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Studies on the effect of heat treatment during processing on the viscosity and stability of high-fat market cream

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Summary. The viscosity of cream increased with increasing fat content, and higher values were obtained if the cream was separated at 38–43 °C rather than above 55 °C, and if the cream was cooled to below 15 °C before storage at 20 °C. The 'type' of viscosity varied with cooling and storage conditions; for example, cream cooled below 20 °C and stored at 20 °C gave increased viscosity readings with increased rates of shear due to churning of the cream, while the behaviour of cream cooled only to 20 °C was Newtonian.

Cream stability as measured by free fat content decreased with increasing fat content above about 42 %, with low temperature separation (38–45 °C), with cooling to below 15 °C before storage at 20 °C, and with increased pressure at temperatures below 15 °C.

Dilatometric study of the amount of fat crystallization indicated that larger amounts of crystallization resulted in higher final viscosity and free fat content.

INTRODUCTION

There are 2 major physical problems in the processing of cream (see Crossley & Rothwell, 1954):

(1) The tendency of high-fat (48 %) market cream to become completely solid under certain conditions. This problem is peculiar to the United Kingdom in that it is the only country in the world where the butterfat content of double cream must be not less than 48 %. In other countries, where 'heavy' creams are sold, the butterfat content may be 42 % or even less.

(2) Considerable variations in the thickness ('body' or 'viscosity') of the final product even from day to day.

Crossley & Hawley (1939, unpublished) observed that during the cold storage of cream there is a slow coalescence of the fat globules which could lead to the cream in time acquiring a 'sensitive' condition, in which case almost spontaneous thickening might occur if the cream was subjected to certain conditions of temperature and agitation.

Sommer & Royer (1927) found that cream plugs formed in the top of cream bottles showed a very high fat content in the plugs and microscopic examination showed that some of the fat had coalesced into irregular masses. They stated that the trouble could be partially overcome by storing the cream at low temperatures around 5 °C, as higher temperatures (above about 20 °C) favoured coalescence.

In 1933, Lyons & Pyne published the results of a series of experiments using 50 % cream. They found that cream viscosity increased with increasing fat content, and homogenizing pressure. It was decreased by increasing temperatures of separation and pasteurization. It also decreased as the temperature at which viscosity was measured increased, 3 % decrease in viscosity for each degF rise in temperature through the range of 52–64 °F being obtained. Homogenization at 250–500 p.s.i. caused some instability, with a marked tendency to set as if partly churned on stirring or agitation.

The results of Babcock (1931), Wallenfeldt (1940), Skelton & Sommer (1940), Marquardt & Dahlberg (1934) and Dahlberg & Hening (1925) dealing with cream of 35–40 % fat content, largely confirm the results of Lyons & Pyne, although there is some disagreement about the effect of separating temperature on viscosity. While Wallenfeldt stated good viscosity cream could be produced by separating at low temperature (35 °C), Skelton & Sommer found this had little effect if the cream was to be pasteurized subsequently. The effect of ageing at 5 °C for 48 h was shown to cause considerable viscosity increases during the first 24 h and a slight increase during the 24–48 h period.

The objective of the work reported in this paper was to obtain a better understanding of the factors affecting the viscosity and stability of double cream.

EXPERIMENTAL METHODS

Material

Cream was obtained by separating bulk raw milk at the required temperatures. The cream was then standardized to 50 % or other fat content as necessary with skim-milk from the same original milk as the cream, and then heat treated at 80 °C for 15 sec in a small plate heat exchanger followed by cooling. Samples of cream were taken from the heat exchanger before and after the holding section, after cooling to about 15 °C (mains water) and after the final cooling to about 5 °C (chilled water). Some cream samples taken after the holding section were cooled in about 3 min in ice-water in the laboratory. Other samples were heated and cooled in water baths in the laboratory and did not undergo any treatment in the heat exchanger.

After being heat treated and cooled samples were stored either in a cold store at 5 °C or in a water bath at 20 °C as required.

Viscosity measurements

At the present there appears to be no known method of obtaining an absolute measure of the viscosity of cream because the method used has some effect on the results obtained. In measuring the 'apparent' viscosity, all the conditions under which measurements are made must be constant. For this investigation the concentric cylinder Couette Viscometer (Ferranti Ltd.) was chosen because of its comparatively wide range and its suitability for allowing flow or hysteresis curves to be constructed.

Free fat measurement

Cream which has become thick under certain processing conditions has been known to contain free fat (Rothwell & Crossley, 1956). The quantity of free fat present in cream was estimated by the method of Webb & Hall (1935), as modified by Rothwell (1962).

Dilatometry

A relative measure of the amount of crystallization in fat can be obtained by dilatometric measurements (Mulder & Klomp, 1956). This method was, therefore, used in attempts to discover the effects of cooling and storage on the crystallization of fat in cream under the various conditions.

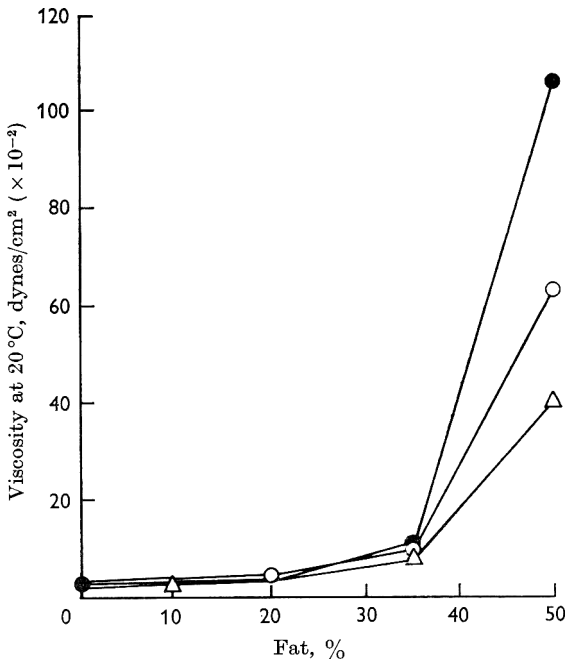

Fig. 1. The effect of fat content and methods of cooling the cream on cream viscosity measured at 20 °C. ●, Cream heated to 80 °C and cooled to 5 °C in heat exchanger; ○, cream heated to 80 °C and cooled to 5 °C in laboratory; △, cream heated to 80 °C and cooled to 20 °C in laboratory.

RESULTS

The effect of some processing conditions on the viscosity of cream

Fat content and method of cooling

The results shown in Fig. 1 are typical of those obtained in 10 experiments with creams of different fat contents produced from the same milk in each experiment. The fat content appeared to have little or no effect on viscosity changes caused by the different cooling methods until a level of about 35 % was reached. Above this level, cream which had been pasteurized and cooled in the heat exchanger had an appreciably higher viscosity than that of cream heated to 80 °C in a heat exchanger,

then cooled to 5 °C in a water bath, and the viscosity of the latter was higher than that of cream heated in the laboratory to 80 °C but cooled to 20 °C.

Temperature to which cream was cooled before being stored at 20 °C

Results obtained when viscosity measurements were made at 20 °C on 48 % fat cream samples which had been cooled rapidly in the laboratory to between 5 and 20 °C after heating to 80 °C are given in Table 1. The cream viscosity appears to be dependent to some extent on the temperature to which the cream was cooled before storage at 20 °C. Although viscosity did not increase with lower cooling temperatures in a regular manner, in all cases cream cooled only to 20 °C had the lowest viscosity, and highest viscosities were usually obtained when the cream was cooled to between 10 and 12 °C.

Table 1. *The effect of the temperature to which cream* (48 % *fat*) *was cooled after heating to* 80 °C *on viscosity at* 20 °C

Cooling temp., °C	Expt. no. 1 Visc.*	2 Visc.	3 Visc.	4 Visc.	5 Visc.	6 Visc.	7 Visc.	8 Visc.	9 Visc.	10 Visc.
5	60	58	68	181	168	56	51	48	55	43
10	60	59	74	205	200	61	56	50	55	44
12	67	60	69	303	358	58	55	53	59	50
15	56	48	62	165	275	53	48	48	53	42
17	53	47	63	133	310	49	46	45	48	—
20	46	42	49	128	135	53	40	37	40	37

* Viscosity in centipoises, measured at 20 °C.
Separation temperatures were 35–40 °C

Table 2. *The effect of separating and cooling temperatures on cream viscosity*

	A		B		C				D		
	Temp. of separation, °C										
Cooling temp., °C	38 Visc.*	72 Visc.	43 Visc.	82 Visc.	32 Visc.	49 Visc.	66 Visc.	82 Visc.	32 Visc.	49 Visc.	71 Visc.
5	53	50	60	54	101	85	57	63	92	51	56
10	55	53	66	53	84	83	55	59	66	49	84
12	52	53	66	57	79	72	51	59	56	45	56
15	50	52	56	48	67	72	45	54	50	33	49
20	41	41	44	42	58	52	42	46	48	35	36

* Viscosity in centipoises measured at 20 °C.

The considerably higher cream viscosities obtained in experiments 4 and 5 cannot be fully explained. They were possibly due to seasonal changes in the milk or to the use of milk from a different source. Despite the higher viscosities, however, the shape of the viscosity–cooling curve is the same as in the other experiments.

Separating temperature

Cream is produced commercially by the separation of milk at temperatures which may vary from as low as 32 to as high as 74 °C. To explore the effect of this wide variation in temperature, cream was produced in each experiment from one batch of milk by separation at 2 or more temperatures. After separation and standardization to

50 % fat, the cream was in all cases heated to 80 °C and then cooled under laboratory conditions as shown in Tables 2 and 3. In general, low separation temperatures produced cream with higher viscosity than high separation temperatures.

Table 3. *The effect of separation temperature and ageing on cream viscosities at 5 °C*

Separation temp., °C	Viscosities at 5 °C, centipoises		
	Fresh	Aged 24 h	Aged 48 h
74	55	89	126
71	38	53	79
68	52	75	87
49	215	290	210
49	264	420	—

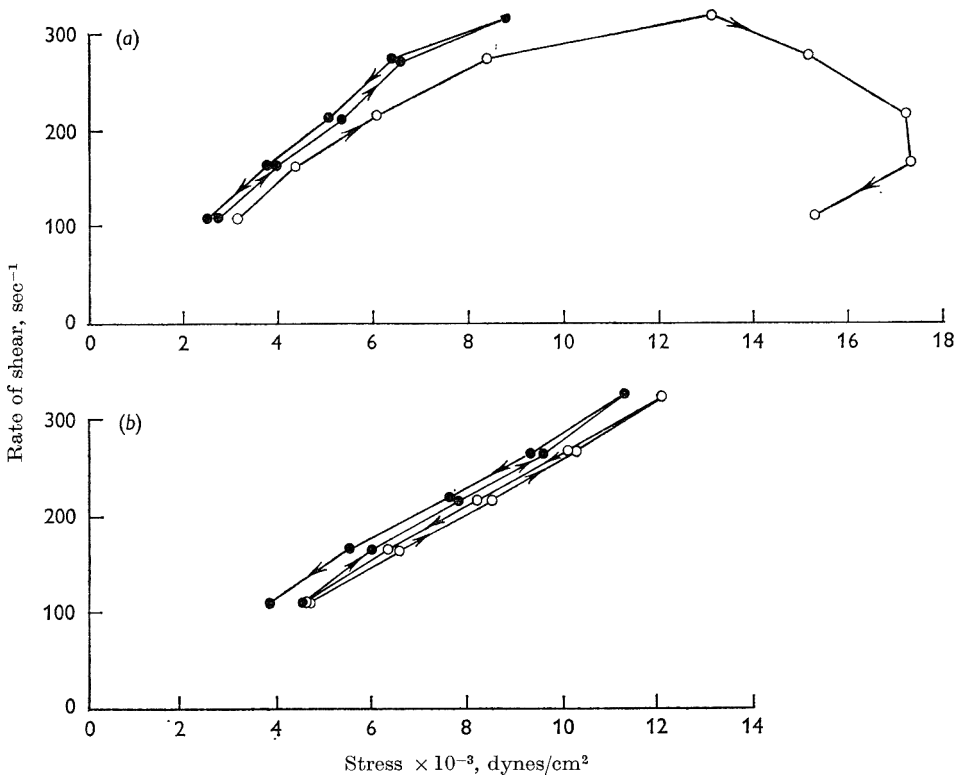

Fig. 2. The effect of cooling temperature on the viscosity 'behaviour' of cream (48–50 % fat) after ageing at 20 °C. (*a*) Cream heated to 80 °C, cooled to 5 °C before ageing at 20 °C; (*b*) cream heated to 80 °C, cooled to 20 °C before ageing at 20 °C. ●, cream aged 1 h; ○, cream aged 18 h.

Cooling temperature and the viscosity 'behaviour' of cream after different times of storage at 20 °C

The results shown in Fig. 2 are typical of those from 10 separate experiments in which cream of 48–50 % fat content was heated to 80 °C, then cooled in the laboratory to temperatures of 5 or 20 °C before storage at 20 °C. From the hysteresis curves it can be seen that cream which was cooled to 20 °C had virtually Newtonian viscosity and

was stable when sheared. However, the cream which was first cooled to 5 °C, after 18 h storage at 20 °C, exhibited a considerable increase in viscosity as the shear rate was increased. This change was not reversible and probably indicated the beginning of churning of the cream; particles of butter were observed on the viscometer cylinders on several occasions. This cream could be described as being in a relatively 'sensitive' condition.

Creams which had been cooled to temperatures between 5 and 20 °C also exhibited the same structural breakdown in roughly decreasing magnitude as the temperature to which the cream was cooled approached 17 °C. Above 17 °C no structural breakdown was observed.

Table 4. *The effect of fat content on the free fat content of cream**

Expt. no.	Fat content, %	Free fat, % of total fat after 48 h storage at 20 °C
1	50	78
	48	29
	45	47
	44	55
	42	3
2	47	7
	42	6
	38	2
3	50	65
	39	1
4	50	51
	40	3

* The cream was cooled to 5 °C after pasteurization at 80 °C, then stored at 20 °C.

Table 5. *The influence of cooling temperature on free fat content of cream*

	Free fat, % of total fat. Cooling temperature before storage at 20 °C for 48 h			
Expt. no.	20 °C	15 °C	10 °C	5 °C
1	2·5	2·5	50	55
2	2	30	45	50
3	2	35	66	66
4	Nil	2	20	33
5	11	54	70	65
6	2	25	61	46
7	2	64	90	90
8	Nil	Nil	9	43
9	Nil	Nil	6	24
10	Nil	23	31	33

Cream was rapidly cooled in the laboratory to the temperatures as indicated before being stored at 20 °C for 48 h.

The effect of some processing conditions on the 'free fat' contents of cream

Fat content

Table 4 shows the results of 4 experiments which indicate the change in free fat as the % fat in cream was decreased. It will be noted that, although the free fat

content was very variable, it was reduced to a low figure when the cream fat content was below about 42%. These figures were confirmed by a series of 17 experiments, in only one of which was a free fat content greater than 5 % obtained at fat contents below 42 % (this one was 10 % free fat at 40% fat content compared with 55 % free fat at 50 % fat content).

Table 6. *The effect of separation temperature on free fat content of cream*

Expt. no.	Separation temp., °C	Free fat, % of total fat. Cooling temperature before storage at 20 °C for 48 h			
		20 °C	15 °C	10 °C	5 °C
1	38	Nil	Nil	10	38
	72	Nil	Nil	1	4
2	38	Nil	Nil	4	25
	72	Nil	Nil	Nil	7
3	32	Nil	Nil	50	50
	82	Nil	7	22	7
4	32	Nil	Nil	35	40
	49	Nil	Nil	14	30
	66	Nil	Nil	1	4
	82	Nil	Nil	1	4
5	32	1	2	4	20
	49	Nil	Nil	2	8
	71	Nil	Nil	6	4
6	32	Nil	Nil	13	18
	54	Nil	Nil	Nil	Nil
	71	Nil	Nil	Nil	Nil
7	32	3	9	26	65
	71	1	4	26	43

All cream was heated to 80 °C after separation and standardizing to 50 % fat content, then cooled as required.

Table 7. *The effect of storage of milk at 5 °C before separation on the free fat content of cream*

Expt. no.	Fat in cream, %	Age of milk*	Free fat, % of total fat after 48 h at 20 °C
1	50	Fresh	Trace
		24 h	49
2	50	Fresh	Trace
		24 h	10
3	50	Fresh	14
		24 h	18
4	47	Fresh	4
		24 h	46
5	50	Fresh	8
		24 h	65
6	50	Fresh	27
		24 h	50

* Bulk raw milk was divided into 2 equal parts—one was separated at 32–38 °C immediately, the other was separated under the same conditions after 24 h storage at 5 °C.

Table 8. *Effect of centrifugal pressure on free fat in cream*

Sample*	Temp. of cooling, °C	Treatment	Free fat, % of total after 48 h at 20 °C
1	5	Direct to water bath	14
2	20	Direct to water bath	15
3	5	2000 *g* for 10 min	67
4	20	2000 *g* for 10 min	10
5	5	1200 *g* for 10 min	44
6	5	400 *g* for 10 min	12

* Samples from the same bulk cream.

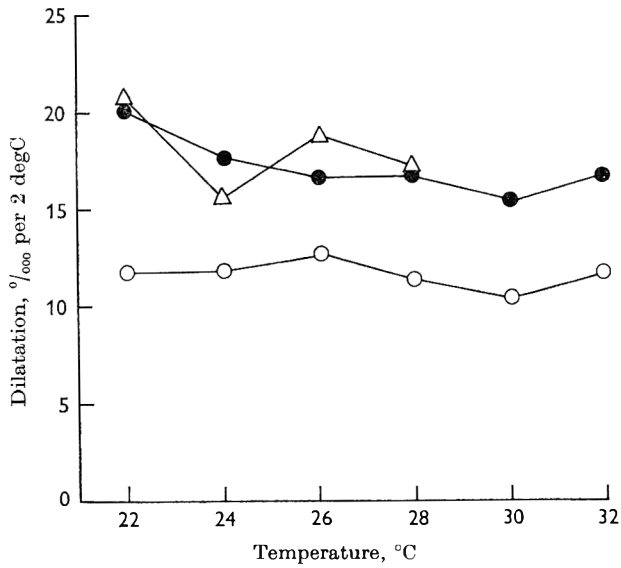

Fig. 3. Dilatometric expansion of cream. ●, held at 3 °C for 4 h; ○, held at 20 °C for 4 h; △, held at 3 °C for 4 h, then at 20 °C for 20 h.

Cooling temperature of the cream

From Table 5, it is seen that in general the amount of free fat increased as the cooling temperature before storage at 20 °C decreased. With one exception, little or no free fat was obtained at the cooling temperature of 20 °C.

Separation temperature

Table 6 shows that the % free fat in 50 % cream is decreased as separation temperature is increased. It appeared that the higher separation temperatures produced a comparatively more stable cream.

Storage of milk at 5 °C

As shown in Table 7, when fresh milk was separated the resulting cream had much less free fat than when a sample of the same milk was separated after 24-h storage at 5 °C.

Centrifugal pressure

Table 8 indicates that increasing the centrifugal pressure on cream after cooling to 5 °C gave much greater free fat than when pressure was applied after cooling to 20 °C only.

The effect of cooling and storage conditions on the amount of crystallization of fat in cream

Results of dilatometric expansions of cream samples which had been cooled and stored in different ways are shown in Figs. 3 and 4. It will be seen that the cream samples which were cooled to temperatures below 20 °C gave considerably greater amounts of expansion for every 2 degC rise in temperature (up to about 35 °C) than did samples which were cooled only to 20 °C. This was the case even after 24- and 48-h storage at 20 °C, after cooling to about 4 °C.

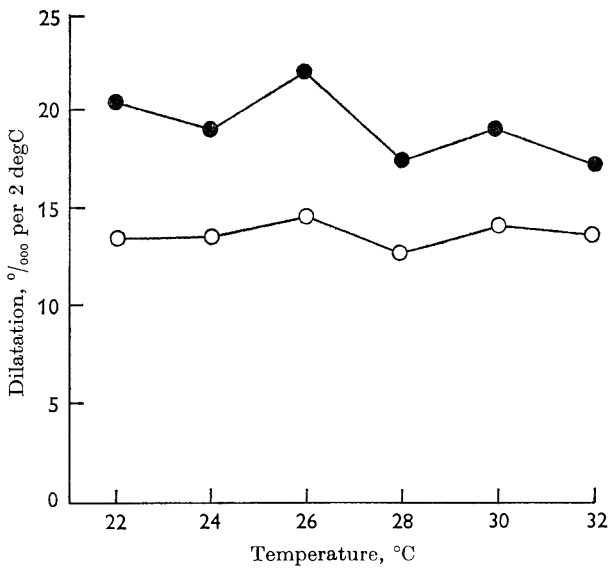

Fig. 4. Dilatometric expansion of cream. ●, held at 3 °C for 1 h, then at 20 °C for 48 h; ○, held at 20 °C for 48 h.

DISCUSSION

Mulder & Klomp (1956) showed that when cream was cooled below a certain temperature a quantity of fat crystallizes. The amount appeared to depend on the composition of the fat, the rate of cooling and whether direct or stepwise cooling was used, and the temperature to which the cream was cooled. The results of the dilatometric experiments reported here are consistent with their results and show that there was likely to be a comparatively large amount of crystalline fat present in cream which had been cooled quickly to temperatures around 5 °C. These crystals were shown to be remarkably stable even if the temperature subsequently rose to 20 °C during storage.

The results also show that if cream is subjected to pressure when it is at temperatures

below about 12 °C, free fat is present in the cream after storage at 20 °C for 48 h. It would appear that the mechanism by which this free fat is produced is that during cooling the fat becomes relatively more solid as the temperature is reduced. If the fat globules are now subjected to pressure, as will be the case in cooling sections of equipment such as plate heat exchangers, it is probable that, instead of being able to flow past each other freely as would occur if only liquid fat were present, damage will occur as hard fat globules collide with each other and with the plates. This would lead to the release of some or all of the fat as 'free fat'.

Also, the fact that relatively hard, stable fat is present in fat globules in these circumstances could account for the higher viscosities obtained when cream was cooled to low temperatures (below about 12 °C).

It is possible that differences in the amount of solid fat present in the cream during separation at various temperatures could lead to the viscosity differences reported here. However, it is clear that this is only a partial explanation for it does not explain why such differences should persist through the whole subsequent processing of the cream.

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The component fatty acids of the colostral fat and milk fat of the sow

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Summary. The fatty acid composition of colostral and milk fat samples representative of the total secretion of the nursing mammae of each of 3 sows was determined by gas–liquid chromatography. In both colostral and milk fats, acids of chain length C_{16} and C_{18} accounted for more than 90 % of the total fatty acids present; colostral fat contained a higher proportion of oleic acid and linoleic acid and less palmitic acid and palmitoleic acid than normal milk fat. Structural studies on milk triglycerides showed that, as in pig depot fat, palmitic acid is preferentially esterified to the secondary alcoholic group (2-position) of the glycerol molecules with the C_{18} acids (stearic, oleic and linoleic acids) being present for the most part in the 1- and 3-positions.

Compared with the many detailed analyses available on the fatty acid composition of cow's milk fat (see Garton, 1963), only limited information regarding the composition of sow's milk fat is recorded. References to the few analyses made before the advent of gas-liquid chromatography are given by de Man & Bowland (1963), who, using this analytical technique, compared the fatty acid composition of colostral fat with that present in milk obtained between the 4th and 5th weeks of lactation from sows given a standard diet or one containing 15 % of tallow. Subsequently, Lindberg & Tollerz (1964) reported the results of gas–liquid chromatographic analyses of samples of milk fat obtained at intervals throughout lactation from sows fed on various diets, the bulk of which consisted of barley or oats or a mixture of the 2 cereals. These workers observed that the fatty acid composition of the post-colostral milk fats of any particular sow varied considerably between samples taken at intervals of a few days, an extreme example being a change from 27·8 to 44·3 % in the oleic acid content of samples taken on the 18th and 21st days of lactation; no regular pattern of variability was noted. In that the analyses were made on very small samples (1–3 ml) taken 'at random' from one of the teats while the sow was nursing its litter, the possibility is not excluded that the analyses might not have been representative of the milk secreted by all the lactating mammae of the sow.

In this paper we report the results of fatty acid analyses of colostral and milk fat samples representative of the total secretion of the nursing mammae of each of 3 sows. The opportunity to obtain these samples was afforded to us by colleagues who

were using these (and other) sows in an experiment primarily concerned with the utilization of dietary nitrogen by the lactating sow and its litter (Elsley & MacPherson, 1966).

EXPERIMENTAL

Animals and diet

Three Large White sows, each having produced its 2nd litter, were used. Throughout pregnancy they were each fed daily with 2·2 kg of a diet consisting largely of barley (62·5 %) together with maize, weatings, white fish meal, soyabean meal and a suitable supplement of minerals and vitamins. During lactation each sow received daily 5·0 kg of a similar diet, except that it included a smaller proportion of barley (47·5 %) which was offset by the inclusion of more weatings and soyabean meal. Drinking water was offered after each feed. The crude fat content of the 'pregnancy' and 'lactation' diets was 2·4 and 2·8 %, respectively. After parturition the sows were put into metabolism cages and the size of the litters reduced to 8 piglets for each sow.

Collection of colostrum and milk

To obtain samples of colostrum and milk the piglets were denied a normal suckling period and each of the 8 nursing mammae of each sow was milked by hand following the intravenous injection of 5 i.u. of oxytocin (Pitocin; Parke-Davis and Co.) to bring about a satisfactory 'let-down' of the secretion. The total volume collected at any one time from each sow was usually 200–300 ml. This was thoroughly mixed and a sample (30 ml) for analysis was taken into a polythene container and frozen at −20 °C, at which temperature it was stored for several weeks. From each sow a total of 7 or 8 such samples was obtained at intervals of up to 40 days after parturition.

Analytical methods

After each colostral or milk sample had been allowed to thaw, a portion (10 ml) was refluxed for 1 h with 200 ml of a mixture of ethanol and ether (3:1, v/v). The resulting extract was filtered and the filtrate distilled almost to dryness; the residue (mainly fat) was saponified under reflux for 1 h with excess 0·5 N-ethanolic KOH. The saponification mixture was made acid with 10 N-H_2SO_4 and the fatty acids recovered in the usual way by extraction with ether. The acids were converted into methyl esters by refluxing with excess methanol containing 1 % (w/w) H_2SO_4 and then analysed, before and after hydrogenation, by gas–liquid chromatography as described by Duncan & Garton (1963).

The positional distribution of the fatty acids in the triglycerides was determined as described by Garton & Duncan (1965) on 2 samples prepared from the milk of one of the sows. The milk (10 ml) was extracted with ethanol–ether as described above and, after removal of the solvent, the residue was extracted with light petroleum (b.p. 40–60 °C). This solution was applied to a column containing 15 g of silicic acid (Mallinkrodt) mixed with 5 g of Hyflo Super-Cel (Johns Manville); following the elution of a very small amount of non-glyceridic material with 1 % (v/v) ether in light petroleum the triglycerides were eluted with 5 % (v/v) ether in light petroleum.

RESULTS AND DISCUSSION

Table 1 records the values for the fatty acid compositions of the colostral and milk fats of the 3 sows; for clarity of presentation the values in this table (and in Tables 2 and 3) are given to the nearest whole number. After the first (colostral) sample from each animal, the rest can be taken to represent normal milk.

Table 1. *Fatty acid composition of sow's colostral and milk fats*

Sow no.	Days after farrowing	Fatty acid, % (w/w) of total fatty acids						
		14:0*	16:0	16:1	18:0	18:1	18:2	Others†
1	1	1	22	5	5	42	20	5
	4	3	30	10	5	35	13	5
	12	3	28	7	5	41	11	5
	13	3	31	8	4	39	10	5
	17	3	38	12	4	29	10	4
	23	3	29	8	4	43	10	3
	30	3	36	13	3	34	9	3
	37	3	38	15	3	29	9	3
	Mean value, days 4–37	3	33	10	4	36	10	4
2	2	2	23	6	6	42	18	3
	6	4	39	10	5	27	12	3
	12	4	44	9	5	25	11	3
	16	4	36	9	5	34	12	2
	22	4	38	12	4	30	11	2
	28	4	40	12	4	28	10	2
	33	3	42	12	4	27	10	2
	38	4	40	13	4	27	10	2
	Mean value, days 6–38	4	40	11	4	28	11	2
3	1	2	24	7	5	41	18	3
	6	4	33	10	4	31	14	4
	16	4	35	11	5	32	12	2
	22	4	36	14	4	30	10	2
	28	4	38	15	3	27	10	3
	33	4	34	12	3	34	10	3
	40	3	32	10	4	35	13	3
	Mean value, days 6–40	4	34	12	4	32	11	3

* Shorthand nomenclature of Dole, James, Webb, Rizack & Sturman (1959) indicating the number of carbon atoms followed by the number of double bonds.

† Including, in colostral fat, traces of 12:0 and 15:0 together with small amounts of 17:1, 18:3 and 20:4 and, in milk fat, traces of 17:0 together with small amounts of 10:0, 12:0, 14:1, 15:0, 17:1, 18:3 and 20:4.

As previously observed by de Man & Bowland (1963), colostral fat of the sow differs from fat subsequently secreted in that it contains a higher proportion of oleic acid (18:1) and linoleic acid (18:2) and a correspondingly lower content of palmitic acid (16:0) and palmitoleic acid (16:1). Lindberg & Tollerz (1964) also recorded a post-colostral decrease in the linoleic acid content of milk fat when the sow's diet consisted largely of barley, but the change was much less marked when oats formed a considerable part of the diet. Oat lipids are a good source of linoleic acid and, in subsequent studies, Tollerz & Lindberg (1965) showed that there was a close association between

the linoleic acid content of the dietary fat and that of the milk fat. It is thus, perhaps, all the more significant that, when given the usual diets in which barley is the main cereal, the sow provides the piglet with fat containing relatively large amounts of linoleic acid during the first day or two of life when this acid may be of particular metabolic importance.

