2) was first reported in the mammary gland by Ganguly (1960) who used a soluble preparation of cow udder which synthesized a spectrum of fatty acids similar to that found in milk. It has since been studied in the mammary glands of the rat (Baldwin & Milligan, 1966) and the rabbit (Smith *et al.* 1966). One problem as yet unresolved is whether different mechanisms are involved in the synthesis of short and long chain fatty acids. Kumar *et al.* (1965) claimed that extracts of goat and rabbit mammary gland synthesized butyrate either directly from β-hydroxybutyrate without prior cleavage or from acetate by a pathway not involving malonyl-CoA which was not inhibited by avidin, an inhibitor of acetyl-CoA carboxylase. Lachance & Morais (1965) also suggested on the basis of changes produced by the ageing of extracts that short-chain fatty acids were synthesized by an independent mechanism in the rabbit gland. Bogin & Katz (1967) confirmed that 2 systems are involved in the rat mammary gland, an NADH-dependent one synthesizing butyrate and an NADPH-dependent one synthesizing the longer acids but both systems were inhibited by avidin and therefore probably involved malonyl-CoA. A more detailed resolution of these 2 systems is still needed.

Another unsolved problem concerns the mechanism determining the pattern of fatty acid synthesis. The formation of fatty acids is a cyclic process involving the addition of 2 carbon units to an enzyme-bound chain and some factor must determine at what stage the free acyl-CoA is released. This is especially important in the ruminant as the milk fat contains an unusually high proportion of short-chain acids. One of the few investigations of this problem, by Bartley, Abraham & Chaikoff (1967), using a cell-free preparation from rat mammary gland, suggested that the level of malonyl-CoA was important as when this compound was supplied to the system the average chain length of fatty acids synthesized was longer than when acetyl-CoA was supplied and the malonyl-CoA generated *in situ*. Thus, high levels of malonyl-CoA apparently direct the synthesis of longer-chain acids. These workers also observed that microsomes, which are the site of the synthesis of triglycerides from acyl-CoA and glycerol-1-phosphate, shifted synthesis in the direction of longer-chain acids. Smith & Dils (1966) also studied the stimulatory effect of rabbit mammary

gland microsomes on fatty acid synthesis and concluded that it was largely due to the removal of inhibitory acyl-CoA by esterification.

The pentose phosphate pathway. In their review Folley & McNaught (1961) summarized the evidence that there was a close association between the pentose phosphate pathway of glucose-6-phosphate oxidation and fatty acid synthesis. The synthesis of fatty acids requires a supply of reductant in the form of NADPH and this was believed to be supplied mainly by the 2 initial reactions of this pathway which are catalysed by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Subsequent work has confirmed this view. McLean (1964) showed that if involution was produced in the rat mammary gland by blocking the teat-duct the metabolic flux along the pentose phosphate pathway fell more rapidly than the activity of the enzymes involved, but could be restored by adding phenazine methosulphate. As this compound allows the re-oxidation of NADPH without concomitant fatty acid synthesis, it was concluded that the flux through the pathway was limited by the rate of this synthesis. Abraham, Katz, Bartley & Chaikoff (1963) used doubly [^{3}H and ^{14}C]-labelled glucose as a substrate for rat mammary gland slices and demonstrated directly that NADPH generated in the pentose phosphate pathway was the major source of reducing equivalents for fatty acid synthesis.

In the lactating rat mammary gland the pentose phosphate and Embden–Meyerhof pathways metabolize approximately equal amounts of glucose (McLean, 1958; Abraham & Chaikoff, 1959), but in the ruminant the pentose pathway assumes greater importance. Wood, Peeters, Verbeke, Lauryssens & Jacobson (1965) calculated that in the perfused cow's udder 30 % of the glucose was utilized via the pentose phosphate pathway and 8 % via the Embden–Meyerhof pathway, the balance being used for lactose synthesis.

Wise & Ball (1964) suggested that in some lipogenic tissues the reaction catalysed by the malic enzyme contributed significantly to NADPH synthesis:

$$\text{L-malate} + \text{NADP} \rightarrow \text{pyruvate} + CO_2 + \text{NADPH}.$$

This enzyme is highly active in the rat mammary gland and declines rapidly on weaning (Matthes, Abraham & Chaikoff, 1963) but its quantitative importance in mammary lipogenesis remains to be determined.

Triglyceride formation

As previously stated, triglycerides are normally synthesized from acyl-CoA and glycerol-1-phosphate by microsomal enzymes, though a pathway involving the formation of monoglycerides from glycerol and acyl-CoA may be of some importance in ruminants (Patton, Mumma & McCarthy, 1966). Glycerol-1-phosphate can be produced from glucose, though not from acetate, by a branch of the Embden–Meyerhof pathway, but its availability may limit triglyceride synthesis, as shown by Howard & Lowenstein (1965) in a rat mammary gland system. Free glycerol is produced by the hydrolysis of triglycerides taken up from the blood and the enzyme necessary for its conversion to glycerol-1-phosphate, glycerokinase, is present in the guinea-pig mammary gland (McBride & Owen, 1964), but the quantitative importance of this source of glycerol as opposed to the synthesis from glucose is not known. The direct transfer of intact or only partially hydrolysed triglycerides from plasma to milk may

occur to a small extent and there is some evidence that glycerol-2-palmitate produced in this way acts as a precursor for high molecular weight triglycerides in the cow and goat (Dimick, McCarthy & Patton, 1965). Little is known of the mechanism by which the fatty acid composition of particular triglycerides is determined though it has been shown that the lipids of ruminant milk do not contain more than one butyryl residue per triglyceride (Dimick & Patton, 1965) and that this residue is the last of the three acyl groups to be added (Patton & McCarthy, 1963).

