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With 1 plate

The prediction of dairy performance of cows from the lactation induced by treatment with oestrogen

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Summary. Lactation was induced in 30 Holstein heifers by treatment with synthetic oestrogen only. The average age of the heifers at the start of treatment was 16·6 months and the average lactation yield 1072 kg of milk with a fat content of 3·43 % given in 241 days. Subsequently, 22 of these animals gave a normal lactation starting at an average age of 31 months, with yields of 3980 kg milk with a fat content of 3·31 % given in 294 days. The induced lactation was slightly associated with reproductive problems and growth of the animals was normal. There was a good within-cow correlation between the yield and composition of the milk obtained in the 2 lactations. The correlation coefficients between the total production of the milk in the normal lactation and the total yield and peak production in the induced lactation were 0·718 and 0·785 and that between the average percentages of fat in the milk obtained in the 2 lactations was 0·718.

It is possible to predict dairy performance from the performance during the induced lactation, but before the procedure can be recommended for general use the question whether it may induce reproductive difficulties requires further investigation.

INTRODUCTION

Individual performance is one of the most important criteria for the selection of breeding stock. But with dairy animals, it is possible to carry it out only on females and, furthermore, progeny already exist by the time the performance of the dam becomes evident since lactation follows parturition. It would be very worth while in such animals to be able to determine the performance of the female at an early age before she becomes a dam. The results could be applied not only to individual selection but also to family selection including earlier progeny testing on males.

The fact that the administration of a small amount of oestrogen induces mammary

development and milk secretion in guinea pigs, ruminants and primates has been reported by many workers. Naito, Yokoyama, Yoshioka, Yokoyama & Yokotsuka (1953) reported that normal dairy performance in goatlings could be predicted from the results of inducing lactation by treatment with synthetic oestrogen. Eaton, Simmons, Sykes, Wrenn & Hall (1953) and Goto & Oshima (1955) have also reported similar results with slight variation in methods.

The economic importance of such a predictive test would be greater with cows than with goats, and therefore the authors have applied this technique to heifers to find whether similar results to those with goats may be obtained.

EXPERIMENTAL

Animals

Thirty Holstein heifers were used during the 10 years from 1953 to 1963 in 3 experimental stations; 10 of the animals were at Fukushima National Stock Farm, 17 at Hokkaido Agricultural Experimental Station and 3 at the Stock Farm of the University of Tokyo. The heifers were kept at each station throughout these 10 years under as constant conditions of feeding, grazing and management as possible.

Treatment for induced lactation

To induce lactation the heifers were given 10 subcutaneous injections in the neck region of 5 mg diethylstilboestrol (Euvestin, The Takeda Pharmaceutical Co. Ltd., Japan) in 1 ml oil, one every third day. The first injection was given 3 days before the expected onset of the next oestrus. The treatment was started at about 16 months of age. Palpation of the udder was started simultaneously with the injection of oestrogen. Heifers were milked once a day. The yield of milk was recorded when the daily yield reached 0·5 kg and stopped when the yield dropped below 2·0 kg.

To measure the effects of the hormone treatment and induced lactation on the growth of the heifers, their growth was related to the standard growth curve of Holsteins in Japan (Holstein Cattle Association of Japan, 1959). For this purpose measurement of height at withers, body length, chest girth and body weight were made each month.

The effects of the treatment on the ovarian activity was also observed during several oestrus cycles after the beginning of injection.

At the end of the induced lactation the heifers were bred by artificial insemination. In the normal lactation after parturition, the animals were milked twice a day.

Recordings

In order to study the trends in production during the induced and natural lactation the following measurements were made: peak daily production, total yield, total butterfat yield, average butterfat percentage, peak period and duration. The peak production, or time taken to reach maximum yield, was measured from successive 5-day average yields. The butterfat content of the milk was measured by the Gerber method on samples taken at intervals of 10 days.

Lactation yields were corrected to 305 days in animals with records over 305 days, but were not corrected in animals with records of less than 305 days. Age corrections were not applied. A 100-days record was also taken because of its high reliability as an index of the total yield in a normal lactation.

Station	Lactation	No. of animals	at start of lactation, months	of lactation, days	Days to peak	Total milk yield, kg	Peak production, kg	Average daily production, kg	Total fat yield, kg	Average fat, %	Milk yield for 100 days, kg
Fukushima	Induced	9	16·9 ± 2·4	301·3 ± 5·1	189·1 ± 88·6	1534·2 ± 408·7	7·03 ± 1·78	5·08 ± 1·32	52·40 ± 13·77	3·44 ± 0·43	421·5 ± 137·1
	Normal		31·1 ± 2·1	305·0 ± 0	33·0 ± 17·3	4533·4 ± 984·4	21·77 ± 3·96	14·86 ± 3·23	151·75 ± 32·27	3·36 ± 0·28	1877·9 ± 296·9
Hokkaido	Induced	12	16·5 ± 2·2	190·7 ± 61·4	62·6 ± 36·0	693·8 ± 466·5	4·90 ± 2·28	3·35 ± 1·61	23·09 ± 15·31	3·34 ± 0·22	365·7 ± 179·5
	Normal		31·6 ± 5·9	285·9 ± 46·9	27·6 ± 17·1	3541·3 ± 1261·4	20·81 ± 4·27	12·17 ± 3·45	114·44 ± 41·99	3·23 ± 0·30	1657·0 ± 438·3
Tokyo	Induced	1	16	305	93	1458·7	6·44	4·78	63·99	4·39	468·6
	Normal		39	305	13	4273·2	22·96	14·01	157·44	3·68	1765·0
Total or average	Induced	22	16·6 ± 2·2	241·1 ± 72·0	115·7 ± 87·3	1072·4 ± 598·4	5·84 ± 2·25	4·12 ± 1·66	36·94 ± 21·02	3·43 ± 0·38	393·2 ± 158·4
	Normal		31·7 ± 4·8	294·6 ± 35·3	29·1 ± 17·0	3980·4 ± 1203·2	21·30 ± 3·99	13·35 ± 3·47	131·65 ± 41·16	3·31 ± 0·30	1752·3 ± 382·3

Table 2. *Coefficients of correlation between results of induced and of normal lactations*†

Induced (X)	Normal (Y)	Fukushima	Hokkaido	All animals
Peak production	Peak production	+0·805****	+0·560*	+0·621****
	Total milk yield	+0·843****	+0·688***	+0·785*****
	Mean daily production	+0·844****	+0·679***	+0·779*****
	Milk yield for 100 days	+0·824****	+0·647**	+0·721*****
Total milk yield	Total milk yield	+0·684**	+0·609**	+0·698*****
Mean daily production	Peak production	+0·708**	+0·511*	+0·558****
	Total milk yield	+0·679**	+0·633**	+0·718*****
	Mean daily production	+0·680**	+0·648**	+0·719*****
Total fat yield	Total fat yield	+0·612*	+0·543*	+0·681*****
Mean fat percentage	Mean fat percentage	+0·898*****	+0·539*	+0·718*****
Milk yield for 100 days	Peak production	+0·267	+0·380	+0·360*
	Total milk yield	+0·052	+0·576**	+0·450**
	Mean daily production	+0·052	+0·560*	+0·419*
	Milk yield for 100 days	+0·112	+0·497*	+0·418*
Duration	Duration	—†	+0·322	+0·407*
Peak period	Peak period	+0·026	+0·579**	+0·262

D.F.: Fukushima, 9 − 2 = 7; Hokkaido, 12 − 2 = 10; all cows, 22 − 2 = 20. Level of significance P: > 0·1, no mark; < 0·1, *; < 0·05, **; < 0·02, ***; < 0·01, ****; < 0·001, *****

Computation

The correlations of various characters between induced and normal lactations and those within the same lactation were calculated.

For 9 animals the full results of the induced and normal lactations could not be obtained and the data on these were omitted; 2 of the animals had died of bloat soon after the hormonal treatment or after parturition; 2 were culled because of accidental wounds; 2 were discarded on account of reproductive disorders and 3 responded only weakly to the treatment as judged by means of Thompson's critical range test on days for daily production attaining 0·5 and 1·0 kg.

RESULTS

The induced and normal lactations

Soon after the start of the injections of oestrogen, the milk obtained resembled lymph fluid, but it rapidly changed to a colostral type of secretion and resembled normal milk after 3–7 days. The average numbers of days to reach daily milk yields of 100, 500 and 1000 g were 15, 22 and 27 days, respectively. The time of onset of milk secretion differed little as between animals at the 3 experimental stations. Results for the 2 lactations are shown in Table 1.

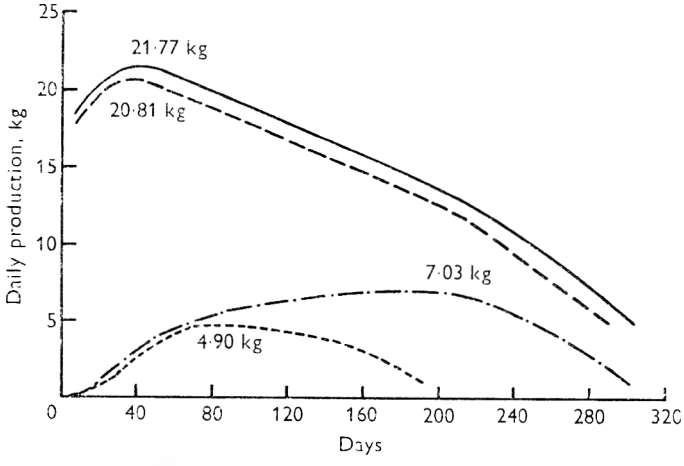

Fig. 1. Lactation curves for induced and normal lactations.

	Mean daily production, kg
——, Normal lactation, Fukushima	14·86
–·–·–, Induced lactation, Fukushima	5·08
– – –, Normal lactation, Hokkaido	12·17
· · · · ·, Induced lactation, Hokkaido	3·15

The total yield, total fat yield and yield for 100 days in the induced lactations were $\frac{1}{3}$–$\frac{1}{5}$ of those in the normal lactation. The peak period came much later in the induced lactations, particularly in the Fukushima herd. There was no significant difference in butterfat percentage between the 2 lactations. The durations of lactation

Induced (X)	Normal (Y)	Fukushima	Hokkaido	All animals
Peak production	Peak production	$Y = 1{\cdot}791X + 9{\cdot}18$	$Y = 1{\cdot}049X + 15{\cdot}67$	$Y = 1{\cdot}102X + 14{\cdot}86$
	Total milk yield	$Y = 466{\cdot}21X + 1256{\cdot}0$	$Y = 380{\cdot}63X + 1676{\cdot}2$	$Y = 419{\cdot}78X + 1528{\cdot}9$
	Mean daily production	$Y = 1{\cdot}532X + 4{\cdot}09$	$Y = 1{\cdot}027X + 7{\cdot}14$	$Y = 1{\cdot}201X + 6{\cdot}33$
	Milk yield for 100 days	$Y = 137{\cdot}44X + 911{\cdot}7$	$Y = 124{\cdot}38X + 1047{\cdot}6$	$Y = 122{\cdot}62X + 1036{\cdot}2$
Total milk yield	Total milk yield	$Y = 1{\cdot}6475X + 2005{\cdot}8$	$Y = 1{\cdot}6467X + 2398{\cdot}8$	$Y = 1{\cdot}4035X + 2475{\cdot}3$
Mean daily production	Peak production	$Y = 2{\cdot}124X + 10{\cdot}98$	$Y = 1{\cdot}355X + 16{\cdot}27$	$Y = 1{\cdot}378X + 15{\cdot}76$
	Total milk yield	$Y = 506{\cdot}37X + 1961{\cdot}0$	$Y = 495{\cdot}94X + 1879{\cdot}9$	$Y = 520{\cdot}42X + 1836{\cdot}3$
	Mean daily production	$Y = 1{\cdot}664X + 6{\cdot}40$	$Y = 1{\cdot}389X + 7{\cdot}52$	$Y = 1{\cdot}503X + 7{\cdot}16$
Total fat yield	Total fat yield	$Y = 1{\cdot}434X + 76{\cdot}60$	$Y = 1{\cdot}489X + 80{\cdot}05$	$Y = 1{\cdot}334X + 82{\cdot}39$
Mean fat percentage	Mean fat percentage	$Y = 0{\cdot}581X + 1{\cdot}36$	$Y = 0{\cdot}745X + 0{\cdot}74$	$Y = 0{\cdot}557X + 1{\cdot}40$

Table 4. *Coefficients of correlations between results within each lactation*

		Induced : Induced			Normal : Normal		
		Fukushima	Hokkaido	All cows	Fukushima	Hokkaido	All animals
Peak production	Total milk yield	+0·906*****	+0·855*****	+0·875*****	+0·900*****	+0·829*****	+0·807*****
	Mean daily production	+0·912*****	+0·962*****	+0·957*****	+0·899*****	+0·817****	+0·809*****
	Mean fat percentage	−0·320	−0·031	−0·022	−0·420	−0·054	−0·081
	Milk yield for 100 days	+0·375	+0·958*****	+0·763*****	+0·969*****	+0·940*****	+0·924*****
	Duration	+0·348	+0·594**	+0·644****	—†	+0·454	+0·377*
	Peak period	+0·651*	+0·110	+0·556****	+0·153	+0·302	+0·229
Total milk yield	Total fat yield	+0·878****	+0·996*****	+0·967*****	+0·906*****	+0·969*****	+0·954*****
	Mean fat percentage	−0·286	−0·090	+0·069	−0·311	−0·103	−0·046
	Milk yield for 100 days	+0·690**	+0·811****	+0·667*****	+0·954*****	+0·951*****	+0·947*****
	Duration	+0·480	+0·852*****	+0·862*****	—†	+0·657**	+0·592****
	Peak period	+0·521	+0·382	+0·692*****	+0·079	+0·377	+0·287
Mean daily production	Milk yield for 100 days	+0·674**	+0·956*****	+0·823*****	+0·954*****	+0·946*****	+0·939*****
Total fat yield	Mean fat percentage	+0·196	−0·023	+0·306	+0·101	+0·127	+0·235

D.F.: Fukushima, $9-2=7$; Hokkaido, $12-2=10$; all cows, $22-2=20$.
Level of significance P: $> 0{\cdot}1$, no mark; $< 0{\cdot}1$, *; $< 0{\cdot}05$, **; $< 0{\cdot}01$, ****; $< 0{\cdot}001$, *****.
† All lactations were of 305 days.

in the 2 types of lactation were similar in Fukushima, but in Hokkaido the induced lactations were far shorter than the normal.

Average lactation curves are shown in Fig. 1. Generally, the values of the various characters in the 2 types of lactation were lower and more variable in Hokkaido than in Fukushima, particularly in the induced lactations. Some examples of udder development are shown in Plate 1. Within cows there are resemblances in udder slope as between the 2 types of lactation.

Correlations of various characters between the induced and the normal lactations

The results are tabulated in Table 2, which shows that most of the correlations were statistically significant. Values in Fukushima were generally higher than those in Hokkaido. There were high correlations between peak production in the induced lactation and various characters of the normal lactation. Similar relationships were observed for total yield, average daily production and total fat yield but the coefficients were lower.

The average fat contents of the milk obtained in the 2 types of lactation were highly correlated. For milk yield for 100 days, peak production, and duration of lactation, the correlations were somewhat lower and in the Fukushima herd they were very low and non-significant. Regression equations between various characters (Y) in the normal lactation and those (X) in the induced lactation are shown in Table 3. Generally, regression coefficients as well as correlation coefficients were higher in the Fukushima than in the Hokkaido herd.

Correlations between characters within each lactation

Correlations between peak production, total yield and average daily production were very high within each lactation (Table 4). The peak production had also a high correlation with the 100-day yield within each lactation except for the induced lactation in Fukushima, and it had a negative but non-significant correlation with average fat percentage. Correlations between yield for 100 days and total yield were higher in the normal than in the induced lactations. Total fat yield had higher correlations with total yield than with average fat percentage within each lactation.

Influence of the treatment with oestrogen upon reproduction

All heifers were examined for the occurrence of oestrus and changes in the ovaries were investigated by rectal palpation over an average of 6·3 cycles (including 1·4 cycles in which there was infertile insemination) after the first injection of oestrogen as shown in Table 5. Accordingly 4·9 oestruses, on average, were examined before the animals were first inseminated. This delayed the age of first calving by about 3 months in comparison with the general age of first calving in Japan.

The influence of the treatment and the induced lactation upon oestrus and ovaries were slight at Fukushima, but 11 heifers out of 17 at Hokkaido and one out of 2 at the Stock Farm of the University showed anoestrus or continuous weak oestrus for 2 or 5 cycles, and in 2 extreme cases follicular cysts were found.

Before treatment
At peak period of induced lactation
At peak period of first normal lactation
Cow no.
1

(*Facing p.* 336)

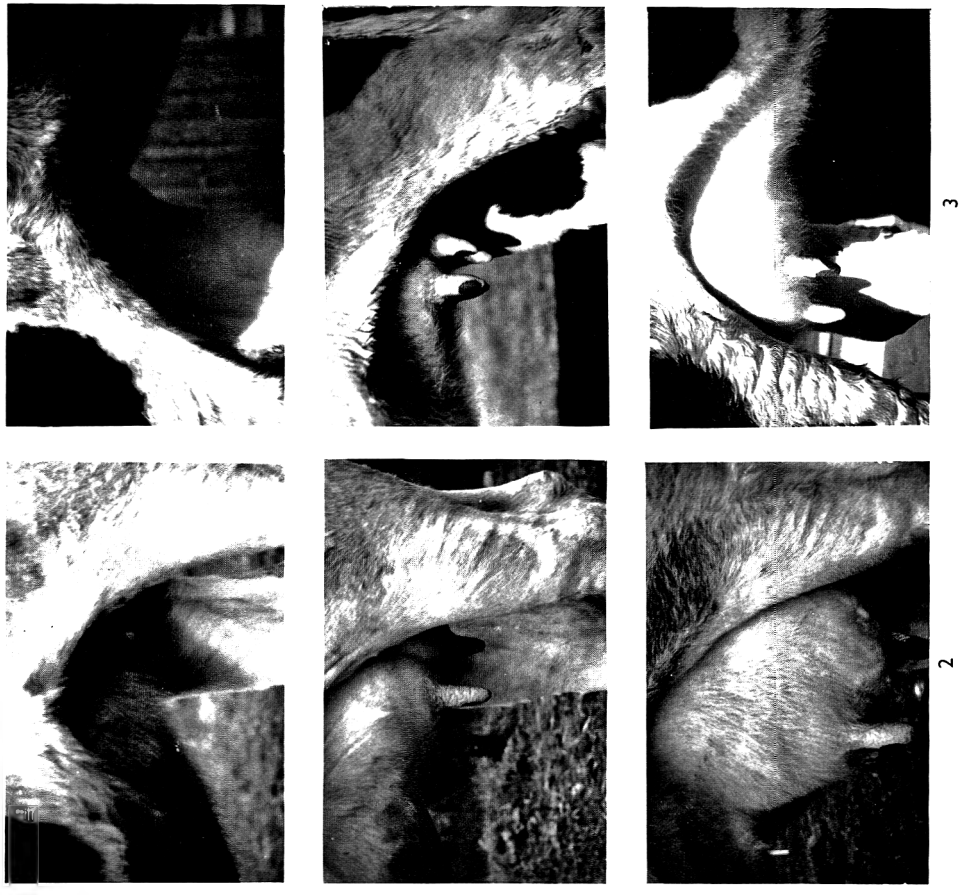
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Table 5. *Influence of oestrogen treatment on reproduction and growth in heifers*

Station	No. of animals	Initial body weight, kg	Age at first injection, months (*A*)	Age at conception, months (*B*)	(*B*) − (*A*), months	Age at first calving, months	No. of recurrences of oestrus before conception	No. of infertile insemination*	Influence upon ovaries and oestrus*†	Influence on growth*†
Fukushima	10	409·1 ± 34·9	16·7 ± 2·3	21·8 ± 2·1	5·1 ± 2·4	30·8 ± 2·2	7·7 ± 3·0	1·0 ± 1·1‡ 0 (4) 1 (5) 5 (1)	*a* (4) *b* (6)	*f* (10)
Hokkaido	17	364·5 ± 42·6	15·9 ± 2·1	22·4 ± 5·2	6·5 ± 3·5	31·4 ± 5·4	5·5 ± 3·1	1·8 ± 1·9‡ 0 (6) 1 (3) 2 (3) 3 (2) 4 (1) 5 (1) 6 (1)	*a* (1) *b* (5) *c* (6) *d* (4) *e* (1)	*f* (2) *g* (8) *h* (7)
Tokyo	2	—	16·5 ± 0·7	25·0 ± 7·1	8·5 ± 7·8	39 (1)*	5·0 ± 1·4	0·5 ± 0·7‡ 0 (1) 1 (1)	*a* (1) *e* (1)	*f* (2)
Total or average	29	381·0 ± 45·0	16·2 ± 2·1	22·4 ± 4·4	6·0 ± 3·5	31·5 ± 4·5	6·3 ± 3·1	1·4 ± 1·3‡ 0 (11) 1 (9) 2 (3) 3 (2) 4 (1) 5 (2) 6 (1)	*a* (6) *b* (11) *c* (6) *d* (4) *e* (2)	*f* (14) *g* (8) *h* (7)

* No. of heifers given in parentheses. † *a*, No influence; *b*, slight disorder in cycle; *c*, weak or absent oestrus for short period; *d*, weak or absent oestrus for long period; *e*, formation of follicular cyst; *f*, no influence; *g*, slow growth; *h*, cessation of growth for short period.

‡ $M \pm a$.

Influence of the treatment upon growth

The growth rate of the animals was normal. In the Hokkaido herd, a slight decrease of growth rate (in chest girth and in body weight) during the early period of the induced lactation was observed in most heifers. In heifers at Fukushima and at the Stock Farm of the University the growth rate was normal.

DISCUSSION

Method of testing

The mammary development and lactation induced by treatment with oestrogen alone are in some respects different from the normal. Structurally some of the alveoli are cystic and there is a general deficiency of glandular tissue (cf. studies with goats by Cowie, Folley, Malpress & Richardson, 1952; Cowie *et al.* 1965 and Benson, Cowie, Cox, Flux & Folley, 1955; and with cows by Sykes & Wrenn, 1951). Even in the well-developed gland, alveoli are larger in cross-section than normal and have elongated and tongue-shaped cells, as has been shown for goats (Cowie *et al.* 1952; Naito, Yokoyama, Yoshioka, Ide & Yokoyama, 1952; Naito *et al.* 1953). Further, the milk yield in goats (Lewis & Turner, 1941, 1942*a*, *b*; Mixner & Turner, 1943; Mixner, Meites & Turner, 1944; Naito *et al.* 1952, 1953; Eaton *et al.* 1953; Goto & Oshima, 1955; Benson *et al.* 1955) and in cows (Day & Hammond, 1945; Sykes & Wrenn, 1951; Turner, 1959; Naito *et al.* 1955, 1960, 1962) is less than normal.

A method suitable for commercial use for predicting the future dairy production of cows must be simple and economic. For field use it is necessary to adopt a simple, cheap and brief treatment for the prediction of normal dairy performance. For these reasons treatment with oestrogen alone was adopted in this work. Further, it was considered that the alternative treatment with various combinations of hormones—oestrogen, progesterone and mammotrophic hypophysial hormones—although giving high production might have a detrimental influence upon the growth of young heifers.

Dosage and the duration of treatment were decided by the comparison of goats and heifers on the basis of body weight and gestation period. Naito *et al.* (1952) found in goats that sufficient differentiation of the alveoli had occurred by the 11th day after the beginning of the injections and they adopted a treatment of 20 days duration. That such short periods of treatment seem to be better than longer periods for the treatment with oestrogen alone is indicated in the results of Cowie *et al.* (1965) who showed with goats that the best yield was obtained after treatment for only 35 days in comparison with 70 and 140 days. Goto & Oshima (1955) showed that injections on alternate days gave similar results to daily injections in goats; thus, an injection every third day was adopted in the present work.

From the economical aspect, the younger the animal can be tested the better. We gave the first injection for the induction of lactation at about 16 months of age; this age was selected on the basis of our studies in goats (Naito *et al.* 1953). If the animal was too young, the milk secretion response to oestrogen was very weak and under these conditions a poor correlation would be expected between the yields of induced and of normal lactation. Thus, it seemed necessary to delay the

treatment with oestrogen until after several oestrus cycles had occurred in order to obtain sufficient response from the mammary gland. Hindery & Turner (1964) have also reported that the response of calves at 7 months of age was very weak.

In our previous work, virgin goatlings were treated in the non-breeding season (Naito *et al.* 1952, 1953) and thus no influence on reproduction was observed. However, in the present work the heifers were already sexually mature and regular oestrus was occurring. Thus, the treatment with oestrogen might influence hormone secretion from the anterior pituitary and might cause luteal cysts (Yamanouchi, Ashida & Inui, 1954). Nagasawa, Kohno, Shoda & Naito (1959) have shown in goats that the oestrogen level in plasma was high for several days after the first injection but decreased gradually even during the period in which injections were continued. Injections were therefore begun 3 days before the expected onset of the next oestrus to avoid a high level of oestrogen in the plasma over the luteal phase.

Three heifers showed a very weak response to the treatment with oestrogen. The daily yield of one of them never attained 1·0 kg and the other 2 took a long time to give yields of 0·5 and 1·0 kg. In such cases extra treatment might be necessary as reported by Goto & Oshima (1955). For practical reasons these heifers were omitted from this work.

Possibility of predicting normal dairy performances from the performance during induced lactation

The possibility of predicting normal dairy performance from the milk records in the induced lactation has been reported for goats (Eaton *et al.* 1953; Naito *et al.* 1953; Goto & Oshima, 1955). The present results show a similar possibility in cows. In particular, as in goats, the peak production is a good measure for the various characters of the normal lactation, considering the high phenotypic correlations with other characters. As shown by Berry (1945), the repeatability of records in 2 successive lactations is a little higher than that for other combinations of calving order, because of the higher environmental correlation in the former. This may be the case with the high correlations of results between the induced and the first normal lactation.

The yields in the normal first lactation in heifers which have experienced the induced lactation may be higher than in those not so treated. For example, mammary developmental response is more sensitive in multiparous than in nulliparous rats (Yamada, Nagai & Naito, 1954). In the present work, however, all the heifers had undergone the same treatment.

The 100-day yield is reported to be a good index in an abbreviated performance test when estimating heritability (see Madden, McGilliard & Ralston, 1956; Johnson & Corley, 1961; Decking, 1964), and this seems to be confirmed in the normal lactation in the present study, which was highly correlated with total yield. In the induced lactation it was not so reliable, having lower correlations with the other characters owing to the late peak period.

Correction for age at the initiation of the injections was tried in the previous interim report (Naito *et al.* 1960) but it did not effectively increase reliability and has not been used in this report.

The experiment lasted 10 years and this is likely to increase experimental error

even though all heifers were kept at each of the stations on a regime as constant as possible so far as feeding and management were concerned. This problem has been discussed by Van Vleck (1966*a*, *b*), who found that total variance increased with time and differed from season to season.

It is important to note the difference in results between the Fukushima and the Hokkaido stations. Most of the yields and correlation coefficients were higher in the former, particularly in the induced lactation. In Hokkaido, the peak period was earlier and the duration of lactation was far shorter in the induced lactation. There was a similar tendency in the normal lactation. These characters in the induced lactation seem, from the present data, to be fairly strongly influenced by genetic factors. The average age of the animals at the start of the injections was about 1 month less in the Hokkaido herd but the ages of the animals were similar in the 2 herds when milk recording began. Average initial body weight was lower and an influence of the treatment on growth was also observed in Hokkaido. Unfavourable environmental factors, particularly feeding conditions in Hokkaido, the most northern part of Japan, may have influenced the growth of the heifers and caused inferior results.

Because of such factors as these it may well be necessary, if high reliability is to be obtained in the prediction of lactational performance, to induce lactation under good environmental conditions, particularly with regard to feeding.

Reproduction and growth

Yamanouchi *et al.* (1954) reported that some cows suffered from ovarian cysts after prolonged oestrogen treatment. In the present study, some of the heifers of Hokkaido and of the University Stock Farm showed anoestrus or follicular cysts. But these heifers had relatives—dam or elder sister—which had not undergone treatment with oestrogen, and which also suffered from similar disturbances. This suggests the participation of genetic factors, as shown by Casida & Chapman (1951). If the method ultimately proves impractical for ordinary farmers to adopt, it may be worth while for breeders to use in order to reveal congenital reproductive disturbances.

In comparison with the influence on reproduction, the influence upon growth was generally slight. No influence was observed at Fukushima and at the University Stock Farm, but at Hokkaido there occurred a reduction or cessation of growth for short periods after the beginning of the treatment, caused by the decrease of appetite resulting from oestrogen treatment. This may be prevented by careful feeding.

In conclusion, it is possible to predict the normal dairy performance from the performance during an induced lactation; in particular, the peak production in the induced lactation is a good index, especially if the treatment is carried out under conditions of good feeding and care. Before the method is more widely used, indications that it may induce reproductive difficulties should be further investigated.

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EXPLANATION OF PLATE

Udder development of cows 1, 2 and 3. Pictures for cow no. 1 show the similarity in the shape of the udder in the induced and in the subsequent normal lactation. Cow no. 2 was a high yielder and cow no. 3 a low yielder.

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Further studies on the effects of dietary cottonseed oil on milk-fat secretion in the cow

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Summary. The effects of the isocaloric replacement of part of the dietary concentrate mixture by cottonseed oil on the yield and composition of the milk fat and on the pattern of rumen fermentation was investigated in 2 feeding experiments with a total of 8 cows in mid-lactation. The concentrate mixtures were given with high- or low-roughage diets that supplied 9·1 or 1·8 kg of hay/day.

In expt 1 the yield of milk fat was not altered by the addition of 10% cottonseed oil to the concentrate mixtures given either with the high- or with the low-levels of dietary roughage. On the low-roughage treatment, dietary cottonseed oil increased milk yield but reduced the fat content of the milk. The change from the high- to the low-roughage diets containing no cottonseed oil resulted in reductions in the yield and percentage of fat in the milk. In expt 2 the yields of milk and milk fat were similar irrespective of whether the concentrate mixture contained 5 or 10% cottonseed oil.

In expt 1 the inclusion of 10% cottonseed oil in the concentrate mixture reduced the yields and percentages of the medium-chain fatty acids (12:0, 14:0 and 16:0) and increased the yields and percentages of the C_{18} fatty acids in the milk fat. In expt 2, when the concentrate mixture contained 5% cottonseed oil, the yields and percentages of all the fatty acids in the milk fat were similar to the values obtained when the concentrates contained 10% cottonseed oil. For any given concentrate mixture, the change from the high- to the low-roughage treatments in both expts 1 and 2 resulted in increases in the percentage of oleic acid in the milk fat. The highest concentration of *trans*-octadecenoic acid was observed in the milk fat of the cows when they were given the high-roughage diet with the concentrate mixture containing 10% cottonseed oil.

In expt 2 the level of cottonseed oil in the concentrate mixture did not influence the pattern of rumen fermentation as measured by the concentrations of the various volatile fatty acids in the rumen liquor. However, the change from the high- to the low-roughage diets reduced the proportion of acetic and increased the proportions of propionic and *n*-valeric acids in the total volatile fatty acids in the rumen liquor.

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In a previous investigation (Steele & Moore, 1968) it was shown that a reduction in the intake of roughage by the cow resulted in decreased yields of all the fatty acids in the milk fat but this reduction in yield was least pronounced with oleic acid. However, these results were obtained from an experiment that involved between-cow comparisons of treatments. It was decided that an attempt should be made to confirm these findings in an experiment that involved within-cow comparisons of treatments. Preliminary observations (Steele & Moore, unpublished) indicated that when cottonseed oil was included in the concentrate mixture given to cows, there was an increase in the proportion of the total octadecenoic acid in the milk fat that occurred as the *trans*-isomer. It seemed possible that a more detailed study of the effect of dietary cottonseed oil on the proportions of the *cis*- and *trans*-isomers of octadecenoic acid in the milk fat might provide some information on the contribution of the C_{18} fatty acids in the diet of the cow to the C_{18} fatty acids secreted in the milk fat. In previous experiments (Steele & Moore, 1968) it was noted that when cows were given a concentrate mixture containing 6% cottonseed oil the fat content of the milk was less than it was when the cows were given a concentrate mixture containing 10% cottonseed oil. Again, it must be pointed out that these observations were made in experiments that involved between-cow comparisons of treatments. With these problems in mind, 2 further feeding experiments have been carried out so that within-cow comparisons of treatments could be made. The first experiment was designed to study the effects of a concentrate mixture containing 10% cottonseed oil in the diet on the yields of individual fatty acids, including *trans*-octadecenoic acid, in the milk fat of cows given high- or low-roughage diets. In the second experiment, a study was made of the yields of total fat and individual fatty acids in the milk of cows given high- or low-roughage diets and concentrate mixtures containing either 5 or 10% cottonseed oil.

EXPERIMENTAL

Experimental animals

Four pedigree Friesian cows were used in expt 1; these animals had calved on average 58 days before the beginning of the experiment. Four cross-bred Friesian cows were used in expt 2; these animals had calved on average 30 days before the experiment began. In both experiments the cows were housed in a byre and were yoked in stalls equipped for individual feeding. The animals were milked twice daily in a parlour at intervals of 15 and 9 h, and immediately after each milking they were yoked and given half of their daily rations. Water was available *ad lib.*

Experimental diets

Expt 1. In this experiment, the cows were given high- or low-roughage diets that supplied 9·1 or 1·8 kg/day, respectively, of a mature ryegrass hay in the long form. The composition (percentages of the dry matter) of the hay, which was the same as that used in expt 2, was as follows: crude protein, 11·8; crude fibre, 33·5; ether extract, 1·5; ash, 8·8; nitrogen-free extractives, 44·4. Two different concentrate mixtures, containing 0 or 10% cottonseed oil, were given to the cows on each of the 2 roughage treatments. The concentrate mixtures that were given to the animals on the high-roughage treatments differed in composition from those that were given

to the animals on the low-roughage treatments. The constituents used in the basal concentrates were the same as those used in previous experiments (Steele & Moore, 1968). The mean daily intakes of the various dietary components on the 4 feeding treatments are given in Table 1, from which it can be seen that the intakes of cottonseed oil were the same on both the high- and low-roughage treatments. All the treatments were calculated to supply the same amounts of energy (Evans, 1960). The daily intakes of energy were maintained at a constant level for each cow throughout the experiment so that the pattern of rumen fermentation would not be altered by variations in the ratio of hay to concentrates.

Table 1. *Mean daily intakes (kg) of hay and of each of the constituents of the concentrate mixtures given to the cows in expt* 1

	Treatments			
	High-roughage		Low-roughage	
	Unsupplemented ration	Ration containing cottonseed oil	Unsupplemented ration	Ration containing cottonseed oil
Hay	9·1	9·1	1·8	1·8
Barley	4·9	2·3	2·5	0·5
Flaked maize	—	—	5·6	5·6
Decorticated extracted groundnut meal	1·8	2·2	1·8	2·2
Bran	0·9	0·9	—	—
Molasses	0·4	0·4	0·4	0·4
Cottonseed oil	—	0·65	—	0·65
Mineral mixture	0·3	0·3	0·3	0·3

Table 2. *Mean daily intakes (kg) of hay and of each of the constituents of the concentrate mixtures given to the cows in expt* 2

	High-roughage treatments with concentrate containing		Low-roughage treatments with concentrate containing	
	5% cottonseed oil	10% cottonseed oil	5% cottonseed oil	10% cottonseed oil
Hay	9·1	9·1	1·8	1·8
Barley	3·1	2·1	1·5	0·4
Flaked maize	—	—	5·3	5·3
Decorticated extracted groundnut meal	1·8	2·1	1·8	2·1
Bran	0·8	0·8	—	—
Molasses	0·4	0·4	0·4	0·4
Cottonseed oil	0·3	0·6	0·3	0·6
Mineral mixture	0·3	0·3	0·3	0·3

Expt 2. In this experiment the dietary constituents were the same as those used in expt 1. Again, the cows were given high- or low-roughage diets, but expt 2 differed from expt 1 in that all the diets contained supplementary cottonseed oil. The cottonseed oil was included at either 5 or 10% of the concentrate mixture. The mean daily intakes of the various dietary components on the 4 treatments are given in Table 2. As in expt 1, all the treatments supplied the same amounts of energy and the daily intakes of energy were maintained at a constant level throughout.

In both experiments the concentrate part of the rations was given in the pelleted form.

Experimental design

The design of each experiment was a 4×4 Latin square that was balanced for carry-over effects. Cows were randomly allocated to the various treatment sequences. The durations of the experimental periods in expts 1 and 2 were 28 and 24 days, respectively, and each change-over between treatments was abrupt.

Sampling and methods of analysis

The sampling of milk and the methods used in its analysis were the same as those reported previously (Steele & Moore, 1968). In addition, the proportions of *cis*- and *trans*-isomers of octadecenoic acid were determined in the samples of milk fat obtained in expt 1. The methyl esters of the total fatty acids were prepared by the method of Stoffel, Chu & Ahrens (1959) and were analysed by gas–liquid chromatography as described previously (Steele & Moore, 1968). Values for the concentrations of total octadecenoic acids were thus obtained. Separation of the *cis*- and *trans*-octadecenoic acids was achieved by chromatography of the methyl esters on thin-layer plates of silica gel impregnated with silver nitrate (Morris, 1962). The methyl esters of the *trans*-octadecenoic acids were eluted from the plates together with the methyl esters of the saturated fatty acids. The resulting mixture of methyl esters was then analysed by gas–liquid chromatography. The proportions of *cis*- and *trans*-octadecenoic acids could then be calculated from the 2 gas–liquid chromatographic analyses.

Since the cows used in these experiments were not provided with rumen cannulas, an alternative method of obtaining samples of rumen liquor was devised. The method used was based on that described by Raun & Burroughs (1962) for obtaining rumen liquor from sheep with a stomach tube. Unfortunately, this method was not adapted for use with cows in time for expt 1, so patterns of rumen fermentation were studied in expt 2 only. Samples of rumen liquor were obtained at 3 and 6 h after the morning feed on days 21 and 22 of each period in expt 2. Each sample was centrifuged at 700 *g* for 25 min and the relative proportions of the individual steam-volatile fatty acids in the supernatant rumen liquor were determined by the modified method of James & Martin (1952) as described by Youssef & Allen (1966). For the determination of total volatile fatty acids, samples of rumen liquor were mixed with equal volumes of 2·5% (v/v) H_2SO_4 in a saturated solution of $MgSO_4$. The samples were then centrifuged at 700 *g* for 15 min and the steam-volatile fatty acids were distilled (Markham, 1942) from a sample of the supernatant fluid. The amounts of steam-volatile fatty acids in the distillate were determined by titration.

The results were analysed statistically by the methods outlined by Cochran & Cox (1957).

RESULTS

All the rations were consumed within 1 h of feeding. Since certain of the treatment effects were not evident in the early part of the experimental periods, the results given in Tables 3–8 are mean values for the last 4 days of each period.

Expt 1. The mean values for the yield and composition of the milk produced

during the last 4 days of the experimental periods are given in Table 3. When the cows were changed from the high-roughage treatments with the unsupplemented concentrate to the low-roughage treatment with the unsupplemented concentrate there were no significant ($P > 0{\cdot}05$) changes in milk yield, solids-not-fat (SNF) yield, or SNF content of the milk. However, there were significant reductions in the

Table 3. *Mean daily yields of milk and the composition of the milk produced during the last 4 days of each period in expt 1*

	Treatments				
	High-roughage		Low-roughage		
	Unsupplemented ration	Ration containing cottonseed oil	Unsupplemented ration	Ration containing cottonseed oil	S.E.M.
Milk yield, kg	13·5	13·7	12·8	14·8	±0·72
Milk fat yield, g	451	447	357	338	±45·1
Milk fat content, %	3·34	3·26	2·79	2·28	±0·079
SNF yield, kg	1·13	1·09	1·09	1·24	±0·049
SNF content, %	8·36	7·92	8·49	8·37	±0·141

Table 4. *Mean weight percentages of the major fatty acids in the milk fat produced during the last 4 days of each period in expt 1*

	High-roughage treatment		Low-roughage treatment		
	Unsupplemented ration	Ration containing cottonseed oil	Unsupplemented ration	Ration containing cottonseed oil	S.E.M.
4:0–8:0	7·5	6·3	7·1	6·0	±0·60
10:0	0·2	0·1	0·2	0·2	±0·15
12:0	1·4	0·6	1·5	0·7	±0·25
14:0	10·7	5·2	9·8	4·8	±0·91
16:0	43·0	30·4	37·6	28·2	±1·38
16:1	1·9	1·5	1·9	1·9	±0·26
18:0	8·7	14·5	7·7	12·5	±0·96
Total 18:1	20·6	34·7	26·5	38·2	±1·65
cis 18:1	19·3	28·2	24·0	34·6	±1·57
trans 18:1	1·3	6·5	2·5	3·6	±0·32
18:2	2·0	3·4	2·9	5·1	±0·60

yield ($P < 0{\cdot}05$) and content ($P < 0{\cdot}001$) of fat in the milk. The only significant effect of including cottonseed oil in the high-roughage diet was a reduction ($P < 0{\cdot}05$) in the SNF content of the milk. On the other hand, the addition of cottonseed oil to the low-roughage diet resulted in significant ($P < 0{\cdot}05$) increases in the yields of milk and SNF and a highly significant reduction ($P < 0{\cdot}001$) in the milk fat content.

The fatty-acid compositions of the milk fat produced by the cows during the last 4 days of each experimental period are given in Table 4 and the yields of individual fatty acids in the milk fat are given in Table 5. In Tables 4 and 5 and elsewhere in this paper the shorthand designation of Farquhar, Insull, Rosen, Stoffel & Ahrens (1959) is used to denote individual fatty acids. When the cows were changed from the high-roughage treatment with the unsupplemented concentrate to the low-roughage treatment with the unsupplemented concentrate, there was a significant

reduction in the percentage of 16:0 ($P < 0.001$) and significant reductions in the yields of 14:0 ($P < 0.05$), 16:0 ($P < 0.05$) and 16:1 ($P < 0.01$) in the milk fat. The decrease in the percentage of 16:0 was counterbalanced mainly by an increase ($P < 0.01$) in the percentage of 18:1 but there were no significant changes ($P > 0.05$) either in the yield of 18:1 or in the yields of the other fatty acids in the milk fat. The addition of cottonseed oil to the high-roughage diet significantly reduced the percentages of 12:0 ($P < 0.05$), 14:0 ($P < 0.001$) and 16:0 ($P < 0.001$) in the

Table 5. *Mean daily yields (g) of fatty acids in the milk fat produced during the last 4 days of each period in expt 1*

	High-roughage treatment		Low-roughage treatment		
	Unsupplemented ration	Ration containing cottonseed oil	Unsupplemented ration	Ration containing cottonseed oil	S.E.M.
4:0–8:0	32·3	26·6	23·9	19·2	± 4·27
10:0	0·9	0·5	0·8	0·8	± 0·26
12:0	6·0	2·6	5·1	2·2	± 0·99
14:0	45·5	21·9	32·9	15·3	± 4·74
16:0	183·0	128·0	127·0	89·6	± 18·0
16:1	8·1	6·4	6·4	6·0	± 0·90
18:0	36·9	61·3	26·0	39·7	± 4·79
Total 18:1	87·5	146·0	89·2	121·0	± 5·95
cis 18:1	81·8	119·0	80·7	110·0	± 5·66
trans 18:1	5·7	27·4	8·5	11·3	± 1·15
18:2	8·6	14·2	9·8	16·3	± 2·41

Table 6. *Mean daily yields of milk and the composition of the milk produced during the last 4 days of each period in expt 2*

	High-roughage treatments with concentrate containing:		Low-roughage treatments with concentrate containing:		
	5% cottonseed oil	10% cottonseed oil	5% cottonseed oil	10% cottonseed oil	S.E.M.
Milk yield, kg	13·9	14·1	13·9	13·2	± 0·56
Milk fat yield, g	457	525	287	286	± 64·8
Milk fat content, %	3·29	3·72	2·06	2·17	± 0·48
SNF yield, g	1·18	1·17	1·20	1·13	± 0·073
SNF content, %	8·45	8·33	8·66	8·54	± 0·421

milk fat; the yields of 12:0 ($P < 0.05$), 14:0 ($P < 0.01$), 16:0 ($P < 0.05$) and 16:1 ($P < 0.01$) in the milk fat were also depressed. The addition of cottonseed oil to the high-roughage diet significantly increased the percentages of 18:0 and 18:1 ($P < 0.001$) and significantly increased the yields of 18:0 ($P < 0.01$) and 18:1 ($P < 0.001$) in the milk fat. The addition of cottonseed oil to the low-roughage diet produced similar changes to those observed on the high-roughage treatments but the reduction in the yield of 16:0 in the milk fat just failed to reach the 5% level of significance and there were also significant increases in the yield and percentage of 18:2 in the milk fat. The results in Table 4 show that the highest concentration of *trans*-18:1 in the milk fat occurred when the cows were given the high-roughage diet with the concentrate mixture containing cottonseed oil. The milk fat produced by the cows when they were given the low-roughage diets contained significantly

higher ($P < 0{\cdot}001$) concentrations of *trans*-18:1 than did the milk fat produced by the cows when they were given the high-roughage diet with the unsupplemented concentrate mixture.