The values for the fatty acid composition of the milk fats of sows 2 and 3 show less variation over the sampling period than those of sow 1, which are of a similar order to those observed by Lindberg & Tollerz (1964), e.g. the values in Table 2 of 29 and 43% for the oleic acid content of the samples taken on the 17th and 23rd days, respectively, after farrowing. It thus appears that the 'single-teat' samples

Table 2. *Mean proportions of the principal fatty acids present in the milk fat of sows given diets having a high content of barley*

			Fatty acid, % (w/w) of total fatty acids					
	Sow no.	No. of samples	14:0	16:0	16:1	18:0	18:1	18:2
*	1	7	3	33	10	4	36	10
*	2	7	4	40	11	4	28	11
*	3	6	4	34	12	4	32	11
†	14	12	5	37	15	3	30	7
†	15	11	5	34	14	3	34	8

* Present investigation.
† From Table 5 of Lindberg & Tollerz (1964).

Table 3. *Principal component fatty acids of the triglycerides of sow milk fat and of the 2-monoglycerides derived therefrom and, for comparison, a similar analysis of pig depot fat*

Values in parentheses, which show the relative proportion of each acid in the 2-position of the triglyceride molecules, are derived from the expression:

$$\frac{(\text{moles \% fatty acid in 2-monoglycerides}) \times 100}{(\text{moles \% same fatty acid in triglycerides}) \times 3}$$

		Fatty acid, moles % of total fatty acids				
	Source of fatty acids	16:0	16:1	18:0	18:1	18:2
*	Milk triglycerides	38	14	3	28	10
*	2-Monoglycerides	72 (64)	11 (24)	1 (8)	8 (9)	5 (18)
†	Milk triglycerides	35	12	4	32	12
†	2-Monoglycerides	57 (55)	15 (42)	< 1 (3)	9 (9)	9 (24)
‡	Depot triglycerides	26	—	12	51	—
‡	2-Monoglycerides	56 (70)	—	5 (12)	22 (15)	—

* Sample from milk secreted on day 22 by sow 3.
† Pooled sample from all post-colostral milk obtained from sow 3.
‡ From Table 1 of Garton & Duncan (1965).

as analysed by Lindberg & Tollerz (1964) are probably representative of the secretion, at any one time, of all the lactating mammae of a sow. As discussed by these workers, the variations in composition from sample to sample may reflect differences in the extent to which the fatty acids of milk fat are derived on the one hand from plasma lipids and on the other from acids synthesized *de novo* in the gland from acetate.

In accord with Lindberg & Tollerz (1964) our results show that there is apparently a reciprocal relationship between the proportions of oleic and palmitic acids in the milk fat. For sows fed on comparable (high-barley) diets the mean values (Table 2) for the principal component acids of the (post-colostral) milk fats analysed by us and by Lindberg & Tollerz (1964) are in good agreement.

It has been known for some time (see Coleman, 1963; Mattson, Volpenhein & Lutton, 1964) that the depot triglycerides of pigs (and also peccaries) are unusual in that, unlike other reserve triglycerides of both animal and plant origin, they show preferential incorporation of palmitic acid in the 2-position of the molecule, with the unsaturated acids occupying the 1- and 3-positions almost exclusively. As the results in Table 3 show, sow milk fat triglycerides are also assembled with a preponderance of palmitic acid in the 2-position and a corresponding preferential esterification of stearic acid and the C_{18} unsaturated acids in the 1- and 3-positions. In comparable studies on cow's milk triglycerides McCarthy, Patton & Evans (1960) found that palmitic acid was distributed at random among the available positions of the glycerol molecules. It thus appears that whatever enzyme systems are present in pig adipose tissue leading to the selective esterification of palmitic acid with the secondary alcoholic group of the glycerol molecule are also similarly active in the lactating mammary gland of the sow.

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With 3 plates

The distribution and characters of coagulase-negative staphylococci of the bovine udder

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Summary. Coagulase-negative staphyloccoci were isolated regularly over a period of 39 weeks from quarter milk samples of 18 cows belonging to one herd. They did not produce a leucocytosis in the milk or high anti-haemolysin titres in the blood of these cows, but such responses occurred in 2 cows infected with pathogenic staphylococci. The coagulase-negative variety was found to colonize the teat duct and surface of the teat in preference to the milk and possessed greater resistance than pathogenic staphylococci to certain long-chain fatty acids. Infection with coagulase-negative staphylococci was thought to antagonize the colonization of the udder by a pathogenic variety present in the herd; 23 % of the coagulase-negative strains were found to elaborate an antibiotic substance inhibitory to the growth of a coagulase-positive staphylococcus. Coagulase-negative strains were characterized by the production of ϵ-toxin and could be divided into 3 main groups according to their effect on gelatin, mannitol and phenolphthalein diphosphate as substrates.

INTRODUCTION

A substantial amount of work has been carried out on various aspects of coagulase-positive staphylococci of the bovine udder, particularly since these organisms have in recent years assumed a more important role in bovine mastitis. Coagulase-negative staphylococci, on the other hand, have received little attention since they are considered unimportant in the disease. The attitude towards these organisms is reflected by Slanetz & Bartley (1953), who stated 'those staphylococci which produce only narrow zones of alpha haemolysis (1 mm or less) are coagulase-negative, weak toxin producers and can be disregarded'. However, coagulase-negative staphylococci have, on occasions, been incriminated as a cause of bovine mastitis (Reid & Wilson, 1959) and considered by Crossman, Dodd, Lee & Neave (1950) to be capable of infecting the udder without causing irritation.

During routine examination of milk samples from one of the Institute herds (herd A described by Edwards & Smith, 1966) staphylococci, which were either non-haemolytic on ox blood-agar plates or produced only narrow zones of haemolysis, and had no power to coagulate sheep or rabbit plasma, were constantly found. At some tests up to 61 % of the milk samples contained these organisms either alone or together with pathogenic staphylococci. It was observed that in cases where large

numbers of coagulase-negative staphylococci were recovered, the pathogenic variety were either absent or present in very low numbers, and reference to the history of individual cows in the herd revealed that persistent carriers of these organisms had usually been free from staphylococcal mastitis.

For these reasons it was decided to investigate their distribution, habitat and relationship to the pathogenic staphylococci present in the herd. Their biochemical and haemolytic characters were also studied, and because of their apparent effect on pathogenic staphylococci they were examined for some active principle which might be antagonistic to the growth of coagulase-positive types.

MATERIALS AND METHODS

Two groups of cows in herd A were studied: group 1 in which the milk only was examined for the presence of coagulase-negative and coagulase-positive staphylococci, and group 2 in which the milk, teat duct, and teat surface were examined.

Cows in group 1

The quarter milk samples of 20 cows were examined on 20 occasions over a period of 39 weeks.

Collection of samples. The udder of each cow was thoroughly washed in a warm sodium hypochlorite solution (500 ppm.). The sampler wore rubber gloves which were dipped in disinfectant solution after sampling each cow. The first stream of milk was examined in a strip cup, after which the teat orifice was cleaned with a cotton-wool swab soaked in 50 % (v/v) ethanol. Approximately 10 ml of milk was then removed into a sterile container.

Isolation of staphylococci. In the laboratory, 0·1 ml of milk was spread over the surface of a blood-agar plate containing 5 % (v/v) thrice-washed ox red cells in an agar base with the addition of Bacto tryptose peptone (2 %, w/v) and glucose (0·1 %, w/v). The plates were incubated at 37 °C for 24 h and selected typical staphylococcal colonies removed and tested for coagulase production by the method described by Edwards & Rippon (1957).

Other determinations. Total leucocyte counts were performed on the quarter milk samples of 10 cows by the technique of Blackburn, Laing & Malcolm (1955). The serum anti-haemolysin titres of the same 10 cows were examined at monthly intervals by the method of Minett (1937).

Cows in group 2

The milk, teat duct, and teat surface of 16 cows were examined for the presence of staphylococci.

Collection of samples. Before sampling, the udders and teats were washed with warm water and the teat duct examined by introducing with a rotating movement a thin sterile moistened cotton-wool swab, prepared on 19-gauge wire, 4 mm into the duct. The teat orifice was then cleaned with a swab soaked in 50 % (v/v) ethanol and a teat syphon introduced into the teat sinus. Milk was allowed to flow for several seconds to flush the syphon before 2 or 3 ml of fore-milk were collected in a sterile Universal container. The skin of the teat was examined by rubbing a sterile moistened swab several times over the teat surface.

Isolation of staphylococci. Swabs of the teat duct and surface were inoculated on the surface of ox blood-agar plates and 0·1 ml of the milk was examined by incubation on the same medium.

Haemolytic activity. The haemolytic pattern and colonial appearance of strains were examined on the blood-agar medium previously described. The effects of specific anti-α-haemolysin and of β-haemolysin on the haemolytic activity of coagulase-negative strains were examined by the plate techniques of Elek & Levy (1950 and 1954, respectively). Sheep and ox cells were employed in the plates, which were incubated in 20 % (v/v) CO_2 for 24 h and for a further 24 h in air. The presence of ϵ-toxin was investigated by the plate method of Fraser (1962) employing a strain of *Corynebacterium haemolyticum* (N.C.T.C. H 8452).

Biochemical tests. These included: fermentation of mannitol; liquefaction of gelatin; production of urease, lecithinase and phosphatase; Voges–Proskauer and methyl red reactions; nitrate and methylene blue reduction. After preliminary examination, strains were tested routinely for mannitol fermentation and gelatin liquefaction at 37 °C for 7 days using media described by Cruickshank (1960), and phosphatase production on nutrient agar plates containing phenolphthalein diphosphate (Oxoid).

Resistance to fatty acids. The susceptibility of coagulase-negative and coagulase-positive staphylococci to various fatty acids, found by Adams & Rickard (1963) to be present in the keratin-like material of the bovine teat duct, was investigated. Sodium salts of saturated and unsaturated fatty acids (C6–C20) were prepared and incorporated at concentrations of 1, 10, 50 μg/ml in a liquid medium composed of 20 g Bacto Casamino acids, 1·29 g KH_2PO_4, 7·06 g K_2HPO_4, 500 ml yeast dialysate, and water to final volume of 1 l. The yeast dialysate was prepared by dialysing a 4 % (w/v) solution against 4 times its volume of distilled water at 4 °C for 7 days.

Production of substances antagonistic to the growth of coagulase-positive staphylococci

Coagulase-negative staphyloccoci were picked directly from primary isolation plates and sown in circular areas on the surface of ox blood-agar plates containing 10 % (v/v) citrated ox blood in an agar base incorporating 2 % (w/v) Casamino acids and 0·4 % (w/v) yeast extract; the final pH of the medium was 7·4. The plates, enclosed in polythene bags, were incubated at 37 °C for 48 h. Growth was then removed and the plates exposed to chloroform vapour as described by Parker & Simmons (1959).

After removal of residual chloroform vapour, 0·1 ml of an overnight broth culture of a staphylococcal strain P 3, belonging to phage type 42 D and typical of the prevailing pathogenic type in herd A, was spread over the surface of the plates which were re-incubated for a further 18 h at 37 °C. They were then examined for inhibition or marked reduction in the growth of strain P 3 in the area surrounding the original position of the coagulase-negative strain.

RESULTS

Cows in group 1

Numbers of staphylococci. In the milk samples of cows 1–18, coagulase-negative staphylococci were present at most of the tests while cows 19 and 20 were infected with a coagulase-positive variety typical of the pathogenic staphylococcus prevailing in the herd. For cows 1–18 the average number of colony-forming units (CFU) of coagulase negative staphylococci in all quarters ranged from 136 to 2756/ml fore-milk, while the count of coagulase-positive staphylococci was usually lower (Fig. 1). In

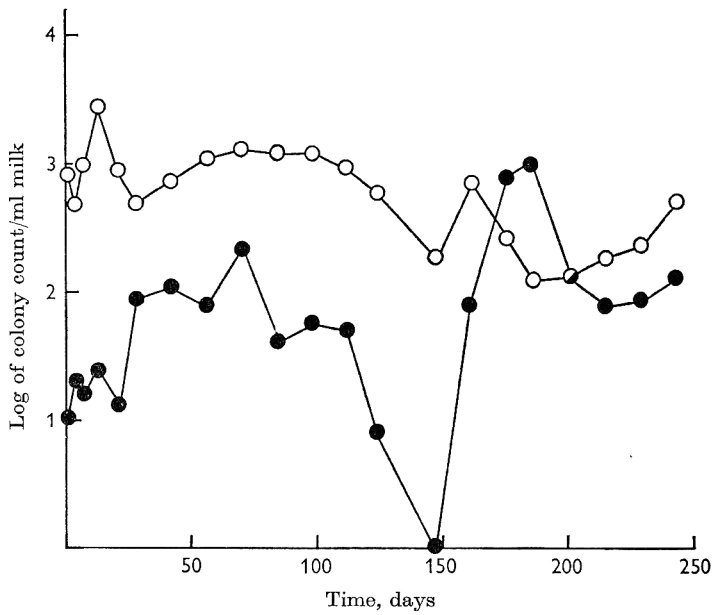

Fig. 1. The average colony counts in fore-milk samples: ○, of coagulase-negative staphylococci; and, ●, coagulase-positive staphylococci in cows 1–18.

only one instance did the count of the latter exceed that of the coagulase-negative variety, this being at the tests carried out between the 160th and 200th day when the count of coagulase-positive staphylococci suddenly rose. This coincided with a sharp rise in the incidence rate of coagulase-positive staphylococci for the whole herd, which temporarily increased from a normal level of 50 % to a temporary level of 89 %.

In cows 19 and 20 the colony counts of coagulase-negative staphylococci were generally lower and very irregular. These organisms were mainly isolated from 2 quarters of 1 cow and from 1 quarter of the other into which coagulase-positive staphylococci from the other 5 chronically infected quarters did not spread. It will be seen in Fig. 2 that the count due to coagulase-positive staphylococci was always high, averaging over 4000 CFU/ml of fore-milk.

Leucocyte count. Fig. 3 shows that the number of white cells in the fore-milk of cows 1–18 was low and on average did not exceed 1×10^6 cells/ml, even in the latter period of testing, when many of the cows were approaching the end of their lactation. The

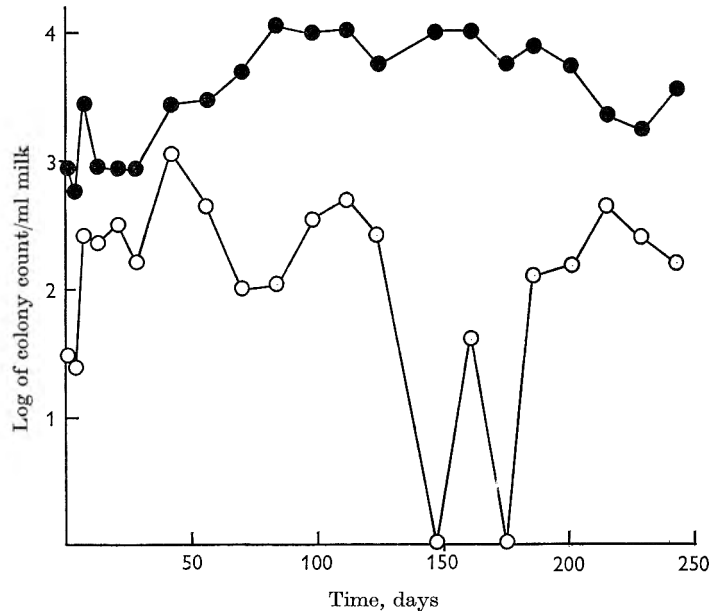

Fig. 2. The average colony counts of staphylococci in fore-milk samples from cows 19 and 20. ○, Coagulase-negative; ●, coagulase-positive.

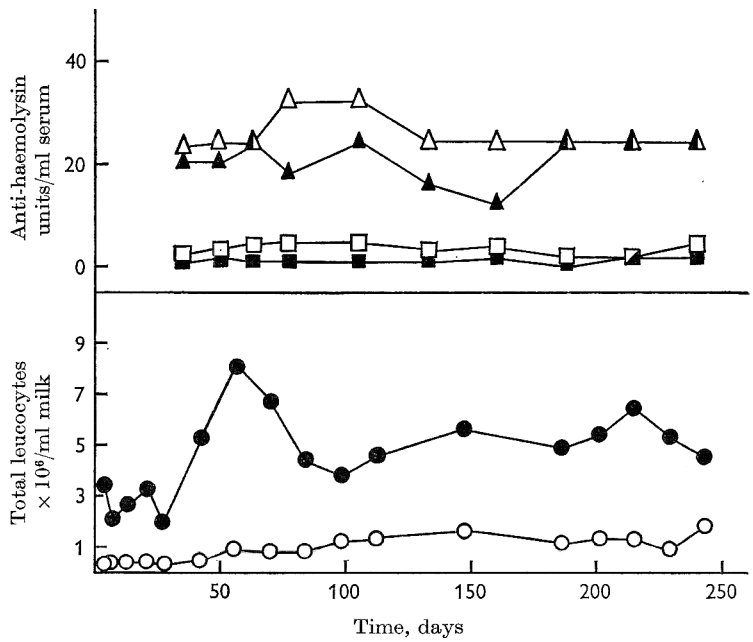

Fig. 3. The average anti-α-haemolysin and anti-β-haemolysin contents of the serum and the leucocyte counts of the fore-milk of cows 1–20. △, Anti-α-haemolysin of cows 19 and 20; □, anti-α-haemolysin of cows 1–18; ▲, anti-β-haemolysin of cows 19 and 20; ■, anti-β-haemolysin of cows 1–18; ●, leucocytes in fore-milk of cows 19 and 20; ○, leucocytes in fore-milk of cows 1–18.

occasional appearance of pathogenic staphylococci in the milk of these cows did not therefore produce established infection resulting in a cellular response.

On the other hand, in cows 19 and 20 the mean leucocyte counts were abnormal and ranged from 2×10^6 to 8×10^6 cells/ml of fore-milk. In one of these cows mastitis, evidenced by clots in the fore-milk, was found at several of the tests.

Serum anti-haemolysin titres. Fig. 3 shows that the anti-α-haemolysin titres of cows 1–18 did not rise above 1 or 2 units/ml and the titre of the anti-β-haemolysin was usually below 0·25 unit/ml. On the other hand, in the cows infected with pathogenic staphylococci, the serum titres were at a high level of 16–32 units/ml for both anti-haemolysins.

Table 1. *The distribution of coagulase-negative and coagulase-positive staphylococci in the milk, in the teat duct and on the teat surface of* 16 *lactating cows*

	Site of colonization (number of sites infected)						
Variety of staphylococcus	Milk alone	Milk and teat duct	Milk and teat surface	Milk, teat duct and teat surface	Teat surface alone	Teat surface and duct	Teat duct alone
Coagulase-negative	0	1	0	7	19	21	9
Coagulase-positive	0	1	1	19	4	6	2

Cows in group 2

Frequencies of isolation of staphylococci from the milk teat duct and teat surface. It can be seen from Table 1 that coagulase-negative staphylococci were isolated with greatest frequency from the teat surface or teat surface and duct together, where they could be found without being present in the milk. The average number of organisms which could be cultivated from swabs taken from the teat duct was 274. On the other hand, coagulase-positive staphylococci were found more often in the milk in association with the other 2 sites. The difference in the frequency of colonization of the 2 species in the milk was significant ($P < 0{\cdot}001$) and the results showed further that coagulase-negative staphylococci, when recovered from milk, were present either in quarters uninfected or carrying only slight infection with the coagulase-positive variety.

The presence of coagulase-negative staphylococci in some of the milk samples may have been due to the flushing of the organisms from the walls of the teat duct during the sampling procedure.

Characteristics of the coagulase-negative strains of staphylococci

Cultural characters. At primary isolation on blood agar, colonies were approximately 0·5–1 mm diam. and surrounded by a narrow clear haemolytic zone 1–1·5 mm diam. (Plate 1); 32 % of strains were non-haemolytic. Of 434 strains examined, the pigmentation of 74 % was yellow, 19·5 % white and 6·5 % yellow to golden. Colonies were usually butyrous in consistency and grew in broth as diplois, tetrads and small clumps. Certain strains lost their haemolytic activity and pigmentation on subculture but regained these characteristics after cultivation on nutrient agar containing 10 % (v/v) milk.

and the number of strains producing inhibitory substance (active strains)

	Group ...	1	2	3	4	5	6	7	8
	Substrates	Mannitol	Mannitol and gelatin	Mannitol and phenol-phthalein diphosphate	Gelatin	Gelatin and phenol-phthalein diphosphate	Phenol-phthalein diphosphate	Mannitol, gelatin and phenol-phthalein diphosphate	None
Haemolytic	No. positive	223	24	10	4	11	2	2	15
	No. active	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
Non-haemolytic	No. positive	19	3	3	0	46	5	66	1
	No. active	(1)	(2)	(3)	(0)	(41)	(0)	(52)	(0)
Totals		242	27	13	4	57	7	68	16

Table 3. *The inhibitory effect of certain long-chain fatty acids on the growth of* 50 *strains of coagulase-negative and* 50 *strains of coagulase-positive staphylococci in liquid medium*

	Strains showing maximum growth in 7 days at 37 °C, % of total	
Fatty acid present (50μg./ml)	Coagulase-negative	Coagulase-positive
Arachidonic	100	44
Linoleic	100	24
Linolenic	50	0
Myristic	100	100

Haemolytic activity. On blood-agar medium containing ox cells, haemolytic activity was more pronounced than on the same medium containing sheep red cells; slight haemolysis occurred with horse red cells.

The haemolysis produced by coagulase-negative strains was not inhibited by anti-α-haemolysin but was potentiated by β-haemolysin. Haemolysis which remained unaffected by anti-α-haemolysin was found to be characteristic of coagulase-negative strains by Elek & Levy (1950) owing to the possession of ϵ-toxin. Confirmation of this was obtained by the plate test of Fraser (1962) in which the haemolysin of haemolytic strains was potentiated, whereas that of the non-haemolytic strains was unaltered except after 48-h incubation (Plate 2).

Biochemical tests. Coagulase-positive and coagulase-negative strains were indistinguishable by these tests.

The use of mannitol fermentation, gelatin liquefaction and phosphatase production made it possible to differentiate the coagulase-negative strains into 3 main groups as shown in Table 2. Mannitol was fermented within 3 days by group 1 strains while group 7 strains did so in 6 days. Gelatin was liquefied within 3 days and phenolphthalein diphosphate was split in 48 h. Haemolytic strains were mainly confined to group 1, while the majority of non-haemolytic strains belonged to groups 5 and 7.

Resistance to fatty acids. The effect of these acids on the growth of 50 strains of each variety is shown in Table 3. Although the growth of all organisms was delayed by the presence of fatty acids in the medium, the coagulase-negative strains were more resistant. Coagulase-positive strains were also slightly inhibited by the presence of lauric, oleic and palmitic acids.

Production of antagonistic substances. Of the 434 strains examined, 99 (23 %) produced an active principle which inhibited the growth of the test organism P 3. The degree of inhibition produced varied considerably, but with all strains zones of inhibition were circular with indistinct margins and populated by a few resistant colonies of the sensitive strain P 3 (Plate 3). The distribution of the active strains in the biochemical groups is shown in Table 2, from which it can be seen that they were all non-haemolytic and were confined mainly to groups 5 and 7.

DISCUSSION

The absence of a response to the presence of coagulase-negative staphylococci in the form of anti-haemolysin in the blood and leucocytosis in the milk of cows 1–18 was probably due to the fact that these organisms populated the teat duct and were only present in the milk as transient invaders. It is possible they were more suited to colonization of the teat duct because of their greater resistance to certain long-chain fatty acids present in the keratin-like material of the teat duct (Adams & Rickard, 1963). At the same time, infection in these cows due to coagulase-positive staphylococci was very low and most of them could be considered to be uninfected with the prevailing staphylococcus, a 42D phage type, and remained free from mastitis for the whole period. By comparison, 2 cows which were consistently infected with very large numbers of pathogenic organisms showed a high leucocyte count in the milk, high anti-haemolysin titres in the blood, but a low and irregular infection with coagulase-negative strains.

An explanation for the results observed could be that the teat duct material of cows 1–18 may have possessed greater quantities of inhibitory substances in the form of non-esterified long-chain fatty acids than that of cows 19 and 20. It would, therefore, be easier for coagulase-negative staphylococci to colonize the teat duct of the former group of cows than it would be for the coagulase-positive variety. Temporary infection with pathogenic staphylococci could occur but such infection would probably be eliminated owing to the relative sensitivity of these strains to the fatty acids present with the resulting recolonization of the site by the coagulase-negative types.

Moreover, it was shown that 23 % of the coagulase-negative strains, mainly isolated from the teat duct, produced *in vitro* an antibiotic-like material active against a typical pathogenic staphylococcus. It is, therefore, conceivable that the active coagulase-negative staphylococci in the teat duct produced some antibiotic material which acted as an additional barrier to infection of the teat duct, and later the milk and udder tissues, by pathogenic staphylococci.

All of the 434 strains examined were classified as non-pathogenic coagulase-negative staphylococci characterized by the production of ϵ-toxin (Elek & Levy, 1950; Fraser, 1964). Biochemically, the coagulase-negative strains were indistinguishable from pathogenic staphylococci but by using mannitol, gelatin and phenolphthalein diphosphate as substrates they could be differentiated into 3 main groups. Haemolytic strains did not produce antibiotic, and 76·5 % of these belonged to group 1, whereas 78·5 % non-haemolytic strains fell into groups 5 and 7 and nearly all these were active antibiotic producers. It appeared, therefore, that at least 2 predominating types existed in herd A represented by group 1 and groups 5 and 7.

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EXPLANATION OF PLATES

PLATE 1

The appearance of coagulase-negative staphylococci on primary isolation on blood agar from the milk samples of 2 cows. The colonies in the upper half of the plate show characteristic haemolysis when few colonies are present; in the lower half the colonies are congested and show little haemolysis.

PLATE 2

The potentiation of the haemolysis of ϵ-toxin-producing strains of coagulase-negative staphylococci in the presence of *C. haemolyticum*. One strain shows only slightly increased haemolysis which is typical of the non-haemolytic variety of coagulase-negative staphylococci.

PLATE 3

The inhibition of a coagulase-positive staphylococcus (P 3) by the preformed antibiotic produced by a coagulase-negative staphylococcus (A 12) using the deferred 'spot' technique. A few resistant colonies can be seen in the zone of inhibition.