LACTOSE SYNTHESIS

The review of Leloir & Cardini (1961) summarized the evidence for a pathway of lactose synthesis which has subsequently proved incorrect. The sequence of reactions described by these authors by which glucose is converted to UDP-galactose has not been challenged.

glucose → glucose-6-phosphate → glucose-1-phosphate→
UDP-glucose → UDP-galactose.

However, the final reaction of the pathway has now been demonstrated to involve not glucose-1-phosphate but glucose:

UDP-galactose + glucose → lactose + UDP.

The involvement of glucose in this reaction was first suggested by Wood, Schambye & Peeters (1957) on the basis of tracer studies which indicated that the 2 hexose moieties of lactose were derived from different pools. Subsequently, Watkins & Hassid (1962) isolated a particulate enzyme, lactose synthetase, from cow udder which catalysed the above reaction and which was inactive when glucose-1-phosphate was substituted for glucose. Bartley, Abraham & Chaikoff (1966*a*) used [^{14}C]glucose labelled in various positions as a substrate for rat mammary gland slices and concluded that their results could only be explained by a terminal reaction involving glucose, not glucose-1-phosphate.

Subsequent investigations of the properties of lactose synthetase have provided some of the most interesting recent discoveries in the field of mammary gland biochemistry. Though the gland enzyme is particulate, a soluble synthetase has been isolated from cow's milk (Babad & Hassid, 1965) and shown to consist of two easily dissociable proteins, the larger A protein and the smaller B (Brodbeck & Ebner, 1966*a*). Brodbeck & Ebner (1966*b*) demonstrated that the B protein could also stimulate the particulate lactose synthetase found in bovine and rat mammary gland though some B protein is tightly bound to these particles. Further investigation established a complete identity between B protein and α-lactalbumin, a well-characterized constituent of milk (Brodbeck, Denton, Tanahashi & Ebner, 1967). The metabolic significance of α-lactalbumin has been reviewed in more detail by Ebner & Brodbeck (1968).

Though the A protein is inactive as a lactose synthetase in the absence of α-lactalbumin, it has been shown that it will act as a galactosyl transferase using not glucose, but *N*-acetyl-glucosamine as acceptor and synthesizing *N*-acetyl lactosamine (Brew, Vanaman & Hill, 1968). This second reaction is inhibited by α-lactalbumin which thus acts to switch the specificity of the galactosyl transferase. Indeed, galactosyl transferase from liver and other tissues can be induced to synthesize lactose

by the addition of α-lactalbumin. The mechanism of this effect, which may be of wide metabolic significance, is of great interest and is currently the subject of intensive study.

It is a point of some importance to determine whether the rate of lactose synthesis in the mammary gland depends on the level of A protein, or α-lactalbumin, or on some other factor. The lactose content of rat mammary gland rises from very low levels 12 h before parturition to a maximum just before suckling commences (Kuhn & Lowenstein, 1967). Lactose synthetase, assayed in the absence of α-lactalbumin, is undetectable until one day pre-partum when a rapid increase in activity commences and persists for the first 6 days of lactation (Kuhn, 1968). If α-lactalbumin is added to the assay system a small amount of activity is detectable pre-partum and some stimulation is seen post-partum but it appears that in the rat a deficiency of α-lactalbumin does not seriously limit lactose synthesis. Though lactose synthetase activities in the rat are far lower than those of the other enzymes of the pathway, UDP-galactose pyrophosphorylase and UDP-galactose epimerase, the activity is adequate to account for the known rate of lactose synthesis.

The nature of the particles which contain lactose synthetase in the gland and the mechanism by which it is rendered soluble in the milk remain to be revealed. As part of the α-lactalbumin of rat and cow mammary gland is tightly bound to the particles while in the milk the A and B proteins are easily dissociated (Brodbeck & Ebner, 1966*b*) there must be a considerable degree of organization. These workers found most of the activity in the microsomal fraction, but a recent report by Coffey & Reithel (1968), describing work in which cow udders frozen in liquid air were used as the starting material, claimed that the intact particles were similar in size to mitochondria but not identical with them. These particles showed no stimulation by α-lactalbumin which suggests they may represent a more intact form of the smaller particles studied by Brodbeck & Ebner (1966*b*).

The demand for glucose in the lactating ruminant can lead to the condition of ketosis, probably due to the low level of oxaloacetate preventing the functioning of the Krebs cycle in the liver (Baird *et al.* 1968) but a detailed consideration of this topic is outside the scope of this review. The mammary gland is not a net producer of ketone bodies (Barry *et al.* 1963; Linzell *et al.* 1967) and slices of cow udder produce only small amounts of β-hydroxybutyrate and acetoacetate on incubation *in vitro* (Baird, 1965). The importance of gluconeogenesis in the mammary gland itself is uncertain though one of the unique enzymes of this pathway, fructose 1,6-diphosphatase, is active in the bovine mammary gland (Baird, 1966) but not in that of the guinea-pig (Krebs & Woodford, 1965). It is possible that in the ruminant gluconeogenesis is important for the reconversion of triose-phosphate produced by the pentose phosphate pathway into glucose.