Expt 2. The mean values for the yield and composition of the milk produced during the last 4 days of the experimental periods are given in Table 6. There were no significant ($P < 0{\cdot}05$) differences in the yields of milk and SNF or in the percentages of SNF in the milk produced by the cows on the 4 dietary treatments. The results given in Table 6 also show that for each level of roughage intake, differences

Table 7. *Mean weight percentages of the major fatty acids in the milk fat produced during the last 4 days of each period in expt 2*

	High-roughage treatments with concentrate containing:		Low-roughage treatments with concentrate containing:		
	5% cottonseed oil	10% cottonseed oil	5% cottonseed oil	10% cottonseed oil	S.E.M.
4:0–8:0	6·4	6·0	5·5	4·6	±0·45
10:0	0·2	0·1	0·2	0·1	±0·05
12:0	0·8	0·7	0·9	0·7	±0·13
14:0	6·6	5·6	6·5	6·4	±0·49
16:0	30·6	31·0	29·7	30·6	±0·20
18:0	16·3	16·5	12·3	11·6	±1·20
18:1	30·6	32·1	34·9	35·3	±0·60
18:2	3·6	3·6	4·3	4·8	±0·66

Table 8. *Mean daily yields (g) of the major fatty acids in the milk fat produced during the last 4 days of each period in expt 2*

	High-roughage treatments with concentrate containing:		Low-roughage treatments with concentrate containing:		
	5% cottonseed oil	10% cottonseed oil	5% cottonseed oil	20% cottonseed oil	S.E.M.
4:0–8:0	27·5	30·1	14·9	12·4	±4·66
10:0	0·8	0·2	0·5	0·4	±0·23
12:0	3·4	3·7	2·5	1·9	±0·51
14:0	28·4	27·5	17·5	17·1	±3·03
16:0	132·0	154·0	80·3	82·3	±18·2
18:0	70·4	81·9	33·3	31·1	±11·8
18:1	132·0	160·0	94·3	94·8	±20·7
18:2	15·4	18·0	11·6	12·8	±1·88

in the level of cottonseed oil in the concentrate mixtures given to the cows did not significantly ($P > 0{\cdot}05$) alter the yield or percentage of fat in the milk. However, irrespective of the level of cottonseed oil in the concentrate mixture, the low-roughage diets significantly ($P < 0{\cdot}05$) reduced both the yield and percentage of fat in the milk. The percentages and yields of the fatty acids in the milk fat produced by the cows during the last 4 days of each experimental period are given in Tables 7 and 8, respectively. When the level of cottonseed oil was increased from 5 to 10% of the concentrate mixture given to the cows on the high-roughage treatment there was a significant ($P < 0{\cdot}05$) increase in the yield of 18:1 in the milk fat (Table 8). On the low-roughage treatments, the higher level of cottonseed oil in the concentrate mixture resulted in a significant ($P < 0{\cdot}05$) increase in the percentage of 16:0 in

the milk fat (Table 7). Irrespective of the level of cottonseed oil in the concentrate mixture, the milk fat produced by the cows on the low-roughage treatments contained significantly lower concentrations of 18:0 ($P < 0{\cdot}01$) and significantly higher concentrations of 18:1 ($P < 0{\cdot}001$) than did the milk fat produced by the cows when they were given the high-roughage diets. Thus, since the low-roughage diets resulted in a marked decrease in total milk-fat output, the yields of all the constituent fatty acids were reduced, but the percentage reduction in the yield of 18:1 was less than that of the other fatty acids.

Table 9. *Mean concentrations (m-equiv./100 ml) of total volatile fatty acids and the mean relative proportions (molar percentages of the total) of the individual volatile fatty acids in the rumen liquor of the cows on the different dietary treatments in expt 2*

	High-roughage treatments with concentrate containing		Low-roughage treatments with concentrate containing		
	5% cottonseed oil	10% cottonseed oil	5% cottonseed oil	10% cottonseed oil	S.E.M.
Total volatile fatty acids (m-equiv./100 ml)	8·33	8·32	8·58	8·82	±0·90
Proportion of individual volatile fatty acids (molar percentages of the total)					
Acetic	65·0	64·5	52·1	50·1	±2·00
Propionic	18·1	17·7	28·1	28·6	±2·74
iso-Butyric	1·3	1·6	1·3	1·6	±0·36
n-Butyric	12·5	12·4	12·7	15·5	±1·75
iso-Valeric	1·7	2·2	1·6	2·8	±0·58
n-Valeric	1·5	1·7	4·1	3·5	±0·73

The concentrations of total steam-volatile fatty acids in the rumen liquor and the relative proportions of the individual steam-volatile fatty acids are given in Table 9. There were no significant ($P > 0{\cdot}05$) differences in the concentrations of total steam-volatile fatty acids in the rumen liquor obtained from the cows on the 4 dietary treatments. On each level of roughage intake, the level of cottonseed oil in the concentrate mixture did not significantly ($P > 0{\cdot}05$) affect the relative proportions of the individual fatty acids in the rumen liquor. However, the low-roughage diets resulted in a significant reduction in the proportion of acetic acid ($P < 0{\cdot}001$) and significant increases in the proportions of propionic acid ($P < 0{\cdot}01$) and *n*-valeric acid ($P < 0{\cdot}05$) in the rumen liquor.

DISCUSSION

Although detailed results are not given for the yields of milk fat produced by the cows at all stages of the treatment periods in expt 1, it was evident that the addition of cottonseed oil to the high-roughage diet resulted in an immediate increase in the yield of milk fat during the first week of the period, but this increase was not maintained as the treatment period progressed. Similar observations have been made by Allen (1934), Garner & Sanders (1938), Allen & Fitch (1941), Davis & Harland (1946) and Steele & Moore (1968). Detailed studies of blood and milk fatty acids and of the production of volatile fatty acids in the rumen will be necessary at all stages of the treatment periods before it will be possible to explain why the

response in milk fat production decreases with the period of time the cows are given diets supplemented with vegetable oils.

The results now reported confirm those obtained by Steele & Moore (1968) in that the reduction in the yield of milk fat observed when the cows were changed from the high- to the low-roughage diets containing no cottonseed oil was associated with a general decrease in the yields of all the fatty acids in the milk fat except oleic acid (expt 1, Table 5). As suggested by Rook (1961), the decreased yields of those milk fatty acids that are synthesized in the mammary gland from acetate and β-hydroxybutyrate (Peeters & Lauryssens, 1964) could be related to the reduction in the proportion of acetate and the increase in the proportion of propionate in the volatile fatty acids in the rumen liquor when the cows were changed from a high- to a low-roughage diet (expt 2, Table 9). In agreement with previous findings (Steele & Moore, 1968), the addition of cottonseed oil to the diet of the cows increased the yield and percentage of octadecenoic acid (18:1) in the milk fat (Tables 4, 5). This could be due to the complete hydrogenation of the dietary C_{18} unsaturated fatty acids by rumen micro-organisms (Tove & Mochrie, 1963), the absorption of the resulting stearic acid and its incorporation into blood triglycerides, which would then be taken up by the mammary gland where oleic acid (*cis*-18:1) is synthesized by the desaturation of stearic acid (Annison, Linzell, Fazakerley & Nichols, 1967; Linzell, Annison, Fazakerley & Leng, 1967). However, the inclusion of cottonseed oil in the concentrate mixture given to the cows on the high-roughage treatment (expt 1) increased the yields of total 18:1 and *trans*-18:1 by 58·4 and 21·7 g/day respectively (Table 5). Thus, 37% of the increased yield of total 18:1 in the milk fat was accounted for by the *trans*-isomer. Ward, Scott & Dawson (1964) have shown that *trans*-18:1 is an intermediate in the hydrogenation of C_{18} polyunsaturated fatty acids by rumen micro-organisms. Therefore, it would appear that hydrogenation of C_{18} polyunsaturated fatty acids in the rumen is far from complete and a certain amount of *trans*-18:1 is absorbed and incorporated into blood and milk triglycerides.

As noted previously (Steele & Moore, 1968), the addition of cottonseed oil to the high- or low-roughage diets given to the cows resulted in a general decrease in the yields and percentages of the milk fatty acids of medium-chain length (Tables 4, 5). As suggested by Moore & Steele (1968), an increased uptake of long-chain fatty acids by the mammary gland might inhibit the *de novo* synthesis in the gland of the medium-chain fatty acids from acetate and β-hydroxybutyrate. An alternative explanation might be that the addition of cottonseed oil to the diet of the cow reduced the production of acetate and butyrate in the rumen and, hence, decreased the rate of uptake of acetate and β-hydroxybutyrate by the mammary gland. Unfortunately, we were unable to study the concentrations of volatile fatty acids in the rumen liquor of the cows in expt 1, but Brown, Stull & Stott (1962) have shown that when cottonseed oil was added to the diet of cows at a level of 6% of the concentrate mixture there was no alteration in the production of the volatile fatty acids in the rumen. The results of expt 2 (Table 9) showed that the concentrations of volatile fatty acids in the rumen liquor were unaltered when the dietary cottonseed oil was increased from 5 to 10% of the concentrate mixture given to the cows.

The results of the previous experiment (Steele & Moore, 1968) showed, somewhat surprisingly, that the addition of cottonseed oil (containing 21% palmitic acid) to the diet of the cow did not significantly alter the yield of palmitic acid in the milk fat. The results now described (expt 1, Tables 4, 5) are even more surprising in that the addition of cottonseed oil to the high-roughage diet given to the cows significantly decreased the yield and percentage of palmitic acid in the milk fat. Feeding experiments with cows given diets containing pure fatty acid might elucidate this problem.

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The effects of mono-unsaturated and saturated fatty acids in the diet on milk-fat secretion in the cow

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Summary. The effects of the addition of either 5 or 10% 'oleic acid' (78% pure) or 10% of a mixture of saturated fatty acids (64% palmitic acid, 31% stearic acid) to the dietary concentrate mixture on the yield and composition of the milk and milk fat and on the pattern of fermentation in the rumen were investigated in a feeding experiment with 8 cows in mid-lactation. The concentrate mixtures were given with a high-roughage diet that supplied 9·1 kg of hay/day.

The addition of 5% 'oleic acid' to the concentrate mixture resulted in increased yields of milk and solids-not-fat (SNF); the percentage of fat in the milk was decreased but the yield of milk fat was unaltered. The addition of 10% 'oleic acid' to the concentrate mixture decreased both the yield and percentage of fat in the milk. In contrast, the concentrate mixture containing 10% of the mixture of saturated fatty acids increased the yield of milk fat.

When the concentrate mixture containing 5% 'oleic acid' was given to the cows, the yields and percentages of the fatty acids from 4:0 to 16:0 (except 12:0) in the milk fat were decreased, but the yields and percentages of 18:0 and 18:1 were increased. Similar but more pronounced effects on the yields and percentages of the fatty acids from 4:0 to 16:0 (except 12:0) in the milk fat were observed when the cows were given the concentrate mixture containing 10% 'oleic acid', but under these dietary conditions the yield and percentage of only 18:1 in the milk fat were increased. The addition of the mixture of saturated fatty acids to the concentrate mixture decreased the percentages of the fatty acids from 4:0 to 14:0 (except 12:0) in the milk fat but decreased the yields of only 10:0 and 14:0; the yields and percentages of 16:0 and 18:1 were increased.

When the cows were given the concentrate mixture containing 5% 'oleic acid' there was a small but significant decrease in the acetic acid:propionic acid ratio in the rumen liquor. A similar but more pronounced change in the acetic acid:propionic acid ratio in the rumen liquor was observed when the cows were given the concentrate mixture containing 10% 'oleic acid', but in this instance there was a significant reduction in the concentration of total volatile fatty acids in the rumen liquor. Apart from a small increase in the relative proportion of propionic acid, the addition of the mixture of saturated fatty acids to the concentrate mixture had no effect on the concentrations of volatile fatty acids in the rumen liquor.

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In a previous investigation (Steele & Moore, 1968*a*) it was found that the addition of tallow to high-roughage diets of cows resulted in a sustained increase in the output of fat in the milk. On the other hand, the addition of cottonseed oil to the same type of diet produced only a transient increase in the output of milk fat. The main differences in the fatty-acid compositions of tallow and cottonseed oil are in the concentrations of stearic acid, which is high in tallow and low in cottonseed oil, and the dienoic acid, linoleic acid, which is low in tallow and high in cottonseed oil. It was of interest therefore to compare the yields and compositions of the milk produced by cows given diets supplemented with saturated fatty acids or a monoenoic acid such as oleic acid. Steele & Moore (1968*a*) observed that when tallow, containing 30% palmitic acid and 60% C_{18} fatty acids, was added to the diet of cows the yields of the C_{18} fatty acids in the milk fat were increased but the yield of palmitic acid was unchanged. It seemed possible that this observation might be explained by the low ratio of palmitic acid to C_{18} fatty acid in the tallow. In the experiment now reported, an investigation was made of the effects of supplementing the diet of cows with a mixture of saturated fatty acids containing 64% palmitic acid and 31% stearic acid.

EXPERIMENTAL

Experimental animals

Eight cross-bred cows were used; they had calved on average 45 days before the beginning of the experiment. The cows were housed in a byre and were yoked in stalls equipped for individual feeding. They were milked twice daily at intervals of 15 and 9 h. and immediately after each milking they were yoked and given half of their daily rations. Water was available *ad lib.*

Experimental diets

The cows were given 9·1 kg/day of a mature ryegrass hay in the long form. The composition (percentages of the dry matter) of the hay was as follows: crude protein, 13·3; crude fibre, 35·2; ether extract, 1·5; ash, 9·7; nitrogen-free extractives, 40·3.

Table 1. *Compositions (weight percentages of the total) of the fatty acids incorporated in the concentrate mixture*

Fatty acid*	'Oleic acid'	Saturated fatty acid mixture
14:0	3·3	—
16:0	6·5	63·9
16:1	5·8	—
18:0	2·4	31·3
18:1	78·0	2·7
18:2	0·8	—

* Shorthand designation of Farquhar, Insull, Rosen, Stoffel & Ahrens (1959).

Four different concentrate mixtures were given to the cows. The basal (low-fat) concentrate mixture contained barley, decorticated extracted groundnut meal, bran, molasses and minerals. The other 3 concentrate mixtures contained, in addition, 5 or 10% 'oleic acid' or 10% of a mixture of saturated fatty acids. The

fatty acids incorporated into the concentrate mixtures were obtained from Harrington Bros. Ltd., Balham, London.

The 'oleic acid' was 78% pure and the mixture of saturated fatty acids contained 64% palmitic acid and 31% stearic acid (Table 1). The mean daily intakes of the various dietary components on the 4 feeding treatments are given in Table 2.

Table 2. *Mean daily intakes (kg) of hay and of each of the constituents of the concentrate mixtures*

	Treatments			
		Concentrate containing		
	Unsupplemented ration	5% 'oleic acid'	10% 'oleic acid'	10% saturated fatty acid mixture
Hay	9·1	9·1	9·1	9·1
Barley	5·0	5·0	5·0	5·0
Decorticated extracted groundnut meal	1·35	1·35	1·35	1·35
Bran	0·75	0·75	0·75	0·75
Molasses	0·20	0·20	0·20	0·20
Minerals	0·25	0·25	0·25	0·25
'Oleic acid'	—	0·38	0·76	—
Saturated fatty acid mixture	—	—	—	0·76

Experimental design

The 8 cows were randomly assigned to the treatment sequences of two 4×4 Latin squares. Each square was balanced for carry-over effects. The duration of each experimental period was 28 days and each change-over between treatments was abrupt.

Sampling and methods of analysis

The sampling of milk and the methods used in its analysis were the same as those reported previously (Steele & Moore, 1968*a*, *b*). Samples of rumen contents were analysed for steam-volatile fatty acids as described by Steele & Moore (1968*b*).

The results were analysed statistically by the methods outlined by Cochran & Cox (1957).

RESULTS

All the rations except the one with the concentrate mixture containing 10% 'oleic acid' were eaten within 1 h of feeding. Most of the concentrate containing 10% 'oleic acid' was eaten within 1 h of feeding and the remainder was eaten slowly during the day. Since certain of the treatment effects were not evident in the early part of the experimental periods, the results given in Tables 3–5 are mean values for the last 4 days of each period.

The mean values for the yield and composition of the milk produced by the cows during the last 4 days of the experimental periods are given in Table 3. The addition of 5% 'oleic acid' to the concentrate mixture given to the cows did not significantly alter the yield of milk fat but it increased the yield of milk and thereby reduced the percentage of fat in the milk. There was also an increase in the yield of SNF but the percentage of SNF in the milk was not significantly changed by the

addition of 5% 'oleic acid' to the concentrate mixture. Compared with the results obtained with the cows on the unsupplemented ration, the addition of 10% 'oleic acid' to the concentrate mixture significantly reduced both the yield and percentage of fat in the milk. The higher level of 'oleic acid' in the diet did not significantly

Table 3. *Mean daily yields of milk and the composition of the milk produced during the last 4 days of each period*

	Treatments				
		Concentrate containing			
	Unsupplemented ration	5% 'oleic acid'	10% 'oleic acid'	10% saturated fatty acid mixture	S.E.M.
Milk yield, kg	13·1	14·8*	13·5	14·3	±0·63
Milk fat yield, g	476	434	343***	563***	±25·7
Milk fat content, %	3·63	2·93***	2·54***	3·94	±0·169
SNF yield, kg	1·16	1·30*	1·21	1·25	±0·057
SNF content, %	8·85	8·78	8·95	8·74	±0·124

*, ***, Significantly different ($P < 0·05$, $P < 0·001$, respectively) from the values obtained with the unsupplemented ration.

Table 4. *Mean weight percentages of the major fatty acids in the milk fat produced during the last 4 days of each period*

	Treatments				
		Concentrate containing:			
	Unsupplemented ration	5% 'oleic acid'	10% 'oleic acid'	10% saturated fatty acid mixture	S.E.M.
Fatty acids†					
4:0–8:0	7·3	6·1***	4·7***	6·7*	±0·27
10:0	0·3	0·2**	0·2**	0·2**	±0·04
12:0	1·4	1·1	0·7	1·0	±0·37
14:0	10·4	7·6***	6·2***	6·4***	±0·55
16:0	39·3	28·2***	27·1***	43·7***	±1·02
18:0	9·1	11·9***	10·2	9·0	±0·66
18:1	22·3	34·8***	40·1***	24·7*	±0·94
18:2+18:3	3·3	3·0	2·8	2·0*	±0·36

*, **, ***, Significantly different ($P < 0·05$, $P < 0·01$, $P < 0·001$, respectively), from the values obtained with the unsupplemented ration.

† Shorthand designation of Farquhar *et al.* (1959).

alter the yield of milk or the yield of SNF. The inclusion of 10% of the mixture of saturated fatty acids in the concentrate mixture gave rise to a significant increase in the yield of milk fat but it did not affect the percentages of fat or SNF or the yield of SNF in the milk.

The fatty-acid compositions of the milk fat produced during the last 4 days of each experimental period are given in Table 4 and the yields of individual fatty acids in the milk fat are given in Table 5. The addition of 5 or 10% 'oleic acid' to

the concentrate mixture given to the cows resulted in reductions in the concentrations (Table 4) and yields (Table 5) of the fatty acids from 4:0 to 16:0 in the milk fat. The reductions in the concentrations and yields of these fatty acids in the milk fat were more pronounced when the cows were given the concentrate mixture containing 10% 'oleic acid'. When 'oleic acid' was included in the concentrate mixture at the 5% level there were increases in the concentrations (Table 4) and yields (Table 5) of both 18:0 and 18:1 in the milk fat. However, the addition of 10% 'oleic acid'

Table 5. *Mean yields (g/day) of the major fatty acids in the milk fat produced during the last 4 days of each period*

	Treatments				
		Concentrate containing:			
Fatty acids†	Unsupplemented ration	5% 'oleic acid'	10% 'oleic acid'	10% saturated fatty acid mixture	S.E.M.
4:0–8:0	32·4	25·4**	15·6***	35·5	±2·34
10:0	1·4	0·9*	0·6***	1·0*	±0·19
12:0	6·2	4·7	2·4*	5·2	±1·58
14:0	45·8	31·6***	20·6***	34·0***	±3·06
16:0	174·1	116·6***	90·0***	232·5***	±10·67
18:0	40·4	49·3*	33·9	47·9	±4·14
18:1	98·8	143·6***	133·1**	131·4**	±9·67
18:2+18:3	14·6	12·6	9·3**	10·4*	±1·79

*, **, ***, Significantly different ($P < 0·05$, $P < 0·01$, $P < 0·001$, respectively), from the values obtained with the unsupplemented ration.

† Shorthand designation of Farquhar *et al.* (1959).

Table 6. *Mean concentrations (m-equiv./100 ml) of total volatile fatty acids and the mean relative proportions (molar percentages of the total) of the individual volatile fatty acid in the rumen liquor of the cows during the last 2 days of each experimental period*

	Treatments				
		Concentrate containing:			
	Unsupplemented ration	5% 'oleic acid'	10% 'oleic acid'	10% saturated fatty acid mixture	S.E.M.
Total volatile fatty acids (m-equiv./100 ml	8·69	8·09	7·63*	8·22	±0·365
	Proportions of individual volatile fatty acid (molar percentages of the total)				
Acetic	68·6	66·1*	60·6***	67·3	±1·19
Propionic	15·6	18·4**	22·9***	17·6*	±0·96
iso-Butyric	1·5	1·5	1·8	1·4	±0·19
n-Butyric	11·4	11·0	11·0	11·1	±0·56
iso-Valeric	1·5	1·3	2·0*	1·4	±0·16
n-Valeric	1·4	1·7	1·5	1·4	±0·30

*, **, ***, Significantly different ($P < 0·05$, $P < 0·01$, $P < 0·001$, respectively) from the values obtained with the unsupplemented ration.

to the concentrate mixture increased the concentration and yield of only 18:1 in the milk fat. When the cows were given the concentrate mixture supplemented with the saturated fatty acids there were increases in the concentrations and yields of 16:0 and 18:1 and decreases in the concentrations and yields of 14:0 and 10:0 and in the concentration of the short-chain fatty acids (4:0 to 8:0) in the milk fat.

The concentrations of total steam-volatile fatty acids and the relative proportions of the individual steam-volatile fatty acids in the rumen liquor of the cows during the last 2 days of each experimental period are given in Table 6. The proportion of acetic acid in the total rumen volatile fatty acids was decreased and the proportion of propionic acid was increased when the cows were given the concentrate mixture containing 5% 'oleic acid'. Similar but more pronounced effects on the relative proportions of acetic and propionic acids were observed when the cows were given the concentrate mixture containing 10% 'oleic acid' and the concentration of total volatile fatty acids in the rumen liquor was significantly decreased. There was a small but significant increase in the relative proportion of propionic acid in the total rumen volatile fatty acids when the concentrate mixture supplemented with the saturated fatty acids was given.

DISCUSSION

Although the addition of 5% 'oleic acid' to the concentrate mixture given to the cows did not significantly affect the yield of total milk fat, there were decreases in the yields of the fatty acids from 4:0 to 16:0 and increases in the yields of stearic and oleic acids in the milk fat. The decreased yields of the fatty acids that are known to be synthesized in the mammary gland from acetate and β-hydroxybutyrate may have been related to the fact that the addition of 5% 'oleic acid' to the concentrate mixture decreased the proportion of acetic acid and increased the proportion of propionic acid in the rumen liquor. However, these effects of the concentrate mixture containing 5% 'oleic acid' on the concentrations of rumen volatile fatty acids, although significant, were very small. It is possible that the increased uptake of C_{18} fatty acids by the mammary gland decreased the rate of the *de novo* synthesis of fatty acids from acetate and β-hydroxybutyrate (Moore & Steele, 1968). The addition of 10% 'oleic acid' to the concentrate mixture given to the cows resulted in a decrease in the yield of total milk fat, pronounced decreases in the yields of the fatty acids from 4:0 to 16:0 and an increase in the yield of oleic acid in the milk fat. Under these dietary conditions it seems more reasonable to associate the decreased yields of the fatty acids from 4:0 to 16:0 in the milk fat with the marked decrease in the concentration of acetic acid and the marked increase in the concentration of propionic acid that occurred in the rumen liquor. The addition of the mixture of saturated fatty acids to the concentrate mixture given to the cows increased the yield of total milk fat but it had little effect on the concentrations of volatile fatty acids in the rumen liquor. Nevertheless, there were decreases in the yields of certain of the milk fatty acids that are derived from *de novo* synthesis from acetate and β-hydroxybutyrate in the mammary gland.

The palmitic acid secreted in the milk fat of ruminants may be derived either from the *de novo* synthesis in the mammary gland from acetate and β-hydroxy-

butyrate or from the triglycerides that circulate in the blood as chylomicrons and low-density lipoproteins (Annison, Linzell, Fazakerley & Nichols, 1967; Barry, Bartley, Linzell & Robinson, 1963). Storry, Rook & Hall (1967) found that when the diet of cows was supplemented with red palm oil (containing 40% palmitic acid and 57% total C_{18} fatty acids) the yields of total milk fat and of stearic and oleic acids were increased, but the yield of palmitic acid in the milk fat was unaltered. In agreement with these findings, Steele & Moore (1968*a*) observed that the addition of cottonseed oil (containing 21% palmitic acid and 76% total C_{18} fatty acids) or tallow (containing 30% palmitic acid and 60% total C_{18} fatty acids) to the diet of cows increased the yields of stearic and oleic acids in the milk fat but did not alter the yield of palmitic acid. In further experiments, Steele & Moore (1968*b*) found that the addition of cottonseed oil to the diet of cows could even decrease the yield of palmitic acid in the milk fat. In order to explain their findings, Storry *et al.* (1967) suggested that palmitic acid may be elongated to stearic acid, which would then be desaturated to oleic acid in the mammary gland. However, there is no evidence that stearic acid can be synthesized from palmitic acid in the mammary gland (Annison *et al.* 1967). In the experiment now reported the addition of the mixture of saturated fatty acids (containing 64% palmitic acid and 34% total C_{18} fatty acids) to the diet increased the yield of palmitic and oleic acids in the milk fat. If, as seems likely, the fatty-acid composition of the diet is reflected in the fatty-acid composition of the triglycerides in the chylomicrons and low-density lipoproteins of the blood, the blood triglycerides that are utilized for milk triglyceride synthesis in cows given diets supplemented with tallow, for example, will contain relatively high concentrations of C_{18} fatty acids and relatively low concentrations of palmitic acid. Under these dietary conditions relatively large amounts of C_{18} fatty acids and relatively small amounts of palmitic acid will be taken up from the blood for milk fat synthesis. If the increased uptake of long-chain fatty acids from the blood inhibits the *de novo* synthesis of palmitic acid in the mammary gland (Moore & Steele, 1968), then the net effect on the total yield of palmitic acid in the milk fat will depend on whether the increased uptake of palmitic acid from the blood is sufficient to offset the decrease in the *de novo* synthesis of palmitic acid in the gland. Thus, when relatively large amounts of total C_{18} fatty acids and relatively small amounts of palmitic acid are taken up from the blood (as in cows given supplementary red palm oil, tallow or cottonseed oil) there may be no change, or even a decrease in the total output of palmitic acid in the milk fat. When relatively large amounts of palmitic acid and relatively small amounts of C_{18} fatty acids are taken up from the blood (as in cows given a mixture of fatty acids containing 64% palmitic acid and 34% total C_{18} fatty acids), the total output of palmitic acid in the milk fat may be increased even though the *de novo* synthesis of palmitic acid in the mammary gland is decreased.

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The effects of a series of saturated fatty acids in the diet on milk-fat secretion in the cow

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SUMMARY. The effects of the isocaloric replacement of part of the dietary concentrate mixture by 5% lauric acid (99% pure) or by 10% of myristic (95% pure), palmitic (96% pure) or stearic (94% pure) on the yield and composition of milk fat was investigated in 2 feeding experiments with a total of 10 cows in mid-lactation. The concentrate mixtures were given with a high-roughage diet that supplied 9·1 or 7·7 kg hay/day in expts 1 and 2, respectively.

In expt 1 the inclusion of myristic acid in the diet decreased the yields of milk and solids-not-fat (SNF), but increased the percentage of fat in the milk without altering the fat yield. When stearic or palmitic acid was included in the diet there was an increase in milk fat yield; palmitic acid caused the greater increase. In expt 2 the inclusion of lauric acid in the diet did not appear to affect the yields of milk or SNF but it caused large reductions in the percentage of fat in the milk and in the yield of fat.

In expt 1 the inclusion of myristic acid increased the yields and percentages of 14:0, 14:1 and 16:1 in the milk fat and decreased the yields and percentages of the short-chain fatty acids (4:0–8:0), 16:0, 18:0 and 18:1. When palmitic acid was included in the diet there were increases in the yields and percentages of 16:0 and 16:1 in the milk fat and decreases in the yields and percentages of 10:0, 12:0, 14:0, 18:0 and 18:1. The inclusion of stearic acid in the diet increased the yields and percentages of 18:0 and 18:1 in the milk fat and decreased the yields and percentages of 12:0 and 16:0. In expt 2 the inclusion of lauric acid in the diet increased the yield and percentage of 12:0, and in 1 cow the content of 14:0 in the milk fat; there were reductions in the yields and percentages of all the other fatty acids in the milk fat. There was no evidence of any marked elongation of the carbon chains of 12:0 and 14:0 in the mammary gland to form 16:0 or 18:0.

In expt 1 the incorporation of myristic acid in the diet decreased the concentration of total steam-volatile fatty acids in rumen liquor. The inclusion of either myristic, palmitic or stearic acid in the diet had little effect on the relative proportions of the individual volatile acids in the rumen liquor. In expt 2 the addition of lauric acid to the diet reduced the acetic acid:propionic acid ratio in the rumen liquor.

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Storry, Rook & Hall (1967) found that when cows were given diets supplemented with red palm oil (containing 40% palmitic acid and 57% total C_{18} fatty acids) the yields of stearic and oleic acids in the milk fat were increased, but the yield of palmitic acid was unaltered. To explain these results Storry *et al.* (1967) suggested that dietary palmitic acid may be elongated to stearic acid which would then be desaturated to oleic acid in the mammary gland. Steele & Moore (1968*c*) observed that when a mixture of fatty acids containing 64% palmitic acid and 31% stearic acid was included in the diet of cows, the yields of both palmitic acid and stearic acid in the milk fat were increased and there was no evidence of chain-elongation of palmitic to stearic acid. To investigate this problem further, experiments have been carried out in which the diet of lactating cows was supplemented with a series of relatively pure saturated fatty acids. It was originally intended to compare the effects of dietary lauric, myristic, palmitic or stearic acids all in 1 experiment, but a preliminary investigation indicated that cows would tolerate only limited amounts of lauric acid in the diet. Therefore, 2 separate experiments were carried out. In one of them an investigation was made of the effects of concentrate mixtures containing myristic, palmitic or stearic acids in the diet of cows on the yield and composition of the milk fat. In the other, the effects of dietary lauric acid were studied separately. The results of these investigations are now reported.

EXPERIMENTAL

Experimental animals

Eight cross-bred Friesian cows that had recently passed the peak of lactation were used in expt 1. Two pure-bred Friesian cows in the eighth month of lactation were used in expt 2. The management of the cows was the same as that described for a previous experiment (Steele & Moore, 1968*c*).

Experimental diets

Expt 1. The cows were given 9·1 kg/day of a mature ryegrass hay in the long form. The composition (percentages of the dry matter) of the hay was as follows: crude protein, 12·6; crude fibre, 31·2; ether extract, 1·5; ash, 6·6; nitrogen-free extractives, 48·1. Four different concentrate mixtures were given to the cows. The basal, low-fat concentrate mixture contained barley, decorticated groundnut meal, bran, molasses and minerals. The 3 high-fat concentrate mixtures were compounded by isocalorically

Table 1. *Compositions (weight percentages of the total) of the fatty acids incorporated in the concentrate mixtures in expts* 1 *and* 2

Fatty acid†	Lauric acid	Myristic acid	Palmitic acid	Stearic acid
12:0	98·6	1·6	—	—
14:0	0·2	94·6	1·5	—
16:0	1·2	1·3	96·4	6·0
16:1	—	0·1	—	—
18:0	—	0·4	1·6	94·0
18:1	—	2·0	0·5	—

† Shorthand designation of Farquhar, Insull, Rosen, Stoffel & Ahrens (1959).

replacing part of the barley with either myristic, palmitic or stearic acids. The dietary fatty acids were incorporated at the level of 10% of the concentrate mixture. The compositions of the added fatty acids (obtained from Harrington Bros Ltd, Balham, London, S.W. 12) are given in Table 1. The mean daily intakes of the various dietary components on the 4 dietary treatments are given in Table 2.

Expt 2. Throughout the experiment, both cows were given 7·7 kg/day of the same hay that was used in expt 1, and during the control period they were given 7·7 kg/day of the low-fat concentrate mixture that was given to the cows in expt 1. As stated previously, diets in which lauric acid constituted 10% of the concentrate mixture were found to be unpalatable to the cows. Hence, during a change-over period, the low-fat concentrate mixture was gradually, but only partially, replaced by a concentrate mixture containing 10% lauric acid. The composition of the concentrate

Table 2. *Mean daily intakes (kg) of hay and of each of the constituents of the concentrate mixtures in expt* 1

	Unsupplemented ration	Ration containing fatty acids
Hay	9·1	9·1
Barley	4·6	2·1
Decorticated extracted groundnut meal	1·7	2·1
Bran	0·9	0·9
Molasses	0·4	0·4
Minerals	0·3	0·3
Myristic, palmitic or stearic acids	—	0·6

mixture containing 10% lauric acid was similar to that of the high-fat concentrates given to the cows in expt 1. It was found, however, that the maximum concentration of lauric acid that the cows would tolerate in the whole concentrate mixture was 5·3%. Thus, during the experimental period, the cows were given 3·6 kg/day of the low-fat concentrate and 4·1 kg/day of the concentrate mixture containing 10% lauric acid. The composition of the lauric acid (also obtained from Harrington Bros Ltd) is given in Table 1.

Experimental design

Expt 1. The 8 cows were randomly assigned to the treatment sequences of two 4×4 Latin squares. Each square was balanced for carry-over effects. The duration of each experimental period was 20 days and the change-over between treatments was always abrupt.

Expt 2. During the control period (days 1–8) the 2 cows were given the low-fat concentrate mixture and, during the change over period (days 9–12), the concentration of lauric acid in the concentrate mixture was gradually increased. During the experimental period (days 13–20) the cows were given the concentrate mixture containing 5·3% lauric acid.

Sampling and methods of analysis

The sampling of the milk and the methods used in its analysis were the same as those reported previously (Steele & Moore, 1968*a*, *b*). Samples of rumen contents

were taken by stomach tube on the last 2 days of each treatment period and were analysed for steam-volatile fatty acids as described by Steele & Moore (1968*b*).

Statistical treatment of results

The results obtained in expt 1 were analysed statistically by the methods outlined by Cochran & Cox (1957).

RESULTS

Expt 1. The low-fat concentrate and the concentrate mixtures containing palmitic or stearic acids were consumed within 1 h of being given to the cows. The concentrate mixture containing myristic acid, although completely consumed, was eaten only slowly during the day. Since certain of the treatment effects were not evident in the early parts of the experimental periods, the results given in Tables 3–5 are mean values for the last 4 days of each period.

Table 3. *Mean daily yields of milk and the composition of the milk produced during the last 4 days of each period in expt 1*

	Treatments				
		Ration containing:			
	Unsupplemented ration	Myristic acid	Palmitic acid	Stearic acid	S.E.M.
Milk yield, kg	12·2	9·9***	11·8	12·7	±0·57
Milk fat yield, g	404	400	492**	459*	±26·4
Milk fat content, %	3·31	4·04***	4·17***	3·61	±0·183
SNF yield, kg	1·04	0·79***	0·98	1·08	±0·050
SNF content, %	8·52	7·98***	8·31*	8·50	±0·088

*, **, ***, Significantly different ($P < 0·05$, $P < 0·01$, $P < 0·001$, respectively) from the values obtained with the unsupplemented ration.

Table 4. *Mean weight percentages of the major fatty acids in the milk fat produced during the last 4 days of each period in expt 1*

	Treatments				
		Ration containing:			
Fatty acids†	Unsupplemented ration	Myristic acid	Palmitic acid	Stearic acid	S.E.M.
4:0–8:0	7·0	5·3***	5·6***	6·9	±0·22
10:0	0·5	0·3	0·1**	0·5	±0·15
12:0	2·1	1·6	0·9***	1·2***	±0·22
14:0	11·4	31·6***	6·3*	9·2	±2·21
14:1	0·5	2·4***	0·4	0·2	±0·30
16:0	38·7	31·8***	60·7***	27·7***	±1·57
16:1	1·3	2·1**	3·5***	1·2	±0·23
18:0	10·1	5·5***	4·3***	18·7***	±0·86
18:1	21·3	15·3***	14·2***	30·1***	±1·02
18:2+18:3	2·9	1·8**	1·7**	1·4***	±0·38

*, **, ***, Significantly different ($P < 0·05$, $P < 0·01$, $P < 0·001$, respectively) from the values obtained with the unsupplemented ration.

† Shorthand designation of Farquhar *et al.* (1959).

The mean values for the yield and composition of the milk produced by the cows during the last 4 days of the experimental periods are given in Table 3. When

myristic acid was included in the concentrate mixture given to the cows there were pronounced decreases in the yields of milk and SNF and in the percentages of SNF in the milk. There was an increase in the percentage of fat in the milk but no change in the yield of milk fat. The inclusion of palmitic or stearic acid in the diet increased the yield of milk fat, but an increase in the percentage of fat in the milk was observed only when the cows were given the diet containing palmitic acid. The percentage of SNF in the milk was decreased when the cows were given the diet containing palmitic acid.

Table 5. *Mean yields (g/day) of the major fatty acids in the milk fat produced during the last 4 days of each period in expt 1*

	Treatments				
		Ration containing:			
Fatty acids†	Unsupplemented ration	Myristic acid	Palmitic acid	Stearic acid	S.E.M.
4:0–8:0	26·6	19·9***	25·9	29·8	±1·59
10:0	1·9	1·1	0·5*	2·2	±0·60
12:0	8·0	6·0*	4·2***	5·2**	±1·01
14:0	43·3	118·8***	29·1*	39·7	±9·51
14:1	1·9	9·0***	1·9	0·9	±1·42
16:0	147·0	119·6***	280·7***	119·5***	±7·98
16:1	4·9	7·9*	16·2***	5·2	±1·19
18:0	38·4	20·7***	19·9***	80·7***	±3·79
18:1	80·9	57·5***	65·6***	129·9***	±4·31
18:2+18:3	11·0	6·8**	7·9*	6·0***	±1·29

*, **, ***, Significantly different ($P < 0·05$, $P < 0·01$, $P < 0·001$, respectively) from the value obtained with the unsupplemented ration.

† Shorthand designation of Farquhar *et al.* (1959).

The fatty-acid compositions of the milk fat produced by the cows during the last 4 days of each experimental period are given in Table 4 and the yields of individual fatty acids in the milk fat are given in Table 5. The inclusion of myristic acid in the diet given to the cows increased the concentrations and yields of 14:0, 14:1 and 16:1 in the milk fat, but significantly decreased the concentrations and yields of all the other milk fatty acids except 10:0. When palmitic acid was included in the concentrate mixture given to the cows the concentrations and yields of 16:0 and 16:1 in the milk fat were significantly increased and, apart from the concentration and yield of 14:1 and the yield of the short-chain fatty acids (4:0–8:0), the concentrations and yields of all the other milk fatty acids were decreased. When the concentrate mixture containing stearic acid was given to the cows there were increases in the concentrations and yields of 18:0 and 18:1 in the milk fat. The concentrations and yields of 12:0, 16:0 and the C_{18} polyunsaturated fatty acids in the milk fat were decreased.

The concentrations of total steam-volatile fatty acids and the relative proportions of the individual volatile fatty acids in the rumen liquor of the cows during the last 2 days of each experimental period are given in Table 6. The effects of the various dietary fatty acids on the volatile fatty acids in the rumen liquor were small. When the diet contained myristic acid, the concentration of total volatile fatty acids in the rumen liquor was decreased and the relative proportion of propionic acid in the total acids was increased. An increase in the relative proportion of

propionic acid was also observed when the cows were given the diet containing stearic acid. The inclusion of either palmitic or stearic acids in the concentrate mixture given to the cows resulted in a decrease in the relative proportion of *n*-butyric acid in the rumen liquor.

Table 6. *Mean concentrations (m-equiv./100 ml) of total volatile fatty acids and the mean relative proportions (molar percentages of the total) of the individual volatile fatty acids in the rumen liquor of the cows during the last 2 days of each period in expt 1*

	Treatments				
		Ration containing:			
	Unsupplemented ration	Myristic acid	Palmitic acid	Stearic acid	S.E.M.
Total volatile fatty acids (m-equiv./100 ml) ...	8·33	7·40*	8·04	7·71	±0·37
Proportion of individual volatile fatty acids (molar percentages of the total)					
Acetic	65·6	63·7	65·7	65·5	±1·44
Propionic	16·1	19·9*	18·2	19·2*	±1·27
iso-Butyric	1·7	1·4	1·6	1·3	±0·19
n-Butyric	13·8	12·2	11·6*	11·5*	±0·89
iso-Valeric	1·5	1·6	1·6	1·5	±0·25
n-Valeric	1·4	1·4	1·3	1·1	±0·30

*, Significantly different ($P < 0·05$) from the values obtained with the unsupplemented ration.