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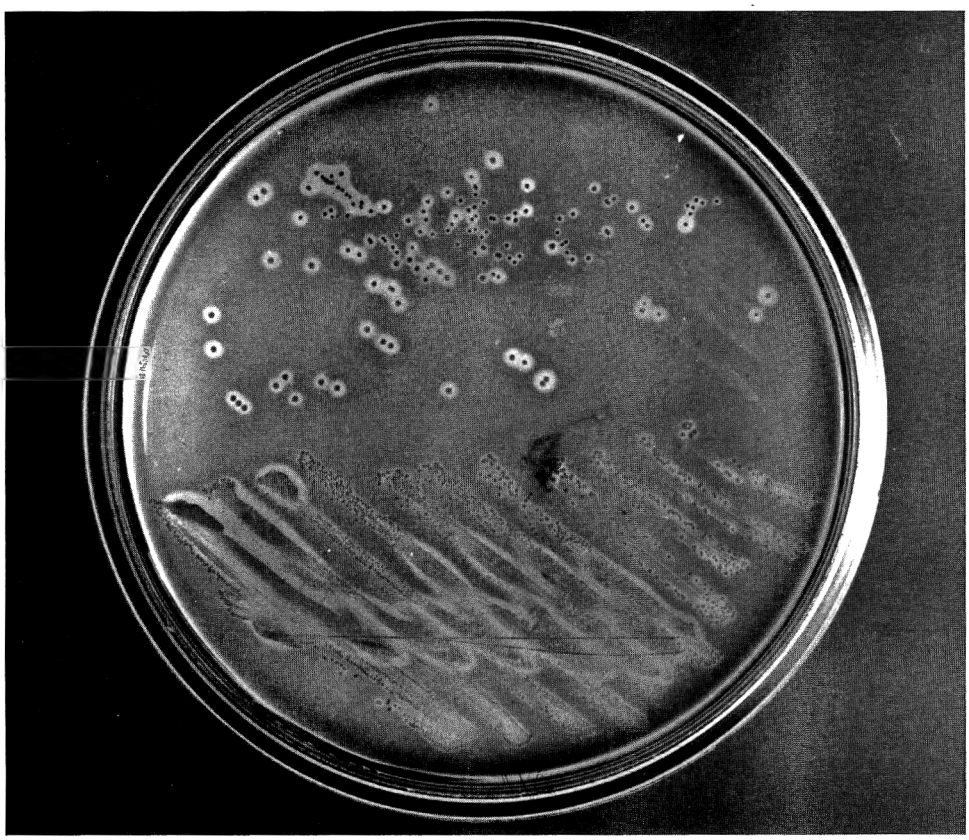

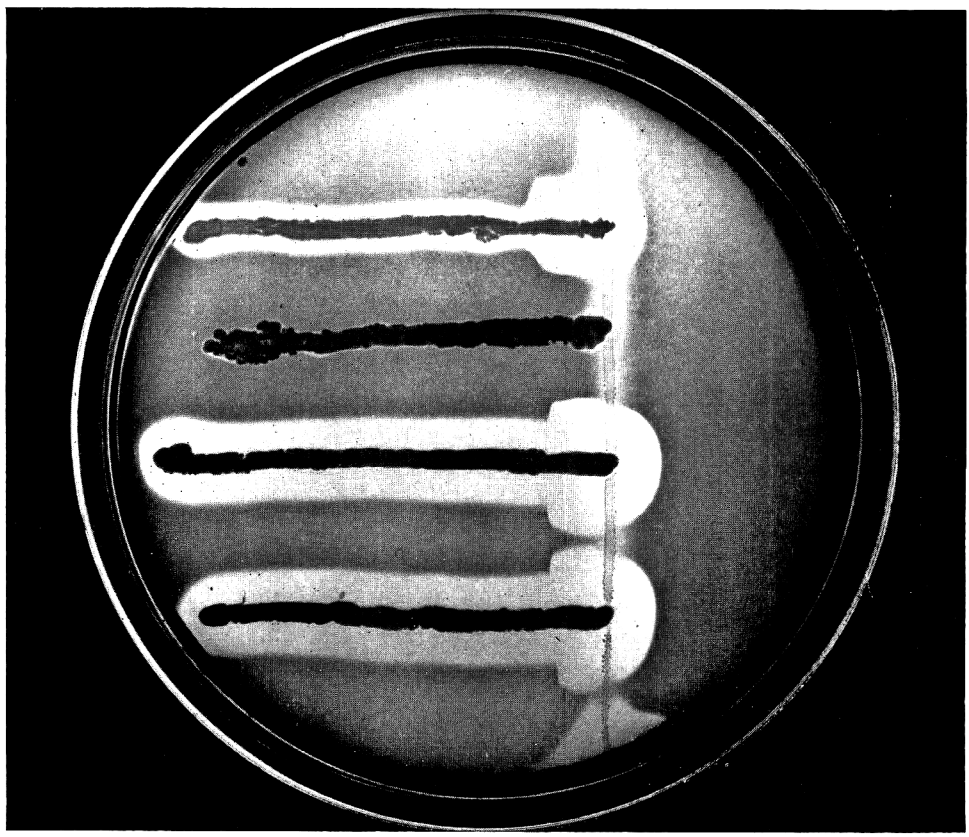

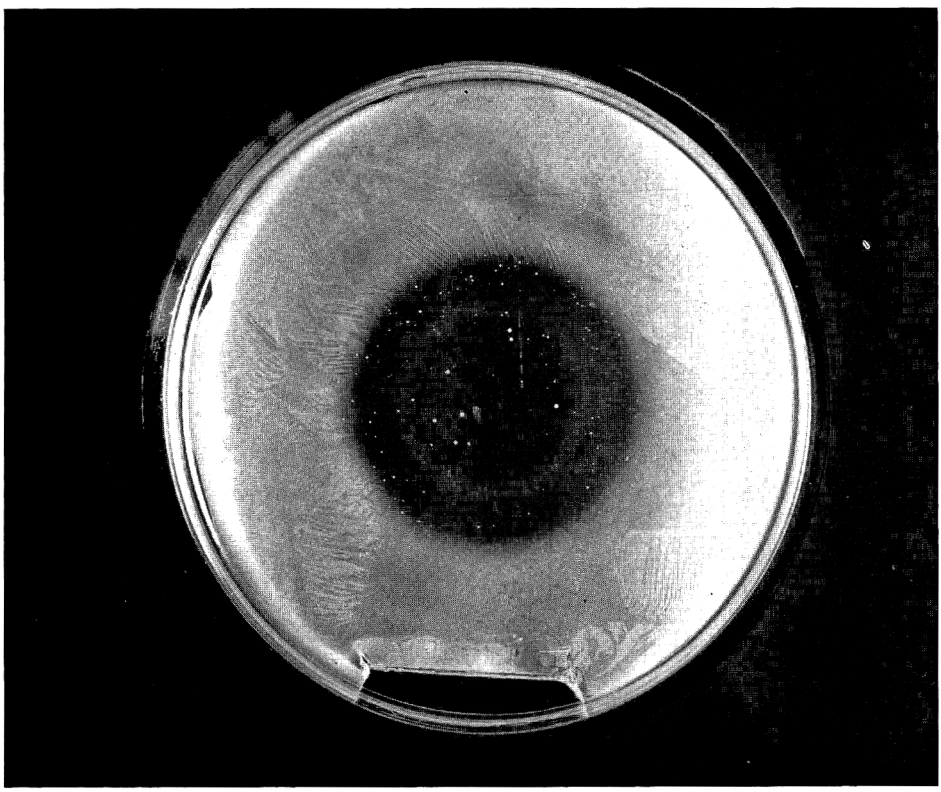

J. Dairy Res. (1966), **33**, 271
With 1 *plate*

Examination of an antibiotic produced by coagulase-negative staphylococci isolated from the bovine udder

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Summary. An antibiotic produced by coagulase-negative staphylococci isolated from the bovine udder was examined by deferred antagonism tests and its inhibitory properties compared with those of other staphylococci. While inhibiting the growth of representative phage types of bovine pathogenic staphylococci, its activity against corynebacteria was more restricted than that of *Staphylococcus aureus* NCTC 6507 and 8004. Only minimal amounts of inhibitory substance were produced in liquid media, larger amounts were produced on solid media and the incorporation of a blood plasma factor further increased production. The inhibitory substance resisted treatment with pepsin and trypsin and withstood boiling for 30 min. Its characters in relation to inhibitory substances produced by other staphylococci are discussed.

Diffusible substances produced by staphylococci and having antagonistic properties against various micro-organisms have been known for many years. The earlier literature on this subject has been reviewed by Florey *et al.* (1949). Gardner (1949) studied the properties of an antibiotic isolated from *Staphylococcus aureus* and Parker, Tomlinson & Williams (1955), Parker & Simmons (1959), and Barrow (1963*a*, *b*) investigated the inhibitory effects against corynebacteria and other staphylococci of phage type 71 staphylococci recovered from impetigo and other superficial vesicular lesions of the human skin. Su (1948) isolated a micrococcus from sewage which inhibited the growth of Gram-positive micro-organisms, and Loeb, Moyer & Murray (1950) obtained a strain of *Micrococcus epidermis* which produced a heat-stable antibiotic substance.

In the previous paper by Edwards & Jones (1966) it was suggested that coagulase-negative staphylococci may have some deterrent action against the infection of the udder by pathogenic types since 23 % of the 434 strains examined produced an antibiotic substance which inhibited the growth of a pathogenic staphylococcus.

The production and some characteristics of the inhibitory substance have now been examined by using 2 active strains, A 5 and A 12, which were selected as typical of these antibiotic-producing organisms.

MATERIALS AND METHODS

Antagonism tests on solid media. Two methods were used for the detection of deferred antagonism employing the Casamino acid–yeast extract medium previously described (Edwards & Jones, 1966) in which the active or test organisms were grown either as a spot or as a streak across the plate at 37 °C for 24–48 h. The growth was then removed and the plates treated with chloroform vapour (Abbott & Shannon, 1958; Parker & Simmons, 1959). In the first method or 'spot method' 0·1 ml broth culture of a sensitive staphylococcal strain, P3, was then spread over the surface of the plate. In the second or 'colicine method' a loopful of test culture of an indicator strain was streaked at right angles to the original inoculum. The plates were then re-incubated overnight at 37 °C.

Antibiotic-producing organisms. The 2 antibiotic-producing strains employed were A5 and A12; these belonged to group 5, previously described (Edwards & Jones, 1966), and were typical producers of large quantities of antibiotic. *Staph. aureus* strains NCTC 6507 (deposited by A. Fleming in 1943) and NCTC 8004 (Gardner, 1949) were also employed for comparative purposes.

Indicator organisms. (*a*) *Staphylococci.* Fifteen strains of coagulase-positive staphylococci (P1–15) representing 15 different phage types were used. These organisms were all isolated from secretions withdrawn from quarters of cows affected with mastitis in the 3 Institute herds.

(*b*) *Corynebacteria.* These included: *Corynebacterium hoffmanii* NCTC 231, 8633 and 8634; *C. xerosis* NCTC 7883, 7929 and 9755 (Gardner, 1949); a diphtheroid Bradford (BDF), and an avirulent strain of *C. diphtheriae mitis* (MC) both used by Barrow (1963*a*); and a strain of *C. pyogenes* from a case of bovine mastitis.

(*c*) *Streptococci.* A strain of *Streptococcus agalactiae* (group B), one of *Str. dysgalactiae* (group C), one of *Str. zooepidemicus* (group C) and one of *Str. uberis* were used.

(*d*) *Escherichia coli.* One strain from a case of bovine mastitis was used.

RESULTS

The result of testing the 15 pathogenic strains against A5 and A12 and the 2 known antibiotic-producing strains NCTC 6507 and 8004 by the colicine method can be seen in Table 1.

All the indicator strains were inhibited by A5 and A12. The inhibition zones were 25–30 mm in diam. with diffuse edges. On the other hand, with the NCTC strains the zones were narrower (about 10 mm) with well-defined edges. The restricted activity in deferred antagonism tests of the latter strains against coagulase-positive staphylococci was also shown by Barrow (1963*a*).

Inhibition of the indicator strains did not take place in tests designed to show direct antagonism (Barrow, 1963*a*) although antibiotic material was present in the medium after only 4 h growth of the 2 active strains.

No inhibition of the growth of 26 coagulase-negative staphylococci, including A5 and A12, occurred when tested against strains P1–15 acting as the 'active strains' in the colicine technique.

Inhibition of corynebacteria and other organisms. The activity of A5 and A12

against the various corynebacterium strains was tested by the colicine method and comparison made with the 2 strains known to be inhibitory (NCTC 6507 and 8004). The results are shown in Table 2.

Table 1. *The activities of coagulase-negative staphylococcal strains A* 5 *and A* 12 *compared with* Staph. aureus *strains NCTC* 6507 *and* 8004 *against pathogenic staphylococcal strains P* 1–15 *when tested by the colicine method*

(+ = inhibition; ± = slight inhibition; − = no inhibition.)

Active strains	Indicator strains														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A 5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A 12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NCTC 6507	±	±	±	−	−	−	+	−	±	+	+	±	−	±	+
NCTC 8004	−	−	±	−	−	−	±	−	+	+	+	+	−	+	+

Table 2. *Comparison of the inhibition of corynebacteria by coagulase-negative staphylococcal strains A* 5 *and A* 12 *with NCTC* 6507 *and* 8004 *employing the colicine method*

(+ = inhibition; ± = slight inhibition; − = no inhibition.)

Active strains	Corynebacteria							
	C. hoffmanii (NCTC)			*C. zerosis* (NCTC)			Others	
	231	8633	8634	7883	7929	9755	BDF	MC
A 5	−	±	−	+	+	±	−	±
A 12	−	±	−	+	+	±	−	±
NCTC 6507	+	+	+	+	+	+	±	+
NCTC 8004	+	+	+	+	+	+	+	+

Only 2 strains of *C. xerosis*, NCTC 7883 and 7929, were inhibited by A 5 and A 12 to any marked degree but in contrast to the inhibitory effect on staphylococci the zones were narrow (10–15 mm in diam.)

On the other hand, the 2 NCTC *Staph. aureus* strains had a wide range of activity in keeping with their accepted character.

No inhibition of streptococci, coliform or *C. pyogenes* strains occurred.

Factors influencing the inhibitory action of active strains

Medium. Casamino-acid–yeast extract agar medium without the addition of blood supported the production of only small amounts of antibiotic. The addition of 10 % (v/v) whole citrated ox blood increased production noticeably but the addition of serum did not. On the other hand, 10 % (v/v) plasma increased antibiotic production to a level comparable to that produced on whole blood-agar medium.

Oxygen tension. The active strains A 5 and A 12 were grown on blood-agar medium under varying oxygen tensions and the degree of inhibition against the indicator strains P 1–15 was tested by incubating under the same oxygen tensions as used for the initial growth of A 5 and A 12. It was found that antibiotic was produced when the gaseous environment ranged from 0 to 20 % (v/v) oxygen though the amount was decreased at the lower oxygen concentrations. The indicator strains were also sensitive

to antibiotic under all the oxygen tensions tested but their sensitivity decreased at the lower oxygen concentrations.

Effect of prolonged incubation on antibiotic production. Using the spot technique, A 5 and A 12 were allowed to produce antibiotic on blood and plain agar media for 1–7 days. The diameter of the spot of growth was measured and the plates treated as previously described. The sensitive strain P 3 was then sown over the plate and after the development of the inhibition zone the diameter was measured and compared as a ratio to that of the growth diameter.

On blood agar the inhibition zone after one day was 36 mm in diam. (ratio 1: 10) and populated with colonies of resistant forms. The zone of inhibition increased up to the 3rd day when it covered the surface of the plate. From the 3rd to 7th days the only indication of continued antibiotic production was the gradual disappearance of the resistant colonies.

After 1 day on plain agar the ratio of the growth diameter to the zone of inhibition was only 1: 5 and was demarcated into a clear inner zone surrounded by zones which were increasingly populated by greater numbers of organisms (Plate 1).

Preparation and properties of the antibiotic

Attempts to demonstrate the presence of the inhibitory substance in different liquid media under varying conditions showed only minimal amounts to be present. It was, therefore, decided to extract the substance from agar plates prepared from Casamino-acid–yeast extract medium without the addition of blood.

Strain A 12 was grown as a lawn on the plates and, after removal of the growth and treatment with chloroform, the agar was cut into slices and frozen at $-20\,^{\circ}C$. The agar slices were thawed slowly at $4\,^{\circ}C$ and the liquid expressed by squeezing in a muslin bag. The liquid was then concentrated approximately threefold by freeze drying. This extract was called the crude antibiotic extract.

The ditch plate method with P 3 as the indicator organism was used for assay; the plates were incubated for 18 h at $37\,^{\circ}C$, after which the zones of inhibition were measured.

Dialysis. The crude antibiotic material diffused rapidly through Visking dialysis tubing when dialysed against distilled water and was found in maximum concentration in the distilled water within 24 h.

Enzyme digestion. At a concentration of 50 mg % (w/v) both pepsin and trypsin acting at pH 2·0 and 8·0, respectively, on the crude antibiotic material, for up to 6 h at $37\,^{\circ}C$, did not reduce the activity of the inhibitor. Pepsin, even after being heat denatured, altered some property of the antibiotic, causing a double inhibition zone to appear on the assay plate.

Heat sensitivity. No loss in activity occurred after heating at temperatures ranging from 45 to $100\,^{\circ}C$ for as long as 30 min.

Antibiotic activity in mixed broth culture. In order to determine if there was any release of inhibitory agent from an active strain in liquid medium, and what effect this would have on the growth of a sensitive strain, A 12 and P 3 were grown together in mixed culture in a Casamino acid–yeast extract broth. The number of organisms present in the medium was determined by the method of Miles & Misra (1938).

The results are shown in Fig. 1, which displays typical growth curves of the 2

strains grown separately and together for 24 h at 37 °C. In mixed culture, both organisms grew normally during the early logarithmic phase but at 3 h the numbers of A 12 fell probably as a result of competition from P 3. Later, some degree of autolysis of A 12 is thought to have taken place with the release of antibiotic from the dead cells. The released antibiotic caused a reduction in the numbers of P 3 which resulted in less competition and therefore increased growth of A 12. Regeneration of A 12 at this stage reduced the numbers of autolysing cells and of the amount of antibiotic released and this, together with the growth of resistant forms of P 3, resulted in the numbers of P 3 increasing and of A 12 decreasing.

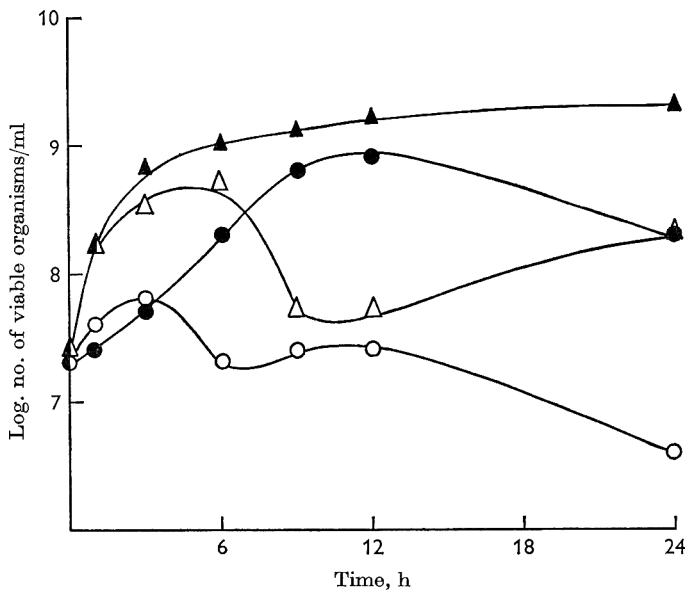

Fig. 1. Growth curves of active staphylococcus strain A 12 alone (●), and of indicator strain P 3 alone (▲); also of A 12 (○) and P 3 (△) when grown together in broth culture.

The use of mixed cultures in which A 12 was initially 100-fold in excess of P 3 resulted in the apparent elimination of P 3 from the culture medium in the early stages of growth. P 3 was again detectable at 7 h but remained at a low and suppressed level for up to 24 h.

DISCUSSION

By deferred antagonism tests it was found that 2 strains of coagulase-negative staphylococci, representative of those isolated from milk samples and other sites on the bovine udder, were capable of suppressing the growth of a wide variety of staphylococci responsible for bovine mastitis by the production of an antibiotic. On the other hand, corynebacteria, which have usually been found sensitive to the action of other staphylococci possessing inhibitory properties such as those belonging to phage type 71 and strains NCTC 6507 and 8004, were relatively resistant.

While the micrococcus described by Loeb *et al.* (1950) was similarly of low activity against corynebacteria, it was found to exhibit inhibition against streptococci. No

inhibition of streptococci by either of the 2 coagulase-negative staphylococci was observed.

Further distinction between the substance which was isolated from the present strains and those described previously is its resistance to proteolytic enzymes and to heat treatment. The substance has a relatively small molecular weight since it diffuses rapidly through dialysis membrane and differs in this respect from the substance described by Gardner (1949), Loeb *et al.* (1950) and by Barrow (1963*b*). A characteristic of the present substance is the readiness with which it is produced in solid but not in liquid media.

In previous work described by Edwards & Jones (1966) it was found that coagulase-negative staphylococci were better suited for 'colonization' of the bovine teat duct than coagulase-positive strains, and it was also shown that 23 % of the coagulase-negative types examined elaborated an inhibitory substance *in vitro* against pathogenic staphylococci. It was suggested that antibiotic production may be of some advantage in assisting these strains to compete against staphylococci pathogenic to the udder in the colonization of the teat duct. However, since some enhancing factor present in blood plasma but not in serum was required to promote its formation *in vitro* it is unlikely that sufficient antibiotic material would be produced in an environment such as the teat duct where reduced oxygen tensions would also lower the amount of antibiotic elaborated.

The explanation suggested in the previous paper that the apparent antagonism between 2 types could be due to their relative sensitivity to the non-esterified fatty acid fraction of the bovine teat duct material is, therefore, more tenable.

It would be of interest, however, to determine whether under strictly controlled conditions the presence of antibiotic-producing strains of coagulase-negative staphylococci in the teat duct could reduce the rate of infection or degree of mastitis in quarters subjected to experimental infection with pathogenic staphylococci.

We wish to thank Dr G. I. Barrow for providing corynebacterium strains BFD and MC, and Mr F. H. Summerfield, A.R.P.S., for preparing the photograph.

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EXPLANATION OF PLATE

The inhibitory effect of antibiotic from an active strain of coagulase-negative staphylococcus grown on plain agar. The plate shows a typical zoning effect against a sensitive staphylococcal strain when low concentrations of antibiotic are produced.

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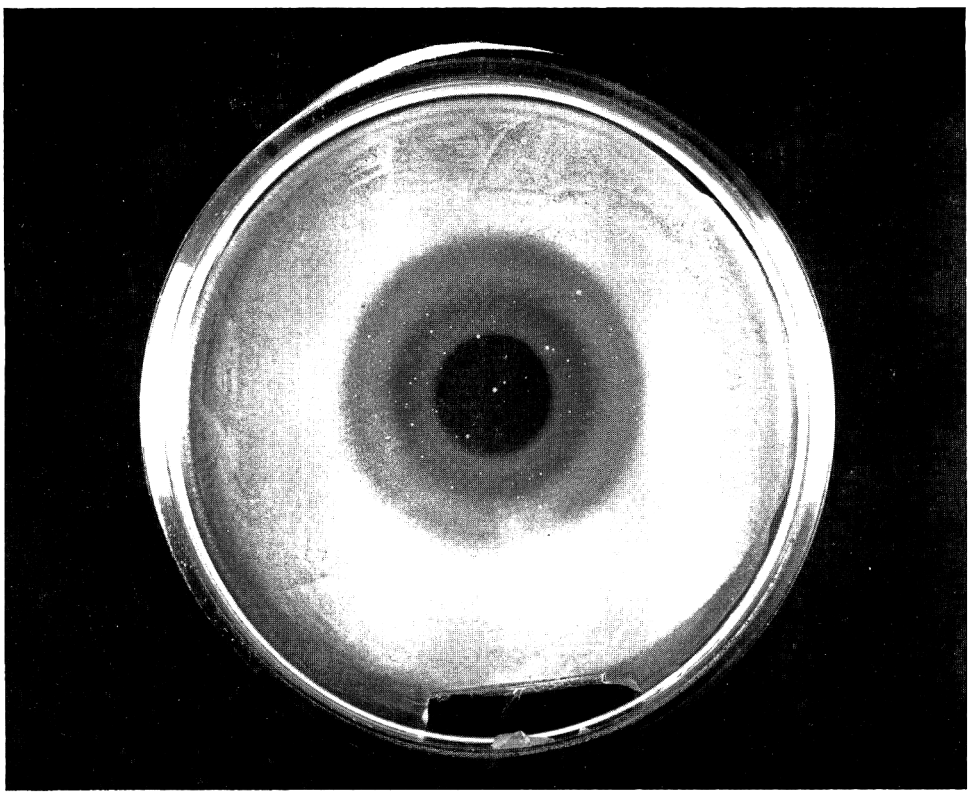

J. Dairy Res. (1966), **33**, 277
With 2 plates

Partition of casein between polymer phases

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Summary. Casein derived from the micelles of milk by removing the calcium has been subjected to partition and counter current distribution between phases containing polyethylene glycol and dextran. The results show that at least 2 kinds of casein complex exist which differ in structure, in size or in both. There are also differences in composition with respect to minor components, though both types contain predominantly α- and β-casein.

The casein–calcium–phosphate micelle of milk was shown to be heterogeneous by electron microscopy after sectioning by Shimmin & Hill (1964) and there have been indications that the surface of the micelle is different from its interior (Sullivan, Fitzpatrick & Stanton, 1959; McGann & Pyne, 1960; Noble & Waugh, 1965; Waugh & Noble, 1965). Waugh & Noble found that the artificial micelles formed by the associations between α- and κ-caseins in the presence of calcium ions could not be represented as products of an equilibrium, but that the results depended on the route by which the final conditions were reached. We have now found the same to apply to associations occurring in the absence of calcium; the casein complexes persisting after removing the calcium (including calcium phosphate) from the micelles of milk are different from those which are formed after the usual methods of precipitation and re-solution. The evidence has been obtained from experiments using partition and counter current distribution. For the examination of complexes it was necessary to avoid solvents which cause disaggregation. Those containing phenol, which were used by Walter (1952) and Signer (1953) to separate α- and β-caseins could not, therefore, be applied to this problem and two of the milder systems described by Albertsson (1960) were investigated. Albertsson showed that pairs of aqueous solutions which had been rendered immiscible with one another by the presence of polymers such as polyethylene glycol and dextran, could be used for separating large molecules, cell fragments, and even cells themselves with the minimum risk of alteration.

The work was started with the idea of finding out whether useful separations of casein fractions could be achieved with these polymer phases. Preliminary orientating experiments were necessary before undertaking counter current distributions and the more important of these are described to give a general impression of how casein behaves in the phases used. The partition coefficients are very sensitive to minor changes and the factors controlling the partition coefficient have not all been disentangled. For this reason, it was never possible to forecast exactly what the partition coefficient of a new casein preparation would be, but it was usually possible to adjust

it to the required value by suitable changes in conditions, e.g. in salt concentration. Then, for this preparation consistent results followed when experiments were carried out within a few days.

Of the 2 systems used in this work, one did produce a partial separation of α- and β-casein, but individual caseins were not observed in any of the fractions separated by the second system.

MATERIALS

Casein solutions

All casein solutions were preparations of first cycle soluble casein, i.e. they were prepared from centrifuged micelles by sequestering the calcium and dialysing as practised by Waugh & von Hippel (1956). Preparations in which the micelles have been rendered soluble by removing calcium as oxalate, that is according to the original method have been designated as casein (via oxalate). The following variations in the method of removing calcium were used. The centrifuged micelles were resuspended by stirring in distilled water equal in volume to approximately half the volume of milk. In the first variation the micelles from 600 ml milk were suspended in 250 ml water and treated with 100 ml (wet volume) of the cation exchange resin Amberlite IR 120 (Na^+). The mixture was continuously stirred and the pH was kept at $7{\cdot}0 \pm 0{\cdot}5$ by titration with N-HCl. The micelles dispersed in about 15 min but the treatment was usually continued for some further time (about 1 h) before decanting the casein solution from the resin. The products made by this process have been designated casein (via resin). In the second variation the calcium compounds were rendered soluble by adding to the micelle suspension an excess of diaminoethanetetra-acetic acid disodium salt (EDTA), and caustic soda solution to keep the pH at $7{\cdot}0 \pm 0{\cdot}5$. The EDTA was at least equivalent to all the calcium in the milk (including the added $CaCl_2$) before centrifuging. As some of the calcium remained in the supernatant the EDTA was more than equivalent to the calcium in the micelles. Casein preparations made in this way are designated casein (via EDTA). The different methods are thought to give the same result but the designations are given in case later work should show the preparations to be different.

Before use the pH, NaCl and buffer concentration of each casein solution was adjusted by dialysis at 3 °C against 100–200 vol. of buffer or buffer + NaCl solution, and if necessary by the addition of extra salt. No bacteriostatic agent was used. The casein solutions were kept at 3 °C and discarded after 2 weeks to avoid using material in which bacterial or enzymic degradation might influence the results. Most of the experiments were done with preparations less than 1 week old.

Dextran

A 20 % (w/v) aqueous solution of Dextran 500 (molecular weight by light scattering = 500000; Pharmacia, Uppsala, Sweden) was used.

Polyethylene glycol

Fifty per cent (w/v) solutions of polyethylene glycol 1500 (PEG 1500) and Polyethylene glycol 4000 (PEG 4000) (molecular weight, 1380–1500 and 3140–4020, respectively; British Drug Houses, Ltd.) were prepared.

Phosphate buffer

A 0·1 M stock solution was prepared, containing 0·05 M-KH_2PO_4 and 0·05 M-K_2HPO_4.

Phases for partition

These were prepared as follows for small experiments and in larger quantities as required. In each case the upper phase was richer in the polyethylene glycol.

(1) *PEG 1500/ammonium sulphate system.* 15 ml 50 % (w/v) aqueous solution of PEG 1500 was mixed with 9 ml 4 M-$(NH_4)_2SO_4$ and 12 ml H_2O and the pH adjusted to the required value with NH_4OH.

(2) *PEG 4000/ammonium sulphate system.* 17 ml 50 % (w/v) aqueous solution of PEG 4000 was mixed with 12 ml 4 M-$(NH_4)_2SO_4$ and 15 ml H_2O and the pH adjusted to the required value with NH_4OH.

(3) *PEG 4000/Dextran system.* This was prepared as described by Albertsson (1960). It had the following composition: 8 ml 20 % (w/v) aqueous solution of Dextran 500; 3 ml 50 % (w/v) PEG 4000; 11 ml H_2O or buffer.

When casein was to be partitioned in one of the systems, a volume of water, equal to the volume of casein solution to be added, was omitted from the system. The same procedure was used for addition of phosphate buffer and sodium chloride.

Preparation of α-, β- and κ-caseins

(1) *α- and β-caseins.* The method of Hipp, Groves, Custer & McMeekin (1952) was used and both fractions were purified by precipitation with acid from 8 M urea (final pH 4·7). Electrophoresis showed α-casein to contain about 10 % β-casein, and β-casein to contain 1–2 % of α- and κ-caseins.

(2) *κ-casein.* The method of Zittle (1962) was used, followed by fractional precipitation with alcohol. The only impurity detected on electrophoresis was a trace of α-casein.

METHODS

Casein determination

The protein concentration was measured by determining the optical density of a suitably diluted sample at a wavelength of 275 mμm. Although α- and κ-caseins contain more aromatic amino acids than β-casein, no corrections were applied because at first, only approximate results were sought and later, during the work with PEG/Dextran system, the relative concentration of α-casein remained approximately constant as may be seen from the results for counter current distribution. When casein was being determined in one of the polymer phases the samples were diluted with water, at least fivefold, usually tenfold. It was always necessary to determine

and subtract the absorption due to the polymers in each phase as the figure varied according to the purity of the polymers.

The extinction was related to total protein as given by micro-kjeldahl nitrogen and confirmed by dry weight. This was done for one preparation only as precise values were not necessary (see Table 1).

Partition coefficients

Mixtures containing the appropriate amounts of polymer solutions, casein solution and water or salt solutions were made at the temperature of the experiment. The mixtures were stirred by gentle inversion for at least 30 sec and then centrifuged. Samples of the 2 phases were then removed and the casein determined. The partition coefficients were calculated from ratios of the optical densities without attempting to relate these to actual concentrations of protein. The concentration of casein was chosen to give a convenient value of optical density. It was usually in the range 0·3–2·0 % in the total mixture of the 2 phases. There was always a residue of undissolved material in the sulphate systems but not in the Dextran system. Under constant conditions (salt concentration, pH, etc.) partition coefficients of any one preparation appeared to be reproducible to within ± 10 %. The results in Tables 1 and 3 confirm this since it is improbable that the changes in time in Table 1 and in concentration in Table 3 would exactly balance the effect of random errors.

Counter current distributions

Initial casein concentrations of about 4 % were used in the PEG 4000/Dextran system with 0·009 M-phosphate and 0·004 M-NaCl. The distributions were effected in 12 centrifuge tubes using the scheme of Bush & Densen (1948) which is well adapted to distributions in which the number of transfers is small. The volume of each phase in each tube was 40 ml. After being mixed by gentle inversion for 30 sec the phases were allowed to stand for at least 5 min to attain equilibrium. During this time no obvious separation occurred and the phases were then centrifuged at 500–1000 ***g*** for 15–20 min. Except for the actual transfers, which were made quickly, all operations were carried out in the cold room at 3–4 °C. The centrifuge was sufficiently well ventilated to prevent a rise in temperature. At the end of the distribution and after the samples for optical density determination had been taken, the casein in each tube was precipitated at pH 4·7 and submitted to electrophoresis.