PROTEIN SYNTHESIS

This section describes recent work on protein synthesis in the mammary gland without exploring the details of the components and genetic variants of the major milk proteins, casein, α-lactalbumin and β-lactoglobulin. In his reviews Barry (1961, 1964) summarized the evidence that milk proteins were synthesized *de novo* in the mammary gland from amino acids absorbed from the blood and subsequent work has

confirmed this. Mepham & Linzell (1966) showed that in the goat the total amino acid uptake by the udder was sufficient to account for the milk protein output, though individual amino acids showed varying balances. Ornithine, though it does not occur in milk proteins, was taken up and there was an excess uptake of arginine and a deficiency of serine. The bovine udder (Verbeke & Peeters, 1965) behaved in a similar way though excess arginine was not absorbed. As glycine is known to be a precursor of serine in some tissues Verbeke, Feteano & Peeters (1967) investigated the fate of [^{14}C]glycine in the perfused ewe mammary gland but found that it appeared almost entirely as glycine in milk proteins. Arginine taken up by the goat udder is partially cleaved to urea and ornithine and the ornithine gives rise to proline in milk proteins (Mepham & Linzell, 1967). Further studies of the amino acid balance of the goat udder (Linzell & Mepham, 1968) demonstrated that glucose provides a considerable proportion of the carbon of serine and alanine and also contributes to glutamate, aspartate and their amides. Work on cell cultures of rat and cow mammary glands (Schingoethe, Hageman & Larson, 1967) showed their ability to synthesize all the non-essential amino acids and also indicated that the rate of β-lactoglobulin and β-casein synthesis was dependent on the concentration of amino acids in the medium. Thus, it appears that provided the supply of α-amino nitrogen and essential amino acids is adequate the mammary gland has the necessary metabolic apparatus to synthesize the particular amino acid mixture required for milk protein production.

From the evidence available protein synthesis in the mammary gland proceeds by a mechanism which does not differ significantly from that now established for a whole range of tissues and species. In brief, proteins are synthesized by polysomes which are complexes of the polypeptide chain-synthesizing units called ribosomes and messenger-RNA. Messenger-RNA, which is produced in the nucleus on the genetic DNA template, determines the type of protein produced by the particular polysome. The various stages of this synthetic process, including the initial activation of the amino acids by combination with transfer-RNA, have been demonstrated in extracts of guinea-pig mammary gland (Fraser, Shimizu & Gutfreund, 1959; Turba & Hilpert, 1961*a*, *b*, *c*). Brew & Campbell (1967) examined the synthesis of a specific protein, α-lactalbumin, in slices and cell-free extracts from guinea-pig mammary glands. Both systems were active and in the cell-free system a large part of the activity resided in the fraction designated 'mitochondrial' on the basis of its centrifugal preparation. However, it was concluded that the main component of this fraction was the so-called 'rough-surfaced endoplasmic reticulum' which consists of membrane-bound polysomes. The particles as isolated contained partially completed polypeptide chains which were further elongated *in vitro*, but chain initiation was also observed. A similar system has been isolated from frozen cow udder (Beitz, Wood & Thomas, 1967) which synthesized uncharacterized protein when supplemented with an ATP-generating system and a complete mixture of amino acids. It was inhibited by ribonuclease which hydrolyses RNA, and puromycin, an inhibitor of polypeptide chain synthesis.

Recent work has confirmed the evidence summarized by Barry (1961) that the phosphorylation of casein takes place after completion of the synthesis of the polypeptide chain. Singh, Dave & Venkatasubramanian (1967) showed that puromycin did not inhibit incorporation of [^{32}P]phosphate into casein by rat mammary gland

slices. This suggested the presence of a pool of unphosporylated casein large enough to maintain phosphate incorporation for the 30-min duration of these experiments. Similar results were obtained by Turkington & Topper (1966) using explants of mouse mammary gland. The enzyme involved in this reaction, phosphoprotein kinase, does not seem to have been studied extensively in the mammary gland since the work of Schmidt & Davidson (1956), and Sundararajan, Kumar & Sarma (1958) who showed it to be associated with a particulate fraction in the rabbit. The mechanism by which the carbohydrate side-chains of κ-casein are added is not known.

In milk, casein exists in the form of complex micelles but it appears probable that these arise spontaneously as a result of the properties of the different casein fractions and the ionic composition of the medium (Waugh & Noble, 1965*a*, *b*) and that no special mechanism is involved.

OTHER ASPECTS OF MAMMARY GLAND BIOCHEMISTRY

General metabolism

In addition to the specialized synthetic pathways reviewed in the preceding sections, the mammary gland possesses the normal complement of enzymes concerned with the production of utilizable energy in the form of ATP. In non-ruminants the principal respiratory fuel is generally assumed to be glucose, in the absence of possible alternatives, but in the ruminant glucose, acetate, β-hydroxybutyrate and triglyceride fatty acids are all possibilities (see Barry, 1964). In the goat, acetate yields about one-third of the total carbon dioxide output of the mammary gland (Linzell, 1960; Annison & Linzell, 1964; Annison *et al.* 1967), but the contribution of free fatty acids is very small (Annison *et al.* 1967). Glucose provides about 22% of the total carbon dioxide output of the fed sheep but this percentage could well vary in different organs (Annison & White, 1961). In the perfused goat udder the contribution of β-hydroxybutyrate is small (Linzell *et al.* 1967) which leaves glucose and possibly absorbed triglycerides to make up the balance of carbon dioxide production.

The initial step in the utilization of glucose (except for the glucose moiety in lactose) involves its conversion to glucose-6-phosphate by the hexokinase enzymes. Rat mammary gland lacks the glucokinase found in liver, where it varies widely in different nutritional states, but 2 other molecular species have been detected (Walters & McLean, 1967*a*). Type I was heat stable and its activity remained constant through the lactational cycle while Type II was heat labile and increased approximately 4-fold during lactation. In common with other tissues a considerable proportion of the rat (Walters & McLean, 1967*a*) and mouse (Bartley, Abraham & Chaikoff, 1966*b*) mammary hexokinase activity is associated with the mitochondrial fraction.