Table 7. *Mean daily yields of milk and the composition of the milk produced by the cows in expt 2*

		Treatments				
		Control period (unsupplemented ration)		Change-over period	Experimental period (ration containing lauric acid)	
	Cow no.	Days 1–4	Days 5–8	Days 9–12	Days 13–16	Days 17–20
Milk yield, kg	1	11·8	13·1	14·1	13·5	14·1
	2	12·0	13·0	10·8	10·9	12·8
Milk fat yield, g	1	437	491	487	379	353
	2	424	456	421	328	332
Milk fat content, %	1	3·70	3·75	3·45	2·81	2·50
	2	3·53	3·51	3·90	3·01	2·59
SNF yield, kg	1	0·95	1·07	1·13	1·12	1·13
	2	0·99	1·10	0·90	0·86	1·04
SNF content, %	1	8·05	8·17	8·01	8·30	8·01
	2	8·25	8·46	8·33	7·89	8·13

Expt 2. During the experimental period (days 13–20) the concentrate mixture containing 5·3% lauric acid was eaten slowly, but completely, during the day. The values for the yield and composition of the milk produced by individual cows during each 4-day period are given in Table 7. There was no indication that the inclusion of lauric acid in the diet given to the cows resulted in any consistent change in the yield of milk or SNF. However, when the cows were given the diet containing lauric acid, there were pronounced decreases in both the percentage and yield of fat in

the milk. The fatty-acid composition of the milk fat and the yields of individual milk fatty acids produced by each cow during the last 4 days of the control and experimental periods are given in Table 8. The inclusion of lauric acid in the concentrate mixture given to the cows resulted in a pronounced increase in the concentration of 12:0 in the milk fat. There was also an indication that the concentration of 14:0 in the milk fat was slightly increased when the diet was supplemented with lauric acid.

Table 8. *Mean weight percentages and daily yields of the major fatty acids in the milk fat produced by the cows during the last* 4 *days of the control period* (*days* 5–8) *and experimental period* (*days* 17–20) *in expt* 2

	Cow no. 1				Cow no. 2			
	Unsupplemented ration		Ration containing lauric acid		Unsupplemented ration		Ration containing lauric acid	
Fatty acids†	Weight percentage	Yield g/day	Weight percentage	Yield g/day	Weight percentage	Yield g/day	Weight percentage	Yield g/day
4:0–8:0	7·2	33·2	5·0	16·6	7·2	30·9	5·0	15·6
10:0	0·5	2·3	0·3	1·0	0·5	2·1	0·4	1·2
12:0	1·4	6·5	12·7	42·1	1·3	5·6	11·4	35·6
14:0	9·2	42·5	12·4	41·1	12·7	54·4	13·5	42·1
16:0	35·2	162·0	33·5	111·0	38·6	165·0	36·3	113·0
18:0	10·6	48·9	7·0	23·2	7·5	32·1	5·3	16·5
18:1	24·6	113·0	21·4	71·0	20·5	87·9	18·5	57·7
18:2+18:3	3·9	18·0	2·0	6·6	3·9	16·7	3·2	10·0

† Shorthand designation of Farquhar *et al.* (1959).

Table 9. *Relative proportions* (*molar percentages of the total*) *of the individual volatile fatty acids in the rumen liquor of the cows during the last* 2 *days of the control period and of the experimental period*

	Cow no. 1		Cow no. 2	
Volatile acids	Unsupplemented ration	Ration containing lauric acid	Unsupplemented ration	Ration containing lauric acid
Acetic	60·1	56·0	57·4	54·0
Propionic	22·2	26·4	25·0	30·2
iso-Butyric	1·9	1·8	1·4	1·1
n-Butyric	11·9	10·3	12·1	9·2
iso-Valeric	0·6	0·9	0·6	1·1
n-Valeric	3·3	4·6	3·5	4·4

In this experiment the concentrations of total volatile fatty acids in the rumen liquor were not determined, but the relative proportions of the individual steam-volatile fatty acids in the rumen liquor of each cow during the last 2 days of the control and experimental periods are given in Table 9. When the diet containing lauric acid was given to the cows, the relative proportions of acetic and butyric acids were decreased and the relative proportions of propionic and valeric acids were increased in the rumen liquor.

DISCUSSION

The fatty acids that are present in the milk fat of the cow are derived from 2 metabolic processes. In the first of these, acetate and β-hydroxybutyrate are taken up from the blood and are then utilized as precursors for the *de novo* synthesis in the mammary gland of the fatty acids up to and including palmitic acid (cf. Peeters & Lauryssens, 1964). In the second process, triglycerides in the chylomicra and low-density lipoproteins of the blood are taken up by the mammary gland. These triglycerides are partially or completely hydrolysed and the resulting fatty acids are incorporated into new triglycerides by the mammary gland (see Barry, Bartley, Linzell & Robinson, 1963). Under normal dietary conditions, the major fatty acids present in the blood triglycerides of the cow are 16:0, 18:0 and 18:1 (Duncan & Garton, 1963), and these are the fatty acids that would be taken up by the mammary gland from the blood when the cows were given the unsupplemented concentrate mixture in expt 1. When the cows were given the diet containing myristic acid, it is reasonable to assume that 14:0 replaced a certain proportion of the 16:0, 18:0 and 18:1 in the blood triglycerides. Thus, there would be an increased uptake of 14:0 and a decreased uptake of 16:0, 18:0 and 18:1 by the mammary gland from the blood triglycerides. This could account for the 3-fold increase in the yield of 14:0 and the decreased yields of 16:0, 18:0 and 18:1 in the milk fat of the cows given the diet containing myristic acid. There was no evidence, therefore, of any substantial chain-elongation of 14:0. Although 14:1 is only a relatively minor component of milk fat, the yield of it was increased when the cows were given the diet containing myristic acid. This mono-unsaturated fatty acid was presumably Δ^9-tetradecenoic acid and was derived from the desaturation of 14:0 (Hilditch & Williams, 1964). It is interesting to note that the yield of 16:1 was also increased when the cows were given myristic acid in the diet. However, under these dietary conditions, the yield of 16:0 in the milk fat decreased so it is unlikely that the increased yield of 16:1 was due to an increased desaturation of 16:0. It seems more likely that the increased yield of 16:1 was due to chain elongation of 14:1 (Δ^9-tetradecenoic acid). If so, the increased yield of 16:1 should have been due to an increased production of Δ^{11}-hexadecenoic acid and not Δ^9-hexadecenoic acid which is normally the major hexadecenoic acid in milk fat and is derived from the desaturation of 16:0 (Hilditch & Williams, 1964). The decreased yields of the fatty acids from 4:0 to 12:0 in the milk fat observed when the cows were given myristic acid in the diet could have been due to the decreased production of volatile fatty acids in the rumen (Table 6) or to a decreased rate of the *de novo* synthesis of fatty acids in the mammary gland (Moore & Steele, 1968).

When the cows were given the diet containing palmitic acid there was presumably a marked increase in the concentration of 16:0 and decreases in the concentration of 18:0 and 18:1 in the blood triglycerides. Such alterations in the fatty-acid composition of the blood triglycerides could account for the 2-fold increase in the yield of 16:0 and the decreases in the yields of 18:0 and 18:1. There was no indication that extensive chain-elongation of 16:0 occurred. The yield of 16:1 in the milk fat was increased when the cows were given the diet supplemented with palmitic acid, but this increase in the yield of 16:1 was presumably due to an increased

synthesis of Δ^9-hexadecenoic acid. It seems unlikely that the decreased yields of the shorter chain fatty acids in the milk fat under these dietary conditions could be due to a decreased uptake by the mammary gland of acetate and β-hydroxybutyrate since increasing the amount of palmitic acid in the diet had little effect on the production of volatile fatty acids in the rumen (Table 6).

The inclusion of stearic acid in the diet of the cows undoubtedly increased the uptake by the mammary gland of 18:0 from the blood triglycerides, but it should be remembered that 18:0 is actively desaturated to *cis*-18:1 in the mammary gland. Hence, the addition of stearic acid to the diet resulted in increased yields of both 18:0 and 18:1 in the milk fat. Certain of the samples of methyl esters prepared from the milk fatty acids obtained from the cows on this dietary treatment were analysed for *cis*- and *trans*-isomers of 18:1 (Steele & Moore, 1968*b*), and it was found that the increased yield of total 18:1 in the milk fat was accounted for entirely by an increased yield of the *cis*-isomer.

It is clear from the results given in Table 5 that the enzyme system in the mammary gland that desaturates fatty acids has a much higher specificity for 18:0 than for 16:0. This difference in specificity presents an important practical drawback to the supplementation of the diet of lactating cows with high levels of palmitic acid, particularly if the milk is used for butter manufacture. It was noted that the butter produced from the milk of the cows given palmitic acid in the diet was relatively hard. In this connexion it is perhaps worth recording that one of the experimental cows produced milk fat containing over 70% of palmitic acid when given the diet containing that acid.

The results of expt 2 are not strictly comparable with those of expt 1 but it is clear that, although the addition of lauric acid to the diet of the cows markedly decreased the total yield of milk fat, it increased the percentage and yield of that acid in the milk fat. These findings support those of Hilditch & Sleightholme (1930), who reported that the addition of coconut oil (rich in 12:0) to the diet of cows increased the concentration of 12:0 in the milk fat. When the diet contained lauric acid there was an indication of a very small increase in the concentration of 14:0 in the milk fat, but it is not possible to conclude whether or not this small increase in the concentration of 14:0 was due to chain-elongation of the dietary lauric acid.

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The digestibility coefficients of myristic, palmitic and stearic acids in the diet of sheep

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SUMMARY. The apparent digestibility coefficients of the various constituents of diets containing myristic, palmitic or stearic acids have been determined in an experiment with 8 sheep.

The inclusion of myristic acid in the diet had no effect on the digestibility coefficient of total dry matter or crude protein, but it decreased the digestibility coefficients of crude fibre and nitrogen-free-extractives and increased the digestibility coefficients of fat and minerals. When palmitic acid was included in the diet there were decreases in the digestibility coefficients of total dry matter, crude fibre and nitrogen-free-extractives. The inclusion of stearic acid in the diet resulted in decreases in the digestibility coefficients of dry matter and nitrogen-free-extractives. There was an inverse relationship between the melting points and the digestibility coefficients of the added fatty acids.

The findings of this investigation are discussed in relation to the results of a previous study on the effects of these dietary fatty acids on the yield and composition of cow's milk.

In a previous investigation (Steele & Moore, 1968) it was found that the inclusion of myristic acid in the diet of cows decreased the yields of milk and solids-not-fat (SNF), but no such effects were observed when palmitic or stearic acids were incorporated into the diet of the cows. The inclusion of palmitic acid in the diet increased the yield of milk fat, but no effect on the yield of milk fat was observed when the diet was supplemented with myristic acid. When the cows were given the diet containing stearic acid, the yield of milk fat was also increased, but this increase was not as marked as when the diet contained palmitic acid. It seemed possible that certain of the results might be explained if these saturated fatty acids had different digestibility coefficients in the diets of the cows. However, little is known about the digestibilities of individual fatty acids in the diets of ruminants. Nevertheless, there is some evidence that the addition of fats to the diets of cows affects the digestibility coefficients of other dietary constituents (Lucas & Loosli, 1944; Nottle & Rook, 1963). Therefore, it was decided to determine the digestibility coefficients of

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the fatty acids and of the constituents in the diets given to the cows in the experiment described by Steele & Moore (1968). Unfortunately, facilities were not available for carrying out digestibility experiments with cows so the experiments were carried out with sheep. The results of these experiments are now reported.

EXPERIMENTAL

Experimental animals

Eight pure-bred Dorset Horn rams were used in the investigation. Each animal was 9 months old and weighed approximately 50 kg. The sheep were fitted with harness and collection bags of the conventional type used in digestibility studies. Throughout the experiment, the sheep were housed in individual metabolism cages. During the experimental periods, the daily rations of hay and concentrate were each given in 2 equal portions at intervals of 9 and 15 h. Water was given *ad lib.*

Experimental diets

Throughout the experiment, all the sheep were given a daily ration of hay that had been chopped to a length of about 5 cm so that spillage from the food containers was reduced to a minimum. Four different concentrate mixtures were given to the sheep. The basal, low-fat concentrate mixture contained barley, decorticated groundnut meal, bran, molasses and minerals. The 3 high-fat concentrates contained myristic acid (95 % pure), palmitic acid (96 % pure) or stearic acid (94 % pure). The detailed composition of the fatty acids added to the concentrate mixtures has been given by Steele & Moore (1968). The mean daily intakes (g) of the various dietary components on the low-fat treatment were: hay, 500; barley, 262; decorticated groundnut meal, 97; bran, 51; molasses, 23; minerals, 17. The mean daily intakes of these dietary components were the same on the 3 high-fat treatments but, in addition, the sheep were given 45 g/day of myristic, palmitic or stearic acids. These experimental diets were thus similar in composition to the diets given to the cows in the experiment reported by Steele & Moore (1968).

Experimental design

The 8 sheep were randomly assigned to the treatment sequences of two 4×4 Latin squares. Each square was balanced for carry-over effects. The duration of each experimental period was 20 days and the change-over between each treatment was abrupt.

Sampling and methods of analysis

The faeces excreted by each sheep during the last 10 days of each experimental period were collected quantitatively. After each daily collection of faeces had been thoroughly mixed, 10 % of each sample was taken and stored in an air-tight container at -12 °C. The remainder of the samples of faeces were then dried at 105° C to constant weight. To allow for any small day-to-day variations in the compositions of the experimental diets, daily samples of food were also taken during the last 10 days of each experimental period. The samples of food were also dried at 105 °C to constant weight. The dried samples of foods and faeces were ground in a mill to pass through a 1 mm sieve before being analysed for crude

protein, crude fibre and ash by the standard methods (Great Britain, Parliament, 1960). For comparison, the crude protein content was also determined in the samples of wet faeces that had been stored at −12 °C. The standard method for the determination of fat (Great Britain, Parliament, 1960) was found to be unsatisfactory for the purposes of the experiment. For instance, it was found that when this method was applied to food or faeces to which known amounts of fatty acids had been added, only 70–75% of the added fatty acids were recovered. The following procedure, which is an adaptation of the technique of Folch, Lees & Stanley (1957), was eventually adopted. A mixture of 15 g dried food or faeces, 75 ml water and 10 ml 5 N-HCl was heated on a water bath at 70 °C for 30 min. After the mixture had been cooled to room temperature, 75 ml methanol and 150 ml chloroform were added with shaking. The mixture was allowed to stand for 1 h and was then passed through a sintered glass filter. The insoluble residues were then re-extracted with 200 ml chloroform–methanol (2:1, v/v) and the 2 chloroform–methanol extracts were combined. The chloroform layer containing the purified lipids was separated from the aqueous methanol layer as described by Folch *et al.* (1957). The lipid content of the chloroform solution was determined gravimetrically after the solvent had been removed under reduced pressure. This method of extracting lipids from samples of food and faeces led to the recovery of 98–100% of added fatty acids.

Statistical treatment of results

The results were analysed statistically by the procedures outlined by Cochran & Cox (1957).

RESULTS

The basal diet and the diets containing palmitic or stearic acids were consumed within 1 h of being given to the sheep but the diet containing myristic acid, although completely consumed, was eaten only slowly during the day. The apparent digestibility coefficients of the diets and dietary components on the 4 feeding treatments

Table 1. *Apparent digestibility coefficients of the constituents of the experimental diets*

	Experimental diets				
		Rations containing:			
	Unsupplemented ration	Myristic acid	Palmitic acid	Stearic acid	S.E.M.
Dry matter	72·1	72·2	69·7**	69·0**	±0·83
Fat-free dry matter	73·6	71·6*	70·3**	71·0**	±0·91
Crude protein					
(1)†	70·2	69·4	71·1	70·4	±1·56
(2)†	71·6	70·8	69·8	72·1	±1·26
Crude fibre	65·9	58·5**	60·6*	62·5	±2·11
Total fat	41·4	74·6***	63·8***	46·4	±3·71
Added fatty acids	—	99·8	81·6	52·7	±7·00
Ash	39·3	46·7*	38·5	42·0	±2·61
Nitrogen-free-extractives	81·1	78·8*	78·1*	77·8**	±1·17

† (1) Values based on analysis of wet faeces. (2) Values based on analysis of dried faeces. *, **, ***, Significantly different ($P < 0·05$, $P < 0·01$, $P < 0·001$, respectively) from the values obtained with the unsupplemented ration.

are given in Table 1. To calculate the digestibility coefficients of the added fatty acids, it was assumed that the addition of myristic, palmitic or stearic acids to the diet did not affect either the digestibility of the fat contained in the basal diet or the excretion of endogenous fat in the faeces.

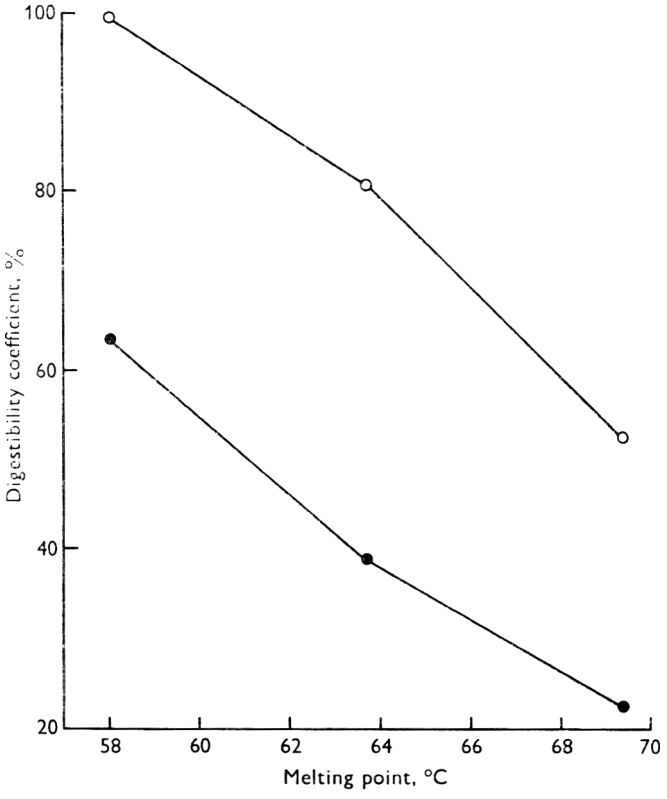

Fig. 1. Relationship between the melting points and digestibility coefficients of myristic, palmitic and stearic acids in the diets of sheep (○) and rats (●). The results obtained with rats are taken from Carroll (1958).

Although the inclusion of myristic acid in the diet did not affect the digestibility coefficient of the total dry matter, it resulted in significant decreases in the digestibility coefficients of crude fibre and nitrogen-free-extractives and significant increases in the digestibility coefficients of fat and minerals. The added myristic acid appeared to be almost completely digestible. The digestibility coefficient of total dry matter was significantly decreased when either palmitic or stearic acids were included in the diet. This decrease in the digestibility coefficient of total dry matter was associated with a significant decrease in the digestibility coefficient of nitrogen-free-extractives when the diet was supplemented with stearic acid, and with significant decreases in the digestibility coefficients of crude fibre and nitrogen-free-extractives when the diet was supplemented with palmitic acid. Stearic acid was less digestible than palmitic acid and palmitic acid was less digestible than myristic acid. There appeared to be an inverse relationship between the digestibility coefficients and the melting points of the added fatty acids (Fig. 1). The results given in Table 1 show

that similar values were obtained for the digestibility coefficients of crude protein irrespective of whether the dried faeces or the wet faeces that had been stored at −12 °C were used for analysis.

DISCUSSION

From the results of experiments in which simple triglycerides, such as trimyristin, tripalmitin and tristearin, and samples of blended and hydrogenated lards were incorporated into the diets of rats, Cheng, Morehouse & Deuel (1949) concluded that there was an inverse relationship between the melting points and the digestibility coefficients of the fats. On the other hand, in an investigation in which a series of saturated fatty acids (from butyric to behenic) and a series of monounsaturated fatty acids (from oleic to nervonic) were included in the diets of rats, Carroll (1958) could find no over-all inverse relationship between the melting points and the digestibility coefficients of the fatty acids. Only when the results for myristic, palmitic and stearic acids were considered, did Carroll (1958) observe an inverse relationship between melting points and digestibility coefficients. For comparison with our results, those obtained by Carroll (1958) for myristic, palmitic and stearic acids with rats are shown in Fig. 1. It may be seen that as the melting point of the fatty acids increased, the decrease in the digestibility coefficients obtained in the experiments with sheep was parallel to the decrease in the digestibility coefficients obtained in the experiments with rats. The differences in the absolute values for the digestibility coefficients of the fatty acids obtained from the experiments with sheep and rats may be accounted for by the fact that in the experiments of Carroll (1958), the daily intake of fatty acids was about 1·3 g/100 g body weight, whereas in our experiments with sheep, the daily intake of fatty acids was only 0·09 g/100 g body weight.

Before the results of the present experiment can be related to those of the previous investigation (Steele & Moore, 1968) it is necessary to assume that the results given in Table 1 are applicable to cows. It is not known to what extent this assumption is justified, but the present findings are consistent with the results of the few experiments that have been reported on the effects of dietary fat on the digestibility coefficients of various constituents in the diets of cows. For instance, Lucas & Loosli (1944) showed that the addition of soyabean oil or corn oil to the diet of cows resulted in decreases in the digestibility coefficients of total dry matter, crude fibre and nitrogen-free-extractives. Nottle & Rook (1963) added beef tallow to the diet of cows and observed decreases in the digestibility coefficients of fat-free dry matter and crude fibre.

In the previous experiment (Steele & Moore, 1968) the decreased yields of milk and SNF observed when the cows were given the diet supplemented with myristic acid can hardly be explained by the effects of myristic acid on the digestibilities of the various constituents of the diet. Although the addition of myristic acid to the diet of the sheep decreased the digestibility coefficient of crude fibre and nitrogen-free-extractives, the decreases in the digestibilities of these dietary constituents were no greater than when palmitic or stearic acid was added to the diet (Table 1). The addition of palmitic or stearic acid to the diet of the cows had no effect on the yields of milk and SNF (Steele & Moore, 1968). At present, no explanation can be

put forward to account for the effects of the inclusion of myristic acid in the diet of cows on the yields of milk and SNF. When the diet of cows was supplemented with palmitic acid the increase in the yield of milk fat was greater than that observed when the diet was supplemented with stearic acid (Steele & Moore, 1968). These different responses in the yield of milk fat could be explained by the differences in the digestibilities of palmitic and stearic acids. If it is assumed that, in the diets given to the experimental cows (Steele & Moore, 1968), the palmitic and stearic acids had digestibility coefficients of 81·6 and 52·7%, respectively, then it may be calculated that, when palmitic acid was included in the diet, the increased yield of palmitic acid in the milk fat was equivalent to 27% of the dietary palmitic acid that had been absorbed. When stearic acid was included in the diet, the increased yields of stearic and oleic acids in the milk fat were equivalent to 28% of the dietary stearic acid that had been absorbed. A certain proportion of the additional stearic acid taken up by the mammary gland would be dehydrogenated to oleic acid (see e.g. Peeters & Lauryssens, 1964).

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The effect of regular intravenous injections of oxytocin at milking time on the proportion of the yield obtained as residual milk in the ewe

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Summary. The changes in the size of the milking and residual fractions following milking intervals of 4, 8, 12, 16, 20 and 24 h were investigated in 18 ewes receiving regular injections of oxytocin at each milking. The observations were carried out using a 6×6 Latin square design in which each treatment interval was repeated 4 times. The milking and the residual fractions were found to increase significantly with the lengthening of the interval. The amount of hand strippings was also related to interval length. When a given interval was repeated there were no changes in the relative sizes of the fractions. A change in the milking interval, however, caused significant changes in the various fractions. It is suggested that in the present experiment the milking fractions consisted in the main of milk that was present in the sinuses prior to milking.

The relationship between the amounts of residual milk and the length of the milking interval has been described by various authors. Turner (1953, 1955) reported a positive correlation between the yield of milk and the amount of residual milk in cows. These observations were made after milking intervals of 10, 14 and 24 h. Elliott (1958), who determined the amount of residual milk and fat after milking intervals of 4, 6, 8, 12, 14 and 16 h, reported a significant increase in residual milk and fat only up to 8 h, after which there were no changes in the size of the residual fraction. Morag & Griffin (1968) found that the amount of residual milk after 16 h was twice that after 8 h. Semjan (1962), who worked with ewes, described a relationship similar to that suggested by the work of Turner (1953, 1955) and of Morag & Griffin (1968). He reported that the amounts of residual milk and residual fat increased with the lengthening of the milking interval up to 20 h and 24 h, respectively.

The regular removal of residual milk after injections of oxytocin leads to the inhibition of the milk ejection reflex both in cows (Donker, Koshi & Petersen, 1954; Morag, 1967) and in ewes (Morag & Fox, 1966). In the studies of Turner (1953, 1955), of Elliott (1958) and of Semjan (1962), several milkings elapsed between the various estimations of residual milk. Thus, it can be assumed that the relationships described by these authors were determined in animals in which the efficiency of milk ejection was similar to those in normally milked cows and ewes.

The present paper reports a study of the changes in residual milk following

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different milking intervals in ewes, in which the milk ejection reflex was severely inhibited by the regular administration of oxytocin.

MATERIALS AND METHODS

Eighteen ewes which had lambed 4–5 weeks previously and had been separated from their lambs within 6 h of parturition were used for this experiment. The animals were divided on the basis of previous milk yield into 3 yield blocks (Table 1). Within each block the ewes were randomly allocated to the 6 treatment sequences of a Latin Square (Table 2). Each treatment consisted of a particular interval (4, 8, 12, 16, 20 and 24 h) repeated 4 times. Treatments followed immediately after each other and the experiment lasted 14 days.

Table 1. *Details of experimental ewes*

Yield blocks*	Daily milk yield, ml: Mean	Daily milk yield, ml: Range	Composition of blocks according to breeds†
High yielding	2058	1958 to 2317	1-PD, 1-HB, 1-CB, 3-DCB
Medium yielding	1671	1520 to 1940	3-HB, 1-CB, 2-DCB
Low yielding	1372	1193 to 1492	4-CB, 2-DCB

All ewes had lambed between 43 and 48 days before the start of the experiment.

* Each yield block consisted of 6 ewes.

† PD, Polled Dorset Horn; HB, Half-bred (Border Leicester × Cheviot); CB, Cross-bred (Dorset Horn × Half-bred); DCB, Dorset Cross-bred (Dorset Horn × Cross-bred).

Table 2. *Experimental design (Latin square)*

	Columns*				
1	2	3	4	5	6
C	F	B	A	D	E
D	A	C	B	E	F
E	B	D	C	F	A
F	C	E	D	A	B
A	D	F	E	B	C
B	E	A	F	C	D

* The treatment members of each column do not coincide in time. Treatments were as follows: 4 milking intervals each of: A, 4 h; B, 8 h; C, 12 h; D, 16 h; E, 20 h; F, 24 h.

Milking was carried out in a herring-bone parlour using a specially constructed Alfa-Laval milking unit (see Morag, Gibb & Fox, 1967). The ewes were brought into the parlour in treatment groups and were offered concentrates in the milking stalls. The udders were not washed or otherwise stimulated, and the teat cups were applied as soon as the ewes had started to eat the concentrates. When the milk flow ceased, the udders were vigorously massaged and machine stripped. When no more strippings could be obtained 5 i.u. of oxytocin (Armour Pharmaceutical Co. Ltd.) were painlessly administered through a semi-permanent nylon cannula which had been inserted into the jugular vein prior to the experiment. The cups were not removed during the injection. When the flow of residual milk had ceased, a further 5 i.u. of oxytocin was given and the ewes were only then handstripped. The milk was collected separately for the period up to the first injection, for the period of machine stripping after the two injections and for the final hand-milking period. In this way 3 fractions were

measured at each milking; (1) a fraction ejected in response to the stimulus of milking—referred to as the *milking* fraction, and (2) and (3), fractions ejected in response to the oxytocin—referred to as the *machine residual* and *hand residual* fractions, respectively.

For 17 days before the beginning of the experiment the ewes had been given 2 injections of 5 i.u. of oxytocin before milking.

The ewes were housed on slats in a barn with a run out to a covered concrete yard. The living area was brightly illuminated throughout the experiment. The ewes were offered water, grassnuts and hay *ad lib.* between milkings and were given 3 kg/day of a concentrate mixture at milking. The daily ration of concentrates was divided into a number of meals equal to the number of milkings which the ewe received on any one day.

As an anti-coagulant 700 μg of Malayan viper (*Agkistrodan rhodostoma* (Boie)) venom was given intravenously every third day.

The experiment was carried out at the University of Reading, England, from 10 April to 11 May 1964.

RESULTS AND DISCUSSION

The yields of milk in the 3 fractions were analysed as in a split-plot design. The 6 milking intervals served as main plots and the 4 repetitions of each interval as subplots.

Table 3 gives the changes in the 3 fractions of milk with the relevant standard

Table 3. *Changes in the amounts of milk in the 3 fractions following intervals of varying lengths*

Experimental milking intervals, h (main plots)	Milking fraction, ml: Subplots (milking order)					Machine residual fraction, ml: Subplots (milking order)				
	1st	2nd	3rd	4th	Mean	1st	2nd	3rd	4th	Mean
4	16	15	17	17	16	212	236	227	239	229
8	212	249	253	253	242	280	279	317	289	291
12	467	527	483	555	508	253	288	311	279	283
16	665	564	594	637	615	379	382	374	408	386
20	699	730	725	849	751	584	471	433	365	463
24	904	854	931	898	897	484	486	514	417	468
Mean	494	490	500	534		365	352	363	333	
S.E., main plots	228					216				
S.E., subplots	21					30				

	Hand residual fraction, ml: Subplots (milking order)					Total milk, ml: Subplots (milking order)				
	1st	2nd	3rd	4th	Mean	1st	2nd	3rd	4th	Mean
4	21·3	24·6	21·9	28·9	24·2	249	276	266	285	269
8	25·7	24·5	31·0	27·8	27·3	518	553	601	570	560
12	30·9	40·9	33·1	39·7	36·2	751	856	827	873	827
16	44·4	35·6	28·9	38·8	37·0	1089	982	997	1038	1038
20	29·4	31·4	39·8	35·4	74·6	1313	1232	1198	1249	1248
24	41·1	70·6	45·1	54·7	52·8	1430	1380	1490	1369	1417
Mean	32·3	37·9	33·3	37·5		892	880	896	905	
S.E., main plots	23·2					292				
S.E., subplots	2·8					35				

errors. The data show that in ewes receiving regular injections of oxytocin, both the amounts of milk removed in response to the milking stimulus as well as those in the machine and hand residual fractions were related to interval length (see main plot values in Table 3). The proportion of the total yield obtained as the milking fraction, however, remained constant after intervals longer than 12 h. The milking fraction represented 6, 43, 61, 59, 60 and 63% of total milk in the 6 intervals in order of increasing length. The repetition of the intervals did not significantly affect the size of the fractions (see subplot values in Table 3).

Table 4. *Changes in the amounts of milk in the 3 fractions following preceding intervals of varying lengths*

Preceding interval length, h	Milking fraction, ml/h*	Machine residual, ml/h*	Hand residual, ml/h*	Total milk, ml/h
4	29·4	35·0	2·46	66·9
8	34·9	37·5	2·11	74·5
12	37·8	29·7	2·98	70·5
16	32·8	32·5	3·11	68·4
20	26·8	27·9	3·19	57·9
24	22·4	29·5	3·45	55·4
S.E.	4·4	4·5	0·56	4·0

* The yields of the fractions obtained have been divided by the lengths of the milking intervals measured in hours. They are, therefore, expressed as ml/h but these quantities should not be interpreted as rates of secretion.

The inhibition of milk ejection in the ewe by exogenous oxytocin reaches a maximum level after 6–7 days of treatment (Morag & Fox, 1966). Oxytocin had been administered for 17 days before the beginning of the present experiment, and the inhibition operated at a maximum and relatively constant level. The amounts of residual milk in the 6 columns of the Latin square were 374, 413, 341, 308, 329 and 352 ml, respectively. With the severe inhibition of milk ejection it is probable, therefore, that the milking fraction was composed mainly of milk that was present in the sinuses prior to milking. Studies carried out in the cow (Zaks, 1955) and in the goat (Tverskoi & Dyusembin, 1955) using teat cannulation techniques show that hardly any milk enters the sinuses during the first hours after milking; from the 4th hour onwards, however, there is a steady accumulation of milk in the sinuses. In the present data for the ewe the trend shown by the milking fraction, both in absolute and relative terms, is similar to that described for sinus milk in the other 2 species. This strengthens the suggestion that the milking fraction is composed in the main of sinus milk.

Zaks (1962) postulated that the passage of milk from the alveoli to the sinuses between milking is under neural control. The flow of milk into the sinuses between milkings in the present ewes did not appear to be inhibited by the exogenous hormone and this lends support to Zaks' suggestion. The movement of milk within the udder between milkings is apparently independent of posterior pituitary activity.

To measure whether, when a change in milking intervals was made, the length of the preceding interval affected the yield of various fractions in the succeeding interval, a further analysis was carried out. The results are shown in Table 4. Whereas

the repetition of any given interval caused no significant changes in the size of the fractions (see subplot means in Table 3), a change in the length of the milking interval affected the size of all 3 fractions.

The level of milk yield was maintained during the 17-day pre-experimental period and during the 14 days of the experiment. This maintenance of a constant level of production, which was independent of the advance of lactation during one month (over a third of the total lactation time in ewes) is a further indication of the galactopoietic effect of the removal of residual milk in ewes with the aid of oxytocin, reported elsewhere (Morag & Fox, 1966).

It is concluded that when milk ejection is inhibited, the size both of the milking and of the residual fractions appears to be related to the interval length. Further work, possibly employing teat cannulation techniques, is required to elucidate the effect of repeated administration of exogenous oxytocin on the efficacy of milk ejection.

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Detection of cow's milk in goat's milk by gel electrophoresis

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Hypersensitivity to cow's milk is not uncommon in humans, particularly babies and infants. Those afflicted may be found to tolerate goat's milk which, in this country, commands a considerably higher price than cow's milk. For economic as well as ethical reasons it is therefore desirable to ascertain that goat's milk offered for sale is free from admixtures of cow's milk. Tests should be sensitive to relatively minor admixtures, since even small additions of cow's milk may undo the benefit which hypersensitive subjects expect to derive from the consumption of goat's milk.

Of the testing procedures described in the literature, that of Solberg & Hadland (1953) is probably the most searching. It is based on the immunological reaction of the proteins of cow's milk with a suitable bovine antiserum. As the proteins of goat's milk respond strongly to antisera to cow's milk, each batch of such antisera has to be adsorbed with goat's milk before it can be used to detect as little as 1 % (v/v) of cow's milk in goat's milk.

Non-immunological procedures of similar sensitivity have now been developed. They are based on the observation that the α_{s1}-caseins of cow's milk (predominantly the genetic variant α_{s1}-B in European cattle) have higher electrophoretic mobilities in urea-containing alkaline gel media than the fastest-moving casein constituent of goat's milk, so that the presence of bovine α_{s1}-casein manifests itself in the appearance of a frontal band missing from the pattern of genuine goat's milk.

EXPERIMENTAL

To detect as little as 1 % (v/v) of cow's milk in goat's milk it is necessary to isolate the caseins and double their original concentration. If, however, a sensitivity level of 2–3 % is considered acceptable, milk can be examined directly by a starch-gel procedure outlined below.

Preparation of caseins. The milk sample is warmed to about 40 °C and centrifuged. Twenty ml of separated milk are measured into a 50 ml centrifuge tube, and an equal volume of hot distilled water is added to bring the temperature to about 35 °C. Two ml of 15 % (v/v) glacial acetic acid are stirred in, followed after a few minutes by 1 ml 1·5 N-sodium acetate. The tubes are then centrifuged for 5 min, the supernatant liquid decanted, the precipitates washed with distilled water, and spun down again. After removal of the wash-water, the caseins are dispersed with the aid of 4 g urea and distilled water, bringing the final volume to approximately 10 ml. The tubes are immersed in warm water to speed dispersion to a smooth, opaque solution.

Gel electrophoresis. Several procedures have been found suitable, and the choice

between them will depend largely upon the facilities available in different laboratories, and the sensitivity requirements.

(1) Polyacrylamide gel electrophoresis. The technique described for the phenotyping of the caseins of cow's milk (Aschaffenburg, 1964), when applied without modification to the caseins isolated from suspect goat's milk, will detect the presence of 1 % (v/v) of cow's milk, as shown in Plate 1. Application of this technique demands facilities for working at low temperature (4 °C). Runs can be conducted conveniently overnight.

(2) Starch-gel electrophoresis. This widely used technique was found to be equally suitable. Like the polyacrylamide technique, our procedure (Aschaffenburg & Thymann, 1965) requires cold-room facilities for phenotyping, but these can be dispensed with for the present purpose. For runs at room temperature (18–20 °C) the voltage should be reduced to a constant 200 V (corresponding to about 15 mA/gel) to avoid overheating; current should be applied until the marker has migrated to a position 10–11 cm from the origin, usually in 5–6 h. Again, as shown in Plate 2, admixture of as little as 1 % (v/v) of cow's milk can be detected.

When a lower degree of sensitivity is considered acceptable, whole milk can be applied instead of the isolated caseins, thereby, of necessity, halving the acuity of the test, since the caseins, as will be remembered, are concentrated twice in the recommended process of isolation. In theory, this reduces the limit to no more than 2 % of cow's milk, but in practice a minimum of 2·5–3 % proved to be a more realistic figure.

The tests here outlined are based on the gel electrophoretic procedures used in our particular laboratory. There is no reason to believe that other versions, of which there are many, could not be adapted equally well to the purpose of detecting the presence of cow's milk in goat's milk.

The technical assistance of Miss J. E. D. Worth is gratefully acknowledged.

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EXPLANATION OF PLATES

PLATE 1

Polyacrylamide gel electrophoresis of the caseins of goat's milk containing known additions of cow's milk. A, Application line. The arrows point to the α_{s1}-casein of cow's milk.

PLATE 2

Starch-gel electrophoresis of the casein of goat's milk containing known additions of cow's milk. A, Application line. The arrows point to the α_{s1}-casein of cow's milk.

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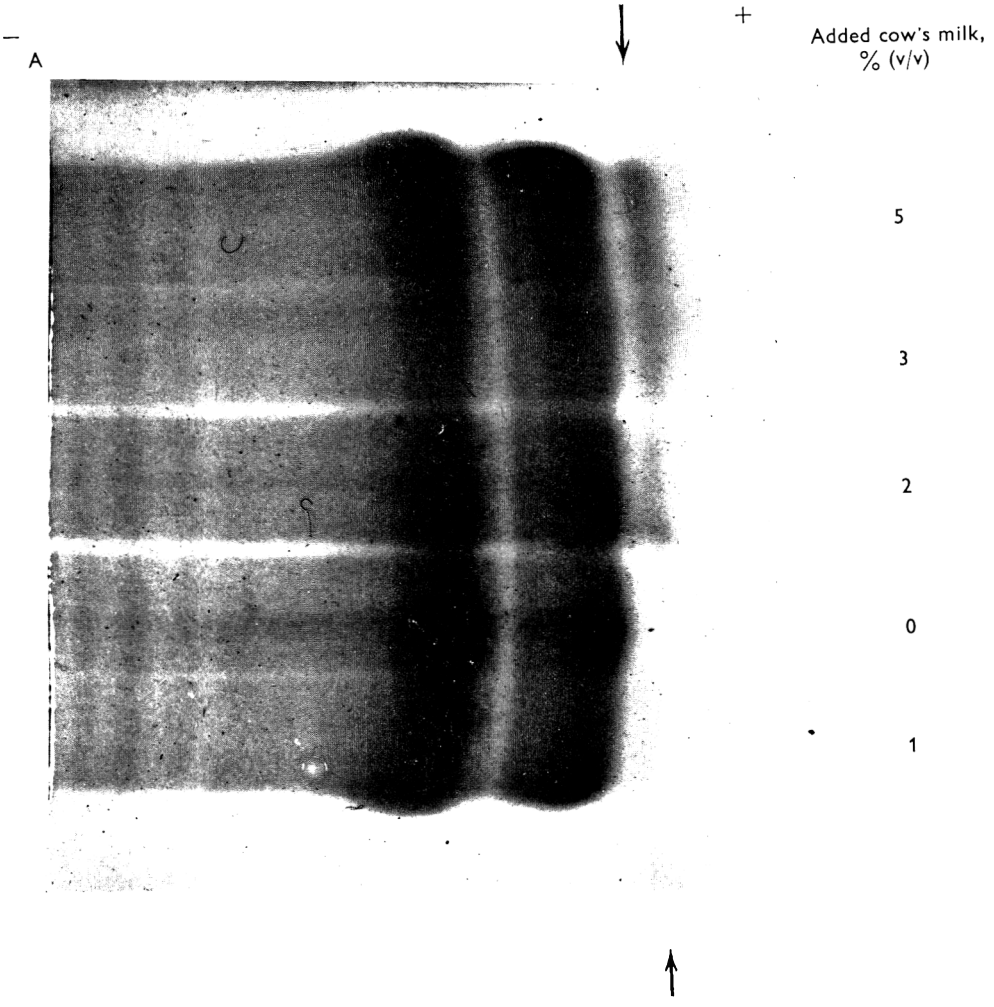

R. ASCHAFFENBURG AND J. E. DANCE

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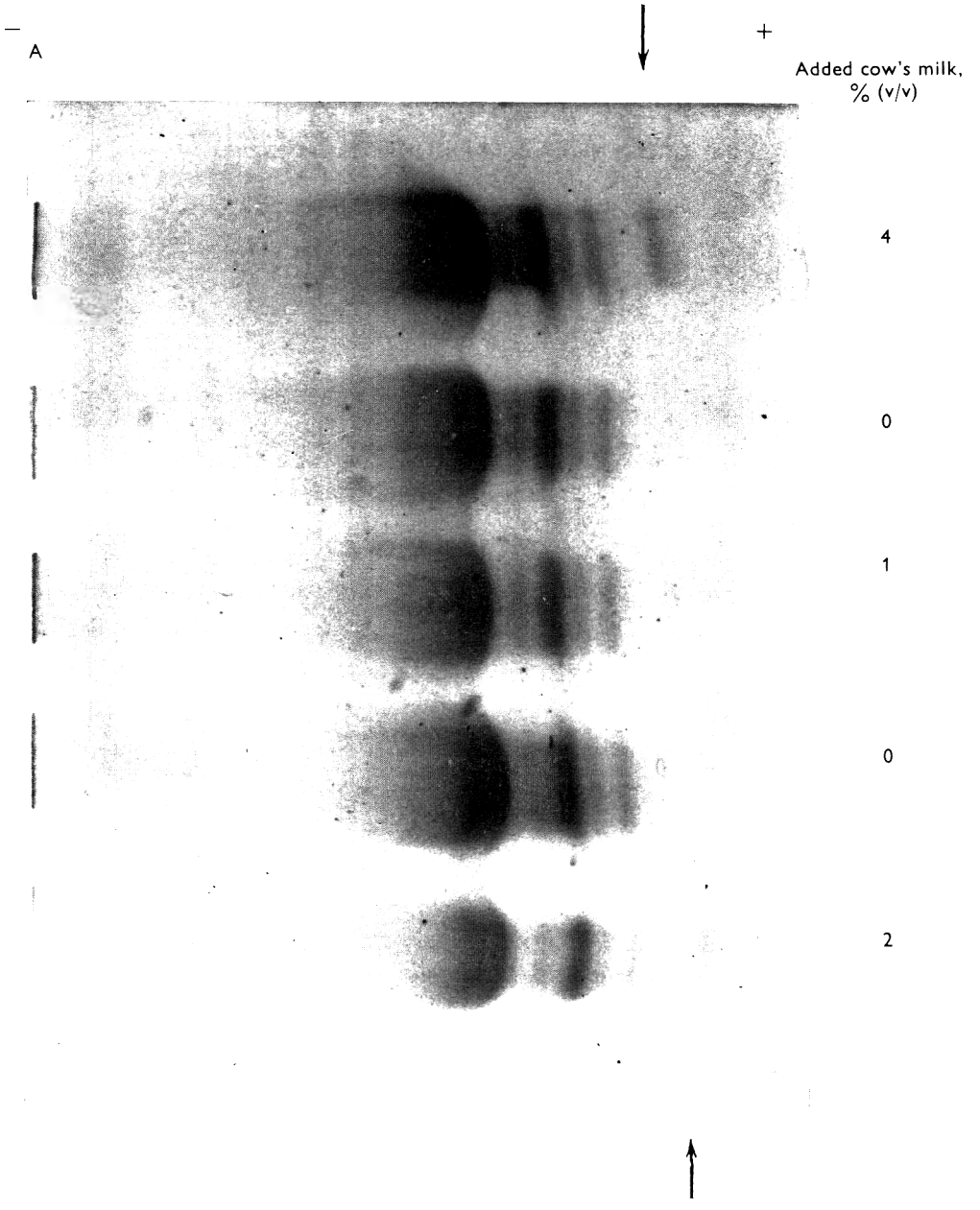
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J. Dairy Res. (1968), **35**, 385

Growth and proteinase production in *Pseudomonas* spp. cultivated under various conditions of temperature and nutrition

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(*Received* 15 April 1968)

Summary. A study was made of the formation of the extracellular proteolytic enzymes during the growth cycle of several species of *Pseudomonas* cultivated under different conditions of temperature and nutrition. Proteolytic activity was not proportional to growth. Expressed per unit of cell dry weight, the proteolytic activity showed a peak in the early logarithmic phase which was greater in cultures grown at 3 than at 28 °C. Proteolytic enzyme was not formed in the absence of organic nitrogen. Of 16 organisms studied, *Pseudomonas aeruginosa* ATCC 10145 was the most prolific producer of proteolytic enzyme.