Polyacrylamide gel electrophoresis

The method was essentially that of Raymond & Wang (1960). However, 7·5 % acrylamide was used and the thickness of the gel layer was restricted to 0·75 mm. The buffer for the gel was 0·05 M 2-amino-2-hydroxymethyl-propan-1:3 diol, adjusted to pH 8·6 with citric acid and containing 5·5 M urea. Electrophoresis was continued for 6 h at 2 °C with a current of 20 mA. Samples were precipitated at pH 4·7 and redissolved in buffer of the same composition as that used for the gel except that 0·3 % (v/v) of β-mercaptoethanol was added (Woychik, 1964). Dialysis against the same buffer was used if necessary.

RESULTS

Effect of polymers on the absorption spectrum of the protein

The presence of a solute in relatively high concentration may affect the conformation of protein molecules and so produce a change in the absorpion spectrum (Herskovits, 1965). It was, therefore, necessary to find out whether the polymers would cause any such change. These possibilities were investigated over the wavelength range of 230–300 mμm using casein dissolved in solutions of polyethylene glycol (12 %) and of dextran (20 %), i.e. at approximately the concentrations present in the phases of the Dextran system. The absorptions determined were the differences between the protein solution and water or the polymer solutions used as solvents. The wavelengths corresponding to the troughs and the peaks in the spectra, i.e. λ = 249 and 275 mμm were not obviously changed but the optical density at all wavelengths increased in the presence of polyethylene glycol. The increase at 275 mμm amounted to 18 % and it increased by a further 3 % on overnight storage.

The measurements were carried out in solutions of relatively concentrated polymers. In order to decide whether the increases found would persist in diluted solutions, an experiment was done with uniform quantities of casein dissolved in polymer solutions of the same composition as before, and in water. The solutions were sampled immediately and after 3 and 64 h (at room temperature and 3 °C) since the previous results indicated some increase in optical density with time. At the time of sampling the samples were diluted tenfold and the optical densities determined immediately. The maximum increase in optical density was observed after 64 h irrespective of temperature. It was not more than 3 %. As the changes and differences observed in most of the experiments to follow were much greater than this, the results from them cannot be attributed to interference by the polymers in the protein determination.

The system polyethylene glycol-ammonium sulphate

Time to reach equilibrium

A solution of casein (via oxalate) in a mixture of the 2 phases (PEG 4000) at pH 7 and 22 °C was kept gently mixed by stirring. Samples were taken at intervals from 0·5 to 8 min and the phases separated immediately by centrifuging. The results are in Table 1. It seems probable that the second result is erroneous. Apart from this, the coefficient diminishes with time but the changes are small compared with those observed in some of the following experiments. Near equilibrium is reached quickly.

Repeated extraction

The partition coefficient of a casein (via oxalate) preparation was determined at pH 8. Samples of upper and lower layers were then re-equilibrated with fresh opposite phases and the new partition coefficients determined. The results of 2 experiments are shown in Table 2. About 50 % of the material remained undissolved after the first partition. When, after expt. 2, the precipitate was added to a fresh sample of mixed phases, part of it dissolved giving a partition coefficient of about 0·2. The residue was again treated in the same way. It dissolved in the lower phase only (partition co-

efficient = 0). A similar result was obtained when the experiment was repeated with a third casein preparation.

A casein (via oxalate) solution was distributed between the 2 phases as for the determination of the partition coefficient and then each phase was washed 3 times with equal volumes of fresh opposite phase. The partition coefficients at the last

Table 1. *The equilibration time necessary for reproducible partition coefficients of casein in PEG-$(NH_4)_2SO_4$ system*

Time of mixing before sampling, min	Net optical density at λ = 275 mμm of diluted (× 10) sample		Partition coefficient
	Upper phase	Lower phase	
0·5	0·083	0·289	0·29
1	0·073	0·234	0·31
2	0·077	0·275	0·28
4	0·060	0·235	0·26
8	0·062	0·244	0·25

The casein concentration in the upper phase was about 0·1 % (w/v) and in the lower phase 0·3 % ($E^{1\%}_{1cm}$ = 8·7).

Table 2. *Changes in partition coefficient of casein in PEG-$(NH_4)_2SO_4$ system on repeated extraction*

Sample	Partition coefficients	
	Expt. 1	Expt. 2
Casein (via oxalate), preparation 1	0·28	—
Casein (via oxalate), preparation 2	—	0·16
Upper layer extracted with fresh lower layer	1·6	1·6
Lower layer extracted with fresh upper layer	0·12	0·14

washings were 5·37 and 0·047. The casein was recovered from the washed upper and washed lower phases by precipitation at pH 4·7 and submitted to gel electrophoresis. Plate 1 shows that the concentration of α-casein and fat-moving components was relatively high compared with β-casein in the material isolated from the upper layer, whereas that from the lower layer shows more β- than α-casein.

A similar result was obtained whether polyethylene glycol 4000 or 1500 was used.

The system polyethylene glycol-dextran

The problem of equilibrium between aggregates

If the components of a mixture are in equilibrium with one another the result of attempts to separate them will depend on the speed with which equilibrium is re-established. For this reason, experiments were done to determine whether the partition coefficients of separated fractions changed significantly during periods of time commensurate with, but longer than, those required for a short counter current distribution.

From a freshly made dialysed solution of casein (via EDTA) having a partition coefficient of 0·66 two fractions were obtained by partition followed by washing each phase twice with an equal volume of opposite phase. At the final washing the values of the partition coefficients were 7·5 and 0·36, representing 30 and 42 %, respectively,

of the original casein. Both fractions were allowed to stand in both phases for 64 h at 3 °C. The 2 phases of each fraction were then remixed, separated and the new partition coefficients were determined. That of the first sample had changed from 7·5 to 6·1 and that of the second from 0·36 to 0·22. Neither of these small changes suggests that a mixture like the original solution is being re-formed sufficiently rapidly to interfere with experiments which are completed in 2–3 days.

The effect of protein concentration

Casein (via resin) was dialysed against 0·025 M-phosphate buffer and samples of different concentration with respect to casein were made by diluting this solution with the same buffer. Distribution coefficients were then determined at 3 °C. The volumes of casein solution and diluting buffer in 22 ml of final mixture are shown in Table 3, together with the resulting partition coefficients. Within the limits of accuracy to be expected and in the range covered, the partition coefficient is independent of protein concentration.

Table 3. *Effect of protein concentration on partition coefficient of casein in PEG-dextran system**

Vol. of casein solution (M/40 in phosphate) (ml)	Vol. M/40 phosphate buffer (ml)	Final casein concentration (%, w/v)	Partition coefficient
1	7	0·104	1·15
2	6	0·208	1·18
4	4	0·416	1·11
6	2	0·621	1·18
8	0	0·832	1·20

* The final mixture in each case had the same basic composition as the system described in Methods and was 0·02 M with respect to NaCl and 0·009 M with respect to phosphate (pH 6·8).

Effect of sodium chloride concentration

This was determined in an experiment similar to that above, but with a different casein preparation and with adjustment of the sodium chloride concentration by adding the solid. The results are in Table 4 (expt. 3). Also included are some figures from other experiments in which the concentration of casein varied as well as the concentration of sodium chloride. While it is clear that different casein preparations give different partition coefficients, in each case the coefficient increases as the concentration of salt decreases. Above 0·2 M-sodium chloride much of the casein was precipitated.

Effect of other salts

Another casein (via resin) preparation was used to compare the effect of sodium chloride and potassium chloride at 2 levels on the partition coefficient (Table 5). The potassium chloride gives a lower partition coefficient in each case.

The effects of $CaCl_2$ and $MgCl_2$ on the partition coefficients were determined using the same casein solution and the phase system already containing 0·01 M-sodium chloride and phosphate buffer (see Table 5). The absence of a large effect is noteworthy.

Effect of precipitation

As already noted under 'Repeated Extraction' the washing of an upper phase containing casein with fresh lower phase removes material with a low partition coefficient leaving in the upper phase a fraction with a high partition coefficient. If this material was precipitated and then dialysed to bring the concentration of salts

Table 4. *Effect of sodium chloride on partition coefficient of casein in PEG-dextran system*

Final concentration of NaCl in phase system (molar)	Partition coefficients*		
	Expt. 1† (casein via oxalate)	Expt. 2† (casein via oxalate)	Expt. 3 (casein via resin)
0·10	—	0·10	*P* 0·08‡
0·04	0·49	0·33	*IL* 0·34§
0·03	1·32	—	—
0·02	3·45	—	1·94
0·01	5·25	—	6·05

* In all cases the phosphate buffer concentration was 0·009 M. The casein concentrations varied within the range 0·1–1·0 %.

† Phase system with 2 ml less water; total volume 20 ml.

‡ *P* = Precipitate.

§ *IL* = Interfacial layer.

Table 5. *Effect of various salts on the partition coefficient of casein* in PEG-dextran system*

Salt	Concentration (molar)	*K*
NaCl	0·03	0·29
NaCl	0·01	1·24
KCl	0·03	0·08
KCl	0·01	0·95
NaCl	0·01 (repeat)	0·72
$CaCl_2$	0·001 + NaCl 0·01	0·86
$CaCl_2$	0·002 + NaCl 0·01	0·61
$CaCl_2$	0·01 + NaCl 0·01	(1·2 precipitate)
NaCl	0·01 (repeat)	0·75
$MgCl_2$	0·002 + NaCl 0·01	0·63

* Casein concentration 1 % (w/v), phosphate concentration 0·009 M.

back to the level used for the partition experiments, and then again subjected to partition, its partition coefficient was now found to be greatly different. The partition coefficient was also changed when casein from a washed lower layer was precipitated and subjected to partition a second time. The results of several experiments carried out in this way are summarized in Table 6. It is clear that precipitation and re-solution alter the partition coefficient.

The partition coefficients of casein from the milk of individual cows

Four samples of milk were collected and first cycle soluble casein (via resin) prepared. The partition coefficients determined, in the presence of 0·01 M-NaCl gave the values 1·08, 1·11, 0·96 and 1·25.

Partition coefficients of separated casein fractions

The partition coefficients of α-, β- and κ-caseins were determined in the presence of different concentrations of sodium chloride, The results are shown in Table 7.

Table 6. *Change in partition coefficient of casein after partitioning, precipitating and repartitioning in PEG-dextran system*

Casein preparation no.	Precipitated by	Partition coefficients: Casein from washed upper phase, Before precipitation	Casein from washed upper phase, After re-solution	Casein from washed lower phase, Before precipitation	Casein from washed lower phase, After re-solution
27/7	$(NH_4)_2SO_4$, saturated	—	—	0·05	0·23
7/9	$(NH_4)_2SO_4$, saturated	6·1	0·31	0·22	0·31
15/9	HCl (final pH 4·6)	12·6	2·0	0·37	0·13
15/9	$CaCl_2$ (pH 7·0)	10·0	1·4	0·39	0·17
15/9	$(NH_4)_2SO_4$, saturated	3·2	2·0	0·48	0·03
15/9	repeat HCL (final pH 4·6)	3·2	17·0	0·48	0·17
15/9	$CaCl_2$ (pH 7·0)	3·2	—	0·48	0·21

Table 7. *Partition coefficients of casein fractions in PEG-dextran system*

	0·1 M-NaCl	0·01 M-NaCl	0·004 M-NaCl
α-Casein	0·028	0·40	0·78
β-Casein	0·12	6·1	12·0
κ-Casein	5·6	75·0	226·0

Phosphate concentration 0·009 M throughout.

Table 8. *Counter current distribution of casein (preparation 2) in PEG-dextran system*

Tube no.	Theoretical*	Experimental	Tube no.	Theoretical	Experimental
1	59	57	7	40	40
2	47	38	8	52	47
3	37	44	9	73	72
4	36	34	10	117	113
5	35	30	11	203	205
6	33	30	12	270	289

* Calculated for 4 components with K values of 0·11, 0·82, 2·3 and 9·0 as in Fig. 1 but in the proportions of 100:200:240:450.

Each unit represents 0·001 of the total casein.

Counter current distribution

The results of a typical experiment are shown in Fig. 1 and Plates 2 and 3. It is clear that aggregates with different partition coefficients were, at least partially, separated. Those with the lower partition coefficients did not differ sufficiently from one another in composition for the differences to be obvious on gel electrophoresis. In contrast, the last 2 fractions of high partition coefficient, did contain enough κ-casein for this to be visible in the electrophoresis gel. Electrophoresis after the action of a minimal concentration of rennin revealed strong bands of para-κ-casein moving towards the cathode in fractions 11 and 12. A weaker band was present in

fraction 10. Mere traces could be detected in most of the other fractions (Plate 3). A similar experiment with a different casein preparation gave a similar result, the separable materials merely being present in different proportions. This is shown in Table 8.

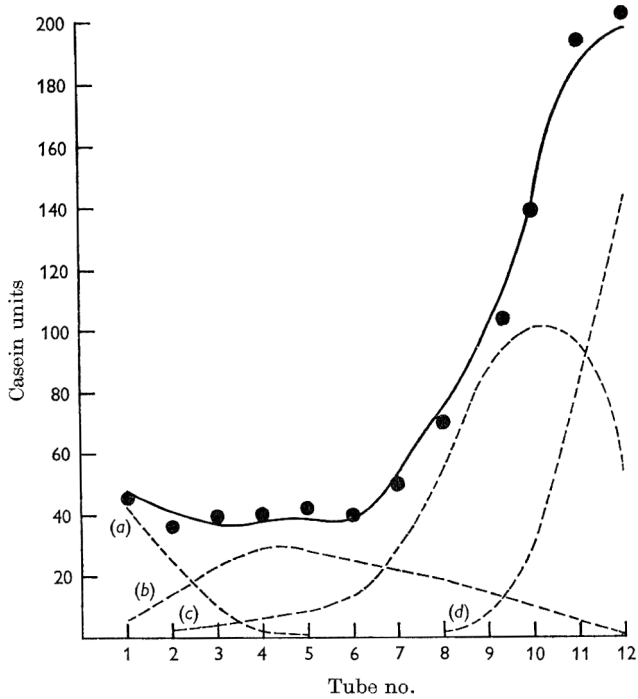

Fig. 1. Counter current distribution of casein, preparation 1. ●. Experimental points. – – – –, calculated distributions for hypothetical single components of a mixture present in the ratios 80:200:450:270. (*a*) 80 units with $p = 0{\cdot}9$ ($K = 0{\cdot}11$); (*b*) 200 units with $p = 0{\cdot}55$ ($K = 0{\cdot}82$); (*c*) 450 units with $p = 0{\cdot}3$ ($K = 2{\cdot}3$); and (*d*) 270 units with $p = 0{\cdot}1$ ($K = 9{\cdot}0$), where p = proportion of solute remaining in lower layer and K = partition coefficient (Bush & Densen, 1948). ———, total distribution for a mixture of the 4 components in the proportions given. Each unit represents 0·001 of the total casein.

DISCUSSION

Although the polyethylene glycol–ammonium sulphate system was interesting for the separation of minor components which move fast during electrophoresis, it was not used a great deal because of the incomplete solubility of casein in the system.

The increase in optical density of casein solutions in polyethylene glycol compared with those in water or dextran may be due, at least in part to an increased scattering of the light. This is suggested by the slightly opalescent appearance of the solutions and by the increase in absolute difference in optical density as the wavelength decreases. The magnitude of the scattering and its several possible causes have yet to be investigated.

The speed with which the phases and protein reach equilibrium was at first surprising but it is probably due to the very fine dispersion of one phase in the other on mixing. This in turn is thought to be due to the low interfacial tension resulting from the similarity of the phases.

In Tables 3–5 the partition coefficients for a given level of sodium chloride concentration do not agree. This is the result of using different casein preparations since in any one preparation repeated determinations of the partition coefficient usually agree to within $\pm 10\%$ providing too long an interval of time has not elapsed. This, however, does not apply to the discrepancy in Table 5 between the first value for NaCl 0·01 M and the other two. No explanation of this discrepancy can be offered.

The values for the partition coefficient of β-casein given in Table 7 show why it was not separated in the polyethylene glycol–dextran system, although it has been consistently observed in first cycle casein in the ultracentrifuge at low temperature. Obviously, however, much of it remained associated with other caseins during the distribution experiments.

The problem of equilibrium between aggregates

Casein exists in ordinary solutions in the form of aggregates (Warner, 1944; Nitschman & Zürcher, 1950) and there is no reason to suppose that these will not also be present in the polymer phases. If the aggregates are in equilibrium with one another or with monomers, and if some have different partition coefficients from others, the behaviour on repeated extraction or counter current distribution will depend on the speed with which equilibrium is established. Repeated extraction was used in the earlier experiments in place of counter current distribution to demonstrate that aggregates with different properties existed and could be separated. This showed that equilibrium between aggregates was not established rapidly. If the equilibrium is established slowly the partition coefficient of a separated fraction would be expected to revert towards that of the original sample. Apart from small changes this did not occur when 2 fractions were stored in both phases for 64 h (7·5→ 6·1; 0·38→ 0·22; original value 0·66) and it appears that one type of aggregate did not change readily into another under the conditions prevailing during separation, and therefore that if there was an equilibrium it was approached very slowly indeed. Payens & van Markwijk (1963) have shown that this is true for β-casein monomers and polymers.

Factors which might be expected to affect the partition coefficient

These are (1) size, (2) composition, and (3) structure.

Size

Albertsson (1960) quotes and confirms the equation of Brønsted (1931) relating the partition coefficient K to the size of the molecules of the solute. The equation is

$$K = e^{M\lambda/kT},$$

where M is the molecular weight, k the Boltzmann constant and λ a characteristic of the solute and the phase system. It follows that K will be above or below 1 according to the sign of λ. Thus, an increase in M will increase the relative amount of solute in one of the phases but it will not cause the bulk of the solute to change from one phase to another. Solutes which favour different phases must, therefore, differ in properties other than size.

Albertsson's derivation of the formula assumes spherical particles of uniform surface. Brønsted considers only groups or pairs of 'isochemical' substances. Since it

is possible that casein aggregates do not fall into either category it is difficult to know how far the formula applies, but some spread of the partition coefficients above or below 1 will occur if there are aggregates which differ only in size.

Composition

When the separation of aggregates was achieved by counter current distribution the differences in composition, as shown by electrophoresis, appeared more clearly than after simple partition experiments. The samples remaining in the lower phase contained detectable amounts of material travelling between the main bands of α- and β-caseins, but nearer α-casein as may be seen from Plate 2. There was also in this group a little more of the fractions moving faster than α-casein. Fractions 11 and 12 (Table 8) which had partition coefficients of about 9 contained κ-casein and a repetition of electrophoresis after the action of rennin showed that samples up to fraction 9 contained only traces of κ-casein. It is, therefore, worth while to consider the possibility that differences in composition may account for the different partition coefficients. One way of doing this is to suppose that one of the assumed constituents (Fig. 1) is in reality an unresolved mixture. For example, we have assumed that tube 12 contained mostly a component with a K of 9. We may, on the contrary, assume that it is a mixture of the main component assumed in (say) fraction 9 with a K of 2·3 (i.e. component (*c*) of Fig. 1) and the extra component, κ-casein. Even if the latter component has a K of ∞ it must be present as [illegible] % of this fraction in order to produce an average K of 9. The same argument would apply if the partition coefficient of an aggregate were a simple function of its composition. As the proportion of κ-casein is $\ll$ [illegible] % it is clear that the differences observed after counter current distribution are not to be accounted for merely by the composition as revealed by electrophoresis. The possibility that aggregates may differ by containing molecules of different solubility but identical mobility must not be dismissed. Further work is required to assess this factor.

Structure

If one or other of the components demonstrated electrophoretically were concentrated on the surface of the aggregate it would be expected to exert a larger effect than other components on the partition coefficient. This might explain the large effect of relatively small amounts of κ-casein on the partition coefficients. That structure could be responsible is indicated in the results for precipitation and resolution of separated fractions (Table 6). The partition coefficients were drastically changed, and not always in the same direction. The process of reformation of aggregates was obviously highly sensitive to procedures which were not standardized.

Waugh & von Hippel (1956) have shown that acid precipitation produces aggregates which are not dispersed again in ordinary aqueous solution except at very high pH values. Other methods of precipitation might have a similar effect, although the same authors used calcium chloride to avoid causing irreversible aggregation.

Sullivan *et al.* (1959) demonstrated a rectilinear relation between the logarithm of the diameter of the micelles in skim-milk and the sialic acid content (approximately equivalent to the amount of κ-casein). This strongly suggested that κ-casein was on the surface as was also assumed by McGann & Pyne (1960) who found a higher

proportion of κ-casein in the smaller micelles. Waugh & Noble (1965) assumed a similar arrangement in order to explain the behaviour of artificially formed micelles of α- and κ-caseins in the presence of varying amounts of calcium. Their hypothesis envisaged the micelle as consisting of calcium caseinate surrounded by a surface layer consisting of a complex of α- and κ-caseinates. Inasmuch as a similar behaviour was observed in solubilized skim-milk and first-cycle casein (Noble & Waugh, 1965) the model may be said to apply also to these mixtures.

It seems, therefore, that size, composition and structure contribute to the partition coefficient. Differences in composition and size would be sufficient to account for the results if both occurred together in the same sense; for example, whereas the incorporation of κ-casein would increase the coefficient by a certain amount the increase would be much greater if the size of the new aggregate were greater. Similarly, composition and structure would be adequate and since the ultracentrifuge experiments quoted by Waugh (1961) indicated a monodisperse complex in first-cycle casein at 25 °C, structure would seem a more likely variable than size at this temperature. Some of the experiments in partition and rewashing of phases reported above were carried out at room temperature with results which indicated complexes of different partition coefficients but similar composition. However, the counter current distribution experiments were carried out at a low temperature. Evidence from ultracentrifuge results at low temperature is not consistent (see Noble & Waugh, 1965) and it is clearly not possible at this stage to assess all the factors controlling the size and composition of the aggregates.

Shimmin & Hill (1964) give pictures of the casein micelles in milk which suggest a matrix of calcium phosphate with numerous casein particles embedded therein. If all, or even most of the κ-casein is on the surface of the micelle the particles liberated from the surface when the calcium phosphate is dissolved will have a different composition from the particles which were originally within the body of the micelle. This would be one reason for the existence of aggregates of different composition and structure, for although it is scarcely to be expected that casein aggregates of different composition would all remain unchanged during prolonged dialysis, casein systems are slow to reach equilibrium and it is possible that some at least of the aggregates separable by counter current distribution may have existed as such in the micelles of the milk. This picture is similar to that of Waugh & Noble (1965) in spite of the fact that they worked with artificial micelles prepared without calcium phosphate.

Until the relationship between the aggregates in polymers and those of the original micelles is defined, the conclusions derived from counter current distribution experiments must remain conditional. However, the results so far are in accord with the assumptions of Sullivan *et al.* (1959) of McGann & Pyne (1960) and of Noble & Waugh (1965), all of whom used different techniques. The experiments with polymer phases suggest that it may be possible to devise systems which would facilitate the isolation and study of unchanged casein aggregates.

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EXPLANATION OF PLATES

PLATE 1

Electrophoresis of casein fractions recovered from a distribution in polyethylene glycol-ammonium sulphate.

PLATE 2

Electrophoresis of casein fractions recovered after counter current distribution. Sample (or tube) numbers 1–12 reading from left to right.

PLATE 3

As Plate 2 after the action of rennin. Sample 1 is missing. The gels have split along the origin.

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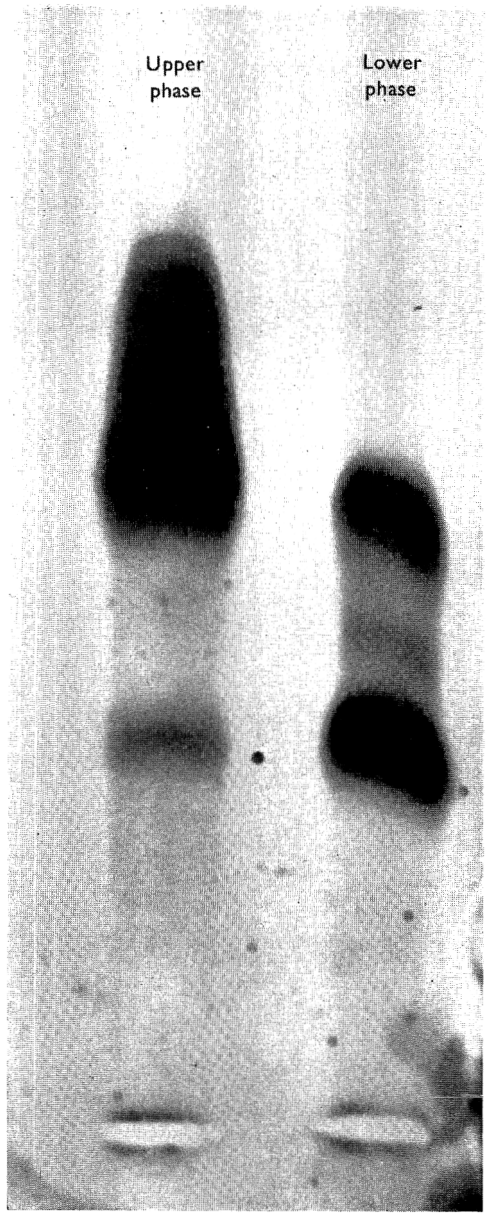
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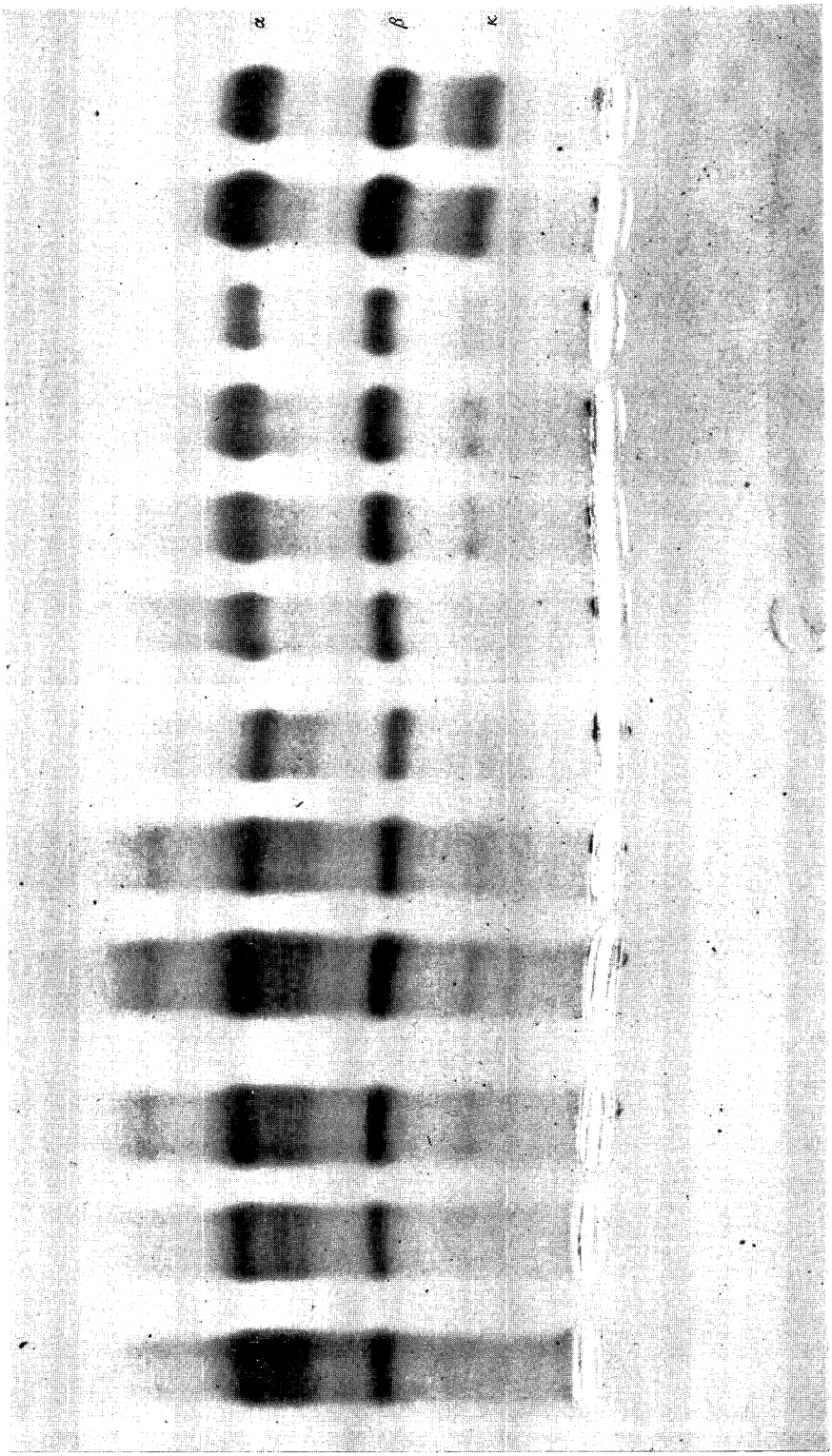

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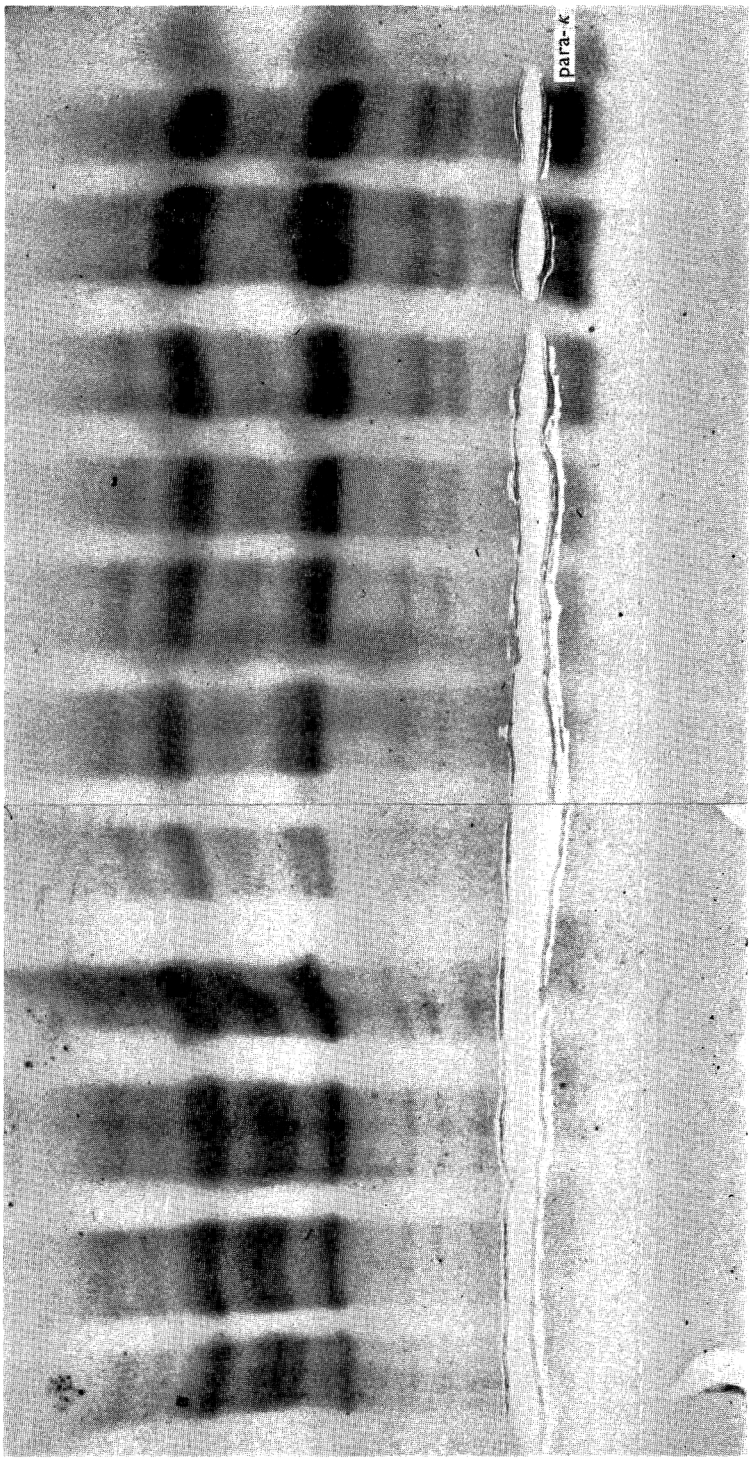
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J. Dairy Res. (1966), **33**, 291
With 1 plate

The induction of ketosis in the lactating dairy cow

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Summary. Injections of L-thyroxine into normal dairy cows at the beginning of lactation resulted in animals fed on a high protein diet becoming ketotic. Some of the symptoms observed in the induced ketotic cows showed many similarities when compared with those associated with the spontaneous condition.