The major source of ATP in all aerobic cells is the oxidative phosphorylation system of the mitochondria, and there is no evidence that the cells of the mammary gland are in any way different. Some properties of guinea-pig mammary mitochondria have been reported (Jones & Gutfreund, 1962, 1963; Chance & Gutfreund, 1963) and, in general, they were similar to those of guinea-pig liver mitochondria with the exception that the enzyme glutamate dehydrogenase was absent in the mammary gland. It was suggested that this absence would prevent the loss of valuable amino acid α-amino groups as ammonia. Butow & Nelson (1964) showed that mitochondria

from involuting guinea-pig glands lost respiratory control, that is the synthesis of ATP was no longer coupled to the oxidation of substrates. This might be one of the factors contributing to the decline of synthetic activity in the involuting gland.

One difference in the metabolism of rat liver and mammary gland, the significance of which is not yet understood, involves the synthesis of the redox co-factor, nicotinamide–adenine dinucleotide (Greenbaum & Pinder, 1968). Judging by the activities of the relevant enzymes, free nicotinamide is involved in the mammary gland but not in the liver.

Enzymes of milk

The enzymes of cow's milk have been reviewed by Shahani (1966) who listed 19 activities that have been detected. At the moment there is no evidence to suggest that they are other than fortuitously present, being eliminated from the secretory cell with the other milk constituents. The mode of milk secretion is outside the scope of this review but it has been suggested that fat globules pass out accompanied by a thin layer of cytoplasm (Kurosumi, Kobayashi & Baba, 1968). However, not all milk contents may originate from the secretory cells as Wheelock & Rook (1966*b*) have suggested that in the cow there is a direct secretion of some substances from blood into milk. In some cases milk is a more tractable source of enzymes than the gland itself and milk lipases have received considerable attention. Downey & Andrews (1965, 1966) separated milk lipase activity by gel filtration into a number of fractions which they concluded were associated with β-casein aggregates. They failed to detect with any consistency an enzyme previously reported by Chandan & Shahani (1963) as isolated from milk clarifier slime, with a molecular weight of 7000. This is of interest, as it appears to be the smallest enzyme yet reported from any source but its mammary gland origin is doubtful (Gaffney & Harper, 1965). The metabolic role of these lipases in the mammary gland is uncertain, but they are presumably involved in the formation of free fatty acids from the triglycerides taken up from the blood.

Milk oligosaccharides

Milk and especially colostrum are rich sources of complex oligosaccharides (see review by Neuberger & Marshall, 1966) and κ-casein contains oligosaccharide side-chains (see review by Jollès, 1966). However, as the metabolic significance of these compounds is obscure this topic, though of great interest to carbohydrate chemists, will not be pursued further.

CHANGES IN MAMMARY GLAND BIOCHEMISTRY AND THEIR CONTROL

In this section I shall consider the changes in the biochemistry of the mammary gland, both those occurring naturally and those artificially induced, and what is known of the mechanisms controlling these changes. The mammary gland is particularly interesting to the biochemist because of the rapid increase in metabolic activity which occurs at the time of parturition. This increase offers the possibility of exploring the relationship between levels of enzymic and metabolic activity and, because the change is largely under endocrine control, of investigating the mode of action of developmental hormones. Some aspects of the hormonal control of mammary metabolism have been recently reviewed (Folley & Jones, 1967).

Three types of investigation have been employed:

(*a*) The biochemistry of the gland has been studied in normal animals through the sequence of pregnancy, parturition, lactation, weaning and involution.

(*b*) The gland has been studied in normal or operated animals in which lactation was induced or maintained by hormone therapy.

(*c*) Mammary gland explants have been cultured *in vitro*, and lactogenesis induced by the addition of appropriate hormones. This offers the advantage of exact control of the milieu and the disadvantage that the small amounts of material available hamper biochemical investigation.

Changes in normal animals

The changes of enzyme and nucleic acid levels in the mammary glands of rats and mice have been reviewed by Munford (1964) and the changes of nucleic acids in a wide variety of species by Denamur (1964). Knowledge of the levels of nucleic acids is important as DNA provides an index of the number of cells present while the RNA/DNA ratio is an index of protein synthetic activity. Though the total enzyme activities are obviously of interest in relation to milk output it is also important to know the enzyme activity per cell as this throws light on the mechanism of the changes. The validity of DNA determination as a measure of the number of cells has been questioned on 2 grounds.

(1) Various milk constituents interfere with some DNA assay procedures but modifications have been suggested to counter this (Slater, 1961; Munford, 1963). Denamur (1964) considered that chromatographic separation of the component nucleotides offered the only reliable procedure but this is time-consuming. Slater (1962) also pointed out that in the involuting gland an apparent rise in DNA content is probably due to a leucocyte invasion. Despite these difficulties the levels of DNA in relation to gland weight reported by different workers in comparable experiments agree reasonably well and so are probably reliable.

(2) The constancy of the cell DNA content of different tissues at different states of development has been questioned. It has been widely assumed that the DNA of cells consists almost entirely of the genetic material in the nucleus which must be constant except during the pre-mitotic phase when a doubling takes place. Multinucleate cells would contain excess DNA but do not seem to constitute a serious problem in the mammary gland. However, recent histochemical determinations of DNA in individual rat mammary gland nuclei (Sod-Moriah & Schmidt, 1968) demonstrated a 2- or 3-fold variation in the nuclear DNA content in any particular section which could not be explained by the number of mitoses. As DNA cannot fall below the amount in the genetic material this variation, if not due to artefacts in the histochemical procedure, indicates the presence of additional non-genetic DNA in some of the cells. This wide within-stage variation was not found when the average values for different stages of lactation were compared, thus confirming earlier work (Tucker & Reece, 1962; Munford, 1963). It seems, therefore, that until further contrary evidence is produced DNA estimation will continue to be used as a reasonably reliable estimate of cellular proliferation.