Refrigerated storage of raw and pasteurized milk to prolong the acceptable life of the product is now a universally accepted principle in the dairy industry. One limiting factor in the storage life of milk under these conditions is the presence among contaminating microflora of psychrophilic bacteria. Undesirable changes occur in the milk stored at low temperature (0–10 °C) as a result of the activity of the latter group of microorganisms.

Extracellular proteinases have been reported in *Pseudomonas putrefaciens* (Van der Zant, 1957), *P. fluorescens* (Peterson & Gunderson, 1960), *P. myxogenes* (Morihara, 1957) and *P. aeruginosa* (Morihara, 1963).

The object of the present study was to investigate the effects of temperature and nutrition on the levels and patterns of production of proteolytic enzymes by various *Pseudomonas* spp.

Methods

Cultures. P. fluorescens CCEB 488, *P. fluorescens* ATCC 13525, *P. aeruginosa* ATCC 10145, *P. aeruginosa* ATCC 14216, *P. maltophilia* ATCC 13637, *P. fragi* ATCC 4973, *P. putrefaciens* NCIB 8615, *P. putida* CCEB 520 and *P. aureofaciens* ATCC 13985 were grown in peptone-yeast extract (P.Y.E.) broth, Koser's citrate medium (Skerman, 1959) or Koser's citrate medium with 0·25 % (w/v) Hammarsten casein. The pH value of the media was adjusted to 7·0. The inoculum for the experiments was prepared from an 18-h culture grown at 28 °C in P.Y.E. broth. The cells were obtained by centrifugation

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and resuspension in 0·85 % saline. This cell suspension was adjusted to a turbidity ($E_{540\ nm}$) of 0·4. The inoculum size was 2 % of the volume of the medium used for the different experiments.

Bacterial growth was followed by measuring the turbidity of the cultures at $E_{540\ nm}$ with a Unicam SP 600 spectrophotometer. A unit of cell dry weight was defined as the dry weight of cellular material per ml present in the culture of the E_{540} value of 1·0.

Proteinase estimation. For the estimation of proteolytic activity in the growth media the cells were removed by centrifugation at 13,000*g* for 10 min and discarded. The supernatant could be stored at 3 °C for several days without loss of activity. The estimation itself was carried out by a modification of the method of Anson (1938). The test substrate employed was 1 % (w/v) Hammarsten casein in borate buffer containing 0·01 M-$CaCl_2$ (Rick, 1963). After adjusting the pH value to 7·5, the temperatures of the buffer substrate and culture supernatant were separately adjusted to 30 °C. Five ml of the substrate were then added to a $6 \times \frac{5}{8}$ in. test tube containing 1 ml of the culture supernatant. The mixture was shaken vigorously for approximately 5 sec and incubated for 10 min at 30 °C in a water bath. Ten ml of 0·3 N-trichloroacetic acid (TCA) were then added and the precipitated undigested casein was filtered off using Whatman no. 1 filter paper. To 5 ml of the TCA filtrate were added 10 ml of 0·5 N-NaOH followed by 3 ml of the phenol reagent of Folin & Ciocalteau (1927) (B.D.H. Ltd.) diluted by addition of 2 volumes of water to 1 volume of reagent. The mixture was shaken for approximately 5 sec and after a further 2–10 min the colour density was measured at 645 nm against a blank consisting of 10 ml 0·5 N-NaOH, 5 ml 0·3 N-TCA and 3 ml diluted phenol reagent. It was found necessary to prepare for each assay an 'assay blank', in which 5 ml of the casein substrate were mixed with 10 ml 0·3 N-TCA and after filtration 1 ml of the culture supernatant was added. The procedure as described above was then followed for the colour development.

The activity was calculated by 'reading off' a standard tyrosine concentration curve and expressed in terms of equivalent amounts of tyrosine. The difference between the amounts of tyrosine present in the filtrate of the digest and of the 'assay blank' represented the proteolytic activity of the culture supernatant. It was expressed as μg tyrosine liberated per ml of culture supernatant and as μg tyrosine liberated per unit of dry cell weight.

RESULTS

A preliminary investigation was made of the growth and proteolytic activity of 6 strains of *Pseudomonas* grown for 18 h at 28 °C on 3 different media. The results are tabulated in Table 1. All 6 strains grew best in P.Y.E. broth. However, the ratio of proteolytic activity detected in the supernatant to the amount of growth in the culture varied greatly. Considering the activity per ml of supernatant of cultures grown in P.Y.E., one of the stronger-growing strains (*P. aeruginosa* ATCC 14216) exhibited the highest activity, followed closely by *P. putida* CCEB 520, which was a comparatively slow-growing organism under the conditions employed. If the comparison was made on unit cell dry weight, *P. putida* CCEB 520 revealed highest proteolytic activity. In Koser's citrate medium, *P. putida* CCEB 520, *P. maltophilia*

ATCC 13637 and *P. aeruginosa* ATCC 14216 grew best. The highest proteolytic activity per unit cell dry weight in this medium was obtained with *P. putida* CCEB 520 and the lowest with *P. aeruginosa* ATCC 14216. When casein (0·25 %, w/v) was added to the Koser's citrate medium, the picture of proteolytic activity again changed. *P. maltophilia* ATCC 13637 now exhibited the highest activity, followed by *P. fluo-*

Table 1. *Growth and proteolytic activity of* 6 Pseudomonas *strains after* 18-*h incubation in* 3 *media at* 28 °*C*

	P.Y.E. broth			Koser's citrate medium			Koser's citrate medium + 0·25% casein		
Organism	Growth*	Activity/ ml of super-natant†	Activity/ unit cell dry weight‡	Growth*	Activity/ ml of super-natant†	Activity/ unit cell dry weight‡	Growth*	Activity/ ml of super-natant†	Activity/ unit cell dry weight‡
P. maltophilia ATCC 13637	0·45	9·0	20·0	0·14	1·5	13·5	0·05	3·5	70·0
P. fluorescens ATCC 13525	0·27	0	0	0·11	1·5	13·5	0·10	3·0	30·0
P. fluorescens CCEB 488	0·36	7·5	21·0	0·07	0·5	9·0	0·07	1·0	14·5
P. aeruginosa ATCC 10145	0·46	4·5	9·5	0·17	3·0	17·5	0·11	2·0	18·0
P. aeruginosa ATCC 14216	0·42	28·0	66·5	0·11	0·5	4·5	0·06	0	0
P. putida CCEB 520	0·26	21·0	80·0	0·15	4·5	30·0	0·14	2·5	18·0

* Expressed as extinction coefficient of cultures at 540 nm.
† μg tyrosine liberated/ml culture supernatant.
‡ μg tyrosine liberated/unit cell dry weight.

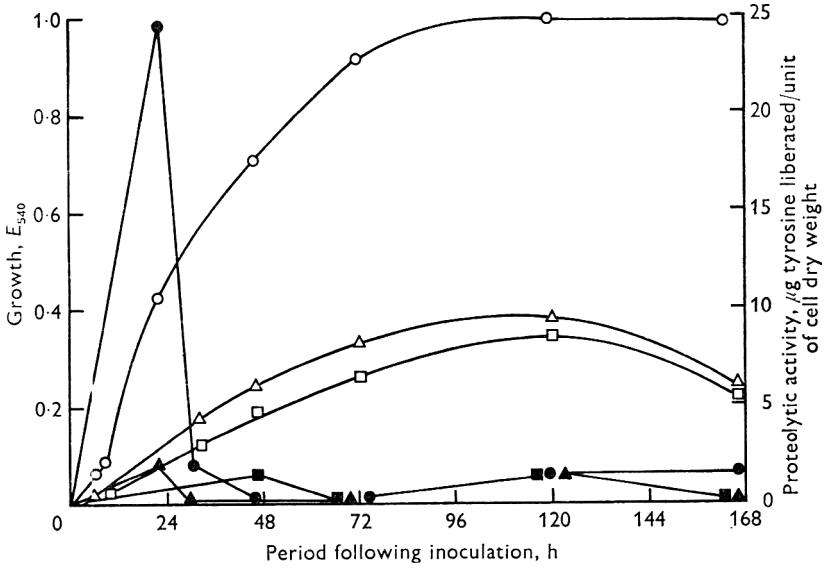

Fig. 1. Growth curves of *P. fragi* ATCC 4973 grown at 28 °C in P.Y.E. broth, Koser's citrate medium and Koser's citrate medium + 0·25% casein, and the proteolytic activity per unit of cell dry weight during the growth period in each of the media. ○, Growth and ●, proteolytic activity, P.Y.E. broth; △, growth and ▲, proteolytic activity, Koser's citrate medium; □, growth and ■, proteolytic activity, Koser's citrate medium + 0·25% casein.

rescens ATCC 13525. *P. aeruginosa* ATCC 14216 showed no activity. These investigations indicate that the proteolytic activity of *Pseudomonas* is highly dependent upon the medium employed and is not necessarily associated with good growth.

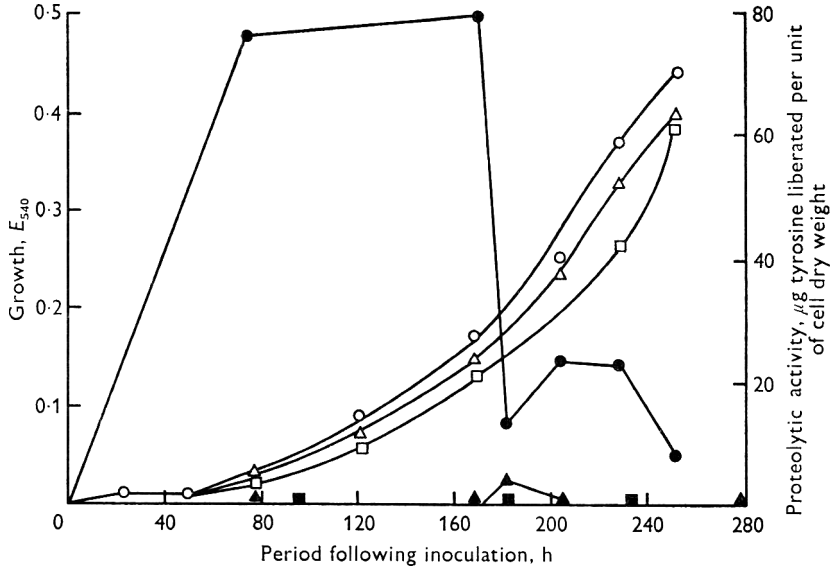

Fig. 2. Growth curves of *P. fragi* ATCC 4973 grown at 3 °C in P.Y.E. broth, Koser's citrate medium and Koser's citrate medium + 0·25 % casein, and the proteolytic activity/unit of cell dry weight during the growth period in each of the media. ○, Growth and ●, proteolytic activity, P.Y.E. broth; △, Growth and ▲, proteolytic activity, Koser's citrate medium; □, growth and ■, proteolytic activity, Koser's citrate medium + 0·25% casein.

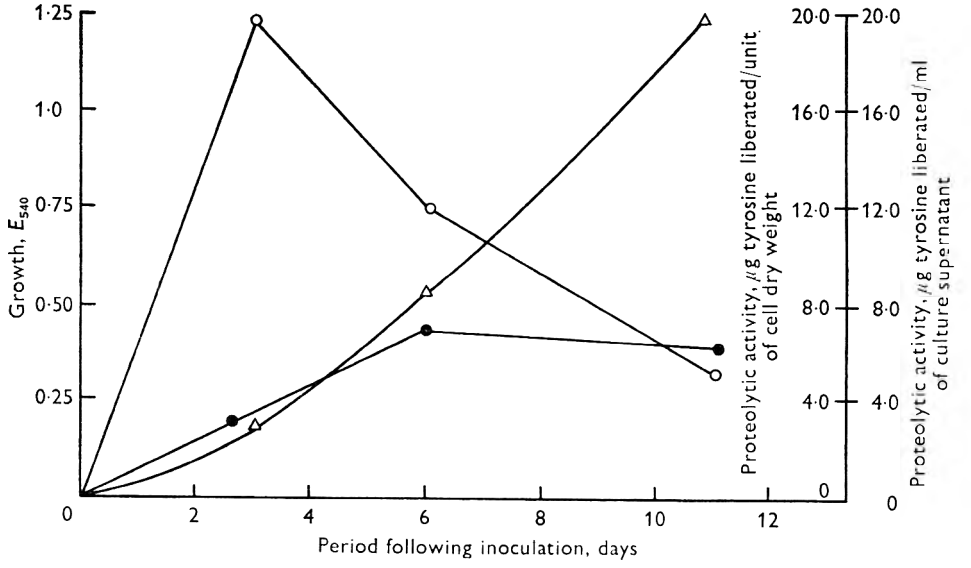

Fig. 3. Growth curve of *P. fluorescens* CCEB 488 grown in P.Y.E. broth at 3 °C, and the proteolytic activity per ml of culture supernatant and per unit of cell dry weight during the growth period. △, Growth; ○ proteolytic activity/unit of cell dry weight; ●, proteolytic activity/m of culture supernatant.

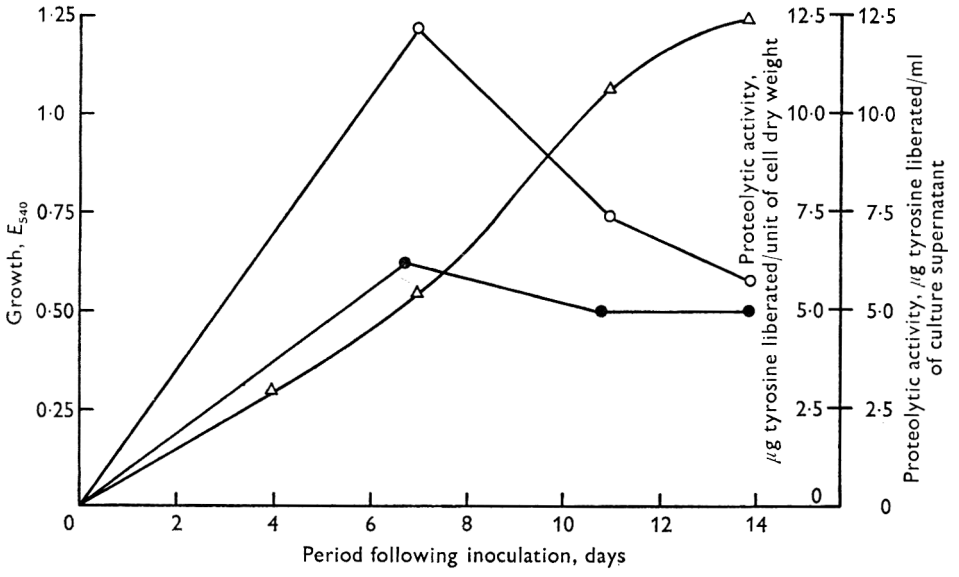

Fig. 4. Growth curve of *P. putrefaciens* NCIB 8615 grown in P.Y.E. broth at 3 °C, and the proteolytic activity per ml of culture supernatant and per unit of cell dry weight during the growth period. △, Growth; ○, proteolytic activity/unit of cell dry weight; ●, proteolytic activity/ml of culture supernatant.

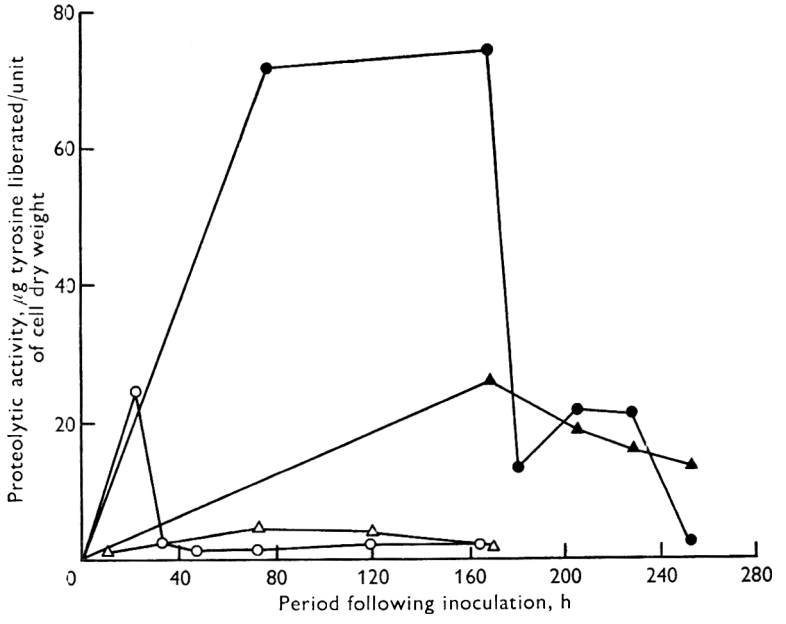

Fig. 5. Proteolytic activity per unit of cell dry weight during the growth period of *P. fragi* ATCC 4973 and *P. fluorescens* CCEB 488 at 3 and 28 °C in P.Y.E. broth. ○, *P. fragi*, 28 °C; ●, *P. fragi*, 3 °C; △, *P. fluorescens*, 28 °C; ▲, *P. fluorescens*, 3 °C.

In further experiments *P. fragi* ATCC 4973 was grown at 28 and at 3 °C in the 3 different media (see above). The growth curves and the proteolytic activity developing per unit of cell dry weight are shown in Figs. 1 and 2. At 28 °C growth in P.Y.E. broth greatly exceeded that in Koser's citrate medium. The addition of 0·25 % (w/v) casein to this latter medium caused a further slight reduction in growth. With all 3 media, but especially with the P.Y.E. broth, proteolytic activity per unit of cell dry weight showed a peak in the early logarithmic phase. Beyond this initial peak, the activity per unit cell material differed little between the 3 media and was comparatively low, with zero activity being recorded on some occasions.

For the cultures grown at 3 °C the growth curves for the 3 media were similar. A marked peak in activity per unit of cell dry weight occurred in P.Y.E. broth commencing in the lag phase and declining following the early logarithmic phase. Activity in Koser's citrate was recorded on only one occasion and this was low compared with the activity in P.Y.E. broth. Activity was not recorded in Koser's citrate in the presence of casein.

A similar experiment was conducted with *P. fluorescens* CCEB 488 and *P. putrefaciens* NCIB 8615, but only P.Y.E. broth and an incubation temperature of 3 °C were employed. The results are shown in Figs 3 and 4. With both organisms, the proteolytic activity in the culture supernatant reached a peak while the cells were in the early logarithmic phase and thereafter declined. With *P. putrefaciens* NCIB 8615, the highest activity per ml of culture supernatant and per unit of cell dry weight occurred at 6 days, while with *P. fluorescens* CCEB 488 the peak activity per ml of culture supernatant occurred at 6 days, and per unit of cell dry weight at 3 days. Maximum growth of *P. fluorescens* CCEB 488 was not attained before 11 days, and of *P. putrefaciens* NCIB 8615 not before 14 days.

Although *P. fluorescens* CCEB 488 and *P. fragi* ATCC 4973 showed very similar growth patterns, the proteolytic activity of *P. fluorescens* CCEB 488 was far lower and was detected later in the growth period when grown both at 28 and at 3 °C (Fig. 5). With both organisms, the activity per unit of cell dry weight in cultures grown at 3 °C exceeded that in cultures grown at 28 °C, throughout the growth period. With *P. fragi* ATCC 4973 the peak of activity in the early logarithmic phase at 3 °C was approximately 3 times that at 28 °C, and the peak at 3 °C for *P. fluorescens* CCEB 488 was approximately 6 times that at 28 °C. The peaks for both organisms occurred later at 3 than at 28 °C on a time-scale basis but earlier in relation to the growth curve.

A further comparison of the effect of temperature on growth and proteolytic activity was conducted with *P. aureofaciens* ATCC 13985 (Figs 6, 7). While activity per ml of culture supernatant of the 28 °C culture (Fig. 6) showed a low peak at the commencement of the logarithmic phase, activity per unit of cell dry weight showed a marked peak at the same point on the time scale. Following the early peak, activity expressed in either way continued at a very low level throughout the growth period, but when the cells reached the stationary phase a slight increase in activity occurred. In Fig. 7 a similar plot is drawn for *P. aureofaciens* ATCC 13985 grown in P.Y.E. broth at 3 °C. Although activity per ml of culture supernatant was low, a slight peak occurred in the early logarithmic phase and a higher peak in activity per unit of cell dry weight occurred at the same time.

During the preliminary investigations *P. aeruginosa* ATCC 10145 was described as being a strong producer of proteolytic activity. When this strain was grown in P.Y.E. broth at 28 °C, 2 peaks of proteolytic activity were obtained (Fig. 8). The first peak occurred at the commencement of the logarithmic phase and was only apparent if the proteolytic activity was expressed in terms of unit cell dry weight. This sharp peak in activity then decreased to parallel the activity of the culture supernatant,

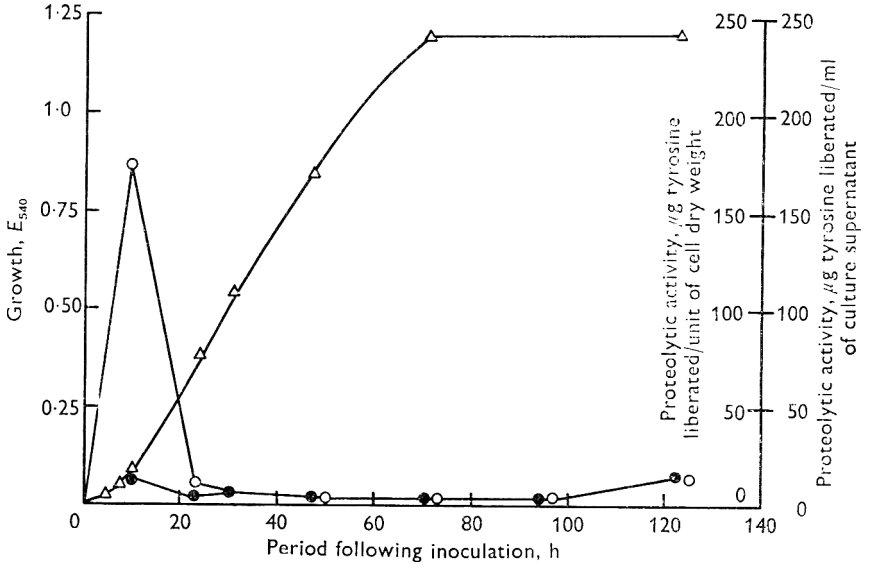

Fig. 6. Growth curve for *P. aureofaciens* ATCC 13985 grown in P.Y.E. broth at 28 °C, and the proteolytic activity per ml of culture supernatant and per unit of cell dry weight during the growth period. △, Growth; ○, proteolytic activity/unit of cell dry weight; ●, proteolytic activity/ml of culture supernatant.

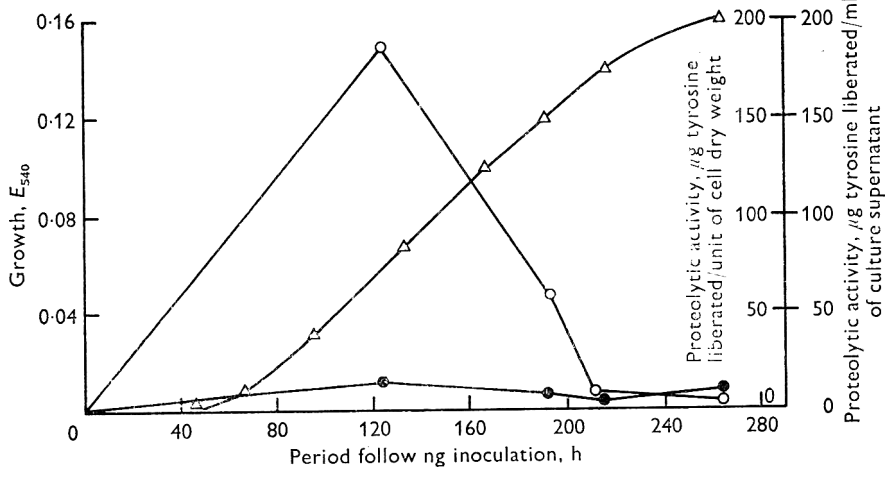

Fig. 7. Growth curve for *P. aureofaciens* ATCC 13985 grown in P.Y.E. broth at 3 °C, and the proteolytic activity per ml of culture supernatant and per unit of cell dry weight during the growth period. △, Growth; ○, proteolytic activity/unit of cell dry weight; ●, proteolytic activity/ml of culture supernatant.

which increased sharply half-way through the logarithmic phase and reached a maximum at the beginning of the stationary phase. This latter peak value was at least 7 times greater than that determined for any of the other pseudomonads, but, as it occurred at such a late stage of growth it was almost certainly due in the main to the increasing autolysis of the bacterial cells. *P. aeruginosa* ATCC 10145, however, exhibited proteolytic activity attributable to an extracellular enzyme earlier in the growth phase, which indicates the importance of expressing the proteolytic activity in relation to unit cell dry weight rather than per ml of supernatant.

Very similar results were obtained with *P. aeruginosa* ATCC 10145 on the other 2 media although the activities were only one-sixth of that shown in P.Y.E. broth.

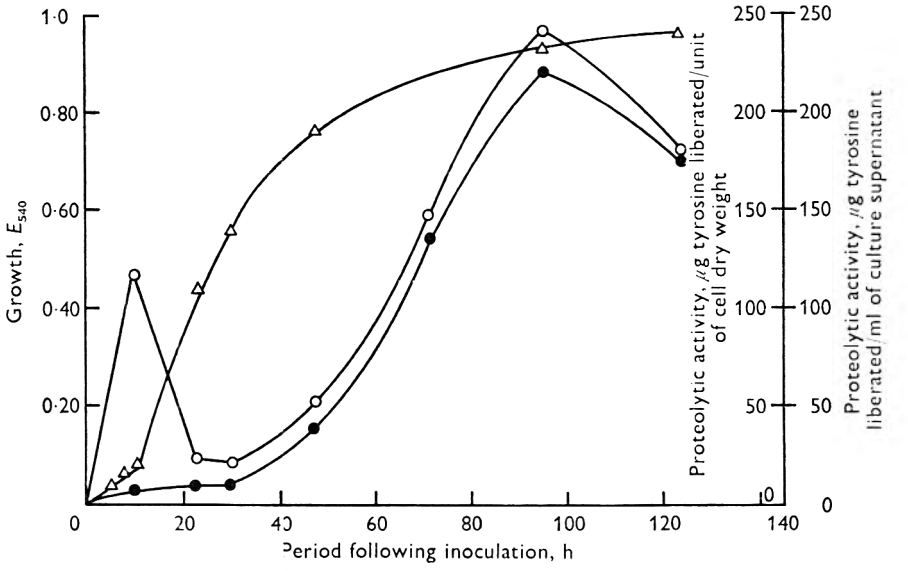

Fig. 8. Growth curve for *P. aeruginosa* ATCC 10145 grown in P.Y.E. broth at 28 °C, and the proteolytic activity per ml of culture supernatant and per unit of cell dry weight during the growth period. △, Growth; ○, proteolytic activity/unit of cell dry weight; ●, proteolytic activity/ml of culture supernatant.

DISCUSSION AND CONCLUSIONS

The liberation of the proteolytic enzyme by young cultures is one of the criteria of extracellularity (Pollock, 1962). On this basis, the extracellularity of the proteolytic enzymes was demonstrated with *P. aeruginosa* ATCC 10145, *P. putrefaciens* NCIB 8615, *P. fluorescens* CCEB 488, *P. fragi* ATCC 4973 and *P. aureofaciens* ATCC 13985. With all these organisms a decrease in the level of activity occurred after the peak was reached, but there was no evidence to provide an explanation for this phenomenon.

When the activity was expressed per unit of cell dry weight there was invariably a sharp rise to a peak of activity as the cells entered the logarithmic phase followed by an equally sharp decline as growth increased further. Thus, the cells apparently commenced proteinase elaboration at a high rate as soon as active division commenced and before any marked increase in growth was observed. Maximum elabora-

tion of proteinase soon after growth was initiated has been reported with *P. fluorescens* (Peterson & Gunderson, 1960).

P. aeruginosa ATCC 10145 exhibited 2 proteinase peaks, one early in the logarithmic phase and one in the stationary phase. It is reasonable to suggest that proteolytic activity produced in the stationary phase may be attributable to intracellular enzyme released by autolysis.

Proteolytic enzyme production per unit of cell dry weight was greater during growth at 3 than at 28 °C. This also occurred with *P. fluorescens* (Peterson & Gunderson, 1960). This effect may be the result of the increased solubility of oxygen at low temperature, but there appears to be no published evidence on this point and until more work is carried out, the effect of oxygen, if any, remains obscure.

The elaboration of extracellular proteinase appears to require the presence of organic nitrogen in the form of amino acids or peptides in the growth medium. Proteinase was not elaborated in a mineral salts medium containing inorganic nitrogen and either citrate or citrate and casein. The 'partial constitutive' theory of McDonald & Chambers (1966) for proteinase biosynthesis in which synthesis is repressed by glucose or other carbon source and induced by end-products may apply to pseudomonads, particularly to the organism *P. aeruginosa* ATCC 10145 in which the effect of nutrition on proteinase production was marked.

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Some properties of the extracellular proteolytic enzymes of the milk-spoiling organism *Pseudomonas aeruginosa* ATCC 10145

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(*Received* 15 *April* 1968)

Summary. The proteolytic enzymes present in the culture supernatant of *Pseudomonas aeruginosa* ATCC 10145 were active in the pH range 5·5–9·0 with a maximum activity at pH 7·3. Heating for 15 sec at 72 °C resulted in a 36 % loss of proteolytic activity whereas heating for 30 min at 63 °C resulted in a 6 % loss. Boiling for 2 min completely inactivated the proteolytic enzymes. At 2 °C the proteolytic enzymes were stable for at least a month and casein was readily hydrolysed at this temperature.

Proteinases elaborated during the growth of *Pseudomonas* spp. in milk are a potential hazard to the integrity of the milk proteins and their activities may bring about alterations in the physical and chemical properties and the flavour of the milk. Thus, properties of the proteinases of *Pseudomonas* spp. which may influence their activity during the handling of whole milk are of interest.

Juffs, Hayward & Doelle (1968) showed that the mesophile *P. aeruginosa* ATCC 10145 when grown in Peptone–Yeast Extract (P.Y.E.) broth produced high levels of extracellular proteinase. In the present study, the pH optima, heat stability and activity at various temperatures of the proteinases of this organism were investigated.

METHODS

P. aeruginosa ATCC 10145 was grown in P.Y.E. broth in batch culture at 28 °C. After 3 days of growth, the culture was centrifuged and the supernatant assayed for its proteolytic activity as previously described (Juffs *et al.* 1968).

For the determination of the relationship between substrate pH and proteinase activity, Hammarsten casein (1 %, w/v) in borate buffer containing 0·01 M-$CaCl_2$ (Rick, 1963) was used as substrate.

Heat was applied to the proteolytic enzymes under a variety of conditions which included simulated milk pasteurization. The supernatant was heated at 63 °C for 30 min, 72 °C for periods from 5 sec to 10 min and 100 °C for 2 min. This was done by immersing $6 \times \frac{1}{2}$ in. test tubes containing the culture supernatant in boiling water to raise its temperature as quickly as possible to one of the temperatures specified above.

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When this was reached the tube was transferred to a constant-temperature water bath set at this temperature. After holding for the required period the tube was removed and cooled in iced water.

RESULTS

A substrate pH-proteolytic enzyme activity curve was established for the proteolytic enzymes present in the supernatant of a 3-day culture of *P. aeruginosa* ATCC 10145 grown in P.Y.E. broth (Fig. 1). The proteolytic enzymes present in the supernatant were active in the pH range 5·5–9·0 with maximum activity at pH 7·3. At pH 6·8, the pH value of milk, the measured activity was 82 % of that at pH 7·3. A 36 % reduction in activity occurred when the substrate was prepared in phosphate instead of borate buffer.

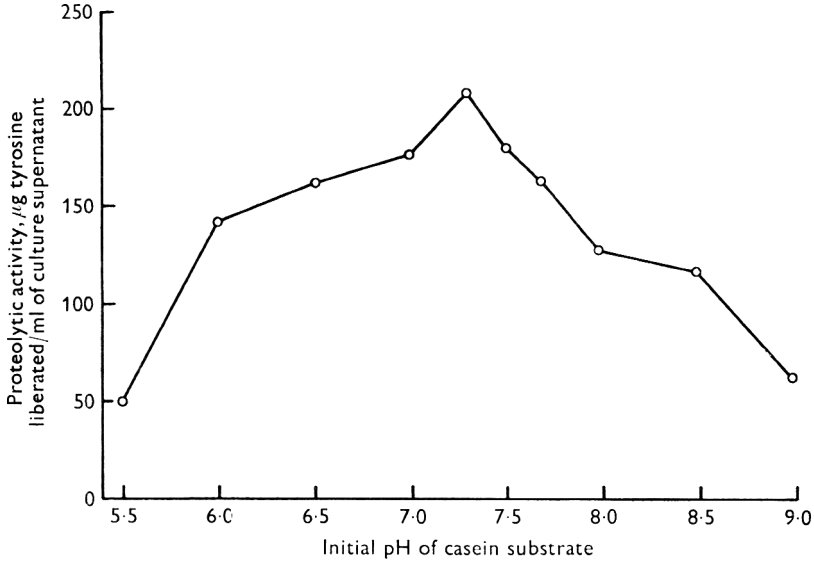

Fig. 1. Substrate pH—activity curve for the proteolytic enzyme present in the supernatant of a culture of *P. aeruginosa* ATCC 10145 grown for 3 days in P.Y.E. broth at 28 °C. The substrate was a 1 % casein solution prepared in borate buffer.

The effect of heat treatment on the stability of the proteinase present in the supernatant was determined. Heating for 15 sec at 72 °C resulted in a 36 % loss of activity, whereas heating for 30 min at 63 °C destroyed only 6 % of the proteinase activity. Boiling for 2 min destroyed the proteinase activity completely.

Investigations into the release of proteolytic activity by bacterial cells showed that heat treatment (72 °C, 15 sec) of cell suspensions in 0·85 % saline did not release proteolytic activity. Pasteurization, therefore, would result in a decrease in extracellular proteolytic activity and not in an increase due to release by the cells. On storage of the suspension at 3 °C no extracellular proteolytic activity was detected until 3 weeks had elapsed.

The sharp decrease in proteolytic activity after heating the supernatant for 15 sec at 72 °C led to studies of the loss of activity with increasing periods of heating at 72 °C for up to 10 min. The culture supernatant was heated at its original pH, 8·3,

and after adjustment of the pH to 6·8, for periods of up to 10 min. Fig. 2 shows the loss of activity (expressed as μg tyrosine liberated/ml culture supernatant) of the supernatant at these different pH values with increasing time of heat treatment. A 29 % loss of activity was associated with the adjustment of the pH value from 8·3

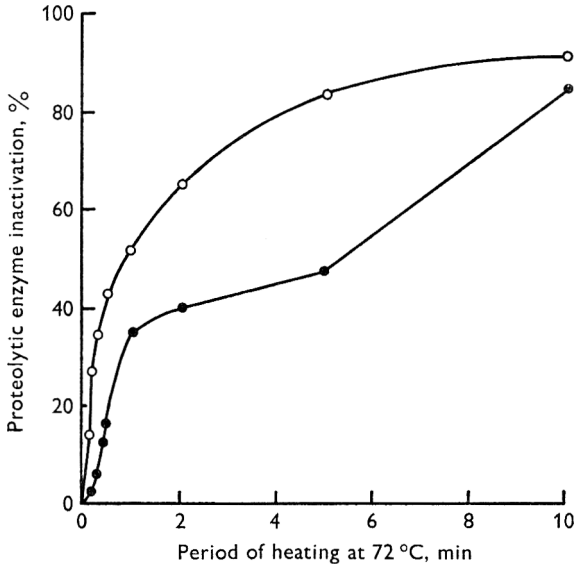

Fig. 2. The effect of period of heating at 72 °C on the stability of the proteolytic enzyme present in the supernatant of a culture of *P. aeruginosa* ATCC 10145 grown for 3 days in P.Y.E. broth at 28 °C. The pH value of the supernatant was adjusted to the levels indicated before heating. Enzyme stability is expressed as % loss of the activity present in the unheated supernatant. ○, Supernatant pH 8·3; ●, supernatant pH 6·8.

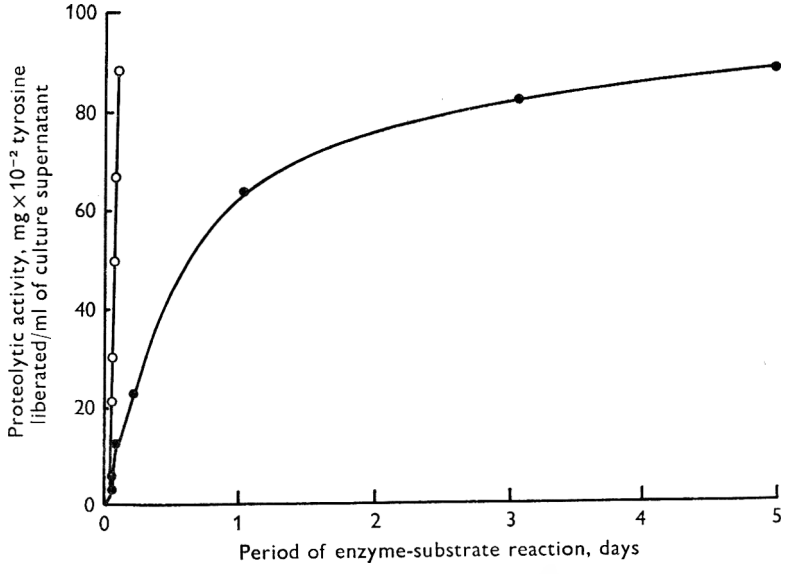

Fig. 3. The effect of the enzyme-substrate reaction temperature on the activity of the proteolytic enzymes present in the supernatant culture of *P. aeruginosa* ATCC 10145 grown for 3 days in P.Y.E. broth at 28 °C. ○, Reaction temperature, 30 °C; ●, reaction temperature, 2 °C.

to 6·8. The inactivation curves at these 2 pH levels do not follow a parallel course. From 1 to 5 min, the rate of inactivation at pH 6·8 was less than that at pH 8·3, but from 5 to 10 min the rate of inactivation at pH 6·8 was greater than that at pH 8·3.

The proteolytic activity of the supernatant using a substrate-enzyme reaction temperature of 30 °C was compared with that at 2 °C. The activity was determined after reaction for up to 2 h at 30 °C and up to 5 days at 2 °C. Fig. 3 shows the course of proteolysis at the 2 temperatures. Although the rate of reaction was less at 2 °C than at 30 °C, there was no loss of activity at 2 °C and the extent of proteolysis measured after 2 h at 30 °C was similar to that measured after 4 days at 2 °C.

DISCUSSION AND CONCLUSIONS

The substrate pH–activity curve for the proteolytic enzymes of *P. aeruginosa* ATCC 10145 (Fig. 1) corresponds closely to that reported for *P. putrefaciens* (Van der Zant, 1957) and also for *P. fluorescens* (Peterson & Gunderson, 1960). All show activity in the pH range 5–9 with peak activity at pH 7–8. At pH 5·5 the casein substrate was partially precipitated and the low activity at this pH may, in part, reflect this reduced solubility in addition to the influence of pH on activity. It is of interest that the enzymes showed 82 % of their peak activity at the pH of milk.

The proteolytic enzymes of *P. aeruginosa* ATCC 10145 retained a high proportion of their activity after simulated pasteurization and are therefore relatively heat-stable with regard to milk pasteurization. Although the pseudomonads themselves are destroyed by pasteurization (Rogick & Burgwald, 1952), any of the proteinases elaborated before pasteurization may continue to be active. However, the loss of activity following treatment at 72 °C for 15 sec was 6 times that after treatment at 63 °C for 30 min. The differing inactivation curves following heating of the supernatant may indicate the presence of more than one proteinase. Morihara (1964) reported that a strain of *P. aeruginosa* produced at least 2 proteinases.

Whole cell suspensions showed no detectable proteolytic activity before or after pasteurization, indicating that no detectable activity was associated with the cell wall prior to or following heat-treatment. If the cells lysed following heating, any intracellular proteinases were either destroyed by pasteurization or were not released in detectable quantities until at least 3 weeks storage at 3 °C had elapsed. Thus, it appears that the cells themselves were not associated with hydrolysis of the milk protein after pasteurization.

P. aeruginosa ATCC 10145 did not grow at 2 °C but its proteolytic enzymes were not denatured by storage at this temperature. Allowing for the reduced rate of reaction, the casein substrate was hydrolysed to the same degree at 2 as at 30 °C.

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The passage of staphylococci through the bovine teat canal

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Summary. In order to determine how bacteria pass through the teat canal into the teat sinus, milk samples were taken by syringe, through the teat wall, from 12 quarters of 7 cows, before and after each milking for up to 16 days. Three of the teat canals were naturally infected before the start of the experiment and the remainder were artificially infected with *Staphylococcus aureus* by the Hadley–Wisconsin swab technique. In 3 of the quarters the inoculum was introduced into the teat sinus through the teat canal by the inoculation technique although the swabs were inserted only 3–5 mm into the canal. The other 9 of the 12 quarters were milked a total of 149 times during the experiment but only once was a colony-forming unit of the bacterium present in a teat canal infection isolated from the milk taken from the teat sinus after milking. One quarter developed an intramammary infection, the bacterium first being detected in the teat sinus prior to the third milking after inoculation of the teat canal. The invasion of the bacteria into the teat sinus therefore occurred between milkings. *Staph. aureus* persisted in teat canals between 5 and 32 or more milkings after inoculation. The experiment showed that bacteria in the teat canal are seldom refluxed into the residual milk in the teat sinus during correctly conducted milking with an efficient machine.