In most cases the levels of the constituents estimated in the blood of the induced ketotic cows were similar to those measured in blood from spontaneous cases. A histological examination of the liver from an induced ketotic cow showed a considerable increase in the fat content, particularly in the centrilobular regions. The fat content of this tissue amounted to 26 % with a concomitant depression of liver glycogen to only 10 % of the normal level.

Diet was found to play an important role in the susceptibility of cattle to the induced ketosis, since animals fed on rations containing a lower level of protein were more resistant. Attempts to induce ketosis in cows 6–8 months after calving were completely unsuccessful.

The part played by the thyroxine and the diet in the induction of ketosis is discussed.

During recent years, attempts have been made by many workers to induce a ketosis in the lactating dairy cow comparable to the spontaneous condition of early lactation. In most cases a ketosis was produced, but many of the accompanying symptoms were lacking. Kronfeld (1965) induced ketone body formation in lactating dairy cows by the administration of growth hormone. However, the characteristic symptoms which accompany the spontaneous condition, such as loss of appetite and reduced milk yield, were not observed. On the other hand, some of these symptoms were produced in cattle without any ketone body formation after the administration of insulin (Kronfeld, 1963). Robertson & Thin (1953) also produced a ketosis in dairy cattle by withdrawing food for 6 days, but when feeding was resumed the total ketone body levels rapidly declined and approached normality within 4 days.

In a preliminary communication the induction of a ketosis was described in dairy cattle maintained under stall feeding conditions, receiving a high protein diet, and injected with L-thyroxine (Hibbitt, 1964). This induced condition showed many of the characteristic symptoms of spontaneous ketosis. The present paper examines this induced ketosis in greater detail. The clinical symptoms, together with some of the biochemical and histopathological changes, are compared with those observed in the spontaneous condition.

METHODS

Animals. Ayrshire and Friesian dairy cattle were used in these experiments. Animals with a previously good record for milk production, and within 3 months of parturition, were selected for the experiment and placed under stall feeding conditions.

Diet. The cows were fed a maintenance ration of medium quality meadow hay which was supplemented during the last 8 weeks of pregnancy (the steaming-up period) with a high protein concentrate cake. The composition of the concentrate cake was as follows: $7\frac{1}{2}$ parts barley, 5 parts decorticated groundnut, 5 parts soya-bean meal, 2 parts molassine meal and $\frac{1}{2}$ part mineral supplement. It contained 90·8 % dry matter which consisted of 31·8 % crude protein, 52·3 % nitrogen free extract, 5·1 % fibre, 3·3 % fat and 7·4 % ash. The ration was fed in gradually increasing quantities from 1 to 2 lb/day at the beginning of the steaming-up period to 15 to 22 lb/day at the time of calving. After calving, the ration was fed in excess of requirements by offering it at the rate of 4 lb/gal of milk produced plus an additional 4 lb/day.

Thyroxine injections. After calving, the milk production was recorded daily. When the daily milk production reached a maximum, 5·0 ml of 0·5 % (w/v) L-thyroxine solution were administered intramuscularly at daily intervals. The thyroxine solution was prepared for injection by vigorously shaking 100 mg L-thyroxine in 15 ml glass distilled water. This suspension was solubilized by the dropwise addition of 0·1 N-NaOH solution with further shaking. The solution was made up to 20 ml with glass-distilled water and stored at 0–4 °C in the dark. Unused solutions were discarded after 3 days.

Tissue biopsies. Cores of liver tissue weighing 300–500 mg were removed by the procedure described by Loosemore & Allcroft (1951). Occasionally, when larger pieces of liver tissue were required, up to 50 g were removed by a laparotomy.

Analytical procedures. Total ketone bodies were estimated in plasma by the method of Thin & Robertson (1952). The levels of acetoacetate and D (−) β-hydroxybutyrate were determined as described by Williamson, Mellanby & Krebs (1962). Glucose was estimated by the glucose oxidase method of Huggett & Nixon (1957) and the previously described procedures of Bach & Hibbitt (1958) were used for the estimation of citrate, pyruvate and α-oxoglutarate in blood. For the investigation of liver glycogen levels, pieces of biopsy tissue were dropped, immediately after removal from the cow, into hot 30 % KOH solution and after homogenization estimated by the method of Kronfeld, Simesen & Dungworth (1960) except that the reducing sugar formed after the hydrolysis of the glycogen was determined by a modification of the Hagedorn & Jensen method described by Baldwin & Bell (1955). For the estimation of liver fat, a portion of tissue was weighed, homogenized in an equal volume of distilled water and extracted with an equal volume of ether. The aqueous phase was re-extracted twice, the extracts were combined, evaporated, and the residue weighed.

RESULTS

Induced ketosis. Eleven cases of moderate to severe ketosis were produced in a group of 15 animals fed on the high protein diet and injected with L-thyroxine Most cases showed a response in the form of increased blood ketone body levels after

the 2nd thyroxine injection, with a complete clinical response after 4 doses. A few cases, however, were more resistant and only showed a full clinical response after 5 or 6 doses (Table 1).

The clinical symptoms, which accompanied the induced ketosis in many animals, followed a constant pattern. Before the administration of the thyroxine, milk production was at its maximum with the animals consuming 22–28 lb/day of high protein cake. After the 2nd injection of thyroxine, a transient increase in milk yield was

Table 1. *The response of normal dairy cows fed on a high protein diet to daily injections of thyroxine*

Cow no.	Breed*	Lactation	No. of thyroxine injections	Total ketone bodies in blood (as acetone), mg/100 ml	Clinical symptoms†
D 83	A	2	4	69	1, 2, 3
E 159	A	2	4	60	1, 2
C 366	A	3	4	50	1, 2
C 226	A	3	6	50	1, 2, 3
C 259	A	3	4	125	1, 2, 3
D 73	A	3	5	30	1, 2
W 292	A	4	4	75	1, 2
D 83	A	3	4	40	1, 2, 3
E 159	A	3	4	55	1, 2, 3
D 208	A	3	4	45	1, 2, 3
D 27	F	3	4	65	1, 2, 3
S 586	F	8	5	15	1
D 199	A	3	5	10	—
C 151	F	4	5	5	—
K 52	A	7	5	15	3

* A = Ayrshire, F = Friesian.
† Symptoms: 1, loss of appetite; 2, decreased milk production; 3, stasis of rumen and intestinal tract.

observed which was frequently accompanied by a reduced appetite. The appetite deteriorated during the next 2 days when further doses of thyroxine were administered. After the 4th thyroxine injection, some animals completely lost their appetite, refusing both hay and concentrates. After the transient increase in milk yield, production fell, in some cases from 60 lb/day to as low as 10–15 lb/day. A rapid loss of weight was observed which was accompanied by the mobilization of depot fats. This was particularly noticeable in the periorbital regions where the eyeballs appeared sunken. Rumination and rumen contractions became very infrequent and after 4–6 days the animal passed only small quantities of hard, dark mucus-covered faeces. In addition to these symptons, high levels of ketone bodies were found in the body fluids together with a marked smell of acetone in the animals breath.

The induced ketotic cow sometimes showed changes which could be attributed to the thyroxine injections. For example, some animals had elevated body temperatures with increased pulse and respiratory rates. Furthermore, in the early stages of the induction, hyperexcitability sometimes preceded the dullness which invariably accompanied the ketosis.

When animals suffering from induced ketosis were left untreated, their recovery

was very slow with the appetite and milk production often taking 3–4 weeks to return to normal. Moreover, some of the more severe cases often showed considerable resistance to treatment.

Changes in the levels of the blood constituents. In addition to the marked clinical symptoms, induced ketosis is also characterized by significant changes in the levels of certain blood constituents. Table 2 shows the levels of 5 blood constituents before the administration of thyroxine and again after 5 days when all the thyroxine injections had been given and the animals were ketotic. It may be seen from this table that the levels of the total ketone bodies and citrate showed highly significant changes. The depression of the blood glucose level, on the other hand, although significant, was less marked than in the spontaneous condition reported previously (Bach & Hibbitt, 1959). Although the mean values for the levels of pyruvate and α-oxoglutarate were slightly elevated in induced ketosis, the changes were not significant at a 5% level.

Table 2. *The levels (mg/100 ml) of some blood constituents of normal cows and of the same cows suffering from an induced ketosis*

Blood constituent	Before thyroxine, mean ± S.E.M.	After thyroxine, mean ± S.E.M.	P*
Total ketone bodies (as acetone)	4·9 ± 0·74 (9)	58 ± 11·9 (8)	< 0·001
Glucose	69·0 ± 3·49 (8)	54·0 ± 5·73 (8)	< 0·05
Citrate	3·18 ± 0·09 (6)	0·85 ± 0·08 (6)	< 0·001
Pyruvate	0·57 ± 0·04 (6)	0·95 ± 0·2 (6)	< 0·1
α-Oxoglutarate	0·22 ± 0·05 (6)	0·38 ± 0·06 (7)	< 0·1

* Probabilities calculated by the t test (Fisher, 1938). The numbers of cows sampled are shown in parentheses.

In an experiment involving 3 animals, the levels of the above-mentioned blood constituents were estimated on samples collected from dairy cows which failed to become ketotic after receiving the high protein diet and the thyroxine injections. With the exception of blood glucose, the levels of the constituents estimated in the blood samples collected before and after thyroxine injections remained the same. Moreover, in contrast to the ketotic group, the resistant animals maintained a normal appetite and showed slightly elevated blood glucose levels after the thyroxine injections.

Analysis of the blood of cows suffering from induced ketosis show the presence of acetone, acetoacetate and β-hydroxybutyrate. A comparison of the levels of the individual ketone bodies was made between spontaneous and induced ketotic cows. Table 3 shows the relative amounts of these compounds in both groups of animals.

Biochemical and histopathological changes in the liver. The liver of the cow suffering from induced ketosis closely resembles that of the cow with the spontaneous condition. It has a pale yellowish colour and a soft friable consistency. Histologically, fatty changes appeared to commence at the centres of the lobules and extend to the periphery. The individual liver cells also show definite changes. Numerous fat droplets appear in the cytoplasm which subsequently coalesce to form large single droplets. The nuclei, which are displaced by the fat to the edges of the cells, often show a deep staining reaction and some appear pyknotic. Plate 1 shows a section of liver

from an induced ketotic cow in which the cells contain numerous cytoplasmic fat droplets stained with Oil Red O.

The increased fat content of the liver of the induced ketotic cow is shown in Table 4 where the mean value for 4 animals amounted to 26 %. This shows a marked increase over the normal value of 4·5 % obtained by Ford & Boyd (1960). Liver glycogen, on the other hand, showed a marked depression with the level in the ketotic liver only 10 % of the normal.

Table 3. *The relative amounts of ketone bodies in the blood of cows suffering from induced and spontaneous ketosis*

(Ketone bodies were estimated in the blood of 4 animals in each group and expressed as mg/100 ml)

Acetoacetate	Ratio, acetone/acetoacetate	Ratio, β-hydroxybutyrate/acetoacetate
	Induced ketosis	
5·6	3·3	4·10
7·2	2·4	4·10
10·9	2·55	3·06
15·2	2·28	3·58
	Spontaneous ketosis	
7·5	2·16	3·40
9·1	1·53	3·30
10·9	1·69	3·75
12·1	1·87	2·60

Table 4. *The levels of glycogen and fat in liver tissue removed from normal cows and cows suffering from induced ketosis*

	Normal, mean ± S.E.M.	Ketotic, mean ± S.E.M.
Glycogen, % (w/w)	1·43 ± 0·2 (6)	0·15 ± 0·02 (6)
Fat, % (w/w)	*4·5 ± 0·56 (12)	~~2·0 ± 4·0 (4)~~ 26.0 ± 4.0 (4)

* Ford & Boyd (1960). The numbers of cows sampled are shown in parentheses.

Table 5. *The response of normal dairy cattle fed on a balanced dairy ration to daily injections of* L-*thyroxine*

Cow no.	Breed	Lactation	No. of thyroxine injections	Total ketone bodies in blood (as acetone mg %)	Clinical symptoms*
F 116	Ayrshire	3	4	15	—
F 143	Ayrshire	3	4	10	—
G 627	Ayrshire	2	4	5	—
U 381	Ayrshire	6	4	25	1
G 20	Ayrshire	2	4	75	1, 2, 3

* Symptoms: 1, loss of appetite; 2, decreased milk production; 3, stasis of rumen and intestinal tract.

The effect of diet on induced ketosis. To investigate the part played by the high protein diet in the production of induced ketosis, an attempt was made to induce the condition in 5 cows maintained under stall-feeding conditions and fed on a concentrate

cake containing 20 % crude protein instead of the 31 % crude protein in the high protein ration. The composition of this diet which was fed at the rate of 4 lb/gal was as follows: 14 parts barley, 1 part molassine meal, 2 parts soya bean meal, $2\frac{1}{2}$ parts decorticated groundnut and $\frac{1}{2}$ part mineral supplement. Table 5 shows that only one cow developed a severe ketosis, the other animals in the group all having blood ketone levels of 25 mg % or less. Three of the cows (F 116, F 143 and G 627) showed no clinical symptoms whatsoever.

The effect of the stage of lactation on the susceptibility to induced ketosis. In view of the higher incidence of spontaneous ketosis in the first few weeks of lactation, the importance of the stage of lactation was investigated on the susceptibility of cows to the induced disease. An attempt was made to induce a ketosis in 6 cows 6–8 months after calving. These animals, maintained under stall-feeding conditions, were fed the high protein diet for 8 weeks before the thyroxine injections. After 4 successive injections the animals remained normal in all respects except for the effects of the thyroxine which produced a slight loss of weight, an elevated body temperature and increased pulse and respiratory rates. However, it must be borne in mind that the milk production of these animals was consideralby lower than that of a corresponding group early in lactation.

DISCUSSION

Emery & Williams (1964) showed an increase in the incidence of ketosis in cattle implanted with triiodothyronine. A detailed description of the ketosis produced was not given, so it must be assumed that the condition was indistinguishable from the spontaneous condition. Excluding the side effects produced by the L-thyroxine injections in the present studies, the clinical symptoms observed in the cases of induced ketosis closely resembled those seen in the spontaneous condition.

The changes in the levels of some of the blood constituents as a result of the induced ketosis followed a pattern similar to that observed in the spontaneous disease. However, the blood glucose levels deserve comment since after the 1st thyroxine injection they were often elevated above normal. At the completion of the thyroxine injections when the animal became ketotic, the blood glucose levels were depressed. This initial elevation was not altogether surprising since it has been reported in the literature that hyperthyroidism may be accompanied by hyperglycaemia (Marks & Rose, 1965). The hyperglycaemia may have followed from the mobilization of liver glycogen since the levels of the latter were considerably depressed in the induced ketotic animal (Table 4). The citric acid content of the blood was low in induced ketosis, the levels closely agreeing with those reported by Bach & Hibbitt (1959) in spontaneous cases. The levels of the individual ketone bodies in the blood were all elevated above normal and with the exception of a small difference in the ratio of acetone to acetoacetate, the relative amounts of the ketone bodies were similar in both forms of ketosis. On the other hand, the blood pyruvate and α-oxoglutarate levels which may be elevated in spontaneous ketosis showed no significant deviation from normal in the induced disease.

The possible role of thyroxine in the induction of ketosis can be considered in the light of various findings reported in the literature. The observation of Folley & White (1936) that thyroxine increases the milk yield has been found in these experi-

ments. Before the thyroxine injections the cows were at peak lactation and consuming the high protein cake and hay in quantities approaching their maximum intake of dry matter per day. Although these animals produced more milk after the thyroxine injections, their food intake did not proportionately increase. Furthermore, Thomas, Moore & Sykes (1949) have shown that feed efficiency does not increase in thyrotoxic dairy cows, therefore it was not surprising to find that animals maintained under these conditions mobilized depot fats with a consequent loss of body weight.

The efficiency of thyroxine-treated cows to conserve energy may also be reduced, since Hoch & Lipmann (1954) have shown L-thyroxine to be an uncoupling agent in rat and hamster liver mitochondria preparations. The energy produced in oxidation in the thyroxine-treated animal is only partially utilized for the formation of high energy phosphate compounds. Furthermore, an elevated basal metabolism following thyroxine injections (Maynard, 1951) provides an additional factor which may contribute to the catabolism of protein and fat.

In addition to the role of thyroxine, diet may play a very important part in the induction of ketosis. The protein content of the diet used in these experiments was in excess of the requirements for maintenance and lactation, so the surplus protein was catabolized. It is generally accepted that protein is wasteful for the production of energy (Blaxter, 1962), therefore, if it is incorporated into the diet in excess of requirements the available energy would be less than that provided by food containing less protein and more carbohydrate. Furthermore, the feeding of excess protein probably contributes directly to ketone body formation since the conversion of glucogenic amino acids to carbohydrate may be accompanied by a corresponding conversion of the ketogenic amino acids to ketone bodies and acetyl coenzyme A. This hypothesis would explain why in a preliminary experiment involving 5 cows, where an attempt was made to induce ketosis in animals fed on a concentrate ration containing less protein, greater resistance was observed with only one cow becoming ketotic.

The stage of lactation has a vital influence on the susceptibility of the cow to induced ketosis. In this respect there is a very close similarity to the spontaneous condition where ketosis is rarely seen later than the 3rd month of lactation. A possible reason for this lies in the fact that in the first few weeks of lactation, when milk yields reach a maximum, the digestible energy intake is often insufficient. High yields are frequently obtained at the expense of the animals own reserves.

The author is indebted to Mr G. Newman for his advice on the composition of the diets, and also to Dr P. Radford and Mr D. Neill of the Department of Animal Husbandry, University of Bristol, for their analysis. The section of liver tissue was kindly prepared by Mr A. Mackenzie and photographed by Mr F. H. Summerfield.

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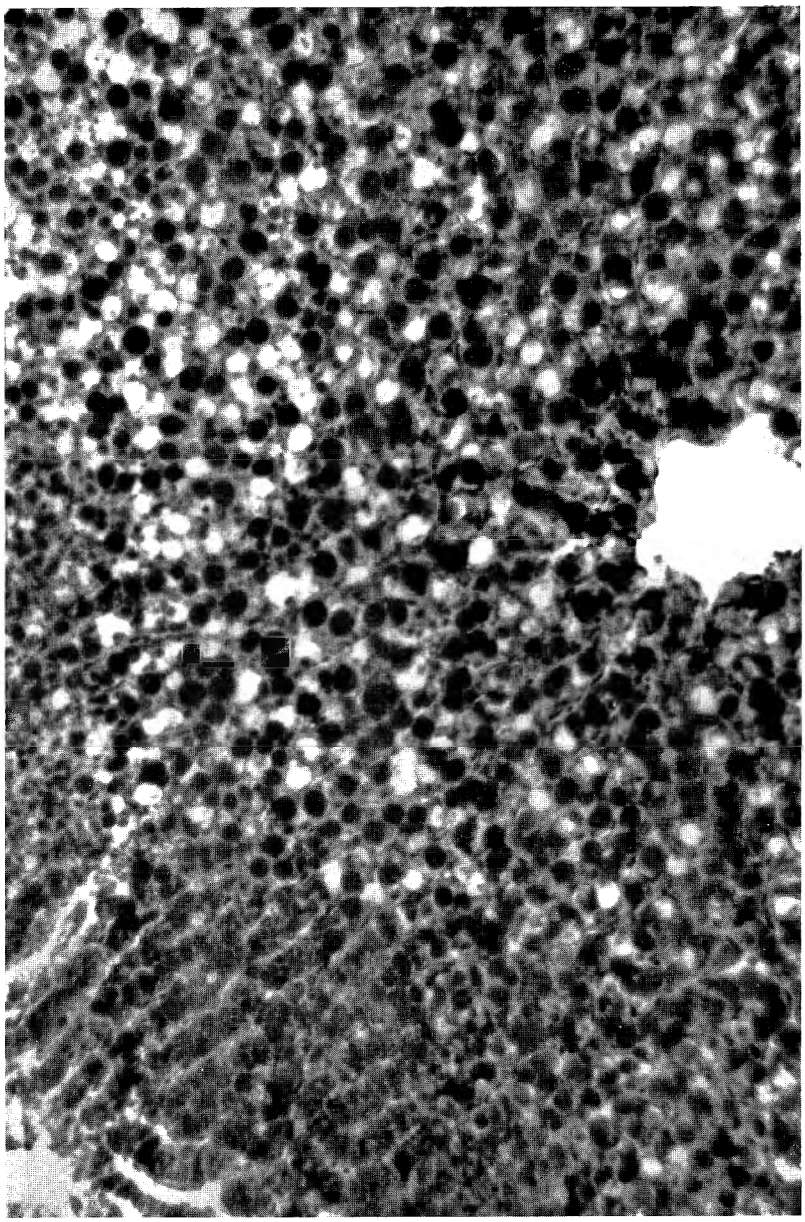
EXPLANATION OF PLATE

A section of liver from a cow suffering from induced ketosis (× 200). Droplets of fat stained with Oil Red O can be seen in the cell cytoplasm.

Printed in Great Britain



Identification of compounds causing symbiotic growth of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in milk*

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(*Received* 6 *April* 1966)

Summary. Strains of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* isolated from yoghurt culture were used to study the cause of the synergistic effect on acid production by mixtures of the 2 cultures. The beneficial effect was essentially limited to the gradual accumulation of factors in the *L. bulgaricus* cultures that promoted more rapid acid production by *Str. thermophilus*. The active factors were identified as glycine and histidine.

The starter culture employed in the manufacture of yoghurt is usually composed of a mixture of *L. bulgaricus* and *Str. thermophilus*. The studies conducted by Pette & Lolkema (1950*a*) and Katrandziev (1954) have shown that acid production was more rapid when mixed cultures of *L. bulgaricus* and *Str. thermophilus* were inoculated in milk than when single strains were used. Pette & Lolkema (1950*b*) also reported that the more rapid acid production by such cultures was attributable to water-soluble, heat-stable growth factors for the streptococcus produced by *L. bulgaricus*. They also indicated that these factors were amino acids and that valine was the most stimulatory one.

The present study was conducted to clarify further the symbiotic relationship existing between the 2 bacterial species in yoghurt starter cultures.

METHODS

Cultures

Single strains of lactobacilli and streptococci were isolated from different yoghurt products or yoghurt starter cultures obtained from commercial sources by plating on lactic agar (Elliker, Anderson & Hannesson, 1956). The isolates were transferred daily in litmus milk using 1 % inoculum and incubating at 45 °C for about 5 h for the lactobacilli and about 6 h for the streptococci. Between transfers the cultures were stored in the refrigerator.

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Identification of cultures

Two isolates used in the characterization of stimulatory factors involved in associative growth were identified as *L. bulgaricus* (strain NYL_1) and *Str. thermophilus* (strain BYS_2). The cultures possessed characteristics prescribed in *Bergey's Manual of Determinative Bacteriology* (Breed, Murray & Smith, 1957) for the representative species.

Interaction among strains

A. *Acid production.* Interactions among strains were tested by measuring acid production of single cultures and their combinations in sterile reconstituted non-fat milk (NFM) containing 10% solids. Combined cultures were prepared using 0·5% inoculum of each of the 2 strains whose associative growth was to be studied. For the single strains, 1% inoculum was used. After inoculation, the tubes of milk were incubated at 45 °C in a water bath until any one of the single cultures or their combination showed coagulation of the milk. This usually required 3·5–4 h. Tubes were then cooled in ice water and the amount of acid produced was determined by titration with 0·1 N-NaOH to pH 8·3.

B. *Colony count.* Growth curves of single strains and of each strain in the mixed culture were determined by plating single and combined strains incubated for different intervals. Lactic agar (pH 6·8) was used for enumerating the single-strain cultures. For mixed strains trypticase soy agar (pH 7·0) and lactic agar adjusted to pH 5·25 by addition of 1·0 N-HCl were used. The lactobacilli grew very well on the acidified lactic agar while the streptococci were totally inhibited. Trypticase soy agar on which the lactobacilli failed to grow was found suitable for the enumeration of the streptococci. To preserve viability of cells during dilution, 0·1 ml of sterile milk was added to each 99-ml dilution blank before use. Plates were incubated at 37 °C and the colonies were counted after 72 h.

Preparation of cell-free filtrates

The culture, after incubation in milk, was centrifuged at 7970 *g* for 1 h at 2 °C. After adjusting the supernatant to pH 7·0 the volume was adjusted to that of the original culture by addition of distilled water. The slight precipitate formed was removed by centrifugation at 27000 *g* for another 25 min. The resultant supernatant was sterilized by passage through a Seitz filter.

Ion exchange chromatography

Seitz filtrates prepared from 1 l of *L. bulgaricus* (NYL_1) culture, which had been incubated at 45 °C for 12 h, was passed through an Amberlite CG 120 (H+) 100–200-mesh column (4 × 60 cm). After collection of the effluent, the column was washed with decarbonated distilled water until the washings were devoid of lactose, as tested by Molisch reagent. Effluent and washings were collected separately. The column was eluted with 2 N-NH_4OH. The eluate, the effluent, and the washings were concentrated separately under vacuum using a flash evaporator. The volume of each concentrate was adjusted to that of the original culture with distilled water; following Seitz filtration the activity was assayed.

Fractionation by use of a Sephadex column

A 4 × 60-cm column of Sephadex G–25 (fine grade) was equilibrated at room temperature for 24 h with 0·1 N-NH_4OH. A 10-ml portion of the eluate from the ion-exchange column was applied and the column developed with 0·1 N-NH_4OH at a flow rate of 2 ml/min. 10-ml fractions were collected and the optical density at 265 mμm was measured using the eluate collected in the 1st tube as a blank. The fractions comprising each peak were mixed separately and concentrated under low pressure at 45 °C.

Paper chromatography

One-dimensional descending chromatography on Whatman 3 MM paper was used with one of the following solvent systems: A, butanol–acetic acid–water (5:1:4, v/v) B, butanol–methyl ethyl ketone–water (2:2:1, v/v); C, butanol–methyl ethyl ketone–water–cyclohexylamine (10:10:5:2, v/v); D, phenol–water (100:20, v/v). After development, the chromatogram was cut lengthwise into 2 portions; one portion was developed with ninhydrin, the other was used for bioautographic assay. For 2-dimentional chromatography, solvent systems A and B were used.

Bioautography

Areas of biological activity on the paper chromatogram were located by the technique of Speck, McAnelly & Wilbur (1958) using a seeded litmus–milk–agar plate incubated at 37 °C.

Elution of biologically active zones from paper chromatograms

The stimulatory fraction obtained from the Sephadex column was applied to 80 paper strips, which were then developed using butanol–acetic acid–water (5:1:4) solvent system. The zones which were shown to be active by bioautography were eluted with water. The eluate was concentrated to 5 ml and was used for further identification of stimulatory components.

Hydrolysis of stimulatory fraction

One volume of the fraction was hydrolysed with 5 vols of 6 N-HCl at 121 °C for 6 h. The HCl was removed by repeated drying on a steam bath and the material adjusted to pH 7·0 with NaOH. The volume of the fraction was made to that of the starting material with distilled water.

Assay of activity of filtrates, fraction or amino acids

One ml of the sterile test material (water in the case of control tube) was added to 9 ml of a 1 % culture of the test organism in NFM (11 % solids). The contents of the tubes were well mixed and incubated in a 45 °C water bath until coagulation of the milk in any one of the tubes occurred. All tubes were then cooled in ice water and the acid produced in each tube was determined by titration with 0·1 N-NaOH to pH 8·3, using a glass electrode.