Much of the recent work on changes in nucleic acid and enzyme levels during the lactational cycle has used rat mammary glands. Baldwin & Milligan (1966) estimated

the activities of 20 enzymes through pregnancy and lactation and also determined gland weights and DNA and RNA levels. In general, their results confirmed and extended findings previously reviewed. Though the wet weight of the glands reached a maximum in late pregnancy and remained fairly constant through lactation (provided allowance was made for the retained-milk content) the DNA content continued to rise during the first days of lactation and approximately half the final DNA was synthesized post-partum. Some workers (Greenbaum & Slater, 1957; Moon, 1962; Munford, 1963) have found that the maximum DNA level was not reached until late lactation, while Kuhn & Lowenstein (1967) found considerable rat-to-rat variation in the DNA changes in the 4-day period about parturition. Recently, Thibodeau & Thayer (1967) claimed that DNA synthesis was complete by parturition. These changes in the total DNA content of the gland reflect both proliferation of secretory tissue and regression of non-secretory tissue so that a constant value does not imply that no increase in the number of secretory cells is occurring. A direct measure of the number of mitoses in rat mammary gland using [^{3}H]thymidine indicated a peak of mitotic activity on the second and third days of lactation followed by a fall to the level found in late pregnancy by the seventh day (Traurig, 1967). Though, therefore, the exact pattern of DNA change is still the subject of some dispute, the following facts seem clearly established:

(1) Lactogenesis is accompanied by a decrease in the adipose and connective tissue content of the gland and a corresponding increase in the content of secretory-epithelial tissue (Rees & Eversole, 1964; Wrenn, DeLauder & Bitman, 1965; Kuhn & Lowenstein, 1967).

(2) The RNA/DNA ratio rises rapidly post-partum from initial values of about 1 to reach 4–5 (Baldwin & Milligan, 1966; Thibodeau & Thayer, 1967).

(3) The great majority of enzymes studied increase with lactogenesis whether calculated as total activity or on the basis of gland DNA. All 20 enzymes studied by Baldwin & Milligan (1966) showed an initial post-partum increase and in most cases the increase persisted up to the twentieth day of lactation. These changes were particularly marked in the case of enzymes known to be concerned with the synthesis of milk constituents, for example glucose-6-phosphate dehydrogenase, UDP-glucose pyrophosphorylase and fatty acid synthetase but were also significant for enzymes less obviously involved in mammary metabolism such as malate dehydrogenase and isocitrate dehydrogenase. Other enzymes shown to increase rapidly post-partum in the rat include aspartate transcarbamylase, which is concerned with pyrimidine synthesis (Thibodeau & Thayer, 1967), acetyl-CoA carboxylase (Howanitz & Levy, 1965) and lactose synthetase (Kuhn, 1968).

The type of change described above occurs in the rat but when Baldwin (1966) investigated a range of species it was apparent that while the guinea-pig behaved similarly to the rat the situation in the cow was completely different. In first pregnancy heifers the activities of 18 enzymes showed no significant changes at 5 different stages from 14 days pre-partum to the fortieth day of lactation. Thus, in the cow there seems little possibility that lactogenesis is associated with a rapid increase in enzyme activities unless it is assumed that key enzymes as yet unstudied are involved.

In these species, where enzyme activities rise at the beginning of lactation, there

is a corresponding fall after weaning (see review by Munford, 1964). Exceptions occur with the lysosomal enzymes cathepsin and β-glucuronidase which increase in activity in the rat gland during involution (Greenbaum & Greenwood, 1954). At this stage lysosomal enzymes pass into the cytosol or at least become less strongly attached to the particles (Greenbaum, Slater & Wang, 1965). Changes similar to those occurring naturally take place if the litter is removed from the mother rat at the height of lactation. Jones (1967) showed in the rat that changes in enzyme activities 24 h after removing the litter were very similar to those occurring 24 h after hypophysectomy and casein synthesis in the rat ceases within 24 h (Ota, 1964). These changes are not due to a decrease in the level of circulating hormones as they occur in glands from which milk removal has been prevented while other glands on the same animal are suckled and function normally (McLean, 1964; Spencer & Lowenstein, 1966). The mechanism by which milk engorgement produces a decline in enzyme activities remains to be determined, but it is possible that the pressure inside the gland leads to a collapse of the alveolar capillaries and hence interrupts the supply of metabolites and hormones (Cross & Silver, 1956). In addition, the synthesis of milk constituents may be subject to feed-back inhibition by accumulating products. Possible examples of this mechanism are the inhibition by long-chain acyl-CoA of acetyl-CoA carboxylase (Numa, Ringelmann & Lynen, 1965) and glucose-6-phosphate dehydrogenase (Eger-Neufeldt, Teinzer, Weiss & Wieland, 1965), both enzymes involved in the synthesis of fatty acids. Inhibition of this type may also be important in regulating the rate of lactogenesis during normal lactation.

In vivo *effects of hormones*. The morphological and biochemical development of the mammary gland occurs under endocrine control (see review by Cowie, 1961) and a number of workers have compared the biochemistry of the normal lactating mammary gland with that of glands in which lactation has been induced by hormonal therapy.