The mechanism by which bacteria invade the mammary gland is little understood. It is suspected that invasion occurs by bacteria growing through the teat canal or that bacteria are refluxed through the canal into the teat sinus during milking. Murphy & Stuart (1953) working with *Streptococcus agalactiae* showed that the teat canal could remain infected for up to 9 days before an intramammary infection developed. In the period during which the infection was confined to the teat canal, the authors suspected significant bacterial growth to the extent that they grew through into the teat cavity, but stressed that more information was needed before such a conclusion could be justified. Newbould & Neave (1965*a*), who inoculated teat canals with *Staph. aureus*, found that when colonization of the teat canal occurred, only rarely did intramammary infections follow. The method by which the bacterium invaded the gland was not established. The evidence for reflux of bacteria through the teat canal in normal conditions is equivocal. Little (1937) thought that bacteria were sucked through the teat canal in his experiments but his methods could not verify this. McEwan & Samuel (1946) demonstrated that bacteria passed through the teat canal into the teat sinus when a broth culture was sprayed onto the teat ends

during milking. The necessary physical conditions for reflux to occur were produced by Witzel & McDonald (1964), who by continuing milking after complete milk removal recorded a residual vacuum in the teat sinus after removal of the teat cups. They suggested that bacteria may enter during pressure equilibration. In a herd in which normal machine milking was practised Forbes & Hebert (1968) showed that teat canals were infected for some time before the development of most intramammary infections, although some infections developed without detectable preceding infections of the teat canal. The results suggested that infection grew through the teat canal in most cases but reflux of bacteria may have occurred in others. The experiment reported in the present paper was designed to determine how bacteria in the teat canal invade the teat sinus.

EXPERIMENTAL

Experimental animals

Cows. Seven animals were used in the experiment. Four were heifers, and 2 in their third and one in its fifth lactation. Twelve quarters were used for teat-canal inoculation study, all of which had intramammary infections of *Corynebacterium bovis* at the start of the experiment. There was no erosion of the teat canals. During the experiment, each animal was milked by a sterile machine operated at 60 c/min at 13 mm Hg vacuum and with a pulsation ratio of 1 : 1. The interval between morning and evening milkings was 8 h.

Teat canal inoculation

Test bacterium. An α haemolytic strain of *Staph. aureus* was used having a phage pattern of 29,110+ (Davidson, 1961). It was isolated from a cow with mastitis. Its phage type and haemolytic pattern distinguished it from other strains of *Staph. aureus* present in the experimental herd.

Inoculation procedure. Preliminary work showed that the Hadley–Wisconsin swab technique (Murphy & Stuart, 1953) was a satisfactory method of introducing a large inoculum into the teat canal. Each teat apex was thoroughly cleaned with sterile cottonwool soaked in methylated spirits. A swab was then loaded with *Staph. aureus* by drawing it through several colonies of a 24-h culture on 5 % sheep blood agar and a teat canal was inoculated by inserting the swab between 3 and 5 mm into the canal.

Uninoculated quarters. At the start of the experiment 3 right forequarters had teat canals naturally infected with *Micrococcaceae*. The right fore teat canal of cow 2240 was infected with a *Staph.* subgroup 6; that of cow 2816 with a *Staph.* subgroup 3 and that of cow 2490 with a mixed infection of *Micrococcus* subgroups 4 and 5. The bacterial types were determined by the classification of Baird-Parker (1963).

Sampling procedure

Immediately before every milking the teat skins and teat apices were thoroughly cleaned with sterile cottonwool soaked in methylated spirits. Before afternoon milking a sterile swab was inserted 3 mm into the teat canal, inoculated into 5 ml of 1 % glucose broth by stirring and then discarded. This was done before the collection of any other samples. Milk samples were taken after similar preparation of the teats immediately before and after every milking. One ml of milk was collected

by syringe from the teat sinus using the method of Murphy & Stuart (1954). This was followed by the normal collection of approximately 10 ml foremilk drawn through the teat canal.

Cultural technique

Samples (0·1 ml) were plated within 30 min of collection onto 5% sheep blood agar plates. Teat-canal swab broths and hand-drawn milk samples were inoculated onto separate plates. Five plates were seeded each with 0·1 ml of the milk collected by syringe through the teat wall. All plates were incubated at 30 °C for 48 h and refrigerated before being read. One colony of the test bacterium from each series of samples (e.g. pre-milking samples) was identified by the tube coagulase test and phage typing in the case of *Staph. aureus* or by biochemical methods for coagulase-negative types (Baird-Parker, 1963).

RESULTS

The 9 quarters whose teat canals were artificially inoculated were sampled between 3 and 7 milkings before inoculation. The milk samples taken by teat wall puncture during the period preceding inoculation produced only *C. bovis* when cultured. Of the

Table 1. *Inoculation of* Staph. aureus *into the lower* 3–5 *mm of teat canals*

Cow	Quarter	Time of teat canal inoculation	Result of inoculation
2816	LF	Before pre-p.m. milking samples	Bacterium introduced into the teat sinus by inoculation
2706	RF		
2240	RH	After post-p.m. milking samples	
2940	RF	Before post-p.m. milking samples	Bacterium persisted in teat canal for: 17 milkings
2939	LF		32 milkings
2939	RH		11 milkings
1880	LF		5 milkings
1880	RF		Intramammary infection at 3rd post-inoculation milking
2567	RF	After post-p.m. milking samples	Bacterium persisted in teat canal for 5 milkings

9 teat canals inoculated with *Staph. aureus*, in 3 the organism was inadvertently inserted through the canal into the teat sinus. This was shown by the fact that in each of these quarters the milk sample taken by teat wall puncture after inoculation contained the test organism. Two of the quarters had the teat wall puncture samples taken immediately after inoculation but in the third, the right-hand quarter of cow 2240, the interval was the 16 h between evening and morning milkings, during which time the bacterium may have penetrated into the teat sinus. Table 1 shows details of the nine quarters which were inoculated. Of the 6 inoculated quarters in which immediate or rapid penetration of the teat sinus did not ensue, 5 were sampled until it was evident that the test bacterium had disappeared. The sixth quarter, the left fore quarter of cow 2939, in whose teat canal *Staph. aureus* persisted, was sampled for 16 days following inoculation and the experiment was then terminated. Two quarters with uninoculated teat canals were sampled for 8 days and one for 16 days.

Staph. aureus was shown to persist for between 5 and 32 milkings following inoculation (Table 1). In those teat canals where *Staph. aureus* persisted for 5 milkings, there was a rapid decline in colony counts in hand-drawn milk and from teat canal

swabs. In the other quarters counts showed an increase in numbers of bacteria and a subsequent decline and eventual disappearance. The left fore teat canal of cow 2939, which was still infected after 16 days, was tested a further 14 days later and the test bacterium had by then disappeared.

Coagulase-negative strains. The mean number of colony-forming units on plates

Table 2. *Development of intramammary infection after inoculation of* Staph. aureus *into the right fore teat canal of cow* 1880

Time of sampling in relation to milking	The colony count of *Staph. aureus* in milk collected before and after milking		*Staph. aureus* colony counts from teat canal swab†
	Teat wall puncture milk*	Hand-drawn milk*	
Day 1			
Pre a.m.	0	0	—
Post a.m.	0	0	—
Pre p.m.	0	0	0
Post p.m.	0	No sample§	—
Day 2			
Pre a.m.	0	7500	—
Post a.m.	0	250	—
Pre p.m.	0	90	10000
Post p.m.	0	0	—
Day 3			
Pre a.m.	8	400	—
Post a.m.	0	0	—
Pre p.m.	0	30	300
Post p.m.	0	0	—
Day 4			
Pre a.m.	30	0	—
Post a.m.	250	10	—
Pre p.m.	80	30	0
Post p.m.	0	10	—
Day 5			
Pre a.m.	400	150	—
Post a.m.	200	30	—
Pre p.m.	500	500	0
Post p.m.	60	0	—
Day 6			
Pre a.m.	100	1000	—
Post a.m.	500	100	—
Pre p.m.	500	750	0
Post p.m.	50	200	—
Day 7			
Pre a.m.	250	150	—
Post a.m.	300	1000	—
Pre p.m.	150	250	0
Post p.m.	40	20	—
Day 8			
Pre a.m.	50	90	—
Post a.m.	500	100	—
Pre p.m.	1000	1000	0
Post p.m.	—	—	—

* Estimated count per ml of milk.
† Number of colonies per swab.
§ Teat canal inoculated before post p.m. samples were taken.

prepared from broths that had been inoculated with teat-canal swabs from the 3 quarters with naturally occurring teat canal infections was 31·5. This indicated the collection of approximately 630 colony-forming units/swab throughout the period of sampling.

Passage of bacteria through the teat canal in inoculated quarters

1. *Reflux of bacteria during milking.* Six quarters with *Staph. aureus* infections in the teat canal were milked a total of 85 times during the course of the infection. Paired milk samples were collected by teat-wall puncture before and after each milking, and only once was the bacterium present in the teat sinus milk. One colony was present on 1 of the 5 plates prepared from the sample taken from the left fore teat of cow 2940 after the third milking following inoculation of the canal. The hand-drawn milk samples taken before and after this milking had colony counts of 300 and 2, respectively, in 0·1 ml. During the 14 subsequent milkings of this quarter when *Staph. aureus* was present in hand-drawn-milk, the bacterium was not again isolated from the teat sinus. Apart from this incident there was no evidence of reflux of bacteria having occurred during milking.

2. *Passage of bacteria between milkings.* The right fore quarter of cow 1880 developed an intramammary infection after teat canal inoculation, and the progress of the infection is shown in Table 2. In the 2 days following inoculation there was a progressive decline in colony counts of *Staph. aureus* both from hand-drawn milk and from teat-canal swabs. Before the third milking after inoculation the test bacterium was isolated from milk taken by teat-wall puncture, and the infection was again present in the teat sinus before the fifth milking. The passage of bacteria into the teat sinus had therefore occurred between milkings. After the fifth milking an intramammary infection became established but the bacterium was no longer recovered from swabs inserted into that part of the teat canal where the inoculum had been deposited. Thus, although the bacterium passed through the teat canal there was little evidence of colonization in the canal before invasion of the quarter.

Traversal of bacteria across the teat canal in uninoculated quarters

The strain of bacterium isolated from teat-canal swabs and milks of the 3 uninoculated quarters was not isolated from any teat-wall puncture samples during the experiment. The 3 quarters were tested for a total of 64 milkings.

DISCUSSION

The Hadley–Wisconsin swab technique was chosen as the method of inoculating the teat canal because it allowed a large inoculum to be used and avoided introducing fluid into the teat canal. Slanetz, Bartley & Allen (1965) thought it improbable that the introduction of small volumes of broth into the teat canal caused penetration into the teat sinus. However, as the inflammatory response was delayed for only up to 1 day by this latter method of inoculation, it did not simulate the natural teat canal infections which preceded intramammary infection (Forbes & Hebert, 1968). Newbould & Neave (1965*a*) also introduced a fluid inoculum 5 mm into the teat canals and, using between 70 and 600 colony-forming units of *Staph. aureus*, were able to recover the bacterium for up to 6 milkings or more. In those quarters which

developed intramammary infections the delay in elevation of cell count was up to 6 milkings following inoculation. In another paper (Newbould & Neave, 1965*b*) describing experiments in which the same strain of *Staph. aureus* was introduced directly into the teat sinus, the leucocyte response was in one case delayed up to the eleventh milking and was never evident before the third post-inoculation milking. Hence, it is possible that in those quarters in which *Staph. aureus* was introduced into the teat canal and which developed intramammary infection the inoculum may have penetrated into the teat sinus at the time of inoculation. In the work now reported a swab inserted 5 mm into the teat canal was sufficient to introduce infection into the teat sinus in 2 quarters.

There is always a delay between bacterial invasion of the mammary gland and the inflammatory response as reflected by an elevation of cell numbers in the milk. Therefore, in order to detect the time of bacterial invasion of the gland, methods which demonstrate the bacteria to be present within the gland must be used. McEwen & Samuel (1946) showed bacteria to have entered the gland by a *post mortem* examination immediately after milking. During milking the teat ends had been heavily contaminated with a motile bacterium whose mobility may have assisted its entry into the gland. In the living animal, the collection of milk by syringe through the wall of the teat is the only way of detecting bacteria in the gland and by comparing the bacteriology of samples taken immediately before and after milking the time of entry of the bacteria can be determined.

In the present study milk samples were plated within 30 min of collection, during which time phagocytosis of bacteria may have occurred. However, Katsube & Blobel (1964) showed that *Staph. aureus*, although phagocytosed by leucocytes, survived and was able to grow when a suspension of bacteria and leucocytes was plated on sheep blood agar. Newbould & Neave (1965*c*), on the other hand, showed that ingestion of the bacteria by leucocytes suspended in milk prevented growth during incubation for 18 h. Only when the leucocytes were disrupted did bacterial multiplication continue. Culturing on solid media will not maintain viable leucocytes and ingested bacteria survive to form normal colonies. A limitation of the cultural methods used in the present investigation may be their insensitivity, as only 0·5 ml milk was cultured and if very small numbers of bacteria penetrated into the sinus they may well have escaped detection.

Little (1937) suspected that, where invasion occurred, bacteria had been sucked through the teat canal. He recognized invasion by the development of mastitis and his methods could not establish that bacteria had been sucked into the teat sinus. Witzel & McDonald (1964) reported a residual vacuum in the teat sinus after complete milk removal by machine, producing conditions in which reflux of bacteria could occur, but Thiel, Clough & Dodd (1965) suggested that the vacuum was an artifact caused by the cluster weight. However, to produce such a vacuum at all would demand extreme overmilking, and during the work now reported the cows were milked normally and were not stripped out. The milk yields of the cows did not drop during the experiment, showing that normal milking was practised. In addition, the machines were operated with an adequate reserve and stable vacuum. Wilson (1958) deduced that a fluctuating reserve vacuum may predispose to mastitis and Nyhan & Cowhig (1967) showed that a low vacuum reserve was associated with a

high prevalence of mastitis. They thought that the fluctuation in vacuum at the end of the teat and the consequent slowing of milking rate might provide the physical conditions necessary for bacteria to gain entry to the teat cistern from the teat canal or the exterior of the teat. The present experiment shows that bacteria in the teat canal are seldom refluxed into the residual milk in the teat sinus during correctly conducted milking with an efficient machine. In only one of 149 milkings was there evidence of bacteria reaching the teat sinus during milking.

The right fore quarter of cow 1880 developed an intramammary infection and the bacterium was first detected in the teat sinus in the sample taken prior to the third post-inoculation milking (Table 2). Judging from colony counts in normal milks after inoculation, there was little multiplication of the bacterium within the teat canal. On the third day after inoculation *Staph. aureus* was not recovered by swabbing that part of the teat canal in which the inoculum was deposited, although the gland itself was then infected. The bacterium was isolated from the teat sinus before the third and fifth milkings after inoculation, after which all teat-wall puncture samples were infected. Invasion of the teat sinus, therefore, probably occurred between milkings but due to the inability of the bacterium to colonize the teat canal it is doubtful if growth occurred through the canal. It is possible that the infection was carried through the canal in the keratin lining as a consequence of pressures imposed on the teat during milking. Thiel, Clough, Westgarth & Akam (1966) showed that at certain pulsation rates the teat liner closes the teat sphincter in the rest phase of milking. This pressure may cause keratin to move in the canal and eversion of keratin may then occur, giving eroded teat orifices in some quarters. It may be that the structure of some teat canals and the conformation of some teats predispose them to an inversion of keratin. If the keratin were infected, the process would then effect the passage of bacteria into the sinus. Pounden & Grossman (1950) found that machine milking removed more keratin from the teat canal than did hand-milking or calf suckling, especially if overmilking was practised. A predisposition to teat erosion in pointed teats and in teats with plate-formed tips was found by Johansson (1957), while Hickman (1964) found a striking trend towards increased incidence of mastitis with increase in teat diameter. He also found that funnel-shaped teats had a lower frequency of mastitis than had cylindrical teats. It may be that mechanical stresses imposed on some teats by machine-milking are a factor in introducing infection into the gland from the teat canal.

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The composition of the milk of young and of old cows

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SUMMARY. The milk produced by old cows frequently contains a lower concentration of solids-not-fat (S.N.F.) than that of young cows, mainly because of a low concentration of lactose. This can result from mastitis or possibly from other factors associated with ageing. The composition of the milk of 2 groups of old cows has been compared with that of substantially disease-free first-lactation cows. A comparison has also been made between the milk from the 4 quarters of each cow.

The first part of the work was done with 8 old cows and 4 first-lactation cows, and the milk was examined bacteriologically, cytologically and chemically at frequent intervals throughout the lactation. There was strong evidence that most of the difference in chemical composition of the milk of the old cows compared with that of the young cows was the result of mastitis, mainly subclinical. The milk from the old cows had a higher incidence of infection, a lower concentration of lactose and casein, higher concentrations of serum albumins, globulins and proteose-peptones and contained more total and polymorph cells. There was also some indication of another effect, not directly associated with disease in the current lactation, that led to a decrease in the lactose concentration of the milk of the old cows after about 80–90 days in lactation.

In the second part of the work, the milk from 23 old and 8 first-lactation cows in 8 commercial herds was examined twice, once after 58 and again after 163 days in lactation. Milk of abnormal composition as a result of disease was produced by 30 % of the quarters of the old cows at the first examination and by 62 % of the quarters at the second examination.

Some indication is given of the probable loss of milk and milk constituents that can be attributed to disease.

There are many reports that the milk of cows which have had several lactations usually contains lower concentrations of fat, lactose and potassium than does the milk of young cows, particularly those in their first lactation, and a higher concentration of sodium and chloride (see, for example, the recent review by Rook & Wheelock, 1967). The total nitrogen content of milk is much less affected by the age of the cow but the proportion present as casein N progressively declines after the first lactation (Waite, White & Robertson, 1956). A lower lactose concentration is therefore the chief cause of a low S.N.F. percentage, although there may also be a reduction of nitrogen content caused by underfeeding (Rowland, 1946). There is evidence that the incidence of subclinical mastitis is higher in older cows (van Rensburg, 1947; Braund

& Schultz, 1963; Blackburn, 1966, 1968) and that this condition is accompanied by changes in the chemical composition of the milk which are similar to those ascribed to increasing age (Rowland, 1938; Barry & Rowland, 1953; Waite & Blackburn, 1963). These changes have been attributed to transudation of blood serum through mammary tissue rendered more permeable by the inflammatory nature of the disease (Barry & Rowland, 1953) or by impairment of the ability of the milk secreting cells to synthesize milk constituents (Garrison & Turner, 1936; Turner, 1946).

Few studies have been reported in which milk composition has been determined through several lactations in disease-free cows. In one such report (Rook & Campling, 1965) the changes in the average concentration of lactose in the milk of 3 cows for each lactation between the first and the third were 0, −0·08 and −0·09 percentage units. These values average only half those found for large numbers of cows in commercial dairy herds between the first and third lactations (Waite *et al.* 1956).

The composition of the milk given by each of the 4 quarters of the udder, in the absence of disease or injury, is very similar throughout the whole lactation, and the proportion of the total milk yield given by each quarter remains relatively constant (Waite, unpublished). When a quarter becomes diseased, the yield of milk and the concentration of lactose and casein falls while the proportion of the total N appearing in the serum protein–proteose peptones fraction increases (Rowland, 1938*b*; Waite & Blackburn, 1963). Thus, regular chemical analysis of the milk from each quarter, coupled with bacteriological and cytological examinations, provides a good measure of the extent to which the composition of the milk from the whole udder has been affected by disease.

The influence of the age of the cow or the number of lactations *per se* on the chemical composition of the milk is much more difficult to assess. The renewal of the milk-secreting cells prior to each lactation, and the progressive decline in their efficiency in converting blood constituents to milk as the lactation progresses (Linzell, 1960) followed by their shedding at the end of the lactation, represents a complete cycle of events that is repeated in each lactation. Ageing of the udder therefore refers mainly to the milk duct system, the supporting tissue and the teats. However, since milk secretion is under hormonal control, changes in the amounts or relative proportions of these hormones as the glands producing them age, may influence milk composition.

As already mentioned, the concentrations of fat, lactose and nitrogen in milk are usually highest, and of serum proteins lowest, in the milk of cows in their first lactation, and the variability in milk composition between such cows is frequently less than between that of older animals. Regular analysis of the milk of a group of first-lactation cows, managed and fed under the same conditions and at the same time as the older cows, therefore provides a reference level of composition from which the effects of age and to a large extent of disease would be absent. This 2-fold comparison of milk composition, between the quarters of the individual cow and between young and old cows, is the subject of the present paper.

EXPERIMENTAL

The investigation was made in 2 parts. For the first, 12 cows of the Institute herd were sampled at intervals of 3 weeks throughout the lactation; for the second, 4 cows in each of 8 commercial herds—32 cows in all—were sampled on 2 occasions only, at approximately 58 and 163 days after calving. All cows were of the Ayrshire breed. Of the 12 cows from the Institute herd, 4 were in their first lactation, 2 in their sixth, 2 in their seventh, 2 in their eighth lactation, 1 in its tenth and 1 in its eleventh lactation. All had been born at the Institute, except the cow in its eleventh lactation, which entered the Institute herd at her second lactation. Of the 4 cows from each of the 8 commercial farms one was in its first lactation and three were old cows in the fifth–eleventh lactation.

The cows were milked with a bucket, which allowed the milk from each quarter to be collected separately. Cows in the Institute herd were milked in this way at consecutive evening and morning milkings and the 2 samples from each quarter mixed in proportion to yield, but with cows in the commercial herds a sample was taken from the evening milking only. The milk was analysed for total solids, fat, lactose and total nitrogen (N) by methods already described (Waite *et al.* 1956). Total N was partitioned into the following fractions (in every third sample only from cows in the Institute herd), casein, total albumins, β-lactoglobulin, globulins, proteose–peptones and non-protein N (NPN), by the methods of Rowland (1938*a*) and of Aschaffenburg & Drewry (1959). A value for S.N.F. was obtained by difference from the values of total solids and fat, and subtraction of β-lactoglobulin N from total albumin N gave a value for the 'residual albumins', a fraction which consists of α-lactalbumin and blood serum albumin (Aschaffenburg & Drewry, 1959). The total number of cells and of polymorph cells were counted by the method of Blackburn, Laing & Malcolm (1955) in milk taken from the same sample that was used for chemical analysis. Fore-milk samples, taken under sterile conditions immediately prior to evening milking, were examined for the presence of bacteria in the milk of the 12 cows of the Institute herd by noting the growth of colonies from 0·01 ml milk on blood agar plates incubated for 18 h at 37 °C (Blackburn, 1956). A milk sample was recorded as infected if 5 or more colonies of the same species developed (Blackburn, 1968), and in Tables 1–5 the amount of infection is recorded as the percentage of the fore-milk samples (usually 13–16/lactation) which showed such infection. Quarters were treated with antibiotic when streptococci or coagulase-positive staphylococci were present in the fore-milk. Fore-milk samples were not taken from the cows on the commercial farms.

All the cows were due to calve in the spring, those from the Institute herd in 1964 and those in commercial herds in 1965, and most calved between March and mid-May. Thus, most of the food requirements during the first 5–6 months of the lactation were provided by grazing. The amount and quality of the feed available would be the same for the cows in each individual herd although there would be variation between herds. In many of the commercial herds a small amount of concentrated food was also given at milking times. After the cows had gone indoors for the winter the feeding regimes also differed somewhat between farms but were generally of a good standard. All the cows were served about 2–3 months after calving and, so far as is known, few failed to conceive, although some required as many as 3 services. Of the 12 cows from the

Institute herd, 1 old cow (Violet) was not pregnant before the end of the lactation under investigation.

RESULTS

Cows in the Institute herd

The results obtained from the 12 cows in the Institute herd will be presented first.

In a previous investigation (Waite *et al.* 1956) the milk of 87 Ayrshire cows in their first lactation was analysed at intervals throughout lactation and the weighted average composition of the milk of the group for the lactation was given. These values can be used to establish that the lactation average composition of the milk of the 4 cows in their first lactation in the present work was normal (Table 1). The yield

Table 1. *A comparison of the average lactation yield and composition of milk from first lactation cows in* 1950–53 *and the corresponding values for the cows used in the present investigation*

	1950–53	1964–5	S.E.
No. of cows	187	4	
Yield, lb	7300	9090	±202
Cell count, cells × 10^{-6}/ml			
Total	0·10	0·08	±0·020
Polymorph	0·05	0·03	±0·011
Infection, % of samples	N.D.*	13	±5·3
Bacteria present	N.D.	m†	
Fat, %	4·11	4·26	±0·106
S.N.F., %	9·01	8·91	±0·092
Lactose anhydride, %	4·72	4·68	±0·012
Crude protein (N × 6·38), %	3·36	3·33	±0·107
N distribution, % total N			
Casein	80·9	79·7	±1·09
β-lactoglobulin	N.D.	8·1	±0·85
Residual albumins	N.D.	4·5	±0·21
Globulins	N.D.	1·7	±0·06
Proteose-peptones	N.D.	1·1	±0·05
NPN	N.D.	4·9	±0·17

* N.D. = Not determined. † m = Coagulase-negative micrococci.

of milk and the concentration of fat were higher, and the concentration of total N and lactose (and hence of S.N.F.) were slightly lower in the milk of the 4 cows than in the milk of the 187 cows, but the differences in composition were small. The percentages of casein in the total N were similar in the 2 sets of results, and were similar also to the level of 79–80 % of the total N, which Rowland & Zein-el-Dine (1938) found for milk from Ayrshire cows with healthy udders. The amount of β-lactoglobulin N in the milk of Ayrshire cows varies, in our experience, from 6 to 10 % of the total N (Waite, Castle & Watson, 1959, and unpublished results) and for these 4 cows the values ranged from 6·8 to 9·6 %. According to Aschaffenburg & Drewry (1959) a typical value for the α-lactalbumin N in milk from a healthy udder is about two-thirds of the residual albumins N, i.e. about 3 % of the total N for these first-lactation cows, so that the serum albumin N would amount to about 1·5 % of the total N. The immune globulins N was slightly higher at 1·7 % of the total N, the proteose–peptones N accounted for a further 1·1 % and NPN for 4·9 % of the total N, the latter a very typical value (Rowland, 1938).

Bacteria, mainly coagulase-negative staphylococci, were present in the fore-milk samples from these cows on approximately one-third of the sampling occasions but the numbers of colonies that developed were small. The cell counts in the milk from

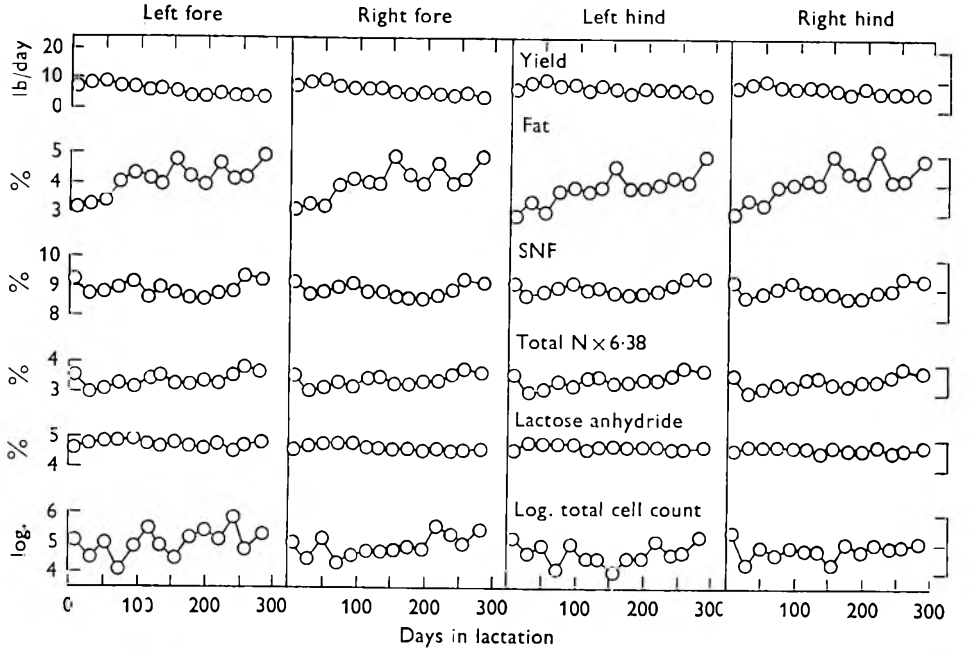

Fig. 1. The yield, chemical composition and cell count of the milk from the 4 quarters of a first-lactation cow.

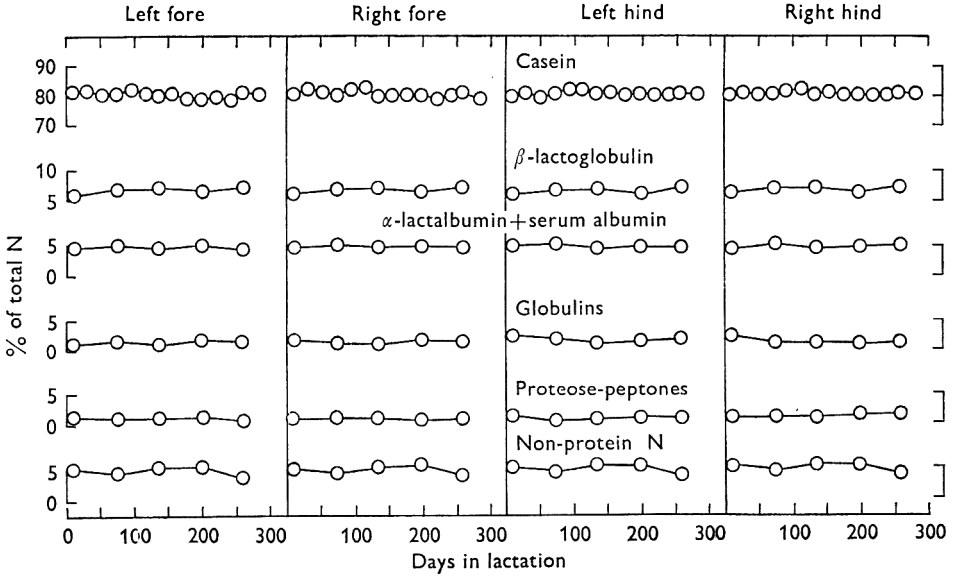

Fig. 2. The N distribution (as % of total N) in the milk from the 4 quarters of the first-lactation cow for which other results are shown in Fig. 1.

all the quarters were low at all stages of the lactation, averaging 80000 cells/ml, of which only 30% were polymorphs. These figures and the low content of serum proteins–proteose peptones (abbreviated to SP–PP in the following text) N of the milks suggest that the bacteria, when present, had caused little tissue damage.

The similarity in chemical composition of the milk from each quarter, and the small variation, except in fat percentage, during the course of the lactation can be seen in Figs. 1 and 2 for the milk of one of the first-lactation cows. The corresponding results for the other 3 cows in their first lactation were similar.

Table 2. *The lactation yield and average composition of the milk of each quarter of 2 cows in their sixth lactation*

Cow	Dawn				Dolphin			
Quarter*	LF	RF	LH	RH	LF	RF	LH	RH
Yield, lb	3112	3024	3459	3252	1840	2542	2559	3166
Cell count, cells × 10^{-6}/ml								
Total	0·16	0·24	0·27	0·21	1·57	2·20	3·40	0·28
Polymorph	0·08	0·13	0·13	0·13	0·99	1·43	2·42	0·14
Infection, % of samples	20	25	30	30	50	40	60	20
Bacteria present†	mS	m	m	mS	SH	SH	mSH	mSH
Fat, %	4·04	4·09	3·99	3·89	3·61	3·92	3·60	3·96
S.N.F., %	8·60	8·40	8·57	8·57	8·47	8·55	8·32	8·79
Lactose anhydride, %	4·50	4·34	4·38	4·44	3·99	4·11	3·84	4·34
Crude protein (N × 6·38), %	3·19	3·22	3·27	3·24	3·47	3·46	3·55	3·55
N distribution, % total N								
Casein	78·1	76·6	77·8	78·6	74·9	76·2	72·6	77·2
β-lactoglobulin	6·6	6·6	6·4	6·3	7·6	7·7	7·4	7·6
Residual albumins	5·8	6·4	6·0	5·7	6·7	5·8	7·3	5·6
Globulins	2·6	3·1	2·7	2·5	2·8	2·3	3·6	2·4
Proteose-peptones	2·1	2·6	2·3	2·1	2·3	2·1	2·9	1·8
NPN	4·8	4·7	4·8	4·8	5·7	5·9	6·2	5·4

* LF, RF, LH, RH refer to left fore, right fore, left hind, right hind quarters.
† m = Coagulase-negative micrococci. S = Coagulase-positive staphylococci. H = Haemolytic.

In the milk from the 8 old cows there were considerable differences in infection and chemical composition, not only between cows, as would be expected, but also between the quarters of individual cows. The results cannot therefore be averaged and are presented briefly for each cow separately in the form of a table giving the lactation yield and the weighted average composition, together with a description of the major variations that occurred during the lactation.

Dawn

This cow was in her sixth lactation and gave 12847 lb milk in 301 days. Reference to Tables 1 and 2 shows that the milk from each quarter of this high-yielding cow had lower lactation average concentrations of fat, lactose, total N and casein than had that of the first-lactation animals, and higher proportions of SP–PP N. Cell counts and infection were higher in the milk from all the quarters, milk from the left fore (LF) quarter being least affected and having the best chemical composition. For the first 85 days of lactation the lactose content in the milk of all the quarters was between 4·7 and 4·8%, which was as good as that of the young cows or even better, but from the 86th until about the 215th day of lactation there was a continuous fall in concen-

tration, a fall that had not occurred in the milk of the young cows. The higher values for the serum proteins and proteose–peptones occurred at about the same time as the decline in lactose concentration and they increased slowly to the end of lactation. These were the major differences that occurred in milk composition during the course of lactation and they could not be identified initially with a rise in infection or cell count, although after the 180th day infection and cell count both increased in the milk from all quarters.

This cow had suffered moderate subclinical mastitis in her fourth lactation but on all occasions the infecting organisms had been coagulase-negative staphylococci.

Dolphin

Sixth lactation, 10107 lb milk in 263 days, Table 2.

This cow suffered from subclinical mastitis in 3 quarters for most of the lactation. Infection and cell counts were high in the milk from all her quarters except the right hind (RH) and were accompanied by lactose and casein concentrations much lower and SP–PP N higher than that in the milk of the first-lactation cows (Table 1). Coagulase-positive staphylococci were present in large numbers in the milk of the 3 affected quarters from about the 40th day of lactation onwards. The lactose concentration in the milk of the best (RH) quarter was about 4·6 % for 80 days after calving, but then decreased slowly during mid-lactation and more rapidly after about 200 days to give an average of 4·3 % for the whole lactation compared with 4·7 % for the young cows. Coagulase-positive bacteria and a high cell count were present in the first sample from this quarter but only once reached infection level again during the lactation. The lactation yields of milk from the 2 most affected quarters, the LF and LH, were 702 and 607 lb less, respectively, than the yields from the 2 corresponding better quarters.

The LH quarter had suffered severe and continuous infection during the fourth lactation and slight infection in the fifth and was, as noted, the most affected quarter in the present lactation.

Gadfly 4

Seventh lactation, 9351 lb milk in 232 days, Table 3.

The milk of all the quarters of this cow was higher than that of the first-lactation cows in fat content, lower in lactose and S.N.F. and almost the same in total N content. The SP–PP N was higher, particularly for the 3 quarters with the higher cell counts. Lactose concentrations differed between the milk of the 4 quarters and the highest value accompanied the least amount of infection and the lowest cell counts. The yield and lactose concentration of the milk of the RH quarter decreased continuously from the beginning of lactation without passing through any maximum, although infection was then absent and the cell count remained below 100000 cells/ml until the 150th day. Infection then appeared to accelerate the fall in lactose concentration, the rise in cell count and the rise in SP–PP N. In the milk from the LF and LH quarters, lactose concentration was about 4·7 % for 80–90 days and then decreased throughout lactation, more quickly in the LH quarter.

There was nothing in this cow's previous history to account for the abnormal

performance of the RH quarter, which gave 861 lb milk less than the LH quarter, although in the 3 preceding lactations the RF quarter had suffered moderate infection with coagulase-positive bacteria and, occasionally, with streptococci.

Table 3. *The lactation yield and average composition of the milk of each quarter of 2 cows in their seventh lactation*

Cow	Gadfly				Veronica			
Quarter*	LF	RF	LH	RH	LF	RF	LH	RH
Yield, lb	1882	1768	3281	2420	2116	1173	2576	2962
Cell count, cells × 10^{-6}/ml								
Total	0·12	0·64	0·13	0·29	0·20	1·24	4·99	0·39
Polymorph	0·04	0·42	0·05	0·15	0·08	0·68	3·59	0·15
Infection, %	8	50	30	16	25	50	40	33
Bacteria present†	m	m	mS	m	m	m	m	mH
Fat, %	4·51	4·66	4·45	4·31	4·63	3·96	4·27	4·27
S.N.F., %	8·74	8·69	8·62	8·61	8·34	7·93	7·99	7·94
Lactose anhydride, %	4·51	4·44	4·41	4·32	4·42	3·97	4·04	4·02
Crude protein (N × 6·38), %	3·33	3·39	3·35	3·44	3·14	3·21	3·18	3·17
N distribution, % total N								
Casein	78·0	76·8	76·7	75·8	71·9	69·2	72·1	72·8
β-lactoglobulin	8·9	8·8	9·0	8·6	8·5	8·0	8·0	8·2
Residual albumins	4·9	5·4	5·4	5·7	6·9	8·3	7·1	6·5
Globulins	2·4	2·8	2·7	3·2	3·2	4·9	3·1	2·7
Proteose-peptones	1·3	1·8	1·7	2·2	3·1	3·8	3·6	3·4
NPN	4·5	4·4	4·5	4·5	6·4	5·8	6·1	0·4

* LF, RF, LH, RH refer to left fore, right fore, left hind, right hind quarters.
† m = Coagulase-negative micrococci. S = Coagulase-positive staphylococci. H = Haemolytic.

Veronica

Seventh lactation. 8827 lb milk in 231 days, Table 3.

This cow suffered from a trampled teat on the RF quarter during the first week of lactation, clinical mastitis in the LH quarter after 50 days, and subclinical mastitis in the latter half of lactation in the other 2 quarters. The chemical composition of the milks reflects these conditions, with abnormally low lactose, S.N.F. and casein concentrations and high proportions of SP–PP in the total N. The lactation yield of milk from the RF quarter which suffered the trampled teat was 943 lb less, and the fat percentage of the milk 0·67 % less, than from the corresponding LF quarter. Streptococci were present in the milk of the quarter in which clinical mastitis developed.

This cow had suffered infection and high cell counts in the milk of all quarters during the 3 lactations preceding the present one, the infecting organisms frequently being coagulase-positive.

Elsa 4

Eighth lactation, 8557 lb milk in 237 days, Table 4.

This cow had 1 quarter, the RH, of which the teat had been damaged by trampling in the previous lactation. The milk from this quarter was infected with coagulase-positive haemolytic bacteria from early lactation and its daily yield of milk rapidly decreased from 10 to 2 lb/day in 100 days. The cell count and the bacterial and chemical results bear evidence of the infected condition, only the total N concentration remaining unaffected. The fat and lactose concentrations were at a low level

and the blood SP–PP values at a high level at the first sampling. The loss of milk, in comparison with the LH quarter, itself diseased, was 1501 lb. Milk from the LH quarter was infected with coagulase-negative, haemolytic bacteria after about 50 days of lactation and this was associated as the lactation proceeded with a moderate rise in cell count, a decrease in lactose concentration, a small rise in the values for blood serum proteins and a more marked rise in the values for proteose–peptones. This quarter had had clinical mastitis in the fifth lactation and moderate subclinical mastitis in the sixth.

Table 4. *The lactation yield and average composition of the milk of each quarter of 2 cows in their eighth lactation*

Cow	Elsa 4				Violet			
Quarter*	LF	RF	LH	RH	LF	RF	LH	RH
Yield, lb	2458	2894	2353	852†	3852	2736	4806	1999†
Cell count, cells × 10^{-6}/ml								
Total	0·12	0·12	1·25	14·71	0·37	1·27	0·36	6·84
Polymorph	0·06	0·07	0·71	10·82	0·23	0·92	0·21	5·24
Infection, %	10	25	33	66	57	57	70	100
Bacteria present‡	m	m	mH	mSH	m	m	mSH	mSH
Fat, %	3·81	3·81	3·48	2·17	3·57	3·39	3·59	2·65
S.N.F., %	8·55	8·54	8·26	6·64	9·00	8·82	9·05	7·98
Lactose anhydride, %	4·50	4·47	4·14	2·59	4·80	4·67	4·89	3·76
Crude protein (N × 6·38), %	3·17	3·22	3·25	3·21	3·40	3·35	3·36	3·32
N distribution, % total N								
Casein	76·4	76·7	75·8	59·0	74·7	73·7	76·1	72·8
β-lactoglobulin	8·8	8·8	8·5	8·0	10·4	10·4	10·4	10·1
Residual albumins	5·1	5·6	5·8	13·0	5·5	5·9	4·9	7·0
Globulins	3·2	2·9	3·3	9·3	2·7	3·1	2·2	3·2
Proteose-peptones	1·9	1·6	2·1	6·2	1·9	2·1	1·6	2·1
NPN	4·6	4·4	4·5	4·5	4·8	4·8	4·8	4·8

* LF, RF, LH, RH refer to left fore, right fore, left hind, right hind quarters.
† Dry before the other quarters.
‡ m = Coagulase-negative micrococci. S = Coagulase-positive staphylococci. H = Haemolytic.

The 2 fore quarters, particularly the left, were relatively free of infection throughout the lactation and their milk had cell counts only a little greater than those in first-lactation milk (Table 1) but, as in the milk of the cow in her sixth lactation (Dawn), the lactose content, which was about 4·7 % for 60 days, decreased slowly during the rest of the lactation.

Violet

Eighth lactation, 13393 lb milk in 409 days, Table 4.

This cow provided somewhat unusual results and was not in calf by the end of the lactation. The weighted average results for the whole lactation for the milk of 2 quarters, the LF and LH, were higher for fat, S.N.F., lactose and total N than the corresponding results for the first-lactation cows (Table 1), although 57 % of these milk samples were infected with coagulase-negative bacteria and, at 2 sampling occasions, with small numbers of streptococci as well. The proportions of total N present as SP–PP were, however, slightly higher. There was no decline in the lactose concentration in the milk of these quarters after early lactation as had occurred in the milk

from the relatively uninfected quarters of the other old cows. By contrast, milk from the RH quarter also contained these types of bacteria in most samples, together with coagulase-positive haemolytic bacteria in 25 % of them, and the quarter had almost stopped lactating (2 lb milk/day) by the 165th day. It produced 2807 lb milk less than the corresponding LH quarter. The cell count, and the values for serum proteins and proteose–peptones of milk from this quarter were abnormally high and the fat, S.N.F. and lactose concentrations abnormally low. The quarter had suffered subclinical mastitis in the preceding 6 lactations with both coagulase-positive and coagulase-negative organisms present in the milk. Milk from the RF quarter was infected after about 50 days in lactation with coagulase-positive bacteria and this was accompanied by a sharp rise in cell count, a fall in casein and lactose concentrations and a rise in the values for SP–PP N.