RESULTS

Interaction of L. bulgaricus *and* Str. thermophilus *in milk*

A total of 118 single isolates were obtained from 5 yoghurt cultures. Forty-two isolates were Gram-positive rods (lactobacilli) and 76 were Gram-positive cocci (streptococci). Nearly all pairs (92 %) consisting of a lactobacillus and a streptococcus revealed stimulation of acid production. The effect of the strains on each other

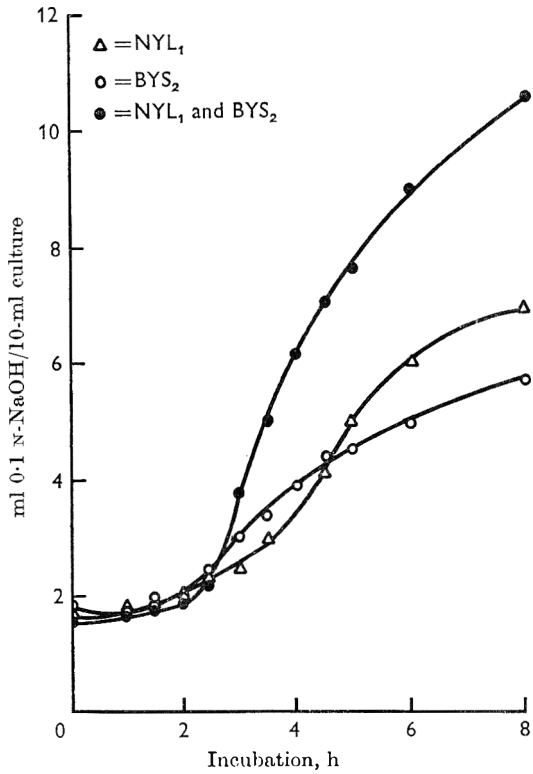

Fig. 1. Acid production by *L. bulgaricus* (NYL_1), *Str. thermophilus* (BYS_2) and their combination.

varied from marked to slight stimulation. The *L. bulgaricus–Str. thermophilus* pair NYL_1–BYS_2 was chosen for further study of the factors involved in their associative growth. Acid production of the single and combined strains and colony counts of each strain during their growth in association were determined after different incubation times. A definite stimulation of acid production occurred with mixed growth of NYL_1 and BYS_2 (Fig. 1). Fig. 2 depicts the growth curves of the same cultures. The data suggested that stimulation of acid production was mainly due to a stimulation of the streptococci by the lactobacilli.

Stimulation by cell-free filtrates

Filtrates of milk cultures of each strain were prepared after various incubation periods by centrifugation, followed by Seitz filtration. Addition of 10 % of the filtrate of *L. bulgaricus* (NYL_1) culture after 6 h incubation to *Str. thermophilus* (BYS_2) growth media was sufficient to produce near maximum stimulation of the latter (Table 1). On the other hand, the Seitz filtrates of *Str. thermophilus* (BYS_2) culture were found to be comparatively ineffective in stimulation of *L. bulgaricus* (NYL_1).

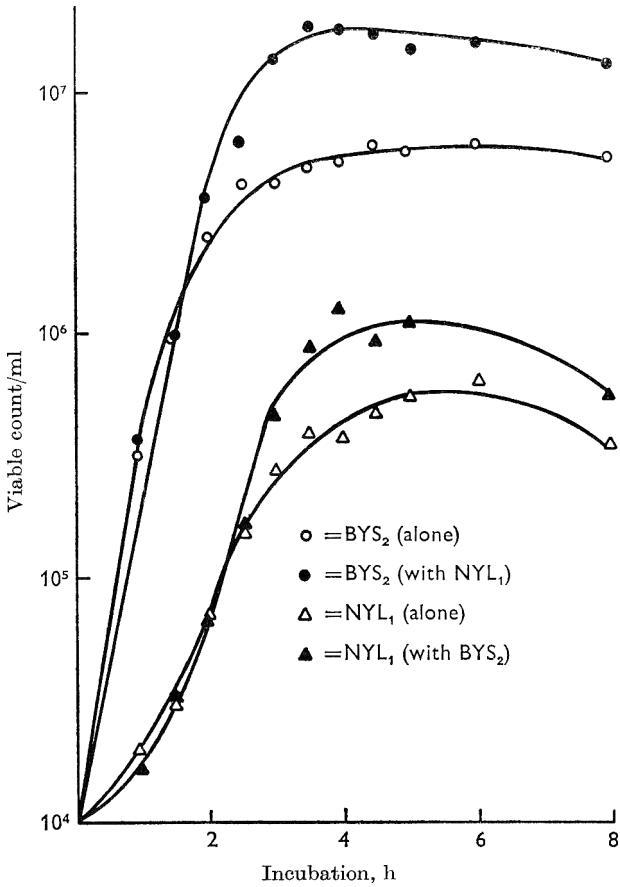

Fig. 2. Colony counts of *L. bulgaricus* (NYL_1) and *Str. thermophilus* (BYS_2), singly and in mixed culture.

Table 1. *Stimulation of* Str. thermophilus (*BYS_2*) *by Seitz filtrates* (10 %) *from milk cultures of* L. bulgaricus (*NYL_1*)

Time *L. bulgaricus* incubated, h	Acid production by BYS_2, ml 0·1 N-NaOH/10 ml culture
0	3·85
2	3·95
4	4·45
6	5·05
12	5·40
24	5·20

Fractionation of culture filtrate of L. bulgaricus *by ion exchange chromatography*

The fractions obtained after chromatography of Seitz filtrates of culture NYL_1 on Amberlite CG–120 were tested for their ability to stimulate acid production by *Str. thermophilus* (BYS_2). The active component(s) was found to be present only in the fraction eluted with 2 N-NH_4OH. The amount of stimulation obtained was equal to that of the original culture filtrate.

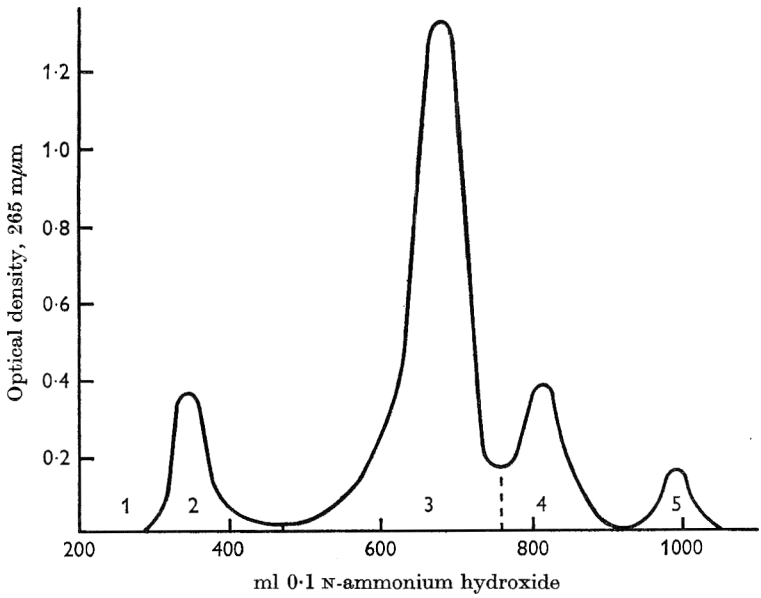

Fig. 3. Elution profile of ion exchange column eluate of *L. bulgaricus* (NYL_1) from G–25 Sephadex column using 0·1 N-ammonium hydroxide.

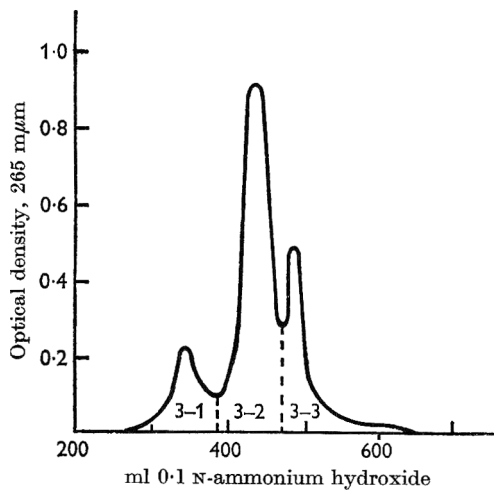

Fig. 4. Elution profile of fraction 3 from G–25 Sephadex column using 0·1 N-ammonium hydroxide.

Gel-filtration of active component(s)

When the ammonia eluate from the ion exchange column was submitted to gel-filtration on Sephadex G–25, a reproducible elution pattern was obtained for each run (Fig. 3). The void volume was designated as fraction 1, and the 4 peaks were designated as fractions 2, 3, 4 and 5, respectively. The major part of the activity stimulatory towards *Str. thermophilus* (BYS_2) was in fraction 3; the other fractions were practically devoid of activity. Fraction 3 was re-cycled through the Sephadex column and gave a reproducible elution pattern (Fig. 4) consisting of 3 peaks (fractions 3–1, 3–2 and 3–3) which were assayed for stimulatory activity after the removal of ammonia. The stimulatory compound(s) was present in fraction 3–2 (Table 2).

One-dimensional chromatography and bioautography

Chromatograms of each of the subfractions obtained from fraction 3 of culture NYL_1 were prepared using 2 solvent systems (A and B). The strips were then tested for the presence of stimulatory zones by bioautography. The resulting bioautograph of fraction 3–2 showed a prominent biologically active zone. When compared to the other half of the strip stained with ninhydrin, the active area was observed to correspond to 2 adjacent strongly ninhydrin-positive zones. The other fractions, tested in the same manner, did not show any biologically active zone.

Table 2. *Stimulation of* Str. thermophilus (BYS_2) *in milk by fractions obtained after passing fraction* 3 *from* L. bulgaricus (NYL_1) *through the Sephadex column*

	Acid production, ml 0·1 N-NaOH/10 ml culture after incubation at 45 °C for	
Fraction	3·5 h	4 h
Control	3·80	3·95
Fraction 3	4·50	4·90
Fraction 3–1	3·90	4·10
Fraction 3–2	4·20	4·75
Fraction 3–3	3·60	3·95

Table 3. *Stimulation of* L. bulgaricus (BYS_2) *in milk by histidine and glycine, singly and in combination*

		Acid production ml 0·1 N-NaOH/ 10 ml culture, incubation at 45° C for	
Concentration, mg/ml	Amino acid	3·5 h	4 h
0·00	Control	3·65	3·80
0·05 each	Histidine, glycine	3·65	3·85
0·10	Histidine	3·65	3·80
0·10	Glycine	3·60	3·80
0·5 each	Histidine, glycine	3·75	4·15
1·0	Histidine	3·75	4·15
1·0	Glycine	3·85	4·10
2·5 each	Histidine, glycine	4·50	4·65
5·0	Histidine	4·25	4·40
5·0	Glycine	3·95	4·20
5·0 each	Histidine, glycine	4·65	4·85
10·0	Histidine	4·15	4·35
10·0	Glycine	3·70	4·00

Activity of the material after elution from the active zones

The stimulatory zones observed on paper chromatograms by bioautography were eluted with water. After concentrating under vacuum, a portion of the eluate was hydrolysed with 6 N-HCl. The untreated and the hydrolysed eluates were then tested for stimulation of *Str. thermophilus* (BYS_2). The eluted material contained essentially all the activity present in the original Seitz filtrate, and acid hydrolysis did not significantly affect its activity.

Identification of the stimulatory factors

These results indicated that the growth of *Str. thermophilus* was stimulated by 2 ninhydrin-positive compounds. The eluate of the 2 active zones was chromatographed 1-dimensionally on paper along with control samples of 22 known amino acids. The colour after development with ninhydrin and the position of the active compounds on the chromatograms indicated that the active compounds present in the eluate were glycine and histidine. This conclusion was confirmed by the use of 2-dimensional chromatography.

Acid production by *Str. thermophilus* (BYS_2) in milk containing added glycine and histidine was measured. Data presented in Table 3 showed that fortification of milk with histidine resulted in increased acid production. Addition of glycine, on the other hand, produced very little added effect on acid production, even though it was observed to be equally stimulatory by bioautography. A mixture of glycine and histidine was slightly more stimulatory than when either of these compounds was tested alone. Very little further stimulation was obtained by increasing the total concentration of amino acids in the mixture from 5 to 10 mg/ml.

To check further whether stimulation by histidine was specific for strain BYS_2, 5 other strains of *Str. thermophilus* isolated during this study were also tested. Besides histidine and glycine, valine was also included, since Pette & Lolkema (1950*b*) reported valine to be the stimulatory amino acid required by *Str. thermophilus*. None of the strains tested was stimulated by valine or glycine. However, fortification of milk with histidine resulted in increased acid production by all strains tested.

DISCUSSION

A number of vitamins, amino acids, peptides, and pyrimidine bases are required for optimal growth of *Str. thermophilus* in synthetic media (Nachev, 1958; Nurmikko & Karha, 1962). Any deficiency of these growth factors in milk would, therefore, be expected to restrict the growth of the organisms in this medium. It could be corrected by suitable additions to milk or alternatively the growth of a suitable symbiotic strain could be used to provide the missing growth factors.

In the present study, the higher acid production by a mixed culture of *L. bulgaricus* and *Str. thermophilus* in milk, as compared with either organism growing alone, was mainly due to the improved growth of the streptococcus resulting from the production of histidine and glycine by the lactobacillus. This implies that *L. bulgaricus* has proteolytic ability. Mair-Waldburg (1953) showed that *L. bulgaricus* was capable of breaking down casein into various nitrogenous compounds. MacDonald (1955) demonstrated that *L. bulgaricus* and other lactobacilli isolated from dairy products

were able to utilize unhydrolysed caseinate. Hamdy, Harper & Weiser (1955) and Chebotarev (1962) also observed that *L. bulgaricus* grown in milk accumulated various amino acids in the medium.

The addition of histidine and glycine to milk resulted in a greater acid production by *Str. thermophilus* growing alone. Clearly, this organism cannot produce these amino acids from milk or produces them in lower concentrations than is optimum for growth. Different results were obtained by Pette & Lolkema (1950*b*). By omission in turn of single amino acids from a mixture added to milk, they observed that *Str. thermophilus* required 6 amino acids in spring milk and 11 amino acids in autumn milk for optimal growth. Furthermore, valine was found to be the most important. However, in our studies, the growth of *Str. thermophilus* was not stimulated by adding valine to milk. This discrepancy may be due to differences in strain as well as differences in milk composition. It is, however, possible that the technique of omitting single acids in turn may result in misleading culture responses due to amino-acid imbalance. Our positive identification of histidine and glycine as the active factors produced in milk by *L. bulgaricus* for growths of *Str. thermophilus* avoids such difficulties.

The present study emphasizes the need for a better understanding of the factors involved in associative growth of yoghurt starter cultures and the value of proper selection of strains for use in the manufacture of yoghurt.

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The transfer of *N*-acetyl-4-aminoantipyrine and of thiocyanate from blood to milk

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Summary. The entry into milk from blood of *N*-aeetyl-4-aminoantipyrine (NAAP) and urea, compounds known to be distributed evenly throughout the whole of body water, and thiocyanate and thiosulphate, the distribution of which is confined largely to extracellular fluid, has been investigated. The concentrations of NAAP and urea in blood and in milk were similar, but the concentrations of thiocyanate and thiosulphate in milk were much lower than the concentrations in blood plasma. A progressive increase in the ratio of the thiocyanate concentration in milk to that in blood occurred with advancing lactation and marked variations in the thiocyanate ratio of the milk of the separate quarters were observed in some cows, and these variations in the ratio were closely related to variations in the chloride content of the milk; similar inter-quarter variations in the ratio for NAAP were not observed. With NAAP, there was a continuous equilibrium between blood plasma and milk within the udder, whereas with thiocyanate the content in milk after its secretion into the ducts and cisterns of the udder appeared to be largely independent of later changes in the concentration of thiocyanate in blood plasma. These results are discussed in relation to the secretion of chloride in milk.

The changes in milk composition characteristically associated with advancing lactation, age and infection of the udder, of an increase in the contents of sodium and chloride and a decrease in the contents of potassium and lactose, have frequently been explained in terms of a dilution of the primary secretion of the alveolar cells with a transudate of blood plasma (Peskett & Folley, 1933; Barry & Rowland, 1953). The milk of heifers in the middle of their lactation, and free from infections of the udder, invariably contains some sodium and chloride but it is uncertain whether this also is partly of extracellular origin or originates wholly in the alveolar cells. Knutsson (1964), and McKenzie & Lascelles (1965) have shown that the infusion of sodium and chloride into the cistern of a lactating quarter is followed by a net transfer of sodium and chloride out of the gland against the concentration gradient. It is not known to what extent this process is active in the regulation of the sodium and chloride contents of milk under normal physiological conditions.

Possibly because of technical difficulties, there is little direct experimental evidence on the physiology of secretion of water and minerals in milk. There is, however,

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circumstantial evidence (Rook & Storry, 1964) that the volume of water secreted in milk is regulated by the osmotic equilibrium between milk and blood and that variation in the rate of synthesis and secretion of lactose, which accounts for a major part of the osmotic activity of synthesized materials, causes variation in the output of water (Rook, Storry & Wheelock, 1965). Also, with cows restricted in their access to drinking water the variations in the osmotic pressure of milk and blood suggest that milk is in osmotic equilibrium with the blood flowing through the udder continuously throughout the period for which the milk remains within the udder and not only during its formation (Wheelock, Rook & Dodd, 1965).

The present work was designed to study the transfer to milk from blood of NAAP and urea, compounds known to be distributed throughout the whole of body water and of thiocyanate and thiosulphate, the distribution of which is confined largely to extracellular fluid. It was found that whereas the concentrations of NAAP and urea in blood plasma and in milk were similar, the concentrations of thiocyanate and thiosulphate in milk were invariably considerably less than those in blood plasma. Further experiments were done to determine whether the entry of thiocyanate into milk varies with the rate of entry of chloride and whether, after entry into milk, there is a further re-equilibration of NAAP or thiocyanate between milk and blood plasma.

EXPERIMENTAL

Animals

Lactating Friesian cows from the Institute herd were housed in a metabolism shed and given a diet of hay and concentrates balanced for milk production.

Experimental procedure

All the experiments were of the same design (Table 1). Immediately before the start of an experiment the animals were milked with a machine designed for the separate collection of milk from each quarter of the udder, and a sample of jugular venous blood was taken through a polythene cannula which had been inserted the previous day. Thiocyanate, thiosulphate and NAAP were administered intravenously as an iso-osmotic solution in water, and urea was offered in admixture with a small amount of a concentrated food which was invariably consumed within about 5 min. The animals were milked and samples of jugular venous blood were taken at intervals throughout the experimental period.

Methods of analysis

Total solids in blood plasma and milk, and chloride in milk were determined as described previously (Wheelock *et al.* 1965). Samples of blood plasma and milk were analysed for NAAP by the method of Brodie (1951), thiocyanate by the method of Eder (1951), thiosulphate by the method of Cardozo & Edelman (1952) and urea by the method of Van Slyke & Cullen (Hawk, Oser & Summerson, 1947).

Table 1. *Details of experiments*

Expt. no.	Length of experiment, h	Compounds* administered at 0 h	Time of blood sampling, h	Time of milking, h	Remarks†
1	5	10 g NAAP; 20 g sodium thiocyanate	0, 2, 5	0, 2, 5	Residual milk was removed after an injection of 10 i.u. synthetic oxytocin (Syntocinon Sandoz Ltd., London, W.1) at the end of last milking of the experiment
2	11	10 g NAAP; 20 g sodium thiocyanate; 50 g sodium thiosulphate; 100 g urea	0, 2, 5, 8, 11	0, 2, 5, 8, 11	—
3	11				
4	8	10 g NAAP; 20 g sodium thiocyanate	0, 2, 5, 8	0, 2, 5, 8	—
5	8				
6 A–D	8	20 g sodium thiocyanate	0, 2, 5, 8	0, 2, 5, 8	Repeat experiments on the same cow 24, 18, 10 days and 1 day from the end of lactation
7	11	10 g NAAP; 20 g sodium thiocyanate	0, 0·25, 0·5, 0·75, 1, 1·5, 2 and then at hourly intervals	0, 2, 5, 8, 11	RH and LF quarters left unmilked at 5 and 8 h.
8	11				
9	24	10 g NAAP; 40 g sodium thiocyanate	0, 3, 18, 21, 24	0, 3, 18, 21, 24	LF and RH quarters left unmilked at 18 and 21 h
10	24				RF and LH quarters left unmilked at 18 and 21 h
11	24				RF and LH quarters left unmilked at18 and 21 h

* NAAP, *N*-acetyl-4-aminoantipyrine.
† RF, right-fore quarter; RH, right-hind quarter; LF, left-fore quarter; LH, left-hind quarter.

RESULTS

Changes with time in the concentration in blood plasma and in milk of intravenously administered NAAP and thiocyanate

The concentrations of NAAP in blood plasma and in milk (Fig. 1) declined exponentially throughout the course of an experiment, and for samples of milk and blood plasma taken at the same time comparable values were invariably obtained. The concentration of thiocyanate in blood plasma showed a sharp fall throughout about the first 2 h but thereafter the concentration decreased slowly, at a rate of a few per cent per h. The concentration of thiocyanate in milk was, however, only about one-third of that in blood plasma.

This distinction between NAAP and thiocyanate in their rate of transfer to milk from blood was invariably observed (Table 2): for 6 cows at 5 and 8 h after the administration of the compounds, the ratio of the concentration in milk to that in blood plasma varied for NAAP from 0·90 to 1·13 and for thiocyanate from 0·22 to 0·37.

In 2 expts., urea with a distribution throughout body water similar to that of NAAP, and thiosulphate with a distribution similar to that of thiocyanate, were also administered and the ratio varied from 1·01 to 1·07 for urea and from 0·30 to 0·36 for thiosulphate.

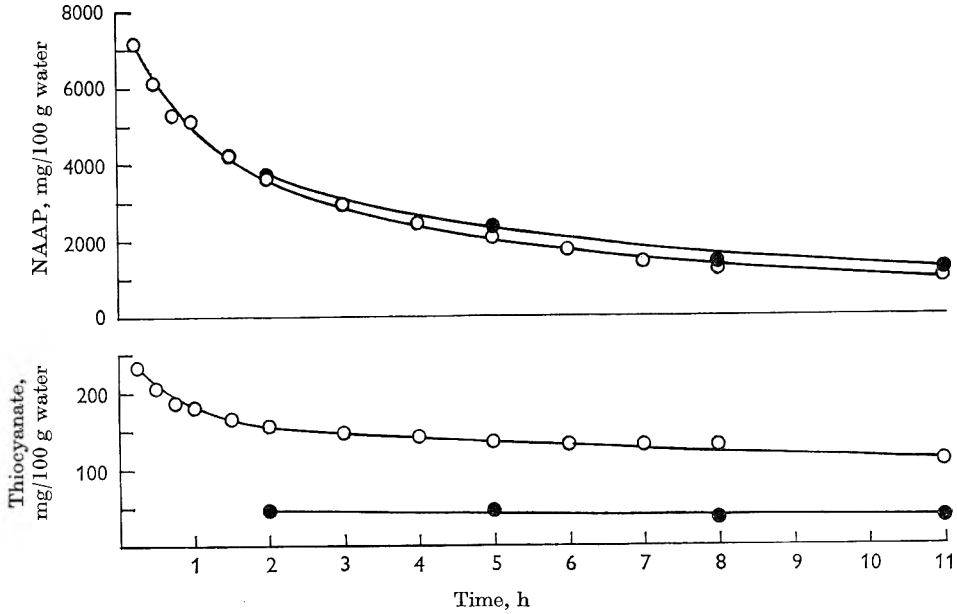

Fig. 1. Expts. 7 and 8. The changes with time in the concentration in blood plasma and milk of *N*-acetyl-4-aminoantipyrine and of thiocyanate after their intravenous administration. ○, blood plasma; ●, milk.

Table 2. *The ratio of the concentration in milk water to that in blood plasma water of N-acetyl-4-aminoantipyrine, urea, thiocyanate and thiosulphate after their intravenous or oral administration*

Expt. no.	Time after administration, h	NAAP	Urea	Thiocyanate	Thiosulphate
2	5	0·95	1·05	0·23	0·30
	8	0·90	1·01	0·37	0·35
3	5	1·03	1·02	0·26	0·31
	8	0·98	1·07	0·30	0·36
4	5	0·95	—	0·27	—
	8	0·94	—	0·34	—
5	5	1·00	—	0·22	—
	8	0·97	—	0·30	—
7*	5	1·13	—	0·34	—
	8	1·07	—	0·37	—
8	5	1·02	—	0·29	—
	8	0·93	—	0·25	—

* Mean values for right-fore and left-hind quarters only.

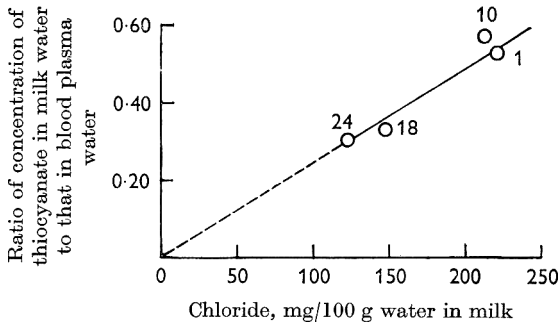

Fig. 2. Expts. 6A–D. The relationship between the ratio of the concentration of thiocyanate in milk to that in blood and the chloride content in the milk of a cow in advanced lactation. Values are the mean for determinations made 5 and 8 h after the intravenous administration of thiocyanate. The numbers indicate the days from the end of lactation. The line is drawn to pass through the origin and the mean value for the four points.

Table 3. *Variation between quarters of the udder in the ratio of the concentration in milk water to that in blood plasma water of N-acetyl-4-aminoantipyrine and thiocyanate together with the chloride content of the milk for the separate quarters (mg/100 g water)*

(Values were obtained 5 or 8 h after the intravenous administration of NAAP and thiocyanate)

Expt. no.	Time after administration, h	Quarter*	Ratio for NAAP	Ratio for thiocyanate	Chloride content of milk
1	5	RF	0·86	0·25	101
		RH	0·90	0·34	128
		LF	0·91	0·26	100
		LH	0·86	0·28	108
		**C	2·36	14·30	11·93
1	5†	RF	0·86	0·26	123
		RH	0·95	0·44	163
		LF	0·87	0·31	123
		LH	0·91	0·34	136
		C	3·80	22·46	13·87
3	8	RF	1·00	0·29	106
		RH	1·01	0·29	105
		LF	0·99	0·29	104
		LH	0·90	0·31	105
		C	5·19	3·39	0·91
4	8	RF	0·92	0·34	125
		RH	0·99	0·32	117
		LF	0·94	0·38	122
		LH	0·90	0·32	113
		C	4·13	8·32	4·47
5	8	RF	1·00	0·32	12
		RH	0·97	0·30	130
		LF	0·07	0·28	114
		LH	0·94	0·31	114
		C	2·53	5·71	6·82

* RF, right-fore quarter; RH, right-hind quarter; LF, left-fore quarter; LH, left-hind quarter.
** Coefficient of variation, %.
† Values for residual milk removed after injection of oxytocin at the end of the last milking of the expt.

Effect of stage of lactation on the transfer of thiocyanate to milk (expts. 6A–D)

In the one cow studied, the ratio of the concentration of thiocyanate in milk to that in blood increased progressively throughout late lactation, from a value of about 0·30 on the 24th day before the end of lactation to about 0·57 at the end of lactation. This increase was proportionate to the observed increase in the concentration of chloride in the milk (Fig. 2).

Table 4. *The effect of leaving milk in the udder on the transfer of thiocyanate and N-acetyl-4-aminoantipyrine to milk*

Expt. no.	Quarter*	Time after intravenous administration, h				Weighted mean value for milked obtained at 18, 21 and 24 h†
		2	5	8	11	
		N-acetyl-4-aminoantipyrine, μg/100 ml				
7	RF	3453	2315	1464	1074	1618
	RH	3093	‡	‡	1074	1074
	LF	3365	‡	‡	980	980
	LH	3197	2225	1470	1030	1575
			**C 4·94	—	4·30	25·30
8	RF	4296	2350	1424	1077	1618
	RH	4395	‡	‡	992	992
	LF	4083	‡	‡	1027	1027
	LH	4206	2283	1344	1014	1547
			C 3·13	—	3·50	25·62
		Time after intravenous administration, h				
		3	18	21	24	
		Thiocyanate, mg/100 ml milk water				
9	RF	82	68	59	47	64
	RH	80	‡	‡	60	60
	LF	72	‡	‡	63	63
	LH	78	65	57	53	62
		C 5·54	—	—	12·83	2·78
10	RF	88	‡	‡	64	64
	RH	86	67	60	50	64
	LF	86	75	59	43	68
	LH	90	‡	‡	78	78
		C 2·17	—	—	9·11	6·50
11	RF	69	‡	‡	44	44
	RH	64	61	42	36	55
	LF	68	56	48	38	52
	LH	70	‡	‡	41	41
		C 2·79	—	—	8·82	13·71

* RF, right-fore quarter; RH, right-hind quarter; LF, left-fore quarter; LH, left-hind quarter.
** Coefficient of variation, %.
† It was assumed that the volume of milk secreted was proportional to the length of the milking interval.
‡ Quarter not milked.

Differences between quarters in the rate of transfer of NAAP and of thiocyanate to milk

The variations between quarters of the udder in the ratio of the concentration in milk to that in blood plasma of NAAP and thiocyanate and in the concentration of chloride in milk are given in Table 3. The coefficient of variation between quarters for the NAAP ratio was similar for all the cows (2·5–5·2) but for the thiocyanate ratio there were marked differences from cow to cow, the coefficient ranging from 3·4 to 22·5; the highest values were observed in cows in which udder quarters differed in their chloride content, and between quarters within an udder the ratio for thiocyanate was related to the chloride content of the milk.

Effect of leaving milk within the udder on the transfer of NAAP and thiocyanate to milk (*Table* 4)

The removal of milk from only 2 quarters of the udder at 2 successive milkings had no effect on the relative concentrations of NAAP in the milk from the 4 quarters at the following milking. However, the milk from the previously unmilked quarters invariably had a higher concentration of thiocyanate than the milk from the other 2 quarters. In 2 of the cows, the weighted mean thiocyanate concentrations for the milk removed from the milked quarters over the 3 milkings were similar to those for the unmilked quarters over the same period, but in the other cow they were slightly higher.