Abraham, Cady & Chaikoff (1960) maintained a 50% normal milk yield in rats hypophysectomized in mid-pregnancy by treatment with prolactin plus cortisol and showed that slices from these glands metabolized [1 and 6]^{14}C glucose in a manner identical with slices of normal glands, the Embden–Meyerhof and pentose phosphate pathways being equally active. Withdrawal of the therapy 4 days before killing caused a reversion to the pre-lactating type of metabolism. Cole & Hopkins (1962), using non-pregnant hypophysectomized rats, induced lactogenesis by treatment with growth hormone, prolactin and cortisol. The RNA and DNA levels and the activities of 3 out of 4 enzymes studied were as in a normal gland immediately before parturition. In a related type of experiment Jones (1967) looked at the changes in 7 enzymes in the lactating rat during the 24-h period following hypophysectomy. All the activities fell rapidly during the first 12 h and then became stable at lower values. In the case of acetyl-CoA carboxylase this low value was less than 5% of the control value. Recently, Baldwin & Martin (1968) also examined rats hypophysectomized during lactation and showed that cortisol plus prolactin were capable of partially maintaining lactation (though not well enough to support litter growth) and fully maintaining the gland weight and nucleic acid content. The enzymes glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and phosphoglucomutase were maintained at near control values while aldolase, phosphofructokinase and malate

dehydrogenase were only poorly maintained. [^{32}P]phosphate incorporation into DNA and RNA was normal and, surprisingly in view of the poor lactational performance, so was [^{14}C]leucine incorporation into casein. These results in the rat suggest that hormones, and in particular cortisol and prolactin, can exert a selective effect on gland metabolism and enzyme levels apart from generally promoting growth and development.

In the rabbit, Chadwick (1962) showed that intra-ductal injection of prolactin into the pseudo-pregnant animal produced a synthesis of lactose within 2–3 days and Heitzman (1968), using prolactin plus cortisol, demonstrated that this synthesis was accompanied by a rise in the activity of 2 enzymes of the lactose synthesis pathway, UDP-glucose pyrophosphorylase and UDP-glucose epimerase.

In the hypophysectomized lactating rabbit a normal milk yield can be maintained by treatment with prolactin alone (Kilpatrick, Armstrong & Greep, 1964), in contrast to the requirements of the rat described above. P. E. Hartmann (1968, personal communication) has recently shown that the restoration of lactation produced by prolactin is accompanied by an increase in the activities of a number of mammary gland enzymes which return to the control levels found in the unoperated rabbit. The connexion between the restoration of enzyme activities and milk yield is not a simple one, however, as bovine growth hormone, which has little effect on lactation in the hypophysectomized animal, does produce significant increases in enzyme activities. As in the cow it seems that factors other than enzyme activities are important in determining milk yield, or that enzymes not yet studied play a crucial controlling role.

Though an adrenal steroid is essential for the maintenance of lactation in the hypophysectomized rat, adrenalectomy, while depressing milk production, has a much less dramatic effect than hypophysectomy (Greenbaum & Darby, 1964; Willmer, 1960; Wilmer & Foster, 1965). Thyroidectomy (Walters & McLean, 1967*b*) and ovariectomy (Barker & Ludwick, 1967) also depress but do not abolish lactation in the rat.

In vitro *effects of hormones*. The work described in the previous section using whole animals is often difficult to interpret because of possible interactions between different endocrine glands and the effect of general metabolic changes on the mammary glands. For example the influence of thyroidectomy on lactation in the rat (Walters & McLean, 1967*b*) was partially due to the depressed food intake. This difficulty can be overcome by using *in vitro* systems where the hormonal environment can be accurately controlled. Some work has been carried out using tissue slices but except with insulin the results have not been easy to interpret and have sometimes been contradictory. The difficulty lies in maintaining the viability of slices over a long enough period for developmental hormones such as prolactin and the adrenal corticoids to act.

The action of insulin in stimulating lipogenesis from glucose and acetate in slices from non-ruminant mammary glands and at the same time increasing the oxidation of glucose via the pentose phosphate pathway has been extensively reviewed by Folley & McNaught (1961). This action of insulin in stimulating fatty acid synthesis is not prevented by actinomycin D (Mayne & Barry, 1965), which is an inhibitor of the synthesis of new messenger RNA, and therefore cannot involve enzyme synthesis. As the effect of insulin in increasing glucose oxidation via the pentose phosphate pathway can be reproduced to a large extent by increasing the concentration of

glucose in the medium (McLean, 1960) it is possible that the short-term effects of insulin on slices are due to an increased uptake of glucose.

The development of the techniques of organ explant culture for the mammary gland (Elias, 1959; Elias & Rivera, 1959) has produced an *in vitro* system which remains viable for several days and which can be used for the study of the control of morphological and biochemical changes by hormones. Most of the work of biochemical interest has used explants from the mammary glands of pregnant mice which show good maintenance of structure over a period of several days when cultured in a complex medium supplemented with the hormones insulin and aldosterone (Rivera, 1964). Aldosterone can be replaced by other steroids including cortisol and corticosterone (Turkington, Juergens & Topper, 1967). When, in addition, prolactin is present the alveolar cells develop vacuoles, the alveoli become filled with a secretion (Rivera, 1964), and casein synthesis can be demonstrated by the incorporation of [^{32}P]phosphate or [^{14}C]-labelled amino acids (Stockdale, Juergens & Topper, 1966; Turkington & Topper, 1966). The roles of the 3 hormones have been studied in more detail without fully revealing the nature of the biochemical changes which occur. Insulin alone stimulates RNA and protein synthesis over a short period (Mayne, Barry & Rivera, 1966) but after 48 h the activities have fallen below their initial values (Mayne, Forsyth & Barry, 1968). Stockdale *et al.* (1966) reported slightly different findings as in their work the presence of hormones did not affect the initial rate of RNA synthesis but did produce a stimulation after 24 h. The reason for these differences remains unexplained. Insulin also stimulates glucose uptake and fatty acid synthesis in explants as it does in slices (Moretti & Abraham, 1966).