Table 5. *The lactation yield and average composition of the milk of each quarter of* 1 *cow* (*Elsa* 3) *in her tenth and* 1 *cow* (*Carnation*) *in her eleventh lactation*

Cow	Elsa 3				Carnation			
Quarter*	LF	RF	LH	RH	LF	RF	LH	RH
Yield, lb	2681	2374	2728	1912	1059	2597	3213	3094
Cell count, cells × 10^{-6}/ml								
Total	0·39	0·68	0·36	0·76	1·67	0·12	0·49	0·13
Polymorph	0·21	0·45	0·17	0·43	1·12	0·06	0·32	0·06
Infection, %	10	25	25	33	65	6	42	12
Bacteria present†	m	m	mS	m	mSH	mSH	m	mSH
Fat, %	4·27	4·22	4·04	4·13	3·65	3·99	3·57	3·65
S.N.F., %	8·48	8·47	8·58	8·56	8·27	8·56	8·39	8·42
Lactose anhydride, %	4·30	4·22	4·40	4·35	3·89	4·41	4·31	4·36
Crude protein (N × 6·38), %	3·31	3·38	3·34	3·35	3·49	3·31	3·24	3·21
N distribution, % total N								
Casein	76·5	76·5	78·2	73·6	70·6	76·1	74·7	75·4
β-lactoglobulin	7·6	7·4	7·5	7·6	7·7	7·6	7·5	7·3
Residual albumins	5·4	5·3	4·9	5·2	7·2	5·5	6·2	6·1
Globulins	3·1	3·7	2·7	3·4	5·4	3·1	3·8	3·4
Proteose-peptones	2·4	2·1	1·8	5·9	3·0	2·6	2·5	2·5
NPN	5·0	5·0	4·9	4·3	4·8	5·1	5·3	5·3

* LF, RF, LH, RH refer to left fore, right fore, left hind, right hind quarters.
† m = Coagulase-negative micrococci. S = Coagulase-positive staphylococci. H = Haemolytic.

Elsa 3

Tenth lactation, 9695 lb milk in 248 days, Table 5.

Coagulase-negative bacteria were present in the milk of all the quarters early in lactation and there were high cell counts which tended to increase as the lactation progressed. The lactation average percentages of fat and total N in the milk were very similar to those in the milk of the first-lactation cows but the average lactose contents for the lactation (and hence the S.N.F. contents) were all lower, despite being at about 4·8 % for the first 90 days of lactation. The concentration of SP–PP N increased towards the end of lactation.

The milk yield from the RH quarter was 816 lb less than that from the LH quarter, itself diseased. This loss was probably the result of infection contracted during the current lactation but may also have been contributed to by the subclinical mastitis that occurred in this quarter in the 8 preceding lactations.

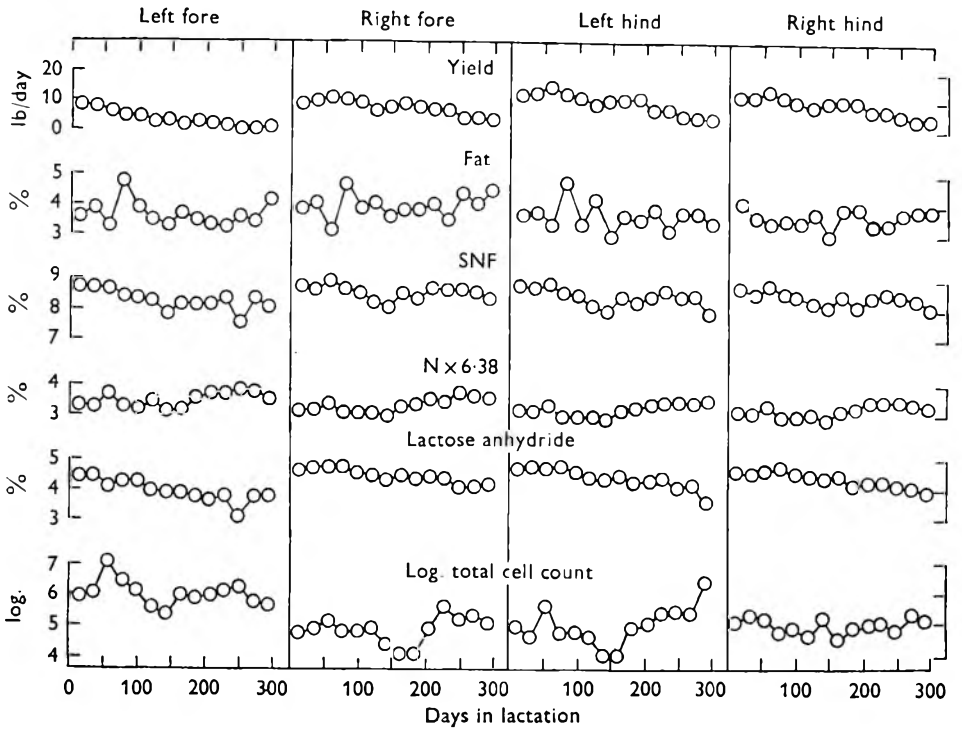

Fig. 3. The yield, chemical composition and cell count of the milk from the 4 quarters of an eleventh-lactation cow (Carnation).

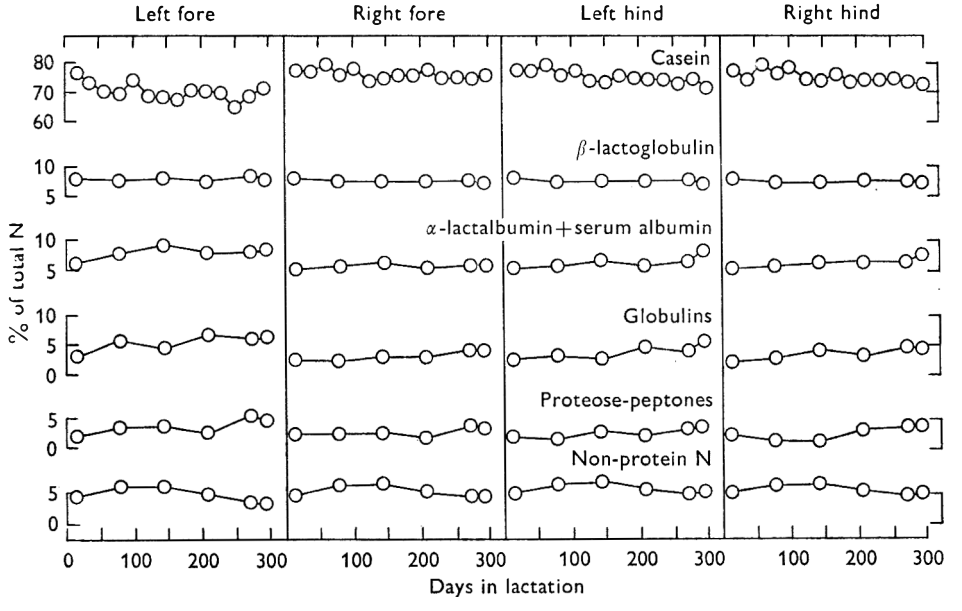

Fig. 4. The N distribution (as % of the total N) in the milk from the 4 quarters of an eleventh-lactation cow (Carnation).

Carnation

Eleventh lactation, 9963 lb milk in 338 days, Table 5.

The results for the whole of the lactation for this cow, the oldest used in the investigation, are shown in Figs. 3 and 4 as a good example of milk from an old cow in which infection in the current lactation was not an overriding factor.

The milk of the 2 right quarters was almost free of infection, with low cell counts, but was lower in its content of fat, lactose, casein and S.N.F. than that of the first lactation cows, and higher in SP–PP N. Milk from the LH quarter, which had suffered severe subclinical mastitis in the 2 previous lactations, had more infection, higher cell counts and poorer chemical composition. The LF quarter was infected throughout the lactation with both coagulase-negative and coagulase-positive haemolytic bacteria and the same condition had existed for most of the preceding lactation. This was associated in the milk with high cell counts, lower fat, S.N.F., casein and lactose concentrations, and higher proportions of the total N were present as residual albumins, globulins and proteose-peptones than in the milk from the other 3 quarters. The milk yield from this quarter was 1538 lb less than that from the corresponding RF quarter. Except in the milk from the badly infected LF quarter the lactose concentration for the first 80 days of lactation was about 4·7 %, but thereafter it fell continuously to the end of lactation, although the cell counts in the 2 right quarters were low.

Cows in commercial herds

At the first time of sampling, on average 58 days after parturition, the milk from all the quarters of 6 out of 8 first-lactation cows was normal but one animal had 1 quarter and one other had 3 quarters affected by subclinical mastitis, as judged by cell count, lactose content and the values for SP–PP N. As the purpose in analysing the milk of first lactation-cows was to provide values for substantially disease-free milk, the analyses from these 4 quarters were omitted from the calculation of the average composition given in Table 6.

The number of quarters of the 23 older cows that were producing milk of low cell count and normal chemical composition at the first sampling was 65, but this number had fallen to 35 at the second sampling 105 days later. The quarters of these cows have therefore been grouped in 3 categories: (*a*) quarters producing milk low in cell count and in blood serum proteins at both sampling dates, (*b*) quarters producing milk low in cell count and blood serum proteins at the first sampling but not at the second, and (*c*) quarters producing milk high in cell count and having higher values for blood serum proteins than the first group at both the first and second time of sampling. The average composition of the milk of the 3 groups of quarters is given in Table 6 together with a measure of the decline in milk yield between the 2 sampling occasions (the 3 groups contained similar numbers of forequarters but the numbers of hind-quarters in groups (*a*), (*b*) and (*c*) were 21, 14 and 11, respectively, making a direct comparison of milk yields inaccurate).

Comparison of the values for the milk of first-lactation cows in Tables 1 and 6 shows that at both sampling dates the animals in commercial herds produced milk which did not differ greatly from the milk of the cows used in the 1950–53 investigation or from the corresponding milk from the Institute herd. At the second

sampling there had been a small drop in lactose concentration but no rise in cell count or serum N. The total N content had increased appreciably.

At the first sampling the milk of the best group of quarters of the cows in their fifth–eleventh lactation was similar in cell count to that of the younger cows from the same herds but it was lower in its content of fat, lactose and total N and had slightly higher values for globulins and proteose–peptones N. At the second sampling, 105 days later, the fat and total N percentages had increased but lactose concentration had fallen by 0·17 percentage units without any significant change in either the cell count or the SP–PP fraction of the N.

Table 6. *The composition of milk from cows in* 8 *commercial herds after* 58 *and* 163 *days in lactation*

	First-lactation cows		Quarters of fifth–eleventh lactation cows: (*a*) Both samples normal		(*b*) 1st sample normal, 2nd not		(*c*) Both samples abnormal	
	58 days	163 days	58 days	163 days	58 days	163 days	58 days	163 days
No. of quarters	28	28	35	35	30	30	27	27
Relative yield, % at 1st sampling	100	85	100	87	100	62	100	60
Cell count, cells × 10^{-6}/ml								
Total	0·06	0·09	0·07	0·09	0·07	0·38	0·76	0·87
Polymorph	0·02	0·03	0·02	0·03	0·02	0·12	0·25	0·31
Fat, %	4·29	4·11	3·94	4·28	3·86	4·44	3·93	4·18
S.N.F., %	8·96	9·07	8·72	8·91	8·74	8·75	8·41	8·59
Lactose, %	4·76	4·62	4·64	4·47	4·64	4·18	4·14	3·97
Crude protein (N × 6·38), %	3·30	3·64	3·21	3·56	3·23	3·70	3·38	3·73
N distribution, % total N								
Casein	80·3	80·0	78·6	78·3	79·4	77·0	75·0	75·4
β-lactoglobulin	7·9	8·0	8·2	8·4	8·0	8·3	8·0	8·3
Residual albumins	4·4	4·3	4·4	4·3	4·4	4·7	5·5	5·1
Globulins	1·5	1·6	2·1	2·2	2·1	2·7	3·5	3·2
Proteose-peptones	1·0	1·2	1·6	1·8	1·4	2·6	3·1	3·1
NPN	4·9	4·9	5·1	5·0	4·7	4·7	4·9	4·9

The milk from the second group of quarters of the old cows was very similar in all constituents to that of the first group at the first sampling, as might be expected, but at the second sampling the average cell count had risen from 70000 to 380000 cells/ml, the lactose content had dropped by 0·46 percentage units and values for the SP–PP fraction of the total N had increased. The fat and total N percentages were markedly higher in the second samples.

The effect of subclinical mastitis on the composition of the milk of the third group of quarters of the old cows was clear even at the first sampling when the milks were found to have high cell counts, low lactose percentages and high SP–PP fractions. The milk quality had deteriorated further at the second sampling.

The milk yield at the second sampling for the first lactation cows and for the best group of quarters of the old cows was 85 and 87 %, respectively, of the yield of these quarters at the first sampling, but in the second and third group of quarters of the old cows the yield at the second sampling had dropped to 62 and 60 % of that at the first.

DISCUSSION

The chemical composition of the milk of the old cows studied in this work was closely connected with the incidence and severity of disease. This was reflected most markedly in the lowered lactose concentration but was also evident in the higher levels observed for serum proteins and proteose–peptones. These changes are consistent with the suggestion of Barry & Rowland (1953) that infected mammary tissue allows a transudation of blood serum into the sinuses or milk duct system with a consequent dilution of the milk. As a lactation progressed it was most noticeable that the chemical changes in the milk of a quarter occurred at much the same time as the presence of infection and a rise in the cell count, particularly of the polymorphs. There was, in fact, a significant inverse correlation between the total cell count and lactose concentration for diseased quarters, the correlation coefficient ranging from $-0{\cdot}7$ ($P < 0{\cdot}01$) to $-0{\cdot}9$ ($P < 0{\cdot}001$).

The lactation average cell count of the milk from the first-lactation cows was about 100000 cells/ml, but in the 8 old cows from the Institute herd only 6 quarters (18%) (Dawn, LF; Gadfly, LF and LH; Elsa 4, LF and RF and Carnation, RF) produced milk which had a lactation average count of that order. The position of the old cows in commercial herds was better as far as the 160th day of lactation, when the milk from 38% of the quarters had cell counts averaging about 100000 cells/ml. Since many of the high polymorph counts occur in the last third of lactation (Blackburn, 1966), the lactation average cell counts for these cows might well have been higher than that recorded at the 160th day. High cell counts and marked changes in chemical composition of the type described above were always associated with the presence of coagulase-positive staphylococci in the milk although on numerous occasions similar but less severe effects were observed when coagulase-negative bacteria were present.

In Tables 1–5 the concentrations of the various N constituents have been expressed as a percentage of the total N and calculated as weighted lactation averages. This was done to enable comparisons to be made between cows but it tends to mask the magnitude of the changes involved. In the RF quarter of Veronica, for example (Table 3), the amount of residual albumins, globulins and proteose–peptones N rose from 25, 10 and 7 mg N/100 g milk to 55, 53 and 22 mg, respectively, under the influence of disease, i.e. 2- to 5-fold increases. A characteristic of the milk of all the old cows, even of the quarters with least infection and lowest cell counts, was the slightly higher value for SP–PP N than was found in the milk of the young cows. Whether this represents a continuing permeability to blood serum of some parts of the mammary tissue infected in earlier lactations is not clear, although it seems highly probable that this could have accounted for the poor milk yield and composition from at least 5 quarters (Dolphin, LH; Elsa 4, RH; Violet, RH; Elsa 3, RH; Carnation, LF).

In the milk from 7 quarters of the old cows in the Institute herd it was, however, noted that the amount of infection and the level of the cell count were not much higher than in the milk of the cows in their first lactation. The chief difference in chemical composition between these 2 types of milk as the lactation progressed was a decline after about 80–90 days in the lactose concentration and a rise in the SP–PP

N value of the milk from the best quarters of the old cows, changes which did not occur in the milk of the young cows. This suggests that there may have been a slightly increased permeability of mammary tissue to blood serum with age that was independent of current disease, although the possibility that some of it resulted from previous infections cannot be eliminated. Walsh, Rook & Dodd (1968*a*, *b*) have recently calculated values for the effect of age on the lactose concentration of the milk of a number of cows and these values show a marked increase after the first lactation. There are, of course, blood serum proteins in the milk from disease-free quarters of first-lactation cows and these may enter through the epithelial layers of the duct system or through the tissue supporting the milk secreting lobules. In view of the renewal of the milk-secreting cells with each pregnancy and some evidence that

Table 7. *Probable loss of milk from infected quarters in* 1 *lactation*

Cow	Recorded lactation yield, lb	Affected quarter	Probable loss, lb
Dolphin	10107	LF	702
		LH	607
Gadfly 4	9351	RH	861
Veronica	8827	RF	943
Elsa 4	8557	RH	1501
Violet	13393	RF	1116
		RH	2807
Elsa 3	9695	RH	816
Carnation	9963	LH	1538
Total	69893		10891 (15·8%)

the milk yield/unit volume of udder tissue is very similar over several lactations (Linzell, 1966) it seems likely that the increased amounts of blood serum constituents in the milk from disease-free quarters of old cows may enter via the same route.

The effect of udder infections on milk yield is difficult to assess because of the possibility of development of compensatory tissue by a healthy quarter on the same side of the udder as a diseased quarter. Blackburn (personal communication), however, is of the opinion, based on post-mortem histological examination of many udders which had been producing infected milk from one or more quarters, that such compensation rarely occurs in the lactation in which the infection first develops. However, a comparison of milk yields from infected and non-infected quarters on opposite sides of the udder (comparing fore- with fore- and hind- with hind-quarter) obviates the need to consider such compensatory growth, and this type of comparison showed large losses of milk from the affected quarters. These are summarized in Table 7, where it can be seen that the lactation loss of milk from 7 cows probably amounted to not less than 15% of the actual production. If the milk so lost had the same composition as that from the quarter of each cow with least infection it would correspond to a loss of some 370 lb fat and 720 lb S.N.F.

In addition to this loss there is also the loss of milk constituents in the milk actually produced, because of the poorer chemical composition of the milk from the affected quarters. A measure of this can be obtained by comparing the weighted

lactation average composition of the milk from the affected quarters with that of the milk from the best quarter, i.e. the quarter having least infection, lowest cell counts and highest chemical composition. (When all 4 quarters are affected the comparison can only be made with the least affected, and hence the loss of constituents is underestimated.) When this comparison was made for the 8 old cows from the Institute herd the range of the losses, in percentage units, were: fat, 0·06–0·41; S.N.F., 0·08–0·38 lactose, 0·11–0·40. There was no loss of total N but there was less casein and more SP–PP N in milk from affected quarters. The weighted mean loss for the 8 cows was 0·18 % fat, 0·21 % S.N.F. and 0·22 % lactose. With the 23 old cows in the commercial herds, the weighted mean loss at the first sampling was: S.N.F., 0·12 %; lactose, 0·16 %. There was no loss of total N and the fat percentage in the milk from the diseased quarters was slightly higher than in the milk of the best quarters. At the second sampling the loss was a little higher: S.N.F., 0·16 %; lactose, 0·18 %. Recently Walsh *et al.* (1968*b*) have given values for the effect of inter-quarter difference and of udder infection on the lactose concentration of the milk of Friesian cows in 8 herds. The herd mean values for inter-quarter difference ranged from 0·07 to 0·25 percentage units of lactose and for disease from 0·04 to 0·21 percentage units. Since both these effects may have stemmed from injury or disease at some time it would appear that the values given above are fairly typical.

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The sterilizing effect against *Bacillus subtilis* spores of hydrogen peroxide at different temperatures and concentrations

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Summary. An investigation was made of the death-rate of *Bacillus subtilis* spores in suspension in hydrogen peroxide concentrations of 10, 15 and 20 % at 25, 50, 60, 70 and 80 °C. Average logarithmic survival curves were plotted against normal time and death-rate constants and Q_{10} values were calculated from the graphically determined decimal reduction times. This treatment of the experimental findings was found to be warranted in the temperature range 60–80 °C. The first-order death-rate constant was ~ 0·1 s^{-1} for 10 % hydrogen peroxide solution at 60 °C and the Q_{10} was about 1·6. Increases in the concentration from 10 to 15 % and from 15 to 20 % each gave an increase of about 50 % in the rate constant. The application of these data to commercial practice is discussed.

In the present Tetra Pak aseptic filling system the paper is sterilized by treatment with a solution of hydrogen peroxide at the ambient temperature followed by radiation heating (Swartling & Lindgren, 1962). To adapt this system to other methods of filling it was desirable to avoid this heat treatment, or to reduce its intensity without at the same time reducing the efficiency of sterilization. It was thought that this might be achieved by raising the temperature of the hydrogen peroxide solution. Investigations by Curran, Evans & Leviton (1940) had shown that the 50 % destruction time for spores of *Bacillus cereus* var. *albolactis* in a 1 % hydrogen peroxide solution could be decreased from 12 min to 1 min by raising the temperature from 30 to 70 °C. The present paper reports a study of the influence of heat-treatment with hydrogen peroxide for different lengths of time, and at different concentrations and temperatures, on the death-rate of *B. subtilis* spores.

MATERIALS AND METHODS

Test organism. The test organism, *B. subtilis* NCDO 736 (ATCC 95244) was obtained from the National Collection of Dairy Organisms, Shinfield, Reading, England. Stock cultures were maintained in nutrient agar (Difco) stabs, which were incubated for 48 h at 30 °C then stored under paraffin oil in the refrigerator.

Media. Nutrient broth was used for the first step in the preparation of spore suspensions. The sporulation medium (HB) was the same as that used by Halvorson (1957) in his work on rapid and simultaneous spore formation. This medium with 1·5 % agar incorporated was also used as a solid medium.

* Now deceased.

The spores were counted by plating on the milk starch agar (MSA) of Grinsted & Clegg (1955) and suspended in the buffer solution suggested by Morrison & Rettger (1930).

Preparation of spore suspension. Nutrient broth was inoculated from the stock culture of *B. subtilis*, and then incubated at 30 °C for about 24 h. Bacterial cells from this culture were then inoculated into a tube of HB medium which in turn was incubated at 30 °C for 24 h. A 10 % inoculum was then transferred from this culture to a fresh tube of HB medium which was heated to 70–80 °C for 10 min, cooled to 30 °C and incubated for 4 h. A new transfer to HB was then made and incubated for 3 h. The resulting culture was used for seeding plates of HB agar. After incubation for 48 h the cells and spores were washed off the agar surface with 0·9 % saline, filtered through cotton, centrifuged, washed 3 times with sterile distilled water and finally suspended in buffer solution (Morrison & Rettger, 1930). The suspension was kept in a refrigerator.

Before use the spore suspension was diluted to contain about 2×10^6 spores/ml, heated to 70–80 °C for 10 min and cooled.

Hydrogen peroxide solutions. Solutions containing 10, 15 and 20 % H_2O_2 were prepared by diluting a commercial stabilized hydrogen peroxide solution (Eka, Bohus) with distilled water. The pH values of the solutions were 2·3, 2·0 and 1·8, respectively. The concentrations of the solutions were checked by titrimetric determination of the hydrogen peroxide. It was also ascertained by this means that the concentration of hydrogen peroxide remained constant during the experiments even at elevated temperatures.

Catalase. Catalase (Sigma Chemical Co. Ltd., St Louis, U.S.A.) was dissolved in 0·9 % saline and filtered through a Seitz filter. The activity of the solution was checked before use.

Procedure. Twenty ml of the hydrogen peroxide solution under test was heated in a test tube immersed in a water bath, which was thermostatically controlled at 25, 50, 60, 70 or 80 °C. When the solution had reached the desired temperature, 1 ml of the diluted spore suspension was added and well mixed in. At intervals, generally after 0·25, 0·5, 1, 2, 3 and 5 min, samples (1 ml) were withdrawn and transferred to another tube containing 0·9 % saline containing enough catalase to decompose all the hydrogen peroxide. The numbers of surviving organisms in the samples and the numbers of spores in the suspension used were determined by plating on MSA agar.

Parallel tests were run using buffer solution instead of the hydrogen peroxide solution.

Calculations. The short exposure time at the elevated temperatures made mixing and withdrawal of samples rather difficult and the accuracy was not good. To compensate for this, all experiments were repeated several times. The average survival counts for any one exposure time and concentration were determined and used for plotting log. 10 survival curves as shown in Fig. 1. The 'tail' of the curves was generally not clearly defined owing to the difficulty of determining exact survival counts for 1 spore or less/ml. Such values were ignored when drawing the curves.

The curves in Fig. 1 are representative of the findings. The curves had a more or less pronounced shoulder, especially at the lower temperatures. Such curves are often met with in this kind of work. The reasons why the death curves were not

strictly logarithmic have been widely discussed (cf. Rotman & Fields, 1966; Humphrey & Nickerson, 1961) and several methods for the treatment of such results have been suggested (Schnell & Ernst, 1962; Wang, Sharer & Humphrey, 1964; Fernelius, Wilkes, De Armon Jr. & Lincoln, 1958). So far, the explanations and the methods proposed seem to be not entirely satisfactory. For our present purpose, it is sufficiently accurate to treat the curves as exponential and beginning with the original number of spores as determined by plating, especially for the high temperatures at which the shoulder was less pronounced.

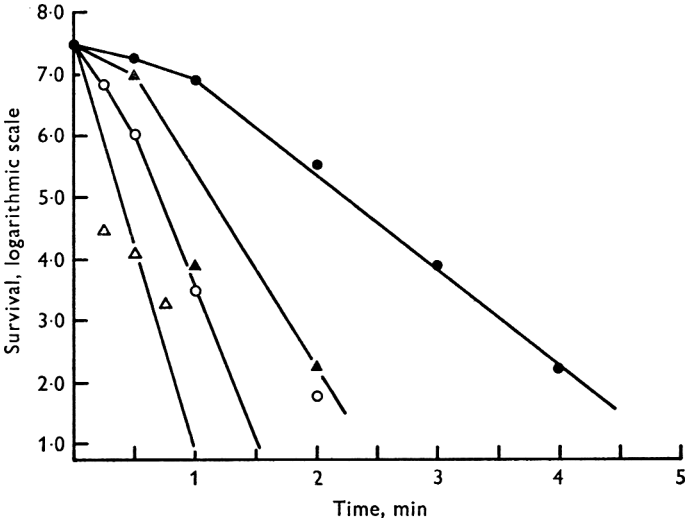

Fig. 1. Survival counts for *B. subtilis* spores in 10 % H_2O_2 solution at 50 °C (●), 60 °C (▲). 70 °C (○) and 80 °C (△).

The results were, therefore, analysed in the following way. The logarithmic survival curves were used for a graphical determination of the time in seconds required for a reduction of 4 log cycles in the original spore count as determined by plating. The first-order death-rate constants and the Q_{10} values were calculated from these values (cf. Meynell & Meynell, 1965). The values obtained were used for extrapolation to temperature–time combinations not tested.

RESULTS

Table 1 gives the time in seconds necessary for a reduction by 4 log. cycles in the spore counts, as determined graphically from the logarithmic survival curves obtained for the various temperatures and concentrations tested.

The values of Table 1 were used for plotting temperatures against the logarithm of the time in seconds for the 3 concentrations of hydrogen peroxide. The diagrams shown in Fig. 2 were obtained.

Between 60 and 80 °C these curves came out as straight lines, indicating that the survival curves could be treated as exponential in this range of temperature without any gross error. At 50 °C, however, the rather broad shoulder at 10 and 15 % hydrogen peroxide introduces a complication.

The first-order death-rate constants found for the temperatures and concentrations tested are given in Table 2. The death-rate constant increased with temperature and with concentration of hydrogen peroxide.

Table 1. *Time (sec) for reduction of 4 log. cycles in the numbers of spores at different concentrations of hydrogen peroxide*

Temp., °C	Hydrogen peroxide concentration, % 10	15	20
25	1640	780	570
50	192	128	66
60	96	53	45
70	60	39	26
80	36	23	15

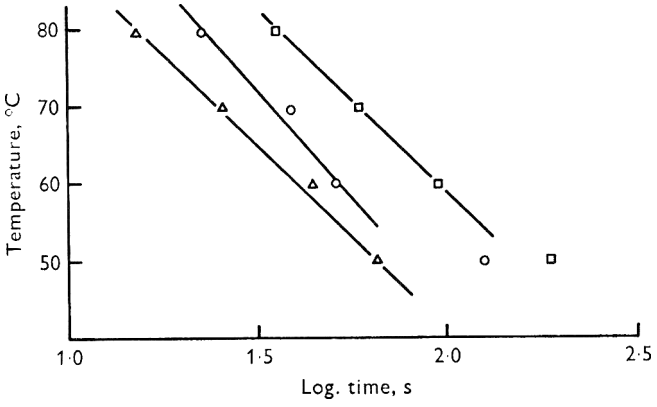

Fig. 2. Exposure time (log. (sec)) for a reduction in the numbers of spores of 4 logarithmic cycles at different temperatures. □, 10 % H_2O_2; ○, 15 % H_2O_2; △, 20 % H_2O_2.

Table 2. *First-order death-rate constants* (s^{-1}) *for* B. subtilis *spores in hydrogen peroxide solution of various concentrations and at various temperatures*

Temp., °C	Hydrogen peroxide concentration, % 10	15	20
50	0·05	0·07	0·14
60	0·10	0·17	0·20
70	0·15	0·24	0·35
80	0·26	0·40	0·61

In the parallel experiments run with buffer solution instead of the hydrogen peroxide, temperature *per se* was found not to influence the spore count during the short times of exposure used in the experiments. The influence of temperature on the death-rate is further demonstrated in Table 3, which gives the temperature coefficients Q_{10} in the temperature range 60–80 °C, where the calculation of Q_{10} seemed justified. As expected from Fig. 2, the Q_{10} values were fairly constant. The average value was 1·63. This value is somewhat lower than the value 2–3 calculated from data of Curran *et al.* (1940). Q_{10} values of this order are considerably lower than those reported

for heat sterilization of bacterial spores (6·6–16) but are similar to those found for chemical or enzymic reactions.

The concentration of the hydrogen peroxide influenced the death rate of the spores. On average, the increases in concentration from 10 to 15 % and from 15 to 20 % each gave an increase of about 50 % in the death-rate constants.

The data presented in Tables 1 and 2 enable calculations to be made of death-rate constants and decimal reduction times for some temperature conditions which are of practical importance though difficult to measure with the rather simple bacteriological technique used in this investigation.

Table 3. *Temperature coefficients,* Q_{10}, *obtained for the temperature range* 60–80 °*C*

	Hydrogen peroxide concentration, %		
Temp., °C	10	15	20
60–70	1·59	1·36	1·74
70–80	1·67	1·69	1·73

Table 4. *Times (to the nearest second) taken to cause reductions of* 3–6 *log. cycles in the numbers of* B. subtilis *spores heated at* 80, 90 *and* 95 °*C in* 15 *or* 20 % *hydrogen peroxide*

Reduction in numbers (logarithmic cycles)	Hydrogen peroxide concentration, %					
	15			20		
	80 °C*	90 °C†	95 °C†	80 °C*	90 °C†	95 °C†
3	17	10	9	11	7	5
4	23	14	11	15	9	7
5	29	18	14	19	12	9
6	35	21	16	23	14	11

* By experiment. † By extrapolation.

Table 4 shows the calculated times for 3-, 4-, 5- and 6-fold decimal reduction of the spore count at 90 and 95 °C, together with the times actually found for 80 °C.

The values given in Table 4 may be used for determining the time–temperature concentration conditions necessary for sterilization by means of a hydrogen peroxide solution. The factors to be considered when using the table are the initial spore count of the material to be sterilized and the degree of sterilization desired.

CONCLUSIONS

If in commercial packaging of sterile products the number of non-sterile packages cannot be allowed to exceed 1 in 1000 packages, the initial spore count on the inner surface of the paper must be reduced to 1 spore or less on the surface of the whole amount of paper used for 1000 packages. For 250-ml Tetra Pak packages the area of the inner surface is about 270 cm^2. With an initial spore count of 0·04/cm^2, a count that is often found on commercial Tetra Pak paper, a decimal reduction of 4 would be just sufficient. In this case, treatment of the paper for about 10 sec in a 20 % hydrogen peroxide solution at 90–95 °C would meet the requirements with a satisfactory margin of safety.

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Chemical methods for assessing lipid oxidation in ultra-high-temperature creams

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SUMMARY. The flavour quality and stability of 3 different UHT creams are described. The rate of oxidation was measured by the thiobarbituric acid (TBA) and peroxide methods as well as by organoleptic assessment. Lipid oxidation developed in some UHT creams on prolonged storage, particularly in creams manufactured during the winter months and stored at 18 °C, while little or no oxidation occurred at 4 °C. Creams manufactured during the summer months were most resistant to oxidation. Creams with no detectable oxidized flavour had TBA values $\leqslant 0{\cdot}08$; values $\geqslant 0{\cdot}16$ and peroxide values $\geqslant 2{\cdot}0$ were associated with pronounced oxidized flavour. These relationships between the chemical and organoleptic assessments were highly significant ($P < 0{\cdot}01$). Low peroxide levels ($< 2{\cdot}0$) were not necessarily indicative of good flavour quality.

Flavour deterioration due to lipid oxidation is encountered in many foods, and considerable costs are often incurred in providing protection against oxidative defects. In some dairy products, particularly dried whole milk, auto-oxidation has been a major obstacle to the development of an acceptable product. Flavour deterioration has been extensively studied in most dairy products (Brown & Thurston, 1940; Greenbank, 1948; Pont, 1960; Day, 1960; Wilkinson, 1964), but there is little published information on the incidence of lipid oxidation in ultra-high-temperature (UHT) sterilized creams.

The present investigation establishes that lipid oxidation is largely responsible for flavour deterioration in some UHT creams. The rates of lipid oxidation under various storage conditions are described and methods for monitoring the rate of oxidation are outlined.

MATERIALS AND METHODS

Three UHT creams (A, B and C) manufactured in different countries were studied. Cartons of cream manufactured during the period October 1966–October 1967 were placed in incubators at 4, 10 and 18 °C as soon as possible after processing. Some cartons were flushed with nitrogen for 2 min at a flow rate of 100 ml/min, under aseptic conditions, before incubation at 18 °C.

Two cartons corresponding with each treatment were removed from the incubators at weekly intervals and analysed both by organoleptic and by chemical methods for the development of off-flavour. The cartons were shaken and their contents transferred to

a beaker and warmed with gentle stirring to 40 °C. Suitable quantities of cream were removed for organoleptic, thiobarbituric acid (TBA), and free fatty acid (FFA) analyses. The remaining portions were subjected to alternate freezing in a salt/ice mixture and thawing by heating to 60 °C, to induce oiling-off of the fat. Peroxide estimations were then performed on suitable samples of the clear oil obtained by centrifuging at 5000 rev/min for 15 min at 45 °C. Considerable difficulty was encountered in oiling-off the creams designated B and on many occasions it proved impossible to do so.

Flavour score

Flavour evaluations were carried out by a panel of trained tasters who scored coded samples at 15 °C as having acceptable, doubtful, or unacceptable flavour characteristics.

Thiobarbituric acid method (TBA)

The TBA method was that of King (1962), with certain modifications. Stoppered test tubes (Quickfit and Quartz Ltd.; cat. no. MF 24/2/6), containing UHT cream (20 ml), were incubated for 15 min in a water bath at 60 °C. Trichloroacetic acid solution (1 ml; 100 %, w/v) was added, the tubes inverted once and returned to the water bath for 15 min. Ethanol (A.R. grade, 2 ml; 95 %, v/v) was added and the tubes shaken vigorously for 5 sec and returned to the bath for a further 15 min. The contents were then filtered through a Whatman no. 42 filter paper and 0·5 ml of TBA solution (prepared by dissolving 1·4 g of 2-thiobarbituric acid (BDH Ltd.) in 95 % (v/v) ethanol to 100 ml) was added to 2 ml of the filtrate and the mixture incubated for 60 min at 60 °C. After cooling, the optical density was read both at 538 nm and at 700 nm in a 1-cm cell. Estimations were done in duplicate, together with blank analyses in which 20 ml of water was substituted for the cream. A slight cloudiness was encountered in some filtrates, and the true optical density at 538 nm was obtained by subtracting the reading at 700 nm from that at 538 nm. The results are expressed as $E^{1\,cm}_{538\,nm}$/20 ml of cream.

Graded quantities of a cream with a pronounced off-flavour were diluted to 20 ml with water and analysed as above. A linear relationship was obtained between the quantity of cream and the optical density (Fig. 1(*a*)).

Peroxide test

Duplicate peroxide analyses were carried out using 0·1 ml quantities of oil prepared from the cream, according to the method of Loftus Hills & Thiel (1946), modified as described by Holloway (1966). Results are expressed as peroxide values (m-equiv. of oxygen/kg of oil). Graded quantities of oil prepared from a cream with a pronounced off-flavour were assayed in duplicate for peroxide development. A linear relationship was obtained between the quantity of oil and the peroxide value (Fig. 1(*b*)).

Free fatty acids (FFA)

Various fatty acid extraction procedures were investigated and optimum recoveries of added fatty acids were obtained by 2 extraction methods similar to those described by Frankel & Tarassuk (1955, 1956) for milk.

Method 1

Equal (2 ml) quantities of cream and 1·0 M-H_3PO_4 contained in stoppered test tubes (Quickfit and Quartz Ltd., cat. no. MF 24/1/5) were shaken vigorously for 2 min on a modified 'Bara' shaker (Baird and Tatlock (London) Ltd.). Diethyl-ether (10 ml) was added and the tubes were shaken for a further 3 min and then centrifuged at 3500 rev/min for 5 min. From the 6 ml total volume of clear solvent layer, a 5-ml sample was removed for titration with 0·01 M alcoholic NaOH in the presence of neutralized ethanol (5 ml) with phenolphthalein as indicator. Blank analyses were carried out on the solvent mixture alone. Results are expressed as ml of 0·01 M-NaOH/ml of cream. By this extraction procedure, up to 80 % of butyric acid added to the cream was recovered, and approximately 30 % of oleic acid.

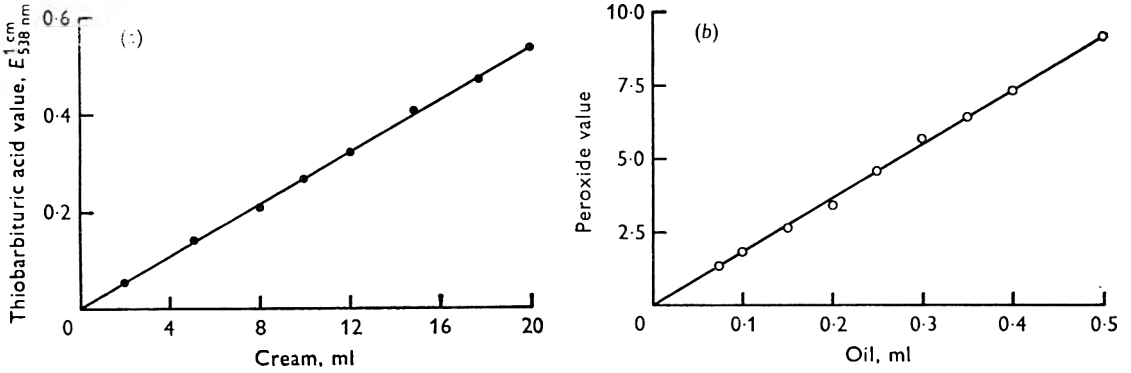

Fig. 1. (*a*) Relationship between quantity of oxidized cream and the thiobarbituric acid value. (*b*) Relationship between quantity of oil prepared from oxidized cream and peroxide value.

Method 2

Equal (2 ml) quantities of cream and of neutralized alcohol were shaken in stoppered test tubes as described above and the fatty acids extracted by the addition of 5 ml of diethyl-ether/petroleum ether (40:60, v/v). From the 4·5 ml total volume of clear extract, 3-ml samples were taken and titrated as in method 1. By this procedure, 70–80 % of added oleic acid was recovered from the cream whereas the recovery of butyric acid was less than 10 %.

RESULTS

Creams of the 'B' series had a chalky grainy flavour which changed little on storage. The 'C' creams had a pronounced cooked flavour which was detectable up to 3 or 4 weeks after processing, when it was replaced by a cheesy flavour. The 'A' creams initially had a slight cooked flavour, but this was generally not detectable after storage for 1 week, when the flavour of the product approached that of fresh cream. On prolonged storage the flavour deteriorated and simultaneously lipid oxidation occurred as indicated by an increase in the TBA and peroxide values of the cream (Fig. 2). The rate of oxidation is markedly influenced by the storage temperatures (Fig. 2(*a*)) and, in general, oxidation was 2–3 times more rapid at 18 than at 10 °C, while little or no oxidation took place at 4 °C. Furthermore, the onset of flavour deterioration was considerably delayed by flushing the cartons with nitrogen immediately after processing. In most of the 'A' creams the TBA values progressively

increased during the initial 6–8 weeks of storage at 18 °C and subsequently declined slowly. The TBA values for samples taken on successive weeks showed considerable fluctuations in those creams which were very susceptible to oxidation (Fig. 2(*b*)) and on prolonged storage the TBA values of such creams stored at 4 °C exceeded those of creams stored at 18 °C. The patterns for peroxide production resembled those observed for the TBA levels (Fig. 2(*b*)). As with the TBA levels, the peroxide values increased during the initial weeks of incubation and then decreased. On prolonged incubation, creams held at 4 °C had higher peroxide values than those stored at 18 °C (Fig. 2(*b*)).

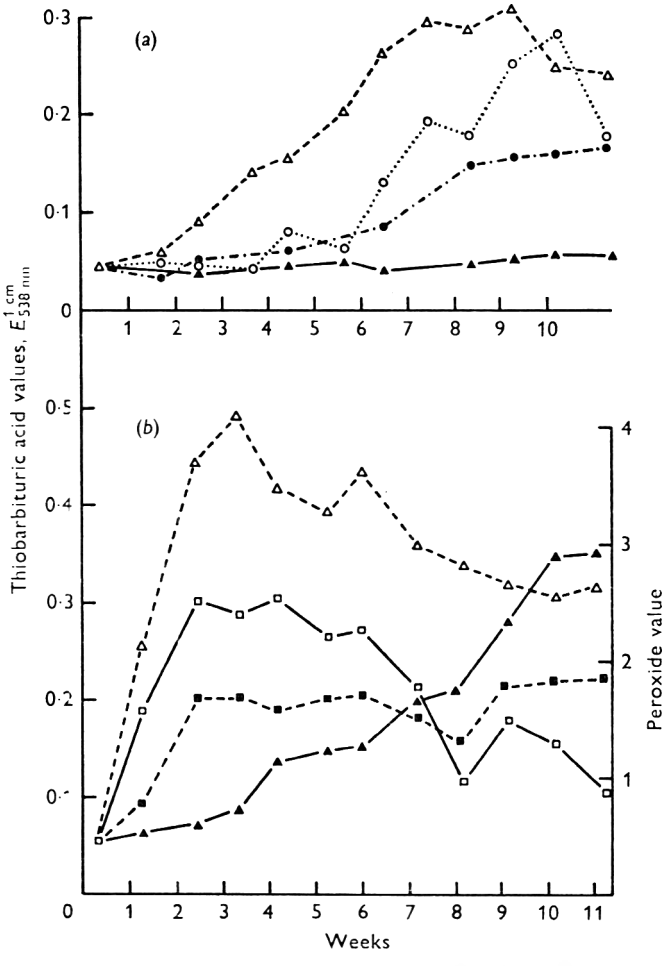

Fig. 2. Rate of lipid oxidation in UHT cream manufactured in July (*a*) and in December (*b*) and stored at 18 °C, △ – – △; TBA values of creams flushed with nitrogen prior to storage at 18 °C, ○ · · · ○; TBA values of creams stored at 10 °C, ● – · – ●, and 4 °C, ▲ — ▲; peroxide values of creams stored at 18 °C, □—□, and 4 °C, ■ – – ■.