DISCUSSION

NAAP is distributed in the tissues of the dog in proportion to their water content and binding to plasma proteins is negligible (Brodie & Axelrod, 1950), and for the cow, Whiting, Balch & Campling (1960) found that the concentration of NAAP in milk water was on average 97 % of that in the water of the blood plasma. The present results confirm that there is a rapid and complete equilibration of NAAP between the water of milk and of blood plasma—the average ratio for NAAP for the results of Table 2 is 0·99—and indicate that the entry of NAAP into the milk is not linked specifically to the secretion of the milk constituents.

Intravenously administered thiocyanate has been shown to have a volume of distribution within the animal body considerably less than that of NAAP and it has been postulated that it diffuses rapidly into extracellular fluid and more slowly into some tissue phases not in equilibrium with the extracellular fluids (Eder, 1951). Also about one-third of the thiocyanate of human blood plasma is partially bound to non-diffusible components—mainly serum albumin. Thiosulphate has a similar volume of distribution within the animal body to that of thiocyanate and is negligibly bound to plasma proteins. The comparable values obtained for thiocyanate and thiosulphate for their distribution between milk and blood suggest therefore that the diffusion characteristics of thiocyanate are more important than the binding to plasma proteins in accounting for the lower concentration in milk than in blood.

The observed results for the distribution of thiocyanate are open to at least 2 interpretations: either a part (20–50 %) of milk water is extracellular in origin, or milk water is wholly cellular in origin and there is a limited entry of thiocyanate into

the alveolar cells. A third possibility, that thiocyanate diffuses across the epithelium of the ducts and cisterns of the udder and that there is a resulting equilibrium of thiocyanate between milk and blood, is inconsistent with the inter-quarter differences observed when 2 quarters were left unmilked (Table 4). It seems unlikely, however, that the close association observed between the chloride content and the entry of thiocyanate into milk, as it varied with advancing lactation (Fig. 2) and in certain cows between the separate quarters of the udder (Table 3), could result from a limited entry of thiocyanate into the alveolar cell. The most probable explanation is that there is a continuous secretion into the udder of a fluid of extracellular origin and that its volume, relative to that of the primary secretion fluid, increases in advanced lactation with other physiological changes within the udder associated with an increase in the chloride content of the milk.

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A comparison between a hot-wire laboratory apparatus and a plate heat exchanger for determining the sensitivity of milk to deposit formation

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Summary. Using several milk supplies, a comparison has been made between the amount of deposit formed under standard conditions in a small plate type ultra-high-temperature heater, and the results from the hot-wire laboratory apparatus described previously. It is shown that the laboratory apparatus reproduces the variations in the sensitivity to deposit formation found using the plate heater. The relationship is linear if the criterion derived from the hot-wire results is taken as the square of that proposed previously. A decrease in the sensitivity to deposit formation during the ageing of fresh milk, and a seasonal variation, are indicated.

A previous paper (Burton, 1965) described a laboratory method for studying the rate of formation of deposits from milk on a heated surface. In this method, a platinum wire carrying an electric current formed the heated surface. As the deposits built up on the wire to form an insulating layer, the differential temperature between the wire and the milk increased. The sensitivity of the milk sample to deposit formation was expressed by the K value, defined as the slope of the line relating the differential temperature to (elapsed time)$^{\frac{1}{2}}$. It was shown that the laboratory method reproduced satisfactorily the effects of certain factors known to influence deposit formation in heat exchangers in practice, e.g. variation in pH and forewarming conditions. However, it was important to compare the results given by the hot-wire apparatus for a range of milks with those for the same milks in a heat exchanger.

Ideally the comparison should be made using a commercial heat exchanger operating under ultra-high-temperature conditions. This was impracticable because of the scale of operation that would be required. The comparison was therefore made using a small scale heat exchanger operating under ultra-high-temperature conditions at a flow rate of about 68 l/h (15 gal/h). The amount of deposit formed was determined by weighing, and in addition pressure differences were measured across some sections of the heat exchanger during operation.

DESCRIPTION AND OPERATION OF THE HEAT EXCHANGER

The heat exchanger used for the experiments was a small plate-type plant (Alfa-Laval type PL 1) modified for the purposes of the experiment. A circuit diagram of the plant is shown in Fig. 1.

In operation, milk is pumped from a balance tank by a centrifugal pump with a small pressure head due to gravity at the pump intake. The first regeneration stage consists of 2 single passes and raises the milk temperature to about 65 °C. Homogenization takes place at this temperature using a Rannie homogenizer (type Lab) modified by the addition of an infinitely variable ratio gearbox to give controllable output. From the homogenizer, milk returns to a second regenerator section, also of 2 single passes, and then enters the final heating section of 3 single passes where it is heated by steam under pressure controlled by hand to give the required final milk temperature. The outgoing milk is cooled in the 2 regenerator sections, and passes through a hand-operated restrictor valve at the plant outlet to apply back pressure to the milk.

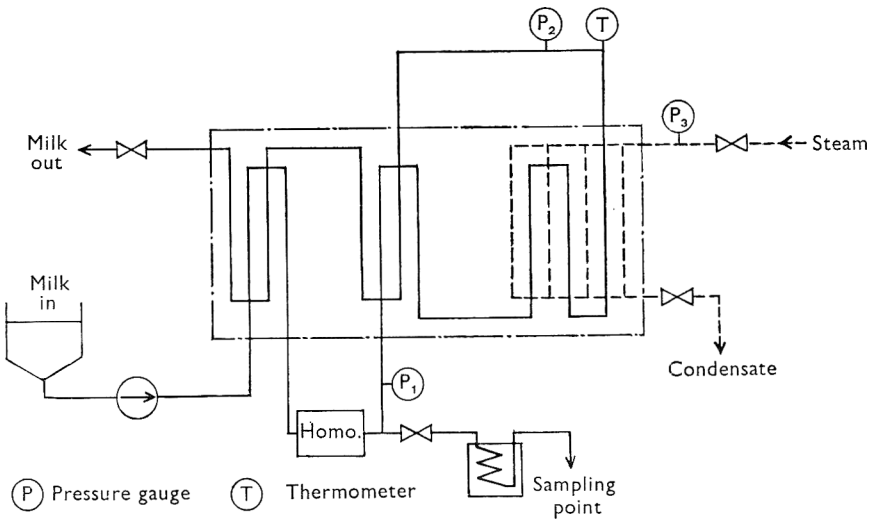

Fig. 1. Circuit diagram of small scale ultra-high-temperature plant.

Pressure gauges are fitted in the milk circuit at the inlet to the second regenerator and at the outlet of the steam heating section. A thermometer is fitted at the outlet of the steam section.

Experience showed that almost all the deposit formation occurred in the steam heating section. Measurement of deposit weight was therefore confined to this section.

For each experiment, 10 gal of raw milk was used. The milk was obtained from the bulked milk of one of the Institute's farms, from the bulked milk of an outside herd, or from a farm collection tanker. A list of the samples used is given with Fig. 3. The milk was at refrigerator temperature except with samples 3, 4 and 14.

Flow through the heat exchanger was established with a supply of distilled water until the required operating conditions were obtained. The homogenizing pressure was set at 2500 lb/in^2, and the speed of the homogenizer was adjusted to give a flow rate of 19–20 ml/sec (68·4–72 l/h). By control of the restrictor valve at the plant outlet, the pressure at the outlet of the steam heating section was adjusted to 62 lb/in^2 (25 lb/in^2 above the standard heating steam pressure of 36–37 lb/in^2) to prevent boiling in the heat exchanger. When the water temperature at the outlet of the steam heating section reached 135 °C, well-mixed milk from the 10-gal experimental sample

was supplied to the plant to replace the distilled water. Timing of the experiment began at this instant.

When milk appeared at the plant outlet, the flow rate was checked and corrected if necessary. The homogenizing pressure, steam pressure and milk pressure at the outlet of the steam heating section were held constant. The milk pressures at the inlet to the second regenerator (about 65 lb/in^2 at the beginning of an experiment) and at the outlet of the steam heating section were recorded throughout the experiment, together with the steam pressure and the maximum milk temperature.

During the first few minutes of the experiment, a sample of about 400 ml homogenized milk was taken for test in the hot wire apparatus. The milk was taken from a sampling point immediately after the homogenizer, cooled by passing it through a stainless steel coil immersed in ice and water and immediately transferred to a refrigerator where it was held for 1–2 h until the hot-wire experiment could begin. The homogenizer speed was temporarily increased while sampling was proceeding in order to maintain constant flow conditions in the heat exchanger after the sampling point.

When all the 10-gal experimental milk sample had passed through the plant, it was replaced by distilled water and the steam supply was shut off. When the plant outlet was running clear and the maximum temperature was below 100 °C, the plant was shut down and drained. The heat exchanger was opened and the plates removed from the steam heating section.

On the reverse side from the deposit layer the plates were dried with a cloth; they were then kept in a gentle current of warm air from a fan heater. The individual plates were weighed at $\frac{1}{2}$–1 h intervals using a torsion balance weighing to 0·01 g, until the weight change for the complete set of plates between successive measurements was 0·3 g or less. This required a drying time of about 4 h. Extrapolation of the drying curve then allowed the total weight of the plates and dry deposit to be obtained to within about 0·1 g. The deposit weight was found by subtraction of the total weight of the clean, dry plates, and was expressed as g/gal of milk treated.

Often it was not possible to treat the whole of the 10-gal experimental sample because excessive build-up of deposit caused the pressure at the inlet to the second regenerator to rise until there was danger of gasket leakage. If the inlet pressure rose above about 95 lb/in^2 the experiment was ended as described above, and the deposit weight measured. The result was then expressed as g/gal of the quantity of milk that had passed through the plant.

OPERATION OF THE HOT-WIRE APPARATUS

The method of using this apparatus was similar to that described previously (Burton, 1965). However, modifications were made to increase the temperature of the hot wire during the experiment in order to represent the conditions in a higher temperature section of the heat exchanger. A 45-mm length of 38 s.w.g. (0·006 in. diam.) pure platinum wire was used, with a heating current of 4·8 A. The initial wire temperature was about 120 °C under these conditions, instead of about 92 °C under the conditions described previously, and an experiment could be completed in less than 40 min instead of 90 min.

Because of the higher operating temperature, the air pressure applied to prevent gas separation at the wire surface had to be increased to 40 lb/in^2, and the milk pre-heating temperature was increased slightly from 65 to 67 °C to maintain constant conditions during the early part of an experiment.

RESULTS

During the operation of the heat exchanger on milk, the final milk temperature changed only slightly with deposit build-up. The pressure difference across the second regenerator and steam heating sections (P_1–P_2, Fig. 1) rose in a characteristic way as the deposits restricted the flow passages in the steam heating section.

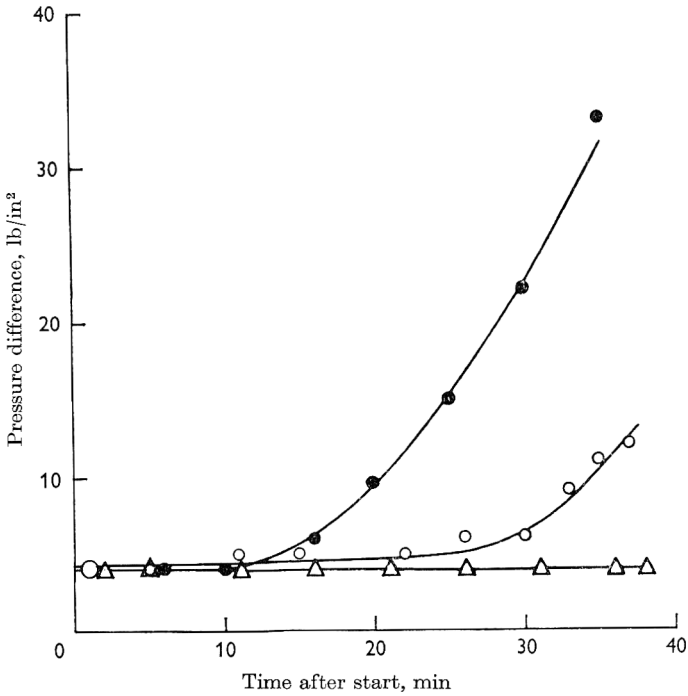

Fig. 2. Variation with time of pressure difference across second regenerator and steam heating sections. ○, sample 2; △, sample 3; ●, sample 8.

The variation of this pressure difference with time during an experiment is shown in Fig. 2 for 3 different milk supplies. The pressure difference remained almost constant for a period, with only a very slight increase, and there was then a relatively quick transition to a much more rapid rise of pressure difference with time.

Quantitatively the pressure difference curves did not reflect sufficiently well the corresponding changes in the amount of deposit found on the plates at the end of the experiment. The comparison of the heat exchanger results with the hot-wire results was therefore based only on deposit weights.

The distribution of deposit weights in the steam heating section for a milk supply giving a moderate amount of deposit is given in Table 1. Normally the maximum amount of deposit was found in the second of the 3 passes. Deposit tended to be

slight at the beginning of the first pass and at the end of the final pass. The general picture agrees with that reported by Lyster (1965) for a commercial plant, where for similar milk pretreatment conditions before the final steam heating section he found the maximum amount of deposit in the second of 5 passes, and virtually no deposit in the final pass.

The relation between deposit weight (in g/gal) and the K value for the corresponding milk as obtained in the hot-wire apparatus is shown in Fig. 3 for all the samples.

Table 1. *Distribution of weights of dried deposit in the steam heating section, for* 10 *gal of milk giving moderate deposits*

Plate no.	Weight, g		Dried deposit, g		
	Before	After	Per plate	Per pass	Total
1*	175·70	176·13	0·43	1·34	
2	178·46	179·37	0·91		
3	185·08	187·03	1·95	4·12	8·42
4	178·43	180·60	2·17		
5	176·81	178·21	1·40	2·96	
6	175·32	176·88	1·56		

* Outlet of steam heating section.
Total deposit weight at final measurement = 8·42 g.
Estimated final deposit weight from extrapolated drying curve = 8·4 g.
Deposit = 0·84 g/gal.

DISCUSSION

Although the pressure difference curves (Fig. 2) could not be used satisfactorily as a measure of the amount of deposit formed in the heat exchanger, their shapes were found to vary in a way indicative of the amount of deposit. In general, variation in the amount of deposit produced by the milk influenced mainly the time at which the transition to a much more rapid rise in pressure occurred. This can be seen by comparing the shapes of the curves in Fig. 2 with the corresponding amounts of deposit formed, as shown in Fig. 3.

It is not possible to say from the available evidence why the pressure difference curves have this shape. It seems unlikely that there is a latent period during which no deposit occurs, and a varying time of transition to a depositing condition. Changes in the hydrodynamic conditions, e.g. Reynolds number and forced turbulence in the inter-plate spaces, with reduction of effective area may be responsible.

There is a strong positive but non-linear relationship between the results of the hot-wire and heat exchanger experiments (Fig. 3). There is some scatter of the experimental results about the mean line estimated by eye. Replicate determinations of the deposit weights and of the K values for the same bulk of milk were not possible because of the time needed to prepare and carry out an experiment. It is, therefore, probable that the scatter is partly experimental. There is some uncertainty in the slope of the line which gives the K value. The accuracy of the determination of the dry deposit weight is high, but the deposit remaining on the plates is not necessarily all the deposit that is formed. Some of the deposit becomes detached and is carried into the cooling sections of the plant. In general, however, the results from the hot-

wire apparatus show whether a certain bulk of milk will give a smaller or greater amount of deposit when used in a heat exchanger.

The non-linear relation between the results of the hot-wire and heat exchanger experiments is probably fundamental and not a chance occurrence. If the results are plotted to give the variation of deposit weight with K^2, as in Fig. 4, a linear relation is obtained and the line passes through the origin. It will be recalled that K is defined as the slope of the experimental line which was found empirically to relate the differential temperature between hot wire and milk ($\Delta\theta$) with (elapsed time)$^{\frac{1}{2}}$ (Burton, 1965). K^2 is therefore the slope of the line that would be obtained if $(\Delta\theta - \Delta\theta_0)^2$ were to be plotted against time, where $\Delta\theta_0$ is the differential temperature

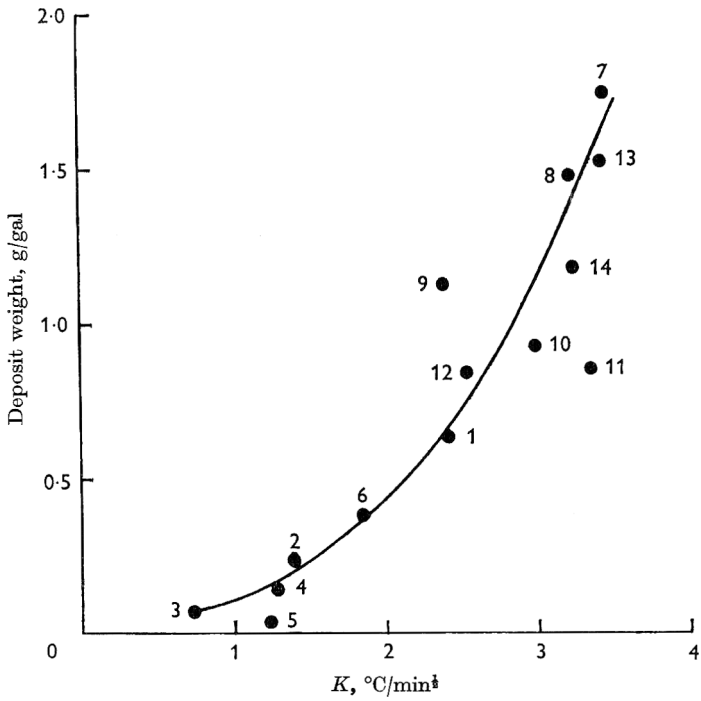

Fig. 3. Relation between deposit weight and K value for several milk supplies detailed below:

Sample no.	Date	Origin	Notes
1	23. iii. 65	Ex-farm bulk tanker	Refrigerated, probably for 24 h
2	4. v. 65	NIRD herd bulk	Fresh
3	5. v. 65	NIRD herd bulk	Stored 24 h at 16 °C
4	6. v. 65	NIRD herd bulk	Stored 48 h at 16 °C
5	2. vi. 65	Ex-farm bulk tanker	Refrigerated, probably for 24 h
6	24. vi. 65	Ex-farm bulk tanker	Refrigerated for at least 48 h
7	16. xii. 65	NIRD herd bulk	Fresh
8	3. ii. 66	NIRD herd bulk	Fresh
9	4. ii. 66	NIRD herd bulk	Refrigerated for 24 h
10	22. ii. 66	Ex-farm bulk tanker	Refrigerated, probably for 24 h
11	23. ii. 66	Ex-farm bulk tanker	Refrigerated, probably for 48 h
12	2. iii. 66	Outside herd bulk	Refrigerated for 24 h
13	15. iii. 66	NIRD herd bulk	Fresh, with addition of 200 mg/10 gal sodium citrate
14	16. iii. 66	NIRD herd bulk	Stored 18 h at 10° C

at the start of the experiment. Therefore, the heat exchanger results are linearly related to a factor which is described by the rate of increase of $(\Delta\theta - \Delta\theta_0)^2$ with time. Unfortunately, $\Delta\theta_0$ cannot be obtained directly from the experimental results, but only by the extrapolation to zero time of the line relating $\Delta\theta$ to $(\text{time})^{\frac{1}{2}}$. It is, therefore, more convenient to derive this new factor as (K^2) from K, where K is as originally defined and determined.

It appears that if a comparison is required of the probable behaviour of 2 milks in the formation of deposits in a practical heat exchanger, the values of K^2 should be compared and not the values of K. The reason why the slope of a line relating $(\Delta\theta - \Delta\theta_0)^2$ and time in the hot-wire experiment should vary linearly with the amount of deposit found in a practical heat exchanger is as yet unknown.

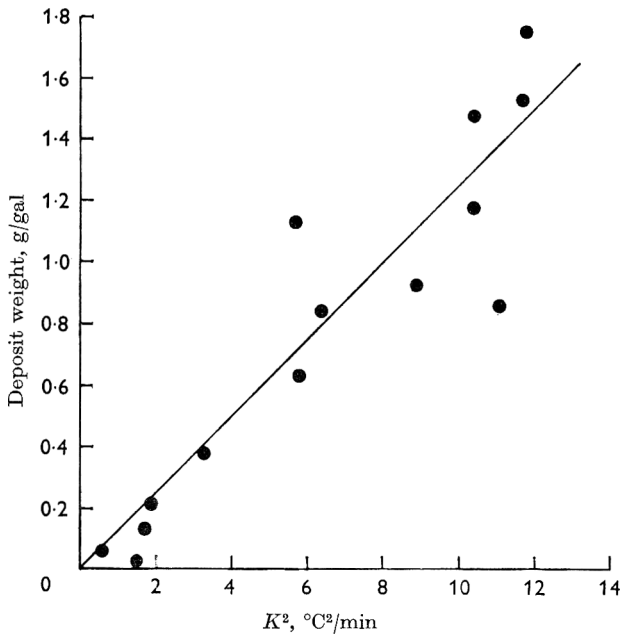

Fig. 4. Relation between deposit weight and K^2 for different milk supplies.

In addition to the comparison between the 2 test methods, the results give preliminary information on some new factors which affect the sensitivity of milk to deposit formation. For example, the results for the milk pairs 2 and 3, 8 and 9 (Fig. 3) show that milk produces less deposit after storage for 24 h than when it is fresh. This effect is shown for milks 2 and 3 in Fig. 2 as well as in Fig. 3. It appears to occur on storage both at refrigerator and ambient temperatures, and it is being studied in other experiments. The sensitivity to deposit formation probably only rises again when bacterial spoilage is proceeding (e.g. sample 4).

A seasonal effect on the amount of deposit formed is also apparent. Samples 2, 7 and 8 from the fresh milk of the Institute's herd showed an increase of about 6 times between early May and the following December–February. Similarly, samples 5 and 10 showed a large increase between early June and the following February for milk

from an ex-farm bulk tanker. Experiments are proceeding to follow this seasonal variation.

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Pediococci in Cheddar cheese

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Summary. Fifty-nine strains of pediococci were isolated as representing the predominant non-starter flora in a series of Cheddar cheeses made over a period of 5 months. All strains had the same physiological characteristics and were identified as *Pediococcus cerevisiae*. A common antigen was found in 54 of the strains, whereas in the other 5 the antigenic component appeared to be related but not the same. Both antigens were located in the cell wall.

All the strains required folinic acid for growth and it was shown with one strain that the small amount of growth which occurred in sterile milk was greatly increased by the presence of the folinic acid producing starter *Streptococcus cremoris* 924.

During a series of experiments with Cheddar cheese, pediococci were frequently isolated in large numbers from control cheeses made in open vats. In 15 out of 16 cheeses pediococci attained numbers as high as 1×10^7/g, whilst lactobacilli, usually the predominant non-starter organisms found in Cheddar cheese, were isolated from only 12 of the cheeses and in smaller numbers. Pediococci have been found previously in Cheddar cheese (Dacre, 1958; Perry & Sharpe, 1960; Franklin & Sharpe, 1963), sometimes forming a high percentage of the lactic acid flora. Their presence over a considerable period of time as the sole or dominant non-starter lactic acid bacteria in cheese has not, however, been reported previously.

Folinic acid has been shown by Sauberlich & Baumann (1948) to be required by *Ped. cerevisiae* (called by them *Leuconostoc citrovorum* strain 8081 and renamed by Felton & Niven (1953)), pasteurized milk containing only sufficient folinic acid to support limited growth of this organism. Møller-Madsen & Jensen (1962) have shown, however, that the starter organisms *Str. lactis* and *Str. cremoris* produce folinic acid and it is suggested by Reiter & Møller-Madsen (1963) that many pediococci found in cheese depend upon starter organisms to provide folinic acid. This hypothesis was investigated and the physiological and serological characters of strains isolated over a period of 5 months were determined.

METHODS

Organisms

Pediococci. Fifty-nine strains were isolated from 16 Cheddar cheeses made in our experimental dairy, which were sampled at 2-, 4-, 12- and 18-week maturation. Colonies of pediococci were picked from acetate agar (Mabbitt & Zielinska, 1956), 1 % tributyrin agar (Franklin & Sharpe, 1963) or from yeast glucose agar. In addition,

2 laboratory strains of *Ped. cerevisiae* (National Collection of Dairy Organisms, NCDO, nos. 813 and 521) were examined. Organisms were cultured in MRS broth (de Man, Rogosa & Sharpe, 1960) and incubated at 30 °C for all tests unless otherwise stated.

Starter. Str. cremoris NCDO 924 was used throughout.

Physiological and biochemical tests

Using methods described by Perry & Sharpe (1960), Franklin & Sharpe (1963) Gunther & White (1961), and Coster & White (1964) strains were tested for the following: catalase production (in MRS broth with only 0·5 % glucose); the presence of a cytochrome containing respiratory system (Deibel & Evans, 1960); the final pH in yeast glucose broth and in MRS broth after 48 h; ability to grow at 45 °C in MRS broth; growth on nutrient agar; growth in yeast glucose broth at pH 9·0 and at pH 4·2; production of ammonia from arginine; hydrolysis of aesculin; production of acetyl methyl carbinol from glucose; reaction in litmus milk; fermentation of arabinose, lactose, dextrin, sucrose and mannitol.

Folinic acid requirement of strains of pediococci and of starter Str. cremoris 924

The casein hydrolysate medium of Eigen & Shockman (1963) supported good growth of the pediococci, but not of *Str. cremoris* 924. However, both species grew well in the complete amino acid medium of Reiter & Oram (1962) which was used for these tests. Unless otherwise stated, the streptococcus 924 was incubated at 22 °C and the pediococci at 30 °C. Daily subcultures were made into litmus milk and MRS broth, respectively. Before assay, each strain was twice subcultured into tryptone broth (0·4 % Difco tryptone being substituted for the amino acids of the defined medium) and 1 drop of the actively growing culture was added to the complete amino acid medium (F) and one drop to the same medium without folinic acid (MF). Absence of growth of a strain after 3 serial transfers in medium MF denoted a folinic acid requirement. The cultures were incubated for 48 h between each subculture.

Production of folinic acid by Str. cremoris 924. After subculturing 3 times in medium MF, a 48-h culture was filtered through sintered glass (porosity 5) and 5 ml of the filtrate diluted with an equal volume of MF medium. Duplicate tubes of this mixture were inoculated with one drop of an 18-h MF culture (first subculture) of a strain of Pediococcus, P3, known to require folinic acid. Controls consisting of uninoculated filtrate and of MF medium without filtrate, inoculated with Pediococcus P3 were included.

Associated growth of Pediococcus P3 *and* Str. cremoris 924 *in defined media.* The first subculture of P3 in medium MF showed a little growth after 18-h incubation due to the transfer of folinic acid with the inoculum. The optical density of this was adjusted to 0·20 with 0·85 % saline and the number of viable organisms determined by surface plating on MRS agar. The number of *Str. cremoris* 924 in medium MF after 18-h incubation was also determined after adjusting the optical density to 0·40 and surface plating on yeast glucose agar (YDA). Fresh 18-h cultures of each organism in MF were then adjusted to 0·2 and 0·4, respectively, and diluted in 0–85 % saline to give a calculated number of 10^3 organisms/ml. Six tubes of medium F (10 ml) and

5 tubes of medium MF were then inoculated with 0·1 ml of each suspension so that each tube contained approximately 10 pediococci + 10 streptococci/ml. The tubes were then incubated at 22 °C. The initial counts of the 2 organisms were checked by plating out a tube of inoculated medium F. Control tubes inoculated with Pediococcus P 3 alone were also prepared.

The numbers of each organism present in each medium during growth was found by plating from a different tube at 6, 24, 48 and 72 h, and 6 days. Total counts were enumerated on YDA and the pediococci were enumerated on the selective acetate agar medium (TAcA) of Rogosa, Mitchell & Wiseman (1951); the numbers of streptococci were determined by dilution counts in litmus milk, as no suitable selective medium was available. An acid clot and a reduction of the litmus was regarded as a positive indication of streptococci, preliminary experiments having shown that pediococci, which produce only slight acid after several days in litmus milk, did not affect the reaction even when streptococci were present in a minority. The TAcA and YDA plates were examined after 5 days at 30 °C, and the LM tubes after 3–4 days at 22 °C.

Associated growth of Pediococcus P 3 *and* Str. cremoris 924 *in milk*

Aseptically drawn sterile raw cow's milk was used as the culture medium. One-day cultures of *Str. cremoris* 924 and of Pediococcus P 3 grown in LM and YDLM, respectively, were diluted 10^{-4} times with 0·85 % saline and 1 drop of each dilution added to 10 ml of milk. Control tubes were inoculated singly with *Str. cremoris* 924 and Pediococcus P 3. All tubes were incubated at 30 °C for 7 days. Where a mixture of organisms was present TAcA and LM were used to enumerate the pediococci and streptococci, respectively. For the control tubes MRSA and YDA were used.

Table 1. *Incidence of pediococci in* 16 *experimental Cheddar cheeses*

Non-starter lactic acid bacteria isolated	No. of cheeses containing pediococci after ripening periods of:			
	2 weeks	4 weeks	12 weeks	18 weeks
Pediococci only	0	5	8	5
Pediococci + lactobacilli	2	2	5	5

Serological methods

Antisera. Antisera were prepared against 2 strains of pediococci (P 3 and P 46) isolated from cheese, using the methods of Sharpe (1955), the organisms being grown in MRS-L broth (Sharpe, Davison & Baddiley, 1964).

HCl extracts of whole cells. Acid extracts of 18-h cultures grown in 10-ml quantities of MRS-Y broth (Sharpe *et al.* 1964) were prepared using Lancefield's (1933) method.

Preparation of cell-wall extracts. Cell-wall preparations of strains P 3, P 46 and P 60 were made using the method of Salton & Horne (1951) as described by Sharpe (1964). Cultures were grown in 400 ml MRS-Y broth and disrupted in the Mickle disintegrator. After separation and washing, lysozyme extracts of the cell walls were prepared (Elliott, 1960) which were then treated with trypsin.

Precipitin ring tests. The method was that of Jones & Shattock (1960).

Gel diffusion test. The slide test as described by Mansi (1958) was used.