The nature of the action of prolactin in producing casein synthesis has been studied in some detail by Turkington, Topper and co-workers. They showed that insulin alone stimulated the synthesis of non-specific soluble protein but that prolactin and cortisol were required for the production of casein, α-lactalbumin and β-lactoglobulin (Lockwood, Turkington & Topper, 1966). The casein produced closely resembled that found in mouse milk judging by the fractions separated by electrophoresis (Turkington, Juergens & Topper, 1965). The action of insulin and cortisol involves a stimulation of DNA synthesis and mitosis (Stockdale & Topper, 1966) which is not further increased by prolactin. If cell proliferation is allowed to take place in the presence of insulin and cortisol and further mitosis is then prevented by colchicine, the addition of prolactin is still capable of inducing casein synthesis (Turkington, Lockwood & Topper, 1967). One role of the corticosteroid appears to be to prevent the dephosphorylation of casein (Turkington, 1968). On the basis of these results it has been postulated that insulin and cortisol stimulate the production of new cells *in vitro* which are subsequently acted on by prolactin in some unspecified way to initiate the synthesis of milk proteins. So far studies of the synthetic activity of explants have involved the incorporation of labelled precursors into protein and fat rather than the estimation of net synthesis and it is possible that the actual amounts of milk constituents being synthesized are small compared with the activity of the lactating gland *in vivo*.

Leader & Barry (1968) showed that in explants taken from mice on the nineteenth day of pregnancy (i.e. shortly before parturition) insulin produced a doubling of glucose-6-phosphate dehydrogenase activity in 24 h which was not dependent on

mitosis but which did require initial RNA synthesis. One of the most interesting aspects of these findings was that the enzyme activity continued to rise after all protein synthesis had been inhibited by cyclohexamide which suggests that an inactive precursor of this enzyme might exist. Whether these findings of Leader & Barry apply to explants taken earlier in pregnancy which have been used in most other work remains to be determined.

Recently Turkington, Brew, Vanaman & Hill (1968) have investigated the changes in the lactose synthetase activity in the mouse mammary gland system previously used for the study of lactose synthesis. The combination of insulin, cortisol and prolactin which induces casein synthesis also increased lactose synthetase activity from low initial levels and within 48 h the amounts of A protein and α-lactalbumin had risen more than 10-fold. However, further increases did not occur and the level of A protein, on the basis of tissue weight, remained about one-fifth of that found in the late pregnant or lactating gland. Thus, it appears that lactogenesis in these explants is not accompanied by the development of full enzymatic activities and that possibly other factors are required for such development.

General considerations

Considering the work reported in the preceding 3 sections together a number of points of interest arise. The rapid change of enzyme levels in the mammary glands of non-ruminants at the time of parturition suggests that it is the low level of these enzymes which limits lactogenesis during pregnancy. However, the absence of these changes in the cow shows that it is possible to have a gland with an apparently full complement of enzymes but unable to synthesize milk constituents and this throws some doubt on the significance of the changes in the non-ruminant. It is possible that synthesis is inhibited by some form of feed-back inhibition which is relieved when suckling commences, but the gland content of lactose in the rat, for example, is already high at the time of parturition (Kuhn & Lowenstein, 1967). Another possibility is that there is a barrier to the uptake of metabolites from blood in the pregnant gland which is broken down at the time of parturition. A knowledge of the actual level of metabolites in the mammary gland before and after parturition would be valuable but this type of experiment is technically difficult. Certainly the comment of Baldwin (1966) that there seems little logical relationship between the ratios of activities of enzymes in mammary glands from different species and the composition of the different milks is a warning against extrapolating from *in vitro* enzyme data to the activity of *in vivo* metabolic pathways.

Another problem is the mechanism of the change in enzyme levels in the mammary glands of non-ruminants. Baldwin & Milligan (1966) suggested that the principal change in the rat is a replacement of non-secretory (mainly adipose) tissue by active epithelial-secretory tissue and that there is no need to invoke the hormonally directed synthesis of specific enzymes. However, the work on mouse mammary explants appears to contradict this view, assuming that the control mechanisms in the 2 species are similar. Lactogenesis can be induced in explants by prolactin in the absence of mitosis and therefore must involve some change in the properties of the individual cell. Emery & Baldwin's (1967) work on the turnover time of rat mammary gland enzymes provided evidence that at least in the case of 2 of them, α-glycerolphosphate

dehydrogenase and glucose-6-phosphate dehydrogenase, there was a real increase in the rate of synthesis above that explicable by the increase in the amount of secretory tissue. The change in enzyme patterns after hypophysectomy and the selective response of enzymes to replacement therapy in the rat also suggest a more precise hormonal control than would be provided by the proliferation or regression of a particular type of tissue. That the biochemical properties of individual mammary gland cells can change is demonstrated by work with single-cell cultures. Bovine mammary cells lose their ability to synthesize lactose after 2 days of culture though they maintain their ability to synthesize β-lactoglobulin for several weeks (Twarog & Larson, 1964). Goat mammary cells also de-differentiate in culture and the pattern of lactate dehydrogenase isozymes shows a characteristic shift (Blanco, Rifé & Larson, 1967). Thus, it still seems possible that prolactin acts, at least in some species, in the way which has been proposed for other developmental hormones (see reviews by Karlson & Sekeris, 1966; Korner, 1967; Tata, 1968). That is, it controls the types of messenger RNA synthesized in the nucleus and thus the types of protein being synthesized by the polysomes. How hormonal control of ruminant lactation is exercised at the biochemical level remains an open question, the answering of which should occupy a good deal of effort in the immediate future.

CONCLUSIONS

The work reviewed here, taken together with earlier findings, provides a reasonably reliable qualitative picture of the main metabolic pathways of the mammary gland. Though the major revision of ideas about the lactose synthetic pathway in recent years suggests caution with respect to other pathways, the evidence seems solid enough to justify the scheme outlined in Fig. 2.

In this figure much detail has been suppressed and individual compounds are only represented when they play a particularly important metabolic role. Within this broad outline questions still requiring clarification include:

(*a*) The relative contributions of plasma triglycerides and *de novo* synthesis to milk fatty acids in non-ruminants.