The cheesy off-flavour observed in the 'C' creams was not due to lipid oxidation since the TBA and peroxide levels of these creams showed no change on storage. Similarly, no products of lipid oxidation were detected in the 'B' creams.

No increase in FFA was observed on incubation of any of the present creams for up to 12 week sat the different temperatures, but the initial concentration of FFA in the 'A' creams, as measured by method 2, showed a marked seasonal variation

(Fig. 3). No such variation was detected by method 1. Furthermore, the concentration of FFA, measured by method 2, was particularly high in the 'C' creams throughout the period of the investigation.

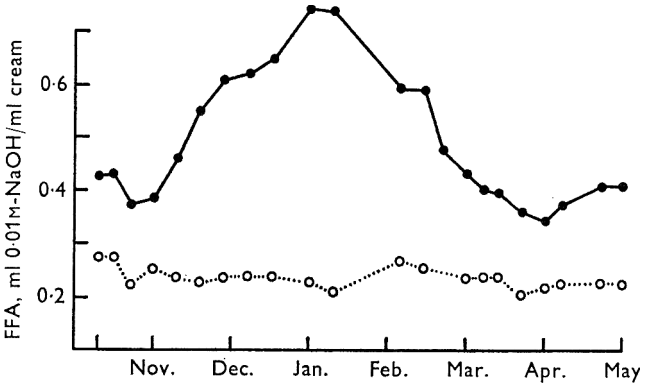

Fig. 3. Seasonal variation in the concentration of FFA extracted by method 1 (○···○) and method 2 (●—●).

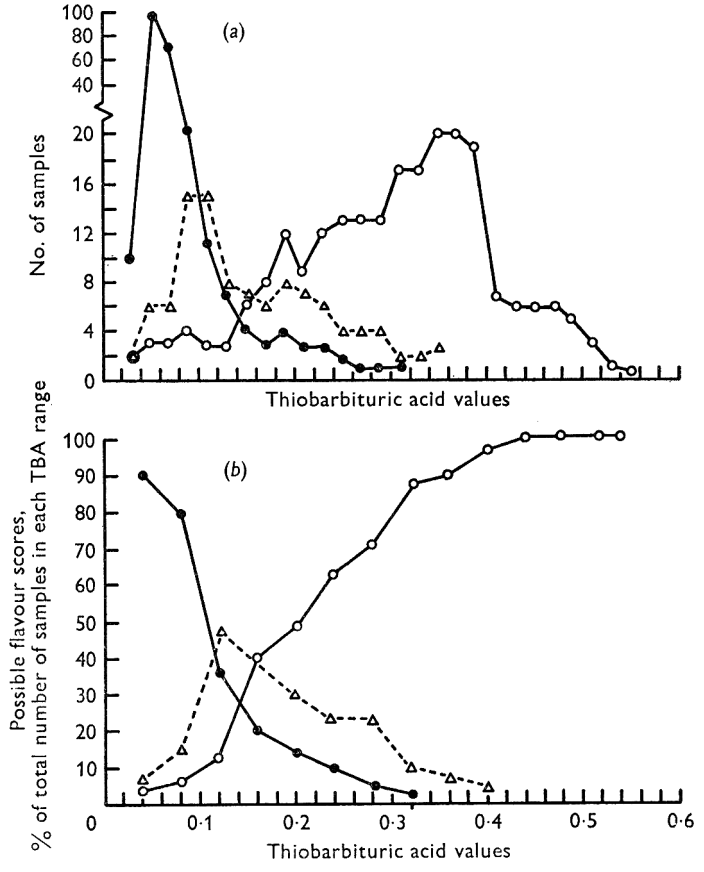

Fig. 4. (*a*) Graph showing numbers of samples with acceptable (●—●), doubtful (△- -△) and unacceptable (○—○) flavour characteristics in each of the various TBA ranges. (Range corresponds to TBA increment of 0·02, see text.) (*b*) Percentage of samples with acceptable (●—●), doubtful (△- -△) and unacceptable (○—○) flavour properties in each of the various ranges. (Range corresponds to TBA increment of 0·04, see text.) Each point in both graphs is located at the centre of its respective range.

The relationships between the 3 flavour scores of the creams and both the TBA and peroxide values are shown in Figs. 4 and 5, respectively. Creams were graded into ranges of ascending order corresponding to TBA increments of 0·02 and according to peroxide increments of 0·166. The number of samples of each of the 3 flavour scores in each individual range was plotted against TBA values (Fig. 4(*a*)) and peroxide values (Fig. 5(*a*)). Each point in both graphs is located at the centre of its respective range. The creams with acceptable or unacceptable flavour constitute well-defined areas of both graphs although some overlapping does occur, particularly with the

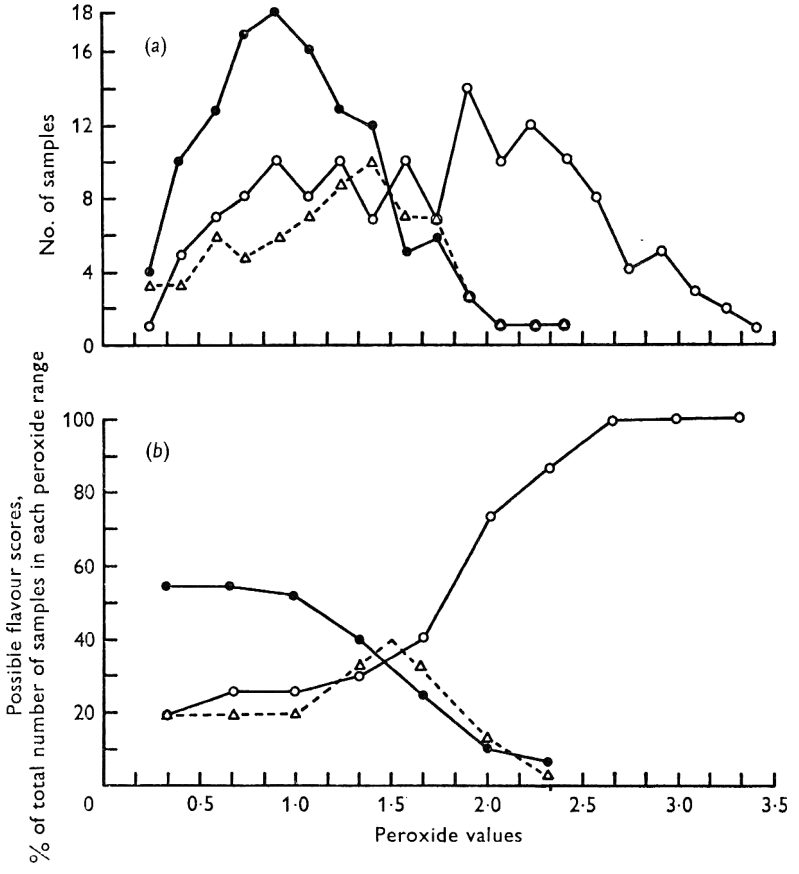

Fig. 5. (*a*) Graph showing numbers of samples with acceptable (●—●), doubtful (△- -△) and unacceptable (○—○) flavour characteristics in each of the various peroxide ranges. (Range corresponds to a peroxide increment of 0·166, see text.) (*b*) Percentage of samples with acceptable (●—●), doubtful (△- -△) and unacceptable (○—○) flavour properties in each of the various peroxide ranges. (Range corresponds to a peroxide increment of 0·33, see text.) Each point in both graphs is located at the centre of its respective range.

peroxide values (Fig. 5(*a*)). In contrast, the areas occupied by the creams of doubtful flavour characteristics are largely masked by those in the acceptable category. The percentage distribution of the 3 flavour scores of the creams in the various TBA and peroxide ranges are shown in Figs. 4(*b*) and 5(*b*). A better percentage distribution pattern was obtained by increasing both the TBA and peroxide increments of each range 2-fold, relative to those used in Figs. 4(*a*) and (*b*), respectively. From these graphs the flavour scores of a cream can be predicted from either its TBA or peroxide value.

Of the creams with TBA values equal to 0·08, 80 % were considered by the taste panel to have acceptable flavour while only 5 % had attained unacceptable levels of off-flavour (Fig. 4(*b*)). The possibility of a cream having acceptable flavour is increased at lower TBA values—over 90 % of all creams with TBA values up to and including 0·08 had acceptable flavours, while only 3·5 % were unacceptable (Table 1). In contrast, of the creams with TBA values equal to 0·16, less than 20 % had acceptable flavour, and as the TBA values increased further, the probability of a cream having acceptable flavour was markedly reduced. In fact, less than 7 % of all creams with TBA values greater than and including 0·16 had acceptable flavour,

Table 1. *Frequency distribution of creams according to flavour score and chemical assessment*

(*a*) Thiobarbituric acid (TBA) ranges

Flavour scores	⩽ 0·08	0·08–0·16	⩾ 0·16	No. of samples
Acceptable	181 (90 %)	49 (44·5 %)	18 (6·8 %)	248
Doubtful	13 (6·5 %)	45 (40·9 %)	48 (18·2 %)	106
Unacceptable	7 (3·5 %)	16 (14·6 %)	198 (75 %)	221
Total	201 (100 %)	110 (100 %)	264 (100 %)	575

(*b*) Peroxide ranges

	< 2·0	⩾ 2·0	No. of samples
Acceptable	118 (43·5 %)	3 (4·9 %)	121
Doubtful	66 (24·4 %)	2 (3·3 %)	68
Unacceptable	87 (32·1 %)	56 (91·8 %)	143
Total	271 (100 %)	61 (100 %)	332

while 75 % were scored unacceptable (Table 1). In the present investigation, creams with TBA values up to and including 0·08 were considered to have good flavour quality, while those with TBA values greater than and including 0·16 were deemed to have attained objectional levels of off-flavour. The flavour quality of creams with TBA values in the range 0·08–0·16 can only be decided satisfactorily by means of a taste panel. As shown by chi-square analysis, the relationship between the 3 flavour scores and the 3 categories of creams as determined by TBA value (Table 1) was highly significant ($P < 0{\cdot}01$; $\chi^2 = 401$).

Similar deductions may be made with the peroxide test (Figs. 5(*a*) and (*b*)). In contrast to the TBA method, a low peroxide value is not necessarily indicative of an acceptable flavour since almost 30 % of creams with peroxide values equal to 1·0 had unacceptable flavour (Fig. 5(*b*)). Further reduction in the peroxide value has very little effect on the possible flavour score of the creams and 28 % of all creams with peroxide values less than 1·0 had unacceptable flavour and only 53 % were scored acceptable. However, a high peroxide value was indicative of poor flavour and 75 % of creams with peroxide values equal to 2·0 had unacceptable flavour and only 12 % were judged acceptable (Fig. 5(*b*)). In the present study, creams with peroxide values greater than and including 2·0 were considered to have attained unacceptable levels of off-flavour and, as shown by chi-square analysis, the relationship between the 3 flavour scores and the 2 categories as determined by the peroxide method (Table 1) was highly significant ($P < 0{\cdot}01$; $\chi^2 = 71$). Over 90 % of all creams with peroxide

values greater than and including 2·0 had unacceptable flavour (Table 1), but over 30 % of all creams with peroxide values less than 2·0 were scored acceptable.

A TBA value of 0·16 and peroxide value of 2·0, being independently derived criteria used to identify creams of unacceptable flavour, do not necessarily reflect the same degree of lipid oxidation. In general, a TBA value of 0·16 is equivalent to a peroxide value of 1·0–1·5. But as it was not possible (Fig. 5) to predict with reasonable confidence the flavour score of creams from peroxide values in this range, the higher peroxide value of 2·0 was selected.

The susceptibility of creams to lipid oxidation, as indicated by the number of weeks required for a cream to reach a TBA level of 0·16 on storage at 18 °C, showed a marked seasonal variation (Fig. 6). The protein content of the creams also varied (McGann, 1968, personal communication) (Fig. 6).

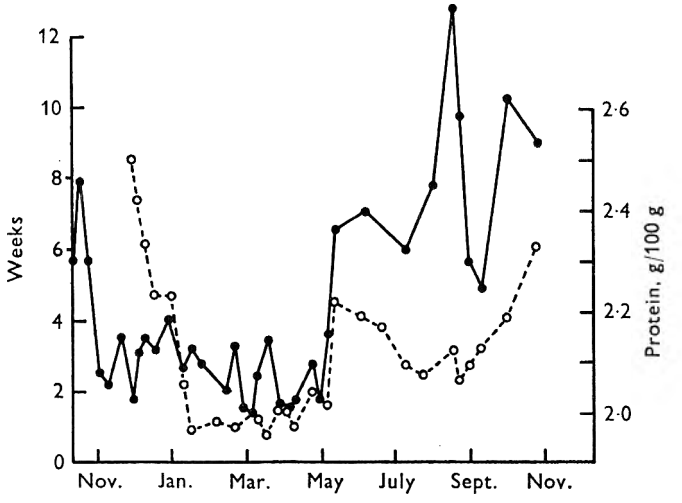

Fig. 6. ●—●, Seasonal variation in the rate of lipid oxidation in creams as measured by the number of weeks required for the cream to reach a TBA value of 0·16 at 18 °C; O--O, Seasonal variation in the protein concentration of creams A.

DISCUSSION AND CONCLUSIONS

The flavours of the 3 creams examined were more acceptable than were those of conventional sterilized cream and in general the flavour of the 'A' creams approached that of fresh cream most closely. The chalky grainy texture of the 'B' creams may be similar to the flavour defect which is often encountered in homogenized milk and which is generally attributed (Trout, 1950) to the use of high homogenization pressures. The pronounced cooked flavour of the 'C' creams is indicative of severe heat-treatment resulting in sulphydryl group activation. The correlation between both the TBA and peroxide methods, which specifically measure lipid oxidation products (Wilkinson, 1964), and the organoleptic assessments indicates that the principal off-flavour detected by the taste panel in the 'A' creams after prolonged storage was largely due to lipid oxidation. This conclusion is supported by the delay in the onset of off-flavour development which resulted from flushing with nitrogen.

Off-flavours from other sources such as protein degradation may also occur in

UHT creams. The cheesy flavour which developed in the 'C' creams after 3–4 weeks storage at 18 °C may have been due to protein hydrolysis, presumably by heat-stable or reactivated proteolytic enzymes. Evidence for proteolysis during storage of UHT milk was reported by Samuelsson & Holm (1966) and similar results have been observed for the 'A' creams (Keogh, 1967, personal communication). The lipases in the original milk were apparently inactivated by the processing conditions, since no increase in FFA was detected on incubation of any of the present creams at the different temperatures. Furthermore, there was no evidence of reactivation similar to that observed for lipases in milk (Nilsson & Willart, 1961) and sterilized ice-milk mixes (Wallander & Swanson, 1965), or for phosphatase in UHT milk (Ashton, 1965). The high levels of FFA detected by method 2 in the 'A' creams manufactured during the winter period (Fig. 3) may be attributed to the use of late lactation milks, which are very susceptible to lipolysis (Fredeen, Bowstead, Dunkley & Smith, 1951). It is difficult to explain why a similar seasonal variation was not detected in the concentration of fatty acids extracted by method 1, though as indicated by the relative recoveries of short- and long-chain fatty acids by both methods, the fatty-acid composition of the extracts are apparently different.

The marked variations in the rates of lipid oxidation in the 'A' creams manufactured during the winter and summer periods apparently reflect compositional changes in the original milks and in the pro- and anti-oxidant content of the processed cream. Numerous investigators (cf. Parks, 1965) have observed that homogenization inhibits the development of lipid oxidation in dairy products. Consequently, the more complete homogenization attainable with summer creams as a result of their higher protein concentration (Fig. 6) could provide increased protection against oxidation. However, other factors such as the bacteriological quality of the original cream may also be involved. The marked oxidative stability of the 'B' creams may also have resulted from more complete homogenization. Furthermore, the elevated levels of sulphydryl groups in summer milks (Ashton, 1965) could inhibit lipid oxidation as was observed by Harland, Coulter & Jenness (1952), who found that in whole-milk powders with relatively high free sulphydryl content and associated cooked flavour the development of lipid oxidation was inhibited during 10 weeks storage. In this connexion it was observed in the present study that creams of the 'A' series which were manufactured during the mid-summer period had a more pronounced cooked flavour, and were particularly resistant to oxidation (Fig. 6). Similarly, the high free sulphydryl content of the 'C' creams, indicated by their pronounced cooked flavour, may contribute to their resistance to oxidation.

The close correlation obtained between the chemical and organoleptic methods for monitoring the development of lipid oxidation permits objective assessment of the flavour quality of some UHT creams by chemical tests (Figs. 4(*b*) and 5(*b*)). Of the 2 methods used in this investigation the TBA method is the more useful since it identifies creams as possessing either acceptable or unacceptable flavours. The peroxide method, on the other hand, will only identify creams with unacceptable flavour, as low peroxide values are not necessarily indicative of low levels of lipid oxidation. Wilkinson (1964) also noted that the relationship of peroxide value to off-flavour does not always hold well in dairy products. Furthermore, the preparation

of oil from UHT cream, which is the initial step in peroxide estimation, is very time-consuming and difficult, particularly with creams which are highly homogenized.

The gradual decline in TBA reactive material on prolonged incubation of the 'A' creams (Fig. 2(*b*)) indicates that malonaldehyde, the compound responsible for colour formation in the TBA test, is not the final product in lipid oxidation in UHT cream. However, since the TBA levels do not decline below 0·2 even on extended incubation, the deduction that creams with TBA levels greater than and including 0·16 have unacceptable flavour is not invalidated. Similarly, the peroxide values fall off on prolonged incubation at 18 °C but do not decline below a peroxide value of 2·0 and in the present investigation creams with peroxide values greater than 2·0 were deemed to have unacceptable flavour quality.

The marked reduction in the rate of lipid oxidation at the lower incubation temperature suggests that creams should be stored in the cold until the time of distribution. In some creams, lipid oxidation is almost completely suppressed by storage at 4 °C (Fig. 2(*a*)). Furthermore, the delay in the onset of lipid oxidation at 18 °C following nitrogen flushing indicates that oxygen should be excluded from the cream. This could be achieved by 'vacuum cooling' (Hand, Guthrie & Sharp, 1938), in which the cream is boiled under reduced pressure and the resultant evaporation of water flushes out the dissolved oxygen. By such procedures, together with storage in the cold, it is likely that lipid oxidation in UHT cream would be completely inhibited.

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With 2 plates

A preliminary study by gel filtration and ultracentrifugation of the interaction of bovine milk caseins with detergents

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Summary. The detergents sodium dodecyl sulphate and octyl phenoxy polyethoxyethanol interact with casein and cause dissociation of the high-molecular-weight casein aggregates. It is presumed that the detergent binds with hydrophobic regions in the casein molecule. The size of the complexes formed between detergents and α_{s1}-casein, β-casein and κ-casein, as estimated by gel filtration and sedimentation velocity experiments, suggests that the caseins were complexed as monomers.

During gel filtration under non-reducing conditions, detergent-κ-casein complexes were separated from other major components because of their conversion through formation of disulphide bonds into high-molecular-weight aggregates. This reaction, which did not occur in sedimentation velocity experiments, was presumably facilitated by the changes in the equilibrium between the individual caseins during gel filtration.

Sedimentation velocity experiments showed that a ratio of about 40 detergent molecules to 1 casein molecule was required to give the smallest casein–detergent complex.

Introduction

Association in α_{s1}-casein and also in β-casein is favoured by rise in temperature and has been suggested by Payens & Schmidt (1965), from the evidence of the values obtained for the enthalpies and entropies of association, to be due to hydrophobic bonding.

In the work now reported this suggestion has been further explored by studying the association behaviour of whole casein and of κ-casein in the presence of detergents under conditions such that the detergent might be expected to react with the hydrophobic regions of the protein and thus interfere with polymerization.

Most of the experiments were done using an anionic detergent, sodium dodecyl sulphate; a few experiments were carried out using a nonionic detergent, octyl phenoxy polyethoxyethanol. The association of casein in the presence of varying amounts of detergent was examined by gel filtration in Sephadex G. 200 and by analytical ultracentrifugation.

The behaviour of whole casein and κ-casein on gel filtration has been reported (e.g. Yaguchi, Davies & Kim, 1968; Yaguchi & Tarassuk, 1967; Nakai, Wilson & Herreid, 1966) and these authors have shown that while separation of casein com-

ponents and aggregates occurs the type of separation obtained depends upon the conditions used for filtration, such as type of gel, temperature, pH of eluting buffer and presence of dissociating agents such as urea.

MATERIALS AND METHODS

Preparation of caseins

Solutions of casein were prepared from acid precipitated casein obtained from skim-milk of an individual Friesian cow having the phenotypes α_{s1}-casein B, β-casein A, and κ-casein A. Acid casein was prepared by precipitation at pH 4·6 with N-HCl, the precipitate being washed and redissolved in water by addition of N-NaOH to pH 7·5–8·0. The resulting solution of sodium caseinate was treated by addition of N-HCl to pH 4·6 and the precipitate was washed with water and stored at −24 °C. Kappa-casein was prepared from milk of the same cow by the method of Zittle & Custer (1963) but without the final ethanol precipitation. When required for use the caseins were dissolved in water by the minimal addition of N-NaOH and dialysed against the selected buffer for 24 h at 4 °C.

Gel chromatography

Gel filtration in Sephadex gel G. 200 (Pharmacia, Uppsala, Sweden) was carried out using 0·05 M Tris-HCl buffer of pH 7·6 containing 0·001 M disodium ethylenediaminetetraacetic acid (EDTA) as the eluting buffer. The Sephadex was washed clean of fines with water, and after establishing equilibrium with buffer it was packed at room temperature into a Sephadex chromatography column (K 20/100) to give a gel column measuring 86 cm × 2·5 cm diam. A flow rate of 12–15 ml/h was maintained by positioning the level of fluid in the buffer reservoir about 15 cm above the top of the column. The samples in 5–20 ml volumes were applied to the gel column using a sample applicator (Pharmacia, Uppsala, Sweden). All the experiments were done at room temperature.

Sodium dodecyl sulphate (SDS; B.D.H. Ltd., Poole, England) and octyl phenoxy polyethoxyethanol (OPP; Triton X-100; Sigma Chem. Co. Ltd., St Louis, U.S.A.) were added to the buffer when casein and detergent interactions were examined. Monomeric forms of κ-casein were obtained by gel filtration using the same buffer system with detergent and containing 0·001 M-dithiothreitol (DTT) as a reducing agent.

The column eluate was collected in 5 ml fractions by use of a fraction collector (Aimer Ltd., London). The presence of material absorbing at 280 nm was detected using a Unicam SP 500 and a 1-cm cell. Selected tubes, as shown in the figures, were pooled and the casein components recovered as precipitates from buffer-containing detergents by first extensively dialysing against distilled water to remove the detergent and then adjusting the pH value to 4·6 with N-HCl and adding solid ammonium sulphate to half saturation.

Rennin treatment

Casein fractions at about 1 % concentration in 0·2 M acetate buffer of pH 6·0 were treated with rennin at a concentration of 1 μg/ml for 30 min at room temperature.

Gel electrophoresis

Starch-gel electrophoresis (SGE) was carried out using thin horizontal gels (18 × 10 × 0·15 cm) with 0·05 M Tris-HCl buffer of pH 8·6 containing 5 M urea and 0·001 M DTT in the gel and 0·38 M Tris-glycine buffer of pH 8·6 in the electrode

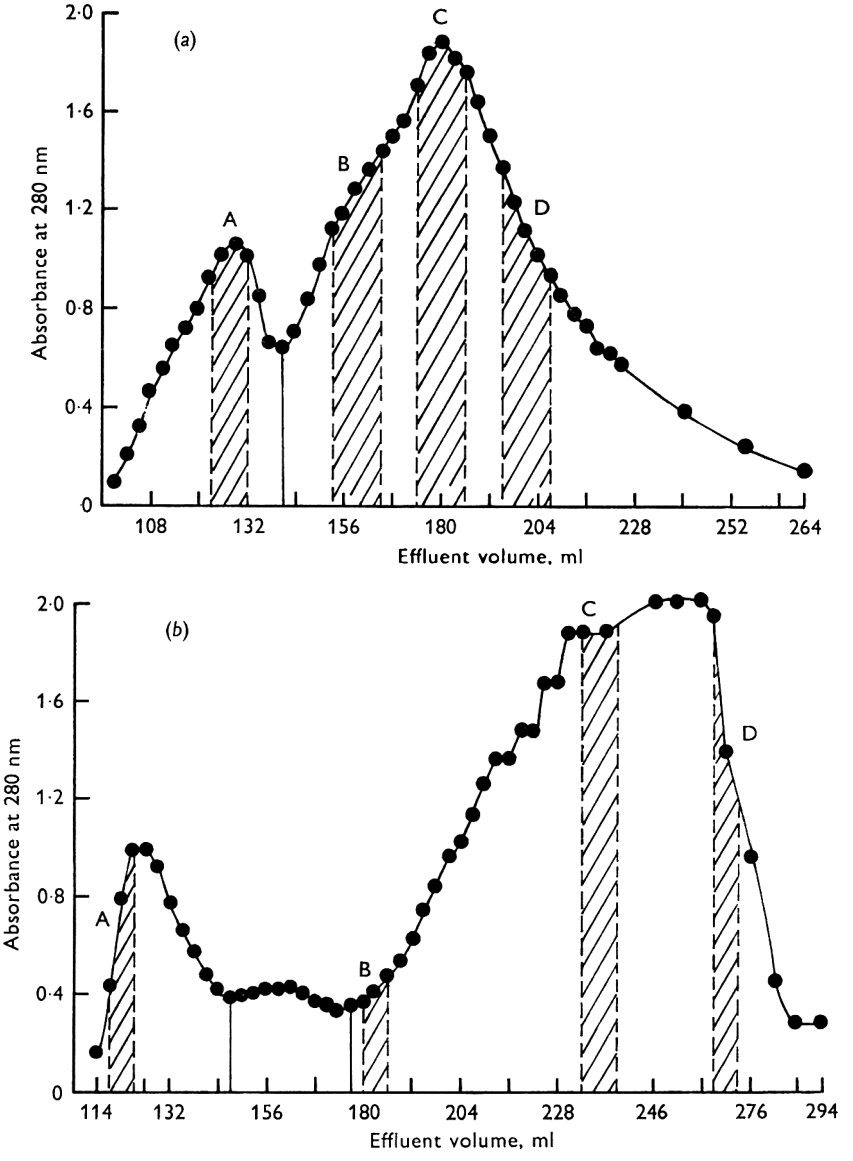

Fig. 1. Elution patterns obtained by filtration in 2·5 × 86 cm column of Sephadex gel G. 200. (*a*) Acid casein: 150 mg casein applied in 5 ml, and eluted with 0·05 M Tris-HCl buffer of pH 7·6 containing 0·001 M EDTA. (*b*) Detergent-treated acid casein: 150 mg casein, applied in 5 ml, and eluted with 0·05 M Tris-HCl buffer of pH 7·6 containing 0·001 M EDTA and 0·02 M sodium dodecyl sulphate. Hatched areas indicate pooled samples, the vertical lines indicate demarcation between peaks for area calculations. Left to right: (*a*): 1st peak 20 %, 2nd peak 80 % of area under the curve. Left to right (*b*): 1st peak 17 %, 2nd peak 8 %, 3rd peak 75 % of area under the curve.

compartment. After electrophoresis for 18 h at 180 V the gels were stained with a solution of amido black (1 %, w/v) in water–methanol–acetic acid (50:40:10, v/v) and washed clean of excess stain with the same solvent.

Analytical ultracentrifugation

Sedimentation velocities were obtained using the Spinco model E analytical ultracentrifuge; rotor AN-D, aluminium single sector cells, at 57980 rev/min and 23 °C. All sedimentation coefficients (S-values) reported are uncorrected.

RESULTS

Gel chromatography of acid casein

The elution pattern obtained by gel filtration of acid casein at room temperature and without detergent is shown in Fig. 1 (*a*) and the corresponding patterns obtained by starch-gel electrophoresis of casein recovered from pooled tubes are shown in Plate 1 (*a*). Sample A, taken from the casein aggregates excluded by the gel, contained α_{s1}-casein together with more κ-casein but rather less β-casein than the acid casein. Samples B and D were similar to the acid casein, while sample C contained relatively more β-casein than did the acid casein. The slower-moving components on SGE were found predominantly in samples A and B. The SGE patterns of these samples after treatment with rennin are shown in Plate 1 (*b*) and suggest that κ-casein was present mainly in sample A and to a lesser extent in B.

Gel chromatography of acid casein in presence of a detergent

The result of gel filtration of acid casein equilibrated with buffer containing sodium dodecyl sulphate is shown in Fig. 1 (*b*) and the SGE patterns of casein recovered from pooled tubes is shown in Plate 2. Sample A now contained relatively pure κ-casein together with some high-molecular-weight impurities. Sample B contained mainly α_{s1}-casein, with a trace of κ-casein. Sample C contained mainly α_{s1}-casein, with a trace of κ-casein. Sample D contained mainly β-casein together with small amounts of α_{s1}-casein and unidentified components in the κ-casein region. Small amounts of slower-moving components were found in all the samples. The SGE patterns of these samples after treatment with rennin confirmed that only traces of κ-casein occurred in samples B, C and D.

Gel chromatography of κ-casein in presence of a detergent

The results of gel filtration of the κ-casein preparation in buffer-containing detergent is shown in Fig. 2 (*a*). The κ-casein occurred in the first peak A and was separated from α_{s1}- and β-casein which were found in the second peak C. The SGE patterns from the pooled tubes of these fractions are shown in Plate 3 (*a*). Some contaminating material occurred with the κ-casein but this was removed by further gel filtration in the presence of DTT. Under reducing conditions resulting from the presence of this reagent the κ-casein was retarded on the gel and was eluted in the volume previously obtained for α_{s1}- and β-casein. The contaminants, with mobilities both faster and slower than κ-casein on SG, were excluded from the gel (Fig. 2 (*b*) and Plate 3 (*b*)).

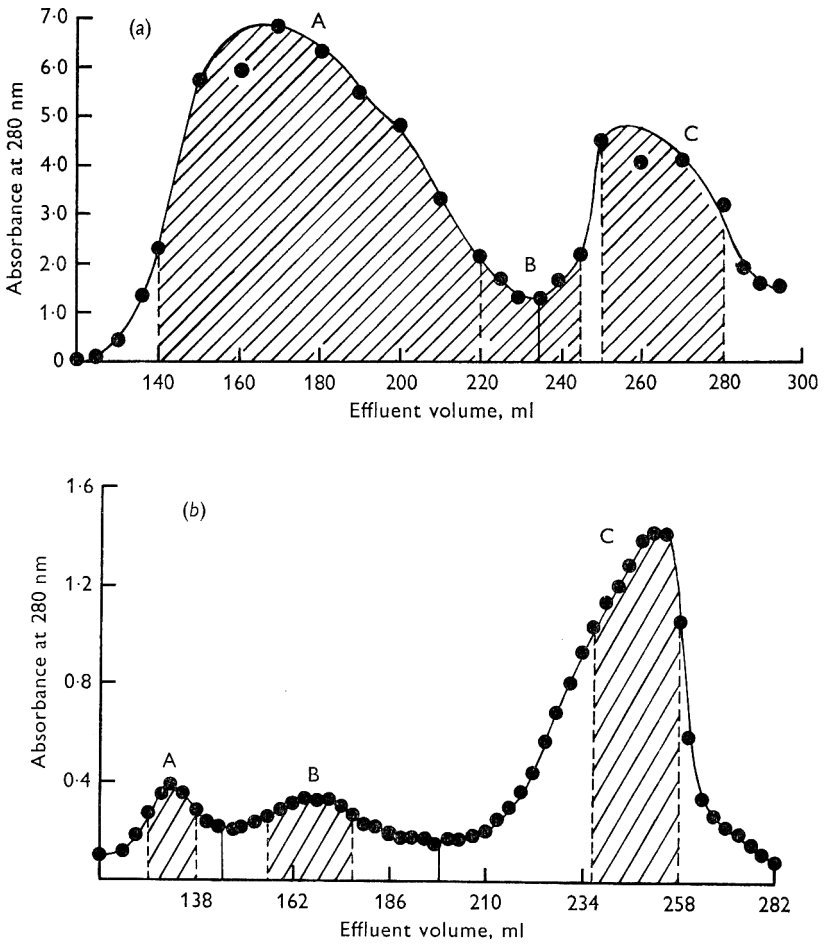

Fig. 2. Elution patterns obtained by filtration in 2·5 × 86 cm column of Sephadex gel G. 200. (*a*) Kappa-casein. 500 mg casein, applied in 20 ml and eluted with 0·05 M Tris-HCl buffer of pH 7·6 containing 0·001 M EDTA and 0·02 M sodium dodecyl sulphate. (*b*) The κ-casein recovered from (*a*) and eluted with the same buffer containing 0·001 M DTT. Hatched areas indicate pooled samples, the vertical lines indicate demarcation between peaks for area calculations. Left to right (*a*): 1st peak 68 %, 2nd peak 32 % of area under the curve. Left to right (*b*): 1st peak 10 %, 2nd peak 20 %, 3rd peak 70 % of area under the curve.

Analytical ultracentrifugation of casein and casein-detergent complexes

A plot of the sedimentation coefficient (S) of casein and the casein–SDS complex against increasing concentrations of SDS is shown in Fig. 3. Under the conditions used for sedimentation a solution of acid casein at approximately 4×10^{-4} M (assuming 25000 M.W.) gave an S value of about 7. The sedimentation coefficient decreased with increase in concentration of SDS to a minimum value of about 2 S at a concentration of $1{\cdot}5 \times 10^{-2}$ M SDS. Examples of the schlieren patterns obtained are shown in Plate 4. The presence of a small amount of material with a high sedimentation rate can be seen in Plate 4 (*a*), which shows the pattern obtained with an SDS concentration of 4×10^{-3} M. The pattern obtained at an SDS concentration of 2×10^{-2} M is shown in

Plate 4(*b*). Experiments in which OPP was used also gave a minimum *S*-value of about 2 *S* at a casein–detergent molar ratio of about 1:40. However, with OPP 2 peaks were obtained at intermediate ratios, the proportion of the 2 *S* increasing as the concentration of OPP increased.

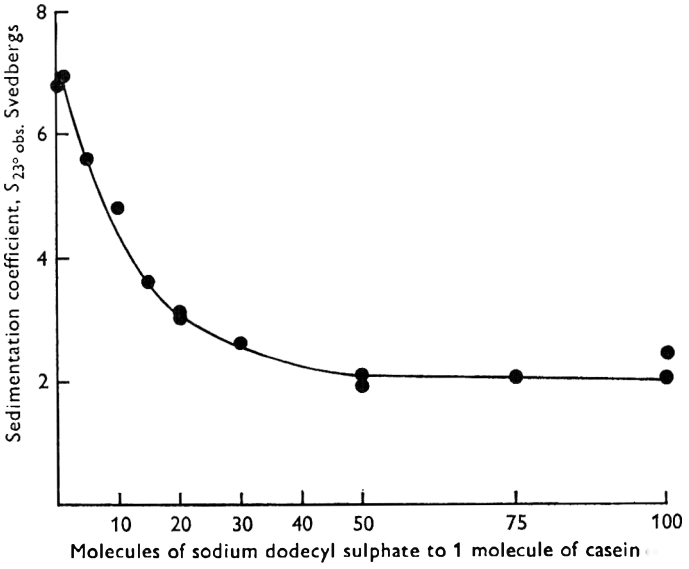

Fig. 3. A plot of sedimentation coefficient of the casein–detergent complex against concentration of SDS. The concentration of casein was about 1 %, w/v (assumed 4×10^{-4} M), in a buffer of pH 7·6 composed of 0·05 M Tris-HCl and 0·05 M sodium chloride, 50 μl of concentrated solutions of SDS were added to 1-ml samples to give the appropriate SDS concentration. Sedimentation velocity experiments were carried out with a Spinco Model E analytical ultracentrifuge operating at 57980 rev/min and 23 °C.

DISCUSSION

The use of detergents to dissociate casein aggregates gives results similar to those obtained with urea or in alkaline conditions. Sodium dodecyl sulphate appeared to give an improved separation of κ-casein from α_{s1}-casein and β-casein on Sephadex gel G. 200, as compared with 6 M-urea which was used as the dissociating agent by Yaguchi & Tarassuk (1967), who found significant amounts of α_{s1}- and β-casein in the fraction enriched in κ-casein. However, the separation of the caseins was also a function of the grade of gel used, and Yaguchi *et al.* (1968), using Sephadex G. 150 and buffers containing urea, obtained good separation of κ-casein from the other components.

The results shown in Plate 3(*a*) and (*b*) demonstrate that when the κ-casein recovered from the first peak (Fig. 2(*a*)) was converted to the reduced and non-aggregated species and again subjected to gel filtration (Fig. 2(*b*)), a significant amount of material giving an amido black reaction on SGE and having ultraviolet absorption at 280 nm was associated with the κ-casein isolated under non-reducing conditions.

The recovery of aggregated κ-casein in good yield in the column void volume was in contrast to the sedimentation velocity experiments carried out at the same casein–

detergent ratios, in which only small quantities of material with high sedimentation rates were observed (cf. Fig. 1(*b*)). These results indicate that the formation of κ-casein aggregates was facilitated during gel filtration. It is possible therefore that a complex between κ-casein and α_{s1}- and β-casein exists even in the presence of detergent but that the equilibrium is disturbed by the removal during gel filtration of low-molecular-weight complexes formed between α_{s1}- and β-caseins and the detergent. With this decrease in amount of α_{s1}- and β-caseins in the casein complex the κ-casein might become unstable and involved in intermolecular disulphide bonding.

The apparent minimum molecular size of the casein–detergent complex occurs at a molar ratio of 40 moles of SDS to 1 mole of casein, when the complex gave an uncorrected sedimentation coefficient of about 2 *S*. The critical micelle-forming concentration of SDS is about $1{\cdot}7 \times 10^{-3}$ M with a micelle composition of about 76 molecules (Anacker, Rush & Johnson, 1964). The micelle has an *S*-value of about 1·8 *S* under the conditions used in our experiments. It has not therefore been possible to detect the presence both of the detergent micelle and the casein–detergent complex in the sedimentation patterns under conditions where excess detergent may have been present in the system.

The different sedimentation velocity results obtained with the non-ionic detergent, octyl phenoxy polyethoxyethanol, suggested that with this detergent 2 complexes existed and that the relative proportions depended upon concentrations of the detergent: this may indicate that the complex of low *S*-value was formed between the detergent micelle and the casein and was not caused by interaction between individual detergent molecules and the casein.

It was possible to use sodium dodecyl sulphate to obtain κ-casein of a high degree of purity by gel filtration using mild preparative treatments. The detergent also acted as a stabilizer for the α_{s1}- and β-casein as is indicated by the finding that complexes of low-molecular-weight probably containing single casein molecules were obtained. This type of complex was formed with κ-casein if an excess of SDS was present, when the casein micelles were solubilized by the removal of calcium. It was also formed by reducing with DTT in the presence of SDS the aggregated κ-casein prepared from acid precipitated casein. The formation of disulphide linkages is inhibited by the presence of the SDS which may interfere sterically with the interaction between κ-casein molecules.

The author is grateful to Mrs J. Jeffcoat and Miss M. A. Raithby for able technical assistance.

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Zittle, C. A. & Custer, J. H. (1963). *J. Dairy Sci.* **46**, 1183.

EXPLANATION OF PLATES

PLATE 1

(*a*) Starch-gel electrophoresis patterns for the caseins recovered from the pooled samples shown in Fig. 1 (*a*). (*b*) As above, but fractions treated with rennin before electrophoresis.

PLATE 2

Starch-gel electrophoresis patterns for the caseins recovered from the pooled samples shown in Fig. 1 (*b*).

PLATE 3

(*a*) Starch-gel electrophoresis patterns for the caseins recovered from the pooled samples shown in Fig. 2 (*a*). (*b*) Starch-gel electrophoresis patterns for the caseins recovered from the pooled samples shown in Fig. 2 (*b*).

PLATE 4

Schlieren patterns obtained with a solution of about 1 % acid casein in 0·05 M Tris-HCl buffer of pH 7·6 containing 0·05 M sodium chloride. Temperature of analysis 23 °C. Terminal speed 57980 rev/min. The solution giving the pattern in the upper photograph contained SDS at a concentration of 4×10^{-3} M. The photograph was taken 34 min after the centrifuge reached two-thirds of its terminal speed. The solution giving the pattern in the lower photograph contained SDS at a concentration of 2×10^{-2} M. The photograph was taken 55 min after the centrifuge reached two-thirds of its terminal speed.

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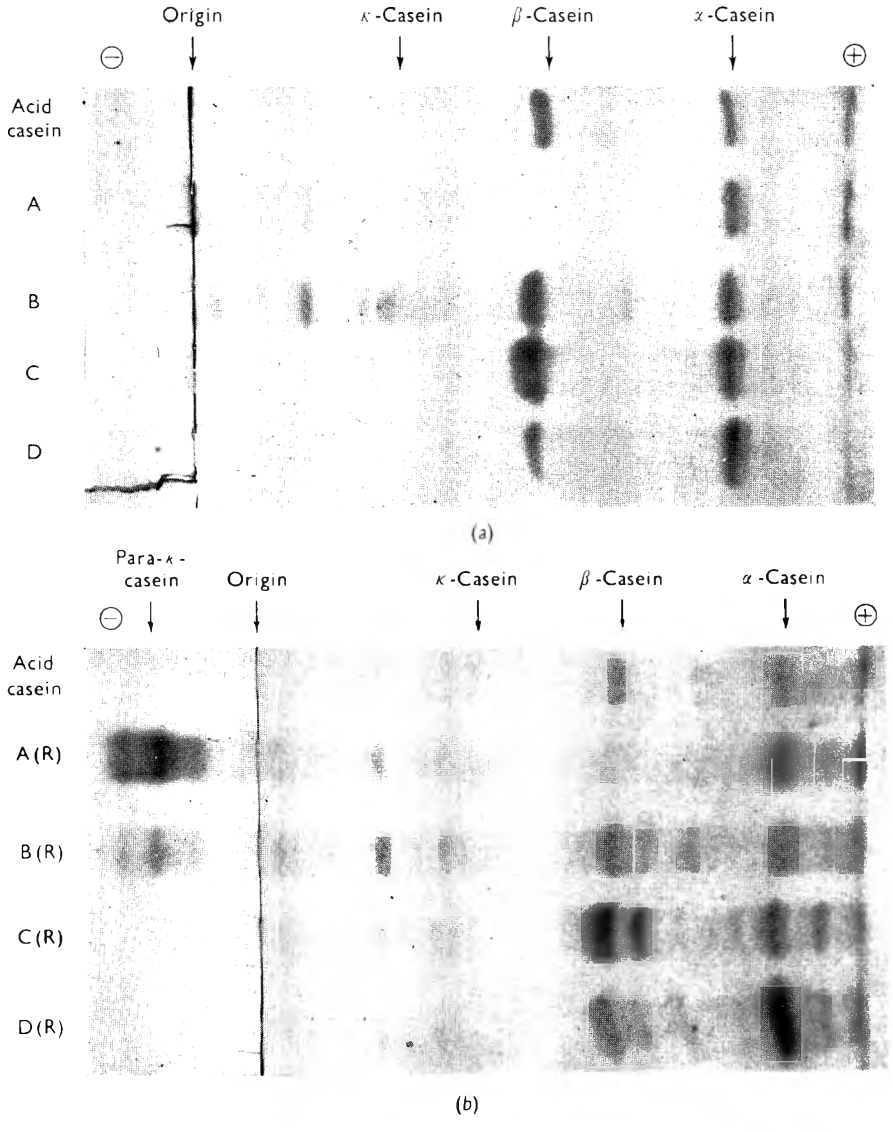
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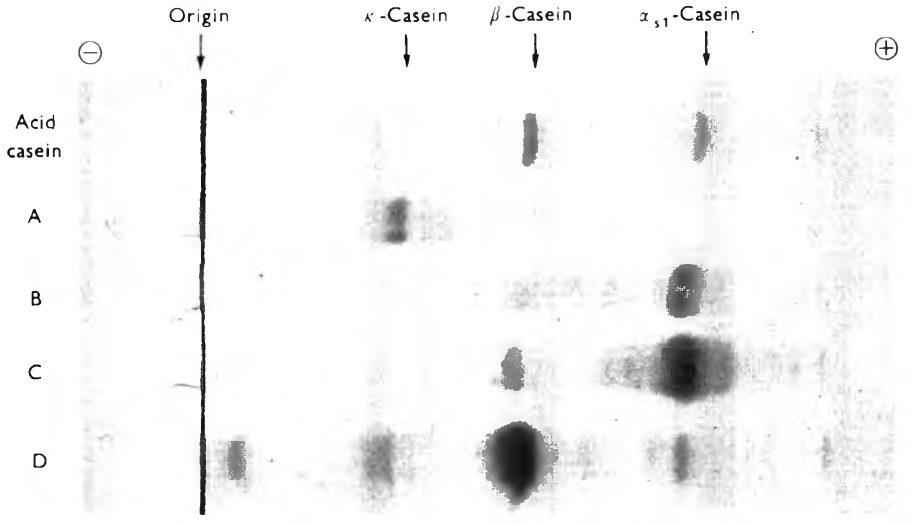
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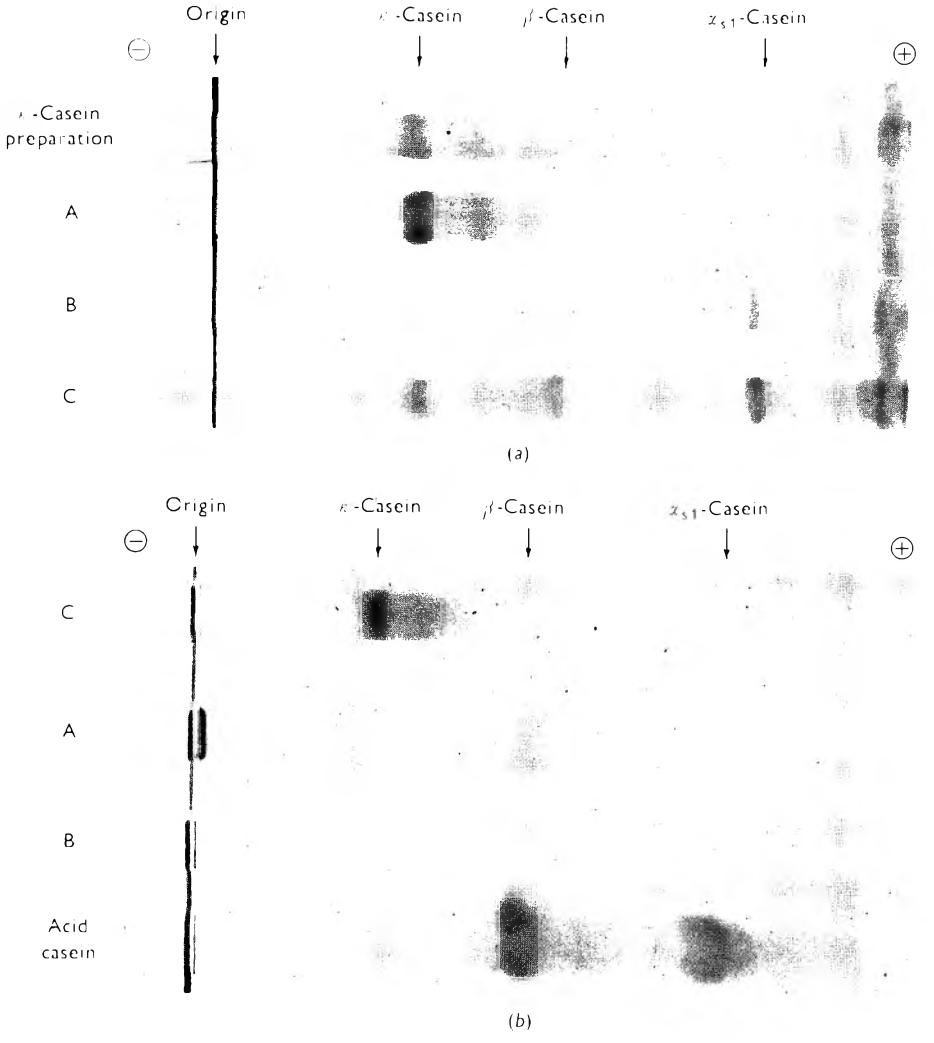
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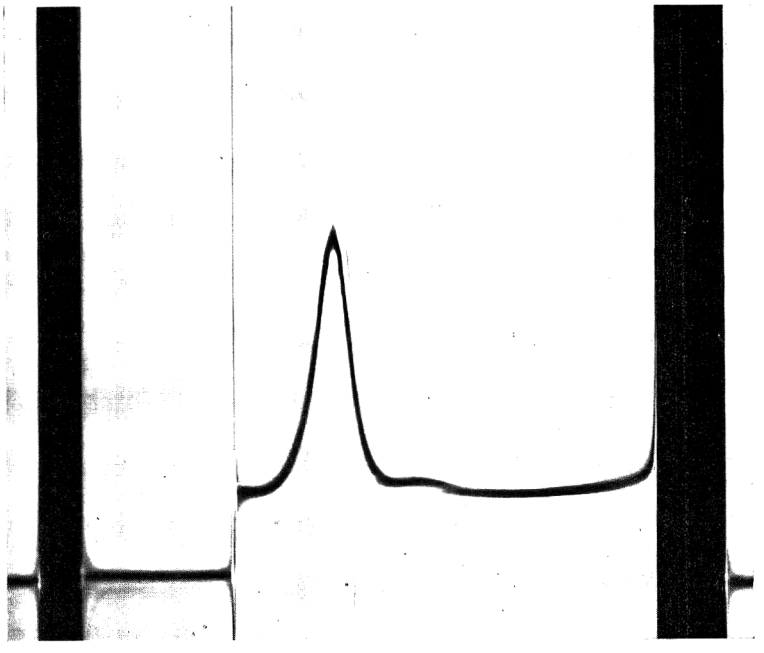
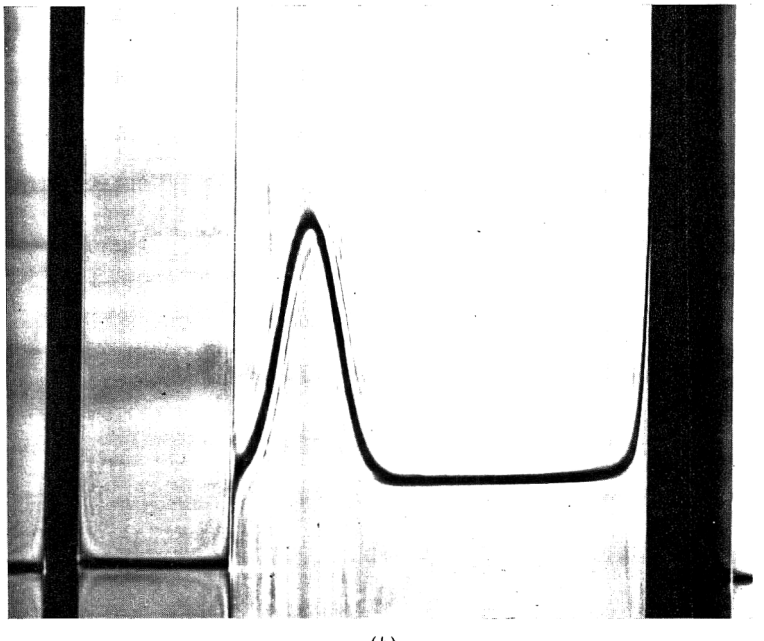


(a)



(b)

Reviews of the progress of dairy science

Section G. Genetics. Genetic variants of milk proteins: their breed distribution

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National Institute for Research in Dairying, Shinfield, Reading

(*Received* 19 *June* 1968)

CONTENTS

INTRODUCTION

Like proteins in general, those synthesized in the mammary gland of the cow and other species have been the subject of intensive research in recent times. So much has been published that, as pointed out by McKenzie (47), it has become almost impossible for a single author to cover comprehensively all aspects of milk protein research even in a review of more than 150 printed pages, such as we owe to his efforts. Thus, it seems justifiable to single out a specific aspect for more detailed scrutiny, as it is intended to do in what follows.

GENETIC VARIANTS OF MILK PROTEINS

All the major protein constituents of cow's milk, α_{s1}-, β-, γ-, and κ-caseins, α-lactalbumin and β-lactoglobulin, are now known to occur in the form of products which reflect the action of autosomal genes transmitted from parents to offspring by straightforward Mendelian inheritance. The products represent co-dominant alleles found either singly, in homozygotes, or paired, in heterozygotes, with the latter clearly distinguishable from the former, so that gene frequencies can be calculated by simple counting. [For general accounts the reader is referred to the relevant chapters in the recent books by Johansson & Rendel (36) and by Buschmann & Schmid (22).] The gene frequencies vary from breed to breed, and while some of the variants are universal, others are restricted in their occurrence. It is the main purpose of this review to collate the gene frequency data scattered widely through the literature, and to discuss their implications. One of the polymorphic systems, γ-casein, has only recently come to light (33), and no data are as yet available for tabulation.

Table 1 lists the established genetic variants and illustrates the rapid rate of development in the past 13 years. In addition to the variants in the table, a sub-variant of β-lactoglobulin has been found in Australian Droughtmaster cattle (47) which consists of a molecule in which a protein of the amino acid composition of the

A-variant is combined with glucosamine and sialic acid. Also, there may be one or two further polymorphic systems amongst the electrophoretically slow-moving components of casein (24,32), but proof that the observed variabilities are genetically determined is still awaited. The red protein (lactotransferrin) may also be polymorphic (34). Some of the variants of restricted occurrence, the rare ones in particular, may well be more widely extant than indicated, as there has hardly been time or opportunity to cover more than a few breeds in the search for them. Nor have many of the numerous breeds present in different parts of the globe been examined at all,

Table 1. *The genetic variants of milk proteins*

Protein constituent	Variant	First reported in: Year	First reported in: Reference	Occurrence	Where found
α_{s1}-Casein	A			Rare	In Holsteins (U.S.A.); R.D.M. (Denmark)
	B	1962	65	In all breeds	Predominant in Western breeds
	C			In all breeds	Predominant in Zebus (India)
	D	1966	30	Rare	In Flamandes (France)
β-Casein	[A	1961	3	In all breeds	Comprising A^1, A^2 and A^3]
	A^1			In most breeds	—
	A^2	1966	40	In all breeds	—
	A^3			Rare	In Holsteins (U.S.A.); Friesians (G.B.); Normandes (France)
	B	1961	3	In most breeds except Zebus	—
	$B_{\frac{1}{2}}$	1968	13	In Zebus only	—
	C	1961	3	Rare	In Guernseys and a number of other breeds, but not in Jerseys
	D	1968	13	Rare	In Deshis (India); Borans (Kenya)
γ-Casein	A	1967	33	In all breeds (?)	Details of distribution not yet known
	B			In most breeds (?)	
κ-Casein	A	1964	53, 60, 67	In all breeds	—
	B			In all breeds	—
β-Lactoglobulin	A	1955	10	In all breeds	Not in Africanders (S. Africa)?
	B			In all breeds	—
	C	1962	15	Rare	In Jerseys
	D	1966	30	Rare	In Montbéliardes (France); Jerseys (Denmark); Simmentals (Germany, Poland)
α-Lactalbumin	A	1958	20	In Zebus only	—
	B	1958	20	In all breeds	—

so that additions to the list of variants are to be expected. Such additions are also likely for another reason: all, or virtually all, the evidence for genetic variation has been obtained through the application of electrophoretic procedures to milk or suitable milk fractions, and reflects primarily charge differences between the variants of a given protein molecule. Interchange of uncharged amino acids, of which there are many in a protein molecule, would not be reflected in the electrophoretic mobility, but would require other methods of detection, methods which are likely to be much more elaborate than the simple electrophoretic techniques, so that progress in this

direction will, one fears, be slow. The only example of a variant discovered by non-electrophoretic means is β-casein B_Z which replaces β-casein B in Zebu cattle, and was detected by means of 'fingerprints' of chymotryptic peptides (13).

PHENOTYPING PROCEDURES

Although, in essence, only 2 types of phenotyping techniques, starch gel and polyacrylamide gel electrophoresis, are at present in general use, a newcomer to the field will, on searching the literature, find himself confronted with a bewildering choice of procedures differing in technicalities. There is little to choose between the 2 gel media, and his selection must depend on his own inclination and the facilities at his disposal. The 2 types have developed on parallel lines, and provide excellent resolution of the casein components when used with gel buffers containing urea and 2-mercaptoethanol at alkaline pH. Techniques have been developed and simplified to the stage at which it is possible to subject sets of milk samples without any fractionation to gel electrophoresis and to obtain phenotyping results for the α_{s1}-, β- and κ-caseins and for the β-lactoglobulins simultaneously (14). The degree of perfection attainable in this manner is well illustrated by the photographs of starch gels in a recent paper by Michalak (50). Electrophoresis in alkaline gels does not resolve β-casein A into its true components A^1, A^2 and A^3 (40), and a second electrophoretic run at acid pH is needed for their resolution (8, 55). This second run can also be utilized for β-lactoglobulin phenotyping (2).

The techniques just described are not suitable for phenotyping of the γ-caseins and α-lactalbumins. Disk electrophoresis of the isolated caseins allows the detection of the γ-casein variants (33), but it may not be necessary to do any γ-casein phenotyping: the A and B variants appear to correspond to the A and B variants of β-casein found in the same milk sample. If confirmed by more extensive work, this would obviate the need for an additional test. Groves & Kiddy (33) made the interesting observation that milk samples containing β-casein C were devoid of a corresponding γ-casein; the D variant remains to be examined. Phenotyping of α-lactalbumin is best done by examination of whey or a whey concentrate. The simple method of paper electrophoresis (5, 20) as well as gel electrophoretic procedures may then be applied. There is, however, hardly any need for α-lactalbumin phenotyping in studies of Western breeds of cattle, since the polymorphism is confined to Zebu cattle [Osterhoff & Pretorius (54) claim to have observed the A-variant in South African cows of European breeds, especially Friesians, in which this variant has never been seen elsewhere].

Though there is little to choose between starch and polyacrylamide as gel media, one aspect should be considered when deciding in favour of starch or, more precisely, partially hydrolysed starch. A standard product is available (Connaught Laboratories, Toronto); results obtained by its use are easy to reproduce. On the other hand, it is much more difficult to repeat the results of those who rely on starch hydrolysed in their own laboratory: batches of the starting material, usually potato starch, vary considerably in their properties, so that much time and effort may be needed to prepare a hydrolysed product of the desired quality. Unless 'homemade' starch is required for a specific purpose, or unless 'Toronto' starch is unobtainable, preference should be given to the use of the latter out of consideration for others who might want to adopt the technique in question.

Table 2. *Gene frequencies of caseins*

Breed	Country	No. tested	α_{s1}-			β-						κ-		Reference
			B	C	Others	A^1	A^2	A^3	A	B	C	A	B	
Jersey	Gt. Brit.	107	—	—	—	—	—	—	0·63	0·37	0	—	—	4
		70	0·79	0·21	—	—	—	—	0·59	0·41	0	0·24	0·76	7
		47	0·57	0·43	—	0·09	0·63	0	(0·72)	0·28	0	0·09	0·91	12
	Gt. Brit., U.S.A.	351	0·72	0·28	—	—	—	—	0·62	0·38	0	—	—	42
	U.S.A.	67	0·81	0·19	—	—	—	—	0·64	0·36	0	—	—	64
		48	—	—	—	—	—	—	—	—	—	0·10	0·90	68
		37	—	—	—	0·22	0·49	0	(0·71)	0·29	0	—	—	40
	Germany	148	0·92	0·08	—	—	—	—	0·66	0·34	0	—	—	49
	Denmark	127	0·93	0·07	—	—	—	—	0·57	0·43	0	—	—	66
		142	0·95	0·05	—	—	—	—	0·65	0·35	0	0·52	0·48	44
	S. Africa	37	0·62	0·38	—	—	—	—	0·60	0·40	0	0·43	0·57	54
Guernsey	Guernsey	18		—	—	—	—	—	0·93	0·02	0·05	—	—	4
	Gt. Brit.	96	—	—	—	—	—	—	0·93	0·02	0·05	—	—	4
		92	0·70	0·30	—	—	—	—	0·95	0·02	0·03	—	—	7
	Gt. Brit., U.S.A.	170	0·77	0·23	—	—	—	—	0·93	0·02	0·05	—	—	42
	U.S.A.	400	0·70	0·30	—	—	—	—	0·98	< 0·01	< 0·02	—	—	64
	U.S.A.	196	—	—	—	0·01	0·98	0	(0·99)	0·01	< 0·01	—	—	4
		47	—	—	—	—	—	—	—	—	—	0·74	0·26	68
Normando	Franco	155	0·82	0·18	—	0·21	0·32	0·02	(0·55)	0·45	0·01	0·34	0·66	31
Holstein–Friesian	Gt. Brit.	231	—	—	—	—	—	—	0·96	0·04	0	—	—	4
		189	0·93	0·07	—	—	—	—	0·95	0·05	0	0·83	0·17	7
		85	—	—	—	0·66	0·24	0·04	(0·94)	0·06	0	—	—	9
	U.S.A.	542	0·87	0·08	A = 0·05	—	—	—	0·98	0·02	0	—	—	64
		138	—	—	—	—	—	—	—	—	—	0·85	0·15	68
		271	—	—	—	0·40	0·54	0·02	(0·96)	0·04	0	—	—	40
		754	0·91	0·08	A < 0·01	0·31*	0·62	0·05	(0·98)	0·02	0·001	—	—	1
	Holland	693	0·99	0·01	—	—	—	—	0·93	0·07	0	0·66†	0·34	61
	Germany	87	0·93	0·07	—	—	—	—	0·96	0·04	0	—	—	49
	S. Africa	34	1·00	0	—	—	—	—	0·77	0·23	0	0·54‡	0·46	54
SLB	Sweden	320	0·87	0·13	—	—	—	—	0·96	0·04	0	—	—	59
SDM	Denmark	139	0·98	0·02	—	—	—	—	0·96	0·04	0	—	—	66
	Denmark	118	1·00	< 0·01	—	—	—	—	0·92	0·08	0	0·68	0·32	44
Ayrshire	Gt. Brit.	64	—	—	—	—	—	—	1·00	0	0	—	—	4
		52	1·00	0	—	—	—	—	1·00	0	0	0·80	0·20	7
		29	—	—	—	0·60	0·40	0	(1·00)	0	0	—	—	9
	U.S.A.	98	1·00	0	—	—	—	—	1·00	0	0	—	—	64
		45	—	—	—	0·72	0·28	0	(1·00)	0	0	—	—	40

Breed	Country	No. tested	α_{s1}- B	C	Others
Shorthorn	Gt. Brit.	64	—	—	—
		115§	0·99	0·01	—
Kerry	Ireland	123	0·94	0·06	—
Gronings Blaarkop	Holland	153	1·00	0	—
MRY	Holland	272	0·98	0·02	—
SKB	Sweden	85	0·84	0·16	—
SRB	Sweden	193	0·98	0·02	—
RDM	Denmark	104	1·00	0	—
		282	0·98	0·01	A < 0·01**
Flamande	France	380	0·87	0·09	D = 0·04
Red Pied Holstein	Germany	137	0·86	0·14	—
Angeln	Germany	201	0·92	0·08	—
Brown Swiss	U.S.A.	203	0·97	0·03	—
		250	0·94	0·06	—
		23	—	—	—
	S. Africa	30	0·93	0·07	—
Montbéliarde	France	350	0·91	0·09	—
German Brown	Germany	126	0·85	0·15	—
German Red	Germany	144	0·88	0·12	—
Spotted Mountain	Germany	290	0·87	0·13	—
Simmental	Hungary	151	0·93	0·07	—
	Jugoslavia	26	0·96	0·04	—
Buša	Jugoslavia	28	0·79	0·21	—
Boran	Kenya	54	0·39	0·61	—
Ankole	Uganda	49	0·40	0·60	—
Africander	S. Africa	38	0·01	0·99	—
Nguni	S. Africa	60	0·86	0·14	—
Pedi	S. Africa	29	0·92	0·08	—
Caprivi–Sanga	S. Africa	20	0·70	0·30	—
Sahiwal	India	76	0·05	0·95	—
Tharparkar	India	71	0·05	0·95	—
Deshi	India	56	0·06	0·94	—
Hariana	India	72	0·21	0·79	—
Red Sindhi	India	21	0·10	0·90	—

* 632 cows. † 164 cows. ‡ 25 cows.
¶ 91 cows. ** 3 A/B cows.

β-						κ-		
A^1	A^2	A^3	A	B	C	A	B	Reference
—	—	—	1·00	0	0	—	—	4
—	—	—	0·97	0·03	0	—		7
—	—	—	1·00	0	0	0·93	0·07	52
—	—	—	0·99	0·01	0	0·72‖	0·28	61
—	—	—	0·92	0·08	0	0·53¶	0·47	61
—	—	—	0·99	< 0·01	0	—	—	59
—	—	—	0·98	0·02	0	—	—	59
—	—	—	0·93	0·07	0	—	—	66
—	—	—	0·95	0·04	< 0·01††	0·74	0·26	44
—	—	—	0·94	0·06	0	—	—	30
—	—	—	0·86	0·14	0	—	—	49
—	—	—	0·94	0·06	0	—	—	49
—	—	—	0·79	0·19	0·02	—	—	64
—	—	—	0·83	0·16	< 0·01	—	—	42
0·14	0·66	0	(0·80)	0·18	0·02	—	—	40
—	—	—	0·67	0·29	0·04	0·70	0·30	54
0·15	0·64	0	(0·79)	0·19	0·02	—	—	31
—	—	—	0·81	0·18	0·01	—	—	49
—	—	—	0·90	0·09	< 0·01	—	—	49
—	—	—	0·76	0·20	0·04	—	—	49
—	—	—	0·81	0·09	0·10	—	—	23
—	—	—	0·90	0·06	0·04	—	—	23
—	—	—	0·80	0·18 B_2	0·02	—	—	23
—	—	—	0·92	0·05	0††	0·66	0·34	13
0·30	0·68	0	(0·98)	0·02	0	0·78	0·22	13
—	—	—	1·00	0	0	0·44	0·56	54
—	—	—	0·97	0·03	0	0·47	0·53	54
—	—	—	0·83	0·17	0	0·26	0·74	54
—	—	—	1·00	0	0	0·78	0·22	54
—	—	—	0·96	0·04	0	0·90	0·10	13
—	—	—	0·95	0·05	0	0·87	0·13	13
—	—	—	0·92	0·05	0‡‡	0·82	0·18	13
—	—	—	0·79	0·21	0	0·83	0·17	13
—	—	—	0·90	0·10	0	0·74	0·26	13

§ Includes non-pedigree animals. ‖ 47 cows.
†† 1 A/C cow. ‡‡ D = 0·03.

PHENOTYPING RESULTS

As a tabulation of all the available phenotyping results would be extremely unwieldy, the results are presented in the form of the gene frequency data derived from them. Table 2 lists the gene frequencies of the casein components (α_{s1}-, β- and κ-), Table 3 those of the β-lactoglobulins and Table 4 those of the α-lactalbumins. As some of the systems and variants have not been known for very long, e.g. the κ-caseins which were not discovered until 1964, or the β-casein variants A^1, A^2 and A^3 which were not detected until 1966, there are still numerous gaps in the tables. Also, in many studies data have only been collected for one or the other of the 5 systems, and there are virtually no results offering complete coverage.

The number of samples examined varies greatly from breed to breed, reaching many hundreds in some of the major breeds, e.g. Channel Island and Holstein–Friesian cattle, while being too small in others to attach much significance to the gene frequency data, which have nevertheless been included in the tables to draw attention to the need for more extensive work. With small numbers in particular there is a danger that the recorded figures are those for 1 or 2 herds rather than for a representative cross-section of a population, but even when numbers seem adequate they may not meet this criterion. The data on the β-lactoglobulins of 448 Australian Jersey cattle (16) provide a good example: they were obtained from only 5 herds of which the 2 largest gave the following phenotyping results:

		Number						Gene frequency		
Herd	Total	A/A	A/B	A/C	B/B	B/C	C/C	A	B	C
II	102	1	14	11	34	31	11	0·13	0·55	**0·31**
III	178	11	58	1	105	3	0	0·23	0·76	**0·01**

There is a startling concentration of the C-allele in herd II, and this is reflected in the figure of 0·10 for the gene frequency recorded in Table 3. Omission of herd II reduces the figure to 0·04, which is much more in line with that for British and Danish Jerseys. Thus, there is no reason to believe that the C-allele is more prevalent in Australia than elsewhere.

Before dealing with the differences in gene frequencies between breeds, the differences within breeds deserve brief discussion. There are as yet few breeds for which relevant data have been collected in different countries. Where this has been done, the results are on the whole fairly concordant, e.g. for the Channel Island and Holstein–Friesian breeds (see Tables 2, 3). Where divergencies occur, they may be due to chance, e.g. in the case of the low frequency of β-casein A in the 34 South African Friesians which represent a single herd. On the other hand, the differences in the α_{s1}- and κ-casein frequencies between the Dutch and the British and American Holstein–Friesians are almost certainly genuine and significant, and the same applies to the differences in these variants between the Danish and the British and American Jerseys. That such divergencies should manifest themselves in animals bred from small foundation stocks imported many years ago is not surprising. For breeds other than those just mentioned, there are unfortunately too few results available to warrant discussion.

Table 3. *Gene frequencies of β-lactoglobulins*

Breed	Country	No. tested	Variant A	Variant B	Variant Others	References
Jersey	Gt. Brit.	80	0·37	0·62	C = 0·01	7
	Gt. Brit.	47	0·34	0·66	—	12
	U.S.A.	39	0·41	0·59	—	41
	Denmark	158	0·56	0·44	—	51
	Denmark	142	0·42	0·58	—	44
	Denmark	159	0·39	0·55	C = 0·04 D = 0·02	44
	Germany	105	0·41	0·59	—	48
	S. Africa	40	0·34	0·66	—	54
	Australia	448	0·26	0·64	C = 0·10	17
Guernsey	Gt. Brit.	27	0·22	0·78	—	11
	Gt. Brit.	84	0·36	0·64	—	7
	U.S.A.	200	0·30	0·70	—	41
	Australia	68	0·44	0·56	—	17
Holstein–Friesian	Gt. Brit.	87	0·40	0·60	—	11
	Gt. Brit.	189	0·43	0·57	—	7
	U.S.A.	406	0·46	0·54	—	41
	Germany	1831	0·40	0·60	—	48
	Australia	108	0·51	0·49	—	17
	S. Africa	41	0·71	0·29	—	54
SLB	Sweden	320	0·62	0·38	—	59
SDM	Denmark	161	0·61	0·39	—	51
	Denmark	118	0·50	0·50	—	44
Ayrshire	Gt. Brit.	52	0·27	0·73	—	7
	U.S.A.	27	0·13	0·87	—	41
	Australia	104	0·38	0·62	—	17
Shorthorn	Gt. Brit.	87	0·11	0·89	—	11
Illawarra Shorthorn	Australia	200	0·08	0·92	—	17
Kerry	Ireland	123	0·24	0·76	—	52
Icelandic	Iceland	52	0·34	0·66	—	20
SKB	Sweden	85	0·19	0·81	—	59
SRB	Sweden	193	0·46	0·54	—	59
RDM	Denmark	293	0·14	0·86	—	51
	Denmark	282	0·10	0·90	—	44
Campine	Belgium	61	0·33	0·67	—	58
Red Pied Holstein	Germany	283	0·34	0·66	—	48
Angeln	Germany	393	0·20	0·80	—	48
Brown Swiss	U.S.A.	24	0·24	0·76	—	41
	S. Africa	30	0·43	0·57	—	54
Montbéliarde	France	364	0·52	0·46	D = 0·02	30
German Brown	Germany	335	0·56	0·44	D < 0·01*	48
German Red	Germany	307	0·34	0·66	—	48
Spotted Mountain	Germany	379	0·50	0·50	D < 0·002†	48
Yellow Franconian	Germany	293	0·53	0·47	—	48
White Fulani	Nigeria	58	0·21	0·79	—	20
Boran	Kenya	52	0·19	0·81	—	5
	Kenya	54	0·07	0·93	—	13
Ankole	Uganda	50	0·02	0·98	—	13
Africander	S. Africa	39	0	1·00	—	54

Table 3 (*cont.*)

Breed	Country	No. tested	Variant A	Variant B	Variant Others	References
Nguni	S. Africa	60	< 0·01	0·99	C < 0·01‡	54
Pedi	S. Africa	27	0·07	0·93	—	54
Caprivi–Sanga	S. Africa	20	0·05	0·95	—	54
Hariana	India	96	0·09	0·91	—	19
Sahiwal	India	15	0·07	0·93	—	19
Deshi	India	27	0·13	0·87	—	19
Gir	India	83	0·24	0·76	—	46
Gavathi	India	20	0·28	0·72	—	46

* 4 cows. † 1 cow. ‡ 1 cow.

Table 4. *Gene frequencies of α-lactalbumin*

Breed	Country	No. tested	Variant A	Variant B	Reference
White Fulani	Nigeria	58	0·15	0·85	20
Boran	Kenya	52	0·13	0·87	5
	Kenya	54	0·05	0·95	13
Ankole	Uganda	50	0·04	0·96	13
Africander	S. Africa	39	0·03	0·97	54
Nguni	S. Africa	60	0·04	0·96	54
Pedi	S. Africa	27	0	1·00	54
Caprivi–Sanga	S. Africa	20	0	1·00	54
Hariana	India	96	0·22	0·78	19
Sahiwal	India	15	0·37	0·63	19
Deshi	India	27	0·22	0·78	19
Gir	India	83	0·38	0·62	46
Gavathi	India	20	0·40	0·60	46

BREED DISTRIBUTION OF GENETIC VARIANTS

As pointed out before, the genetic variants differ from breed to breed in their occurrence and frequency of occurrence. These differences will now be discussed in turn for the variants in each of the 5 systems for which the results have been tabulated.

α_{s1}-*Caseins*

The A variant. This allele is very rare. Discovered in Holstein cattle in the U.S.A., it appears there to be confined to a single blood-line originating in the state of Michigan (39). A protein of the same electrophoretic mobility has been found in 3 heterozygotic Red Danish (RDM) cows (40), and may be identical to the American variant, although it is difficult to see any connexion between the red Danish breed, 'originating from North Slesvig Red(+ Angeln and Ballum) × local island' (45), and the black-and-white Holsteins of Michigan. The amino acid composition of the A variant differs markedly from that of the other alleles of α_{s1}-casein (27), probably as the result of a deletion of part of the amino acid chain (37), an event which may have taken place comparatively recently.

The B and C variants. The B allele predominates in all Western breeds of cattle, while its frequency is lower in some of the few African Zebu breeds so far examined and reaches very low levels in some Indian breeds. The converse applies to the C-variant, as discussed in a recent publication (13). Amongst Western cows the gene frequency of α_{s1}-casein B exceeds 0·90 in many breeds, and reaches 1·00 in some, e.g. the Ayrshire. Notable exceptions are the Channel Island, Normande and Swedish SLB and SKB breeds in which the C allele occurs in a fair proportion of animals. The B and C variants have almost identical amino acid composition; a substitution of a glycine for a glutamic acid residue in C appears to be the only difference between the 2 molecules (26, 43).

The D variant. This allele was recently discovered in Flamande cattle, and shown to occur in low frequency (30). A wider search for it in adjoining territory, e.g. in Belgium, is desirable. According to de Koning (43) it differs in amino acid composition from α_{s1}-casein B only in the replacement of a serine residue by one of proline.

β-Caseins

The A variants. Most of the available information on the A-variant has been obtained by alkaline gel electrophoresis, and requires revision, now that it is known that, by acid gel electrophoresis, resolution into 3 variants, A^1, A^2 and A^3, can be accomplished (40). However, it is clear from the table that the 3, regardless of their proportions, add up to the predominant β-casein variant in all breeds so far examined. The A^3 allele appears to be rare in the Holstein–Friesian and Normande breeds, the only 2 in which it has as yet been detected, but a more widespread search is desirable. A^2 seems to have a higher frequency than A^1 except possibly in Ayrshires, of which, however, only a small number has been tested. In Guernseys the frequency of A^2 is very high indeed, and the same was observed for Indian Zebu cattle belonging to the breeds listed in Table 2 (13). Here, 124 of 126 animals producing β-casein A were found to be A^2-homozygotes. The molecules of the 3 variants differ in their histidine content (56), but the precise differences in their amino acid composition remain to be determined.

The B (B_Z) variant. In the majority of breeds this allele is low in frequency, the most notable exceptions being the Jersey, Normande, Brown Swiss and other mountain breeds amongst Western cattle, and the Hariana amongst Zebu cows in which, as mentioned before, B_Z takes the place of B. The marked contrast between Jerseys and Guernseys is noteworthy. The amino acid composition of β-casein B and the other variants is not known with the degree of precision required to differentiate between mutational changes and analytical errors, possibly because of difficulties in the purification of samples. Results of a collaborative study by French and Dutch teams (57), discussed by de Koning (43), indicate this clearly, but suggest that in the final analysis the molecules of the β-casein variants will turn out to differ in only 1 or 2 amino acids from each other. B_Z, isolated from the milk of Indian cows, differs from B (63), though the 2 are indistinguishable by gel electrophoresis. That the B allele found in African Zebus is B_Z and not B is presumed, but still requires experimental proof.

The C variant. This allele is rare and restricted in its occurrence. First observed in Guernsey cattle (3), it has never been found in Jerseys of which over 1000 have been

examined. The variant appears to be a characteristic of Brown Swiss and other mountain breeds, one of which may be the source of it in the Guernsey breed. The C variant is unlikely to be a true constituent of Holstein–Friesian milk, although its presence in very low frequency has been reported in one large-scale investigation (1). Similar doubts must be entertained regarding its occurrence in pure-bred Normande and Danish RDM cows.

The D variant. Little is as yet known about this variant which was recently shown to be present in the milk of a few Deshi and Boran cows (13), thus indicating a link between Indian and African Zebu cattle. According to Thompson & Gordon (63) D differs from β-casein B in its lysine, arginine and histidine content.

κ-Caseins

The A and B variants. Though κ-casein is probably the most complex casein component in its properties and functions, it is up till now the simplest from the genetical aspect, with 2 variants of which both are present in all the breeds examined. The A-allele tends to predominate in the majority of breeds, except in the Jersey, the Normande and some South African Zebu cattle. Danish Jerseys were found to have a much higher frequency of A than those tested in Britain and the U.S.A. They also differ, as pointed out before, in the distribution of the alleles of α_{s1}-casein. There are striking similarities in the gene frequencies of the α_{s1}- and β- as well as the κ-caseins of Normande and Jersey cattle, suggesting close ties between these 2 breeds. The amino acid compositions of the 2 κ-caseins are very much alike; A contains one more residue of aspartic acid and threonine than B, whilst B contains one more residue of alanine and isoleucine than A (43, 69). Both substitutions occur in the soluble portion of the protein chain, split off by the action of rennin, so that the insoluble para-κ-caseins are of uniform composition (43).

Genetic linkage of the casein systems

Although not strictly within the scope of this review, brief mention might be permitted here of one of the most fascinating developments emerging from the studies of casein phenotypes—that of the linkage relationships of the α_{s1}, β- and α-systems. Evidence has been obtained for close linkage between the α_{s1}- and β-caseins (28, 42) and for a marked, though less close, linkage between κ-caseins and the α_{s1}- and β-systems, respectively (29). [For a summary see (31).] The non-independent occurrence of these casein components has been confirmed by Danish workers (44) who oppose the concept of close linkage, and prefer an interpretation based on multiple allelism at a single locus. Further confirmatory evidence of linkage has just been reported by a group of American workers who also examined the relationship between the caseins and other systems, e.g. serum transferrins and various bloodgroups, with negative results (35). Interesting results were obtained by Arave (1) whose phenotyping data are for the separate A^1, A^2 and A^3 variants of β-casein: 47 of 51 cows carrying the A^3 allele also carried the α_{s1}-casein C allele, and the 6 cows homozygous for β-A^3 were likewise homozygous for α_{s1}-C. From recent work on Indian Zebu cattle (13) it appears that in these it is the β-A^2 allele which is closely associated with α_{s1}-C, the predominant α_{s1}-casein variant in Indian cows. Since, as mentioned before,

there also exists close association between the γ- and β-casein alleles (33), it can be concluded that all the known genetic systems of cow's casein are linked to each other and, presumably, located on the same chromosome.

β-Lactoglobulin

The A and B variants. The β-lactoglobulin polymorphism was the first to be discovered in cow's milk (10, 11), and the distribution of the 2 variants in the breeds of various countries is by now well documented. As shown in Table 3, both alleles have been found in all the cow populations examined, with one exception: the 39 Africander cattle tested by Osterhoff & Pretorius (54) were all of phenotype B/B. However, as Zebu breeds tend to be low in A frequency, this 'anomaly' will probably disappear on examination of larger numbers of Africander cows. Though the frequency of the A allele reaches quite high levels in some Western breeds, it is the B allele which predominates in most cases. Variations within breeds are not, in general, greater than one would expect taking the size of the populations examined, often small, into consideration.

As the β-lactoglobulins are easy to purify and crystallize, they have been the subject of numerous chemical and physico-chemical studies, summarized and discussed at length by McKenzie in his recent review (47). Since this was written, the sequence of the 162 amino acids in the protein chain has been established by Frank & Braunitzer (25), who have shown that the 2 amino acid differences between the A and B molecules are located at positions 68 (valine in A; alanine in B), and 120–122 (aspartic acid in A; glycine in B). That these apparently small differences in structure have a large effect on the properties of the 2 variants has been pointed out in an earlier review (6).

The C variant. Discovered by Bell (15) in Australian Jersey cattle, this allele has not been detected in any other breed except South African Nguni, in which it was observed in the heterozygous form in 1 out of 60 animals tested (54). Whether the variant is, in fact, as restricted as the data in Table 3 suggest, is questionable: a number of techniques used for β-lactoglobulin phenotyping fail to detect C, some because C migrates only fractionally slower than B upon electrophoresis, others because with procedures applied without removal of the caseins, C is liable to be concealed behind β-casein A, e.g. in the method of simultaneous phenotyping described by the reviewer (14). That C is an exclusive characteristic of the Jersey breed can therefore not be stated with conviction. It is, however, unlikely that C will be found in Guernsey or Holstein–Friesian milks, since methods suitable for its detection have been applied to fair numbers of samples of both breeds. The occurrence of the C allele in the Jersey and its absence from the Guernsey is another distinguishing factor of the 2 Channel Island breeds which, as pointed out earlier, also differ in their caseins, e.g. in β-casein C, found in the Guernsey, but not in the Jersey, and in the frequency distribution of the κ-caseins. These divergencies add up to a strong suggestion that the 2 breeds differ in origin and reached the islands by different routes, with the Guernsey possibly arriving from the south, remembering that β-casein C is a constituent of many mountain breeds, e.g. the Brown Swiss and Simmental.

The only amino acid difference between β-lactoglobulins C and B is the replace-

ment of a glutamine residue by a histidine (18,38). This is a permissible point mutation in accordance with the genetic code. McKenzie (47) is mistaken in his statement (p. 184) that a change from glutamic acid to histidine can likewise be accomplished in a single step.

The D variant. Information on the distribution of this recently discovered allele is still scanty. It was first seen in low frequency in Montbéliarde cattle (30), and has since been found in 2 German mountain breeds and in Polish Simmental cows (48,50). The D allele thus appeared to be a characteristic of cattle of the Simmental type, and the report of its occurrence in Danish Jerseys (44) was therefore rather unexpected, and suggests that the variant may be more widely distributed than the early findings indicated.

α-Lactalbumin

The A and B variants. Until recently it was generally accepted that α-lactalbumin polymorphism was restricted to Zebu cattle in which 2 variants were shown to exist, whilst only one of these, the B allele, was found in Western breeds of cattle. However, Osterhoff & Pretorius (54) reported the presence of the A variant in South African cows of European descent, with frequencies of 0·08 in Brown Swiss (30 animals tested), 0·12 in Holstein–Friesians (41 animals) and 0·05 in Jerseys (40 animals). These disconcerting results require confirmation, avoiding untried methods, e.g. the addition of preservative to the milk samples, and starch-gel electrophoresis of wheys prepared by rennet action. In the meantime, a further general search for the possible occurrence of the A allele in Western cattle is desirable.

The data on Zebu cattle, though not very extensive, show a markedly higher level of the gene frequency of α-lactalbumin A in Indian as compared to African breeds. But even in the Indian cows, it is the B variant which predominates, as it does throughout the rest of the world.

The molecules of the 2 variants are identical in amino acid composition, except for the absence of the solitary arginine residue of B which is replaced by a residue of glutamic acid or glutamine in A (26), more likely the latter, as a substitution of glutamine for arginine can be accomplished in a single step according to the genetic code. The sequence of the 123 amino acids forming the α-lactalbumin B chain has recently been determined by Brew, Vanaman & Hill (21). As there happens to be only one arginine in the molecule, it follows that the site of the mutation can be pinpointed without further work: it is located at position 10. When combined with the A-protein of lactose synthetase, the 2 variants of α-lactalbumin are indistinguishable in their enzymic activity (62).

FUTURE WORK

In surveying the present state of knowledge of the breed distribution of genetic variants of milk proteins, attention has been drawn to various shortcomings in the hope that this will act as an inducement to further investigations. But there is not only a need to fill the existing gaps concerning the breeds listed in the tables, but also to extend the studies to the numerous cattle populations for which no phenotyping data have yet been collected at all. No information is, for instance, available on the cattle of Switzerland, thought to be the cradle of many of the European breeds, nor on those of the Mediterranean countries and their possible association

with the cattle of North Africa. Numerous breeds of African, Asian and South American domicile remain to be investigated, and the examination of native breeds is a matter of urgency, as the desire for improvements through cross-breeding tends more and more to reduce the chances of being able to test genuine representatives of the local cattle. Much then has to be done before one can hope to obtain a clear picture of the origin and interrelationships of present-day breeds. As pointed out recently (13), future surveys might to advantage be combined with those of other polymorphic systems, e.g. of the serum transferrins and bloodgroups.

Milk being a perishable commodity, phenotyping is best done 'on the spot'. However, with the facilities of speedy air transport nowadays available, the setting up of 1 or 2 phenotyping centres might be worth considering in all seriousness. Such centres could also act as 'referees' in dubious cases, and subject new variants to detailed biochemical investigations for which facilities might well be lacking at the place of origin.

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