(*b*) The nature of the metabolic fuel which provides energy for the synthetic processes of the mammary gland. In ruminants glucose, acetate and plasma triglycerides all appear to contribute, but little is known of the situation in non-ruminants.

(*c*) Whether a special pathway exists for the synthesis of short-chain fatty acids, and if so, whether this pathway can utilize 4 carbon units without prior cleavage.

Turning to the control of the activities of these various pathways in the mature gland, one is confronted by an almost total lack of knowledge. By analogy with other organs it seems unlikely that a crude control by limitation of precursors exists, and in general the activities of different metabolic pathways seem to be determined by pace-maker reactions, that is, reactions which occur more slowly than others in the sequence and therefore limit the overall flux through the pathway (see Krebs, 1963; Bücher & Rüssmann, 1964). These pace-maker reactions are often catalysed by enzymes which are subject to complex regulatory influences including feed-back inhibition by immediate or ultimate products, and are thus crucial in the process of

metabolic control. Simple determination of enzyme activities *in vitro* is insufficient to locate pace-maker reactions as *in vivo* conditions often differ widely from those used for the assay. The location of these control points requires the determination of the intracellular concentration of metabolites in conditions of changing flux, and this type of study has not been carried out on the mammary gland. Therefore one is reduced to speculation based on *in vitro* enzyme assays and analogy with other tissues. With these reservations in mind it seems that in the mammary gland the Embden–Meyerhof glycolytic pathway is limited by phosphofructokinase activity, the pentose phosphate pathway by the rate of re-oxidation of NADPH, fatty acid synthesis by acetyl-CoA carboxylase activity and lactose synthesis by lactose synthetase activity. The precise nature of the control of protein synthesis has not been worked out for any tissue.

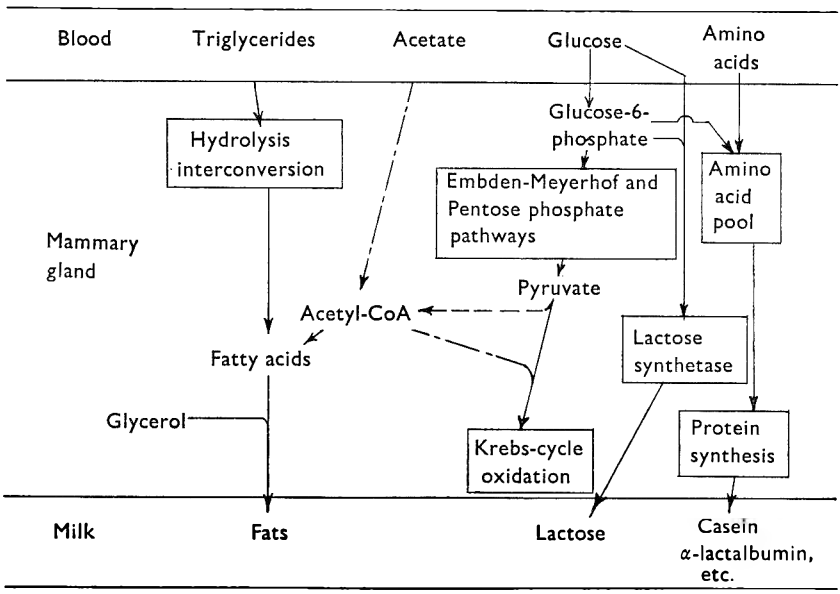

Fig. 2. Principal metabolic pathways of the mammary gland. Pathways marked —·—, are important only in ruminants, and those marked --- only in non-ruminants.

If the pace-maker hypothesis is correct, it follows that only changes in these particular enzymes have an immediate effect on gland metabolism, and that therefore they should be the special subject of inquiry. However, the different enzymes of a particular metabolic pathway tend to maintain constant ratios under different conditions (Klingenberg & Pette, 1962) and therefore a change in the pace-maker is reflected by changes in the other members of the group, though these may be actually present in large excess. Thus, UDP-glucose pyrophosphorylase, an enzyme of the lactose pathway which is present in large excess relative to lactose synthetase, shows rapid response to hormone treatment in the pseudo-pregnant rabbit (Heitzman, 1968). Further developments in the field of mammary gland metabolic control wait on the determination of the levels of key metabolites and the estimation of the activities of presumed pace-maker enzymes, especially at the crucial period of lactogenesis.

Considering the biochemical changes associated with lactogenesis the most striking fact is that as lactose synthesis commences some hours pre-partum both in the rat (Kuhn & Lowenstein, 1967) and in the cow (Wheelock & Rook, 1966*a*), suckling itself is not concerned. In the non-ruminant, it seems possible that a rapid synthesis of pace-maker enzymes, for example lactose synthetase (Kuhn, 1968), is the initiating event. In the ruminant, where enzyme changes have not so far been reported, the biochemical basis of lactogenesis remains wholly obscure.

The increase in enzyme activities which occurs in the non-ruminant immediately before parturition is probably the result of a change in the balance of circulating hormones. Whether the immediate cause of this increase is a proliferation of secretory tissue (Baldwin & Milligan, 1966) or a more specific stimulation of the synthesis of a small group of pace-maker enzymes remains to be determined. It is possible that both mechanisms are involved with the specific induction of enzymes being responsible for the rapid onset of lactogenesis over a period of several hours pre-partum and the replacement of non-secretory by secretory tissue maintaining the increase in enzyme activity which occurs throughout much of lactation. It seems probable that the extensive work being carried out currently with the mammary gland explant system will clarify some of the problems of lactogenesis in the non-ruminant in the near future. However, the investigation of developmental changes and the control of lactogenesis in the ruminant appears a more impenetrable problem which offers one of the main challenges to the lactational biochemist and physiologist in the coming years.

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