RESULTS

Tables 1 and 2 show the incidence and number of pediococci in the 16 experimental cheeses.

Physiological and biochemical characteristics

All the 59 strains of pediococci from cheese were *Ped. cerevisiae* according to the tests used by Gunther & White (1961, 1962) and Coster & White (1964). The 2 NCDO strains differed only in that they did not ferment lactose.

Table 2. *Numbers of pediococci in experimental Cheddar cheese*

No. of cheeses having counts/g in the range of	Age of cheese in weeks			
	2	4	12	18
10^1–10^2	—	1	1	1
10^2–10^3	—	1	—	1
10^3–10^4	1	1	1	1
10^4–10^5	1	1	6	1
10^5–10^6	—	3	5	1
10^6–10^7	—	—	—	5

Requirement of the pediococci and Str. cremoris 924 *for folinic acid*

All the 59 strains of pediococci from cheese and one of the laboratory strains, NCDO 813, required folinic acid for growth, whilst the NCDO strain 521 and the streptococcus had no requirement.

Production of folinic acid by Str. cremoris 924

There was no growth of *Ped. cerevisiae* P 3 in the control second subculture in MF, nor in the uninoculated *Str. cremoris* 924 filtrate. Growth occurred, however, in the MF medium containing *Str. cremoris* 924 filtrate showing that the streptococcus was producing the necessary folinic acid.

Associated growth of Ped. cerevisiae P 3 *and* Str. cremoris 924

Enumeration of *Str. cremoris* 924 in media M and MF was possible up to 72 h using the YDA plates alone as the count of the pediococcus was sufficiently less. Where a mixture of colonies was present they were readily distinguishable. Fig. 1 shows that *Str. cremoris* 924 multiplied at equal rates in media F and MF whereas *Ped. cerevisiae* P 3 multiplied more rapidly in the medium F although the maximum count obtained after 6 days was the same in both media. After 6 days, no viable streptococci were present in the mixed culture in either medium. In medium MF, without streptococci present, the number of viable organisms of *Ped. cerevisiae* P 3 decreased during the 6 days incubation.

Associated growth of Ped. cerevisiae P 3 *and* Str. cremoris 924 *in milk*

When *Ped. cerevisiae* P 3 was grown alone there was only a 60-fold increase in count after a week, whereas in association with *Str. cremoris* 924 there was a 3×10^5-fold increase in count.

Serological reactions

Precipitin ring tests showed that HCl extracts of 54 of the 59 strains reacted strongly with both the *Ped. cerevisiae* P3 and *Ped. cerevisiae* P46 antisera, whilst the other 5 strains reacted only with the P46 antiserum. Results of gel diffusion tests confirmed these findings, extracts of 54 of the strains producing a line of identity with the P3 antisera and extracts of all 59 strains producing a line of identity with the P46 antiserum. Extracts of both NCDO strains gave weak precipitin ring reactions with antiserum P46, but no reaction with the gel diffusion test. Neither extract reacted at all with antiserum to P3.

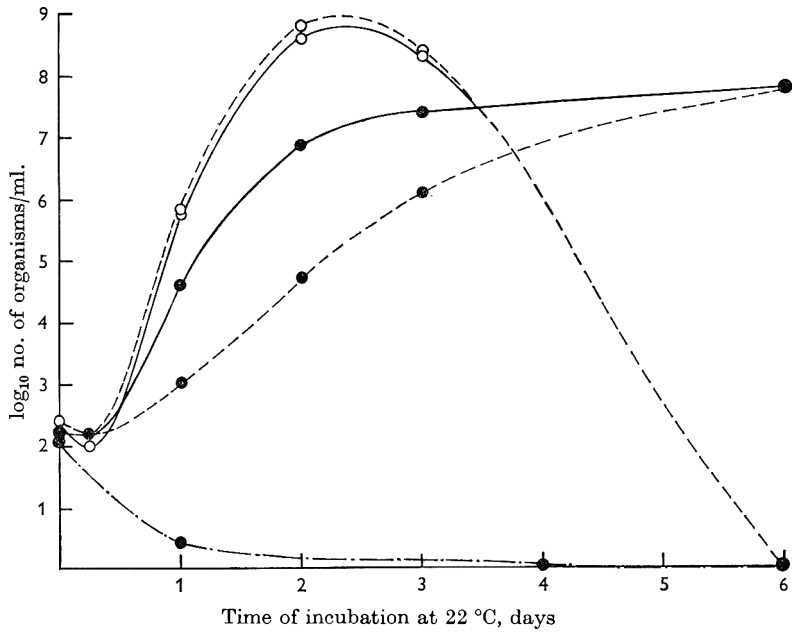

Fig. 1. Counts of *Str. cremoris* 924 and *Ped. cerevisiae* P3 grown in association in a defined medium with and without folinic acid. ○, *Str. cremoris*; ●, *Ped. cerevisiae*; —, plus folinic acid; ----, minus folinic acid; —·—, *Ped. cerevisiae* alone in defined medium without folinic acid.

Location of antigens

Lysozyme extracts of cell walls of *Ped. cerevisiae*, strains P3 and P46 reacted strongly with their respective antisera and continued to do so after treatment with trypsin. Positive results were obtained with both ring precipitin and gel diffusion tests.

DISCUSSION

The morphological, physiological and biochemical characteristics of the 59 strains of pediococci isolated from cheese were identical with each other, and differed from the 2 NCDO strains only in fermenting lactose. All strains were identified as *Ped. cerevisiae* (Coster & White, 1964). Serologically, 2 slightly different groups were apparent, all of the 59 strains possessed a common antigen but 5 strains were further

differentiated from the others in reacting only with the antiserum of *Ped. cerevisiae* P46 and not with that of *Ped. cerevisiae* P3. When extracts of cell walls were prepared by the milder lysozyme treatment and using a much larger quantity of cells an extract of strain P46 reacted, although somewhat weakly, with P3 antiserum. The intensity of the ring precipitin reaction and gel diffusion test and the absence of a reaction with the extracts of the NCDO strains suggest that a type antigen is concerned in these reactions rather than a group reaction. Four of the 5 strains which did not react with antiserum to *Ped. cerevisiae* P3 were isolated from the same cheese and the other one from a cheese made a week later. Strains reacting with antiserum to *Ped. cerevisiae* P3 were also isolated from these 2 cheeses so that this type was present in all 15 cheeses made over a 5-month period.

Associated growth studies using both defined media and milk showed that the starter organism *Str. cremoris* 924 considerably enhanced the growth of *Ped. cerevisiae* P3. Small quantities of folinic acid are almost certainly present in milk and in cheese, although as we have shown for milk the quantity is extremely limiting to the growth of *Ped. cerevisiae* P3, as reported by Sauberlich & Baumann (1948). As postulated by Reiter & Møller-Madsen (1963) the results show that pediococci found in cheese almost certainly depend upon the starter organisms for a major part of their folinic acid requirement. Although the starter organisms which can form folinic acid from suitable amino acids are undoubtedly the chief source of folinic acid, strains of lactobacilli likely to be present in cheese have also been shown by Hendlin, Koditschek & Soars (1953); Mitbander & Sreenivasan (1954); Dighton & Bond (1960) and Zygmunt *et al.* (1962), to convert suitable precursors such as folic acid and compounds related to pteroylmonoglutamic acid (PGA) to folinic acid. Folic acid has been shown by Shahani, Hathaway & Kelly (1962) to be present in Cheddar cheese in the range 4–21 μg/100 g of cheese.

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Phytanic acid and other branched-chain fatty acid constituents of bovine rumen bacteria

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Summary. Phytanic acid, 3,7,11,15-tetramethylhexadecanoic acid, which hitherto had been isolated and identified from several natural sources including butterfat, ox perinephric fat and cow plasma, has now been found present in small amounts (2·9%) in the total fatty acids extracted from the rumen bacteria of a fistulated dairy cow fed a diet of clover–grass hay. This C20 multibranched fatty acid was not detected in the dietary clover–grass hay, and it is considered to have been derived from phytol by enzymic activity of the rumen bacteria.

Phytanic acid was first reported in natural sources by Hansen & Shorland (1951, 1953) who isolated trace amounts of this acid from butterfat and incompletely identified it as a saturated C20 multibranched fatty acid. Subsequently, mass spectra analyses by Bjurstam, Hallgren, Ryhage & Ställberg-Stenhagen (referred to by Stenhagen (1961), by Sonneveld *et al.* (1962) and by Hansen, Shorland & Morrison (1965)) independently identified this constituent as 3,7,11,15-tetramethylhexadecanoic acid. Further interest in phytanic acid was stimulated by the investigations of Klenk & Kahlke (1963), Kahlke (1963, 1964), and subsequently by Steinberg *et al.* (1965), and Hansen (1965*a*) which revealed its presence in substantial amounts in the tissues, blood and urine of humans afflicted with the rare disease described as Refsum's syndrome (Heredopathia atactica polyneuritiformis). Phytanic acid has also been isolated and identified from ox perinephric fat (Hansen, Shorland & Cooke, 1958; Hansen, 1965*b*), ox plasma (Lough, 1963, 1964; Duncan & Garton, 1963), sheep fat (Hansen, 1965*c*) and crude petroleum (Cason & Graham, 1965). The purpose of the investigation now reported was as follows:

(1) To determine if the C20 and C19 multibranched acids occurred in the rumen bacteria of a cow on a diet of clover-grass hay.

(2) To attempt to elucidate the biosynthetic origin of these 2 terpenoid acids.

A note on this work recording the presence of phytanic acid in the rumen bacteria of a cow, was published earlier (Hansen, 1966*a*).

MATERIALS AND METHODS

Rumen bacteria from a fistulated cow and a sample of the clover–grass hay fed to the cow were kindly supplied by Dr R. W. Bailey, Plant Chemistry Division, D.S.I.R., Palmerston North.

Rumen bacteria. Rumen bacteria (14·13 g), free from protozoa, were obtained by removing 2–3 l of rumen liquor periodically from a fistulated cow fed clover–grass hay, and straining it through muslin. The liquor was then centrifuged for 5 min at 500–800 rev/min and the residue of protozoa and large plant debris was discarded. The cloudy supernatant liquid was centrifuged at 25000 ***g*** and the clear supernatant liquid removed. The residue consisted of a slimy yellow layer of mixed rumen bacteria on top of a darker layer of bacteria plus plant debris. The slimy yellow layer was carefully scraped off and freeze dried. The yield was about 1 g of freeze-dried solid/l of crude rumen liquor.

The rumen bacteria (14·13 g) were extracted 3 times with 10 vol. chloroform:methanol mixture (2:1, v/v) and yielded 3·10 g total lipids, the light petroleum (b.p. 60–80 °C) soluble part (1·18 g) of which was saponified with ethanolic KOH. The unsaponifiable matter (0·34 g) was removed with ethyl ether. The soaps were converted to fatty acids (0·64 g) and these were refluxed for 4 h with methanol containing 3 % (w/v) conc. H_2SO_4 to yield 0·47 g methyl esters.

As the small amount of material available precluded the possibility of isolating and identifying the constituent branched-chain fatty acids, low-temperature crystallization from solvent and column chromatography were employed to provide fractions enriched in branched-chain acids. The bacterial methyl esters (0·47 g) were dissolved in 20 vol. acetone and crystallized at −39 °C to yield a soluble fraction (0·30 g) and an insoluble fraction (0·16 g). The soluble fraction was crystallized from 20 vol. acetone at −44 °C, and gave a soluble part (0·27 g). In order to separate saturated from unsaturated constituents and also to concentrate the branched-chain components, the acetone-soluble methyl esters (0·27 g) were converted to mercuric acetate adducts by the method reported by Kishimoto & Radin (1959). This adduct mixture (0·20 g) was extracted with light petroleum to remove the unconverted esters (saturated and branched-chain) and then applied to a column 4 cm × 1·2 cm containing 2·6 g activated Florisil, and eluted with 400 ml light petroleum. The mercuric acid adduct which contained the unsaturated components was dissolved in a mixture of ethyl ether–chloroform–hydrochloric acid (10:8:1, v/v), transferred to the Florisil column and eluted with 57 ml of this solvent mixture.

The saturated normal and branched-chain methyl esters emerged from the Florisil column as 4 fractions (total weight 0·09 g) while the unsaturated material was collected as one fraction (0·10 g).

Clover–grass hay (*P* 419)

The hay sample comprised clover and grass. Approximately 2·25 kg of hay was milled in a Christie Norris mill and then extracted 3 times in a Waring blender with a mixture of chloroform:methanol (2:1, v/v) to yield 182·0 g extract. After saponification, the lipids were freed of unsaponifiable matter, converted to fatty acids and then esterified by refluxing for 4 h with methanol containing 3 % (w/v) conc. H_2SO_4. The methyl esters were dissolved in light petroleum (b.p. 60–80°) and decolorized with activated charcoal. To facilitate identification of the minor and trace fatty acid constituents, concentrates of these components were prepared. The methyl esters were first crystallized from 20 vol. acetone at −44 °C and a sample of the acetone-soluble fraction was hydrogenated and then recrystallized from 20 vol. acetone at −40 °C.

The resulting acetone-soluble and acetone-insoluble fractions, respectively, were further resolved by crystallization from 20 vol. acetone at −40 °C. All fractions were gas chromatographed and comparison of the chromatograms of the acetone-soluble esters before and after hydrogenation facilitated the identification and determination of the percentage content of the constituent fatty acids.

The analytical gas-liquid chromatograph used in the present investigation was constructed in this laboratory and incorporates the ^{90}Sr detector introduced by Lovelock, James & Piper (1959). Glass columns 2·4 m in length and 6·5 mm in internal diam. were employed, and were packed with celite 545, 30–80 mesh (B.D.H. England), impregnated with (*a*) 20% (w/w) polyethylene glycol adipate and (*b*) 5% (w/w), Apiezon L. Polyester columns were siliconized but the Apiezon L columns were not. All gas chromatograph analyses were carried out with both liquid phases. In addition, to confirm the identity and relative proportions of the higher molecular weight n-saturated fatty acids from C19 to C26, relevant fractions were applied to a glass column (2·4 m × 6·5 mm) packed with glass beads (80-mesh) coated with 0·25% (w/w) silicone high vacuum grease prepared for this purpose by the method of Nelson & Milun (1960). Argon was used as carrier gas and the operating temperature was 207 °C. Relative retention times (V_R) were determined on methyl esters and are relative to methyl stearate.

Identification of peaks was based on relative retention times earlier established for the methyl esters of n-saturated and n-unsaturated acids (Farquhar, Insull, Rosen, Stoffel & Ahrens, 1959), singly branched iso and anteiso acids (Hawke, Hansen & Shorland, 1959) and for multibranched acids (Hansen & Morrison, 1964; Hansen *et al.* 1965). Calculations of percentage fatty acids composition were based on component peak areas, and these were measured with a planimeter.

RESULTS

Fatty acid composition of total lipids of rumen bacteria (*P*418)

In Table 1 is recorded the fatty acid composition determined by gas-liquid chromatography of the total fatty acids extracted from the sample of rumen bacteria. In order to facilitate identification of minor peaks, particularly those representing branched-chain and lower n-odd-numbered acids, all fractions were chromatographed including those in which certain components had been concentrated by techniques described in the experimental section. The first fraction eluted from the Florisil column was found to include 46·6% of C15 branched acids (iso and anteiso), 15·0% phytanic acid, 12·0% C17 branched acids and 9·0% n-pentadecanoic acid. Evidence corroborating the identity of the phytanic acid peak was provided by adding to the fraction a measured amount of phytanic acid previously isolated from ox perinephric fat and identified as 3,7,11,15-tetramethylhexadecanoic acid (Hansen, 1965*b*), and applying the mixture to both polyester and Apiezon L columns. In each case the added component emerged with the peak thought to represent phytanic acid in the rumen bacterial lipids.

The most marked features of the composition of the total fatty acids extracted from rumen bacteria (see Table 1) were as follows:

(1) The fatty acids contained 2·8% phytanic acid and a total of 25·7% of con-

stituent C13, C14, C15, C16, C17, C18 and C20 branched-chain fatty acids. It is presumed that the C14, C16 and C18 components were iso-acids since anteiso even-numbered fatty acids have not been isolated from natural sources. The odd-numbered C13, C15 and C17, respectively, branched-chain acids probably contained both the iso and the anteiso isomers, for both have been isolated from natural fats, and in ruminant body and milk fats (Hansen, Shorland & Cooke, 1953, 1958; Shorland & Hansen (1957) they were found to occur together. From wool grease, however, Weitkamp (1945) reported only even-numbered iso acids.

(2) An appreciable proportion (11·4 %) of n-odd-numbered saturated fatty acids was present. This series extended from C13 to C25, the C15 component (8·4 %) being the major one, followed by C17 (1·8 %) and C13 (1·1 %). The C19–C25 constituents were present in trace amounts only.

(3) A very high content (16·0 %) of C15 branched-chain fatty acids was detected.

(4) The more commonly distributed fatty acids, stearic (6·9 %), oleic (11·3 %), linoleic (4·1 %) and linolenic (1·2 %), were present in relatively small proportions.

(5) Palmitic acid occurred to the extent of 30·0 % of the total fatty acids, while

Table 1. *Percentage composition of total fatty acids of rumen bacteria and of dietary clover–grass hay, determined by gas-liquid chromatography* (^{90}Sr *ionization detector*) *with polyethylene glycol adipate liquid phase* (*EGA*) *at* 207 °C

Fatty acids*	V_R (20% EGA 207 °C)	Rumen bacteria (P 418), %	Clover-grass-hay (P 419), %	Fatty acids*	V_R (20% EGA 207 °C)	Rumen bacteria (P 418), %	Clover-grass hay (P 419), %
8:0	0·06	—	0·3	18 Br.	Iso 0·87	0·1	—
10:0	0·10	—	Trace	18:0	1·00	6·9	9·3
11:0	0·14	—	Trace	18:1	1·12	11·3	5·1
12:0	0·18	0·7	0·6	19:0	1·33	Trace	0·2
13 Br.	0·21	0·7	Trace	18:2	1·34	4·1	18·9
13:0	0·24	1·1	Trace	18:3	1·71	1·2	26·6
14 Br.	Iso 0·27	1·7	Trace	20:0	1·75	0·5	2·8
14:0	0·32	4·1	0·7	20:1	1·96	1·4	0·4
15 Br.	Iso 0·37	16·0	0·1	20:2	2·25	Trace	0·1
	Anteiso 0·39			21:0	2·29	Trace	0·1
15:0	0·42	8·4	0·3	20:3	2·64	0·9	0·5
15:1	0·48	—	Trace	20:4	2·91	0·2	—
16 Br.	Iso 0·49	1·1	0·1	22:0	3·02	0·2	1·7
16:0	0·57	30·0	24·5	23:0	4·00	Trace	1·1
16:1	0·65	—	1·0	24:0	5·27	0·2	1·4
17 Br.	Iso 0·66	3·3	0·2	25:0	6·92	0·1	1·0
	Anteiso 0·69						
20 Br.	0·72	2·8	—	26:0	9·12	0·7	1·4
17:0	0·75	1·8	1·4	Unidentified	—	0·5	—
17:1	0·85	—	0·2	Above 22:0	—	—	—

* In designation of fatty acids, the numeral preceding the colon indicates the number of carbon atoms, and that after, the number of doublod bonds. Br. = branched.

palmitoleic acid (16:1) was absent. This may be significant of hydrogenation in the rumen, as has been established by Reiser (1951), Hartman, Shorland & McDonald (1954) and Shorland, Weenink, Johns & McDonald (1957).

(6) A relatively low content (19·1 %) of unsaturated fatty acids was present in contrast to the unusually high content (25·7 %) of branched-chain fatty acid constituents. Included among the unsaturated acids detected was a trace amount (0·2 %) of arachidonic acid (20:4). This acid had formerly been found, *inter alia*, in protozoa (Haines, Aaronson, Gellerman & Schlenk, 1962).

(7) The C19 multibranched acid 2,6,10,14-tetramethylpentadecanoic acid was not detected.

Fatty acid composition of total lipids of clover–grass hay (*P* 419)

The methyl esters of the total lipids of clover–grass hay (see Table 1) when examined by gas chromatography were found to contain as major constituents, palmitic acid (24·5 %), linolenic acid (26·6 %), linoleic acid (18·9 %) and oleic acid (5·1 %). Examination of the concentrates of branched-chain and lower n-saturated acids, and of higher molecular weight n-saturated acids prepared by hydrogenation and low-temperature crystallization from acetone, established that the sample of clover–grass hay (P. 419) contained only trace amounts (0·4 %) of branched-chain fatty acids, and small proportions (9·7 %) of high molecular weight n-odd- and even-numbered components from C19 to C26 inclusive. Also present, were other n-even- and odd-numbered saturated and unsaturated fatty acids and volatile fatty acids C8–C12 (0·9 %). Gas chromatography of the acetone-soluble concentrate indicated the presence of (1) C13, C14, C15, C16 and C17 branched-chain acids (2) C8, C10, C11 and C12 volatile n-saturated fatty acids (3) C11, C13, C15, C17, C19, C21, C23 and C25 n-odd-numbered acids and (4) C8, C10, C12, C14, C16, C18, C20, C22, C24 and C26 n-even-numbered saturated fatty acids. The content of palmitic acid (24·5 %) approximates that normally found in ruminant body fats. Although the C15 branched acid was shown by the polyester liquid phase to be a mixture of 2 components incompletely resolved, viz. the C15 anteiso-acid (+)-12-methyltetradecanoic acid which predominated in amount, and the C15 iso-acid 13-methyltetradecanoic acid, the other odd-numbered branched acids were not resolved and appeared as one peak only, with relative retention times which suggested their respective identities as being C13 (iso) and C17 (anteiso). However, as isomeric odd-numbered branched-chain fatty acids have been found in natural fats with the side methyl in both the iso and anteiso positions, and as both isomers have normally accompanied each other, it is possible that in the present studies small proportions of one isomer were obscured by large proportions of the other. Even-numbered iso-acids, but not even-numbered anteiso-acids, have been isolated from natural sources (Weitkamp, 1945; Hansen *et al.* 1958), so it is probable that the C14 and C16 branched-chain acids detected in trace amounts in this investigation of hay fatty acids were both of the iso-configuration. The C20 multibranched fatty acid, 3,7,11,15-tetramethylhexadecanoic acid, was not detected in the lipids of clover–grass hay, nor was the C19 homologue 2,6,10,14-tetramethylpentadecanoic acid.

It may be of significance that one of the fractions of clover–grass hay (P 419) fatty acids which resulted from hydrogenation and repeated low-temperature crystalliza-

tion from acetone, and in which the liquid branched-chain acids were concentrated, was shown by gas chromatography to contain small amounts of a constituent whose retention times corresponded with those of a mixture of 8–14-methoxystearic acid isomers recently isolated and identified from sheep perinephric fat (Hansen, 1966*b*, Hansen & Smith, 1966). In sheep fat, this mixture of 8–14-methoxystearic acid isomers was postulated to have arisen as an artifact by rigorous esterification of total fatty acids which were presumed to have contained small amounts of hydroxy constituents.

DISCUSSION

Phytanic acid, which formerly had been found, *inter alia*, in the fat of oxen and the blood and milk of dairy cows, has now been shown to be present in the rumen bacteria of a cow. This tetramethyl fatty acid was not detected by gas-chromatography in the clover–grass hay on which the fistulated experimental cow was being fed at the time of withdrawal of samples of rumen digesta, thus indicating that it was not derived by direct assimilation from the diet.

The occurrence of branched-chain fatty facids in bacterial lipids has been the subject of a number of reviews (cf. Anderson, 1941; Asselineau & Lederer, 1953; Markley, 1960; Shorland, 1963; Garton, 1963; Hilditch & Williams, 1964). The branched fatty acids earlier discovered in bacterial lipids, e.g. tuberculostearic acid (Anderson & Chargaff, 1929) lactobacillic acid (Hofmann, Lucas & Sax, 1952), mycolic acid (Asselineau & Lederer, 1953) possess unique structures, in most cases peculiar to the species of bacteria to which each belongs, but unlike those of the iso- and anteiso- (Weitkamp, 1945; Hansen, Shorland & Cooke, 1952, 1958; Shorland & Hansen, 1957) and the multibranched acids (Hansen & Shorland, 1951, 1953; Hansen & Morrison, 1964; Hansen *et al.* 1965) found in wool grease and in body and milk fats. Akashi & Saito (1960), however, in their studies on microbial lipids, reported the occurrence of the optically active C15 anteiso-acid in a strain of *Sarcina*, and Saito (1960) reported the C15 iso- and the C17 iso-acids in *Bacillus subtilis*. Whereas the corresponding acids were present in trace amounts only in ruminant milk and body fats, in the microbial lipids reported they represented substantial proportions of the total fatty acids. In *B. subtilis* the C15 iso-acid constituted approximately 50% of the total fatty acids and the C17 iso-acid approximately 15%. Allison, Bryant, Katz & Keeney, (1962), identified the major fatty acid in the vegetative cells of *B. subtilis* as the C15 anteiso-acid rather than the C15 iso-acid. Likewise, Kaneda (1963) found the major components to be C15 and C17 anteiso-acids). Akashi & Saito (1960) suggested that the iso-and anteiso-branched-chain acids found in minute amounts in the body lipids of sheep and oxen (Hansen *et al.* 1953, 1958) may have originated partly from the lipids of micro-organisms residing in the gut of the host. Subsequent investigations by Macfarlane (1961*a*, *b*, 1962), Lennarz (1961), Keeney, Katz & Allison (1962), Allison *et al.* (1962), Kaneda (1963), Etemadi (1963), Ballio, Barcellona & Boniforti (1965) and others, further extended the number of species of micro-organisms whose lipids contained the C15 and the C17 iso- and anteiso-acids as major constituents. Other branched acids reported as being detected in the lipids of micro-organisms were the C13, C14 and C16 components. Furthermore, Keeney *et al.* (1962) and Allison *et al.* (1962) reported the

presence of the C13, C14, C15, C16 and C17 branched-chain fatty acids in a strain of *Ruminococcus flavefaciens*, a cellulose-decomposing bacterium from the rumen of sheep and cattle. Multibranched fatty acids, however, were not detected in their investigations.

The studies now reported on rumen bacterial lipids disclosed the occurrence of the C18 iso-acid (0·1 %) and the C20 multibranched acid (2·8 %) in addition to those branched-chain constituents already referred to above.

From experiments with a lactating cow, Keeney *et al.* (1962) deduced that rumen digesta were the major sources of the singly branched-chain acids found in ruminant milk and body fats, and that these acids were derived from volatile fatty acids by bacterial synthesis. These authors estimated that the amount of C15 branched acid in the cow's microbial flora could account for more than half of the C15 branched acid in butterfat, and they interpreted their work as supporting the suggestion of Akashi & Saito (1960) that the branched-chain acids of animal fats are derived from bacterial lipids in the gut. Similarly, Kuzdzal-Savoie (1964), who made detailed gas chromatographic analyses of the food consumed by dairy cows and of the butterfat produced during the experimental period of feeding, reported that a particular ration comprising beet, ensilage, hay and a grain-based concentrate contained in its total fatty acids trace amounts of C12 (0·1 %), C13 (0·3 %), C14 (0·2 %), C15 (0·2 %), C16 (0·3 %) and C18 (0·06 %) branched-chain acids, while the milk fatty acids contained considerably larger proportions of C13 (0·2 %), C14 (0·3 %), C15 (0·8 %), C16 (0·5 %), C17 (1·1 %) and C18 (0·3 %) branched acids. The conclusions drawn from this work were that the quantity of branched-chain acids secreted in the milk is greater than the amount ingested from the diet, and that dietary sources could be reponsible for only a very small fraction of the branched acids found in milk fat.

In the investigation now reported, the content of branched-chain acids in the sample of rumen bacteria analysed, represented 25·7 % of the total weight of fatty acids, and is comparable with that reported by Keeney *et al.* (1962), whose figures when extrapolated indicate approximately 15·6 % branched-chain acids in the lipids of the rumen bacteria and an additional 5 % in the rumen protozoa. In the present work, the rumen protozoa were required for another project and examination of their fatty acid composition was not carried out. The clover–grass hay fatty acids (P419), however, contained a total of only 0·4 % branched-chain acids and this level is in line with the traces detected in rye grass (Shorland, 1961), lucerne (Vandeweghe & Kuzdzal-Savoie, 1964) and other grasses (Kuzdzal-Savoie, 1964). In ensilage, Kuzdzal-Savoie (1964) found that branched acids comprised approximately 2 % of the total weight of fatty acids, but this higher figure the author attributed to changes brought about by microbial fermentation.

The C20 multibranched fatty acid, 3,7,11,15-tetramethylhexadecanoic acid, and its C19 homologue 2,6,10,14-tetramethylpentadecanoic acid, possess a chemical structure unlike that of the singly branched acids, but one which bears a close relationship with the isoprenoid alcohol phytol which constitutes about 30 % of the chlorophyll molecule of green herbage.

The C19 multibranched fatty acid 2,6,10,14-tetramethylpentadecanoic acid, which has been isolated and identified from butterfat (Hansen & Morrison, 1964),

sheep perinephric fat (Hansen, 1965*d*), and from crude petroleum (Cason & Graham, 1965), was not detected in either the rumen bacteria or the clover–grass hay. Since it has the same basic isoprenoid structure as phytanic acid, and since in butterfat, sheep fat and crude petroleum, both the C19 and C20 tetramethyl fatty acids have been found present together, the absence of the C19 fatty acid from rumen bacteria suggests the possibility of its biosynthesis being different from that of its C20 multi-branched homologue. The optical rotatory dispersion values shown in Table 2 support the feasibility of different biosynthetic mechanisms for these 2 fatty acids, for whereas the C20 acid from butterfat and from ox perinephric fat has a small positive value, the C19 acid from butterfat and from sheep perinephric fat exhibits a negative rotation. It may also be of significance that phytanic acid from cow plasma has a negative optical rotation (Lough, 1964) as has the synthetic phytanic acid prepared in this laboratory from phytol (see Table 2). The C20 multibranched fatty acid from Refsum's syndrome tissues (Hansen, 1965*a*), on the other hand, has a positive rotation and is of greater magnitude than that from either butterfat or ox fat.

Table 2. *Optical rotatory dispersion measurements* of C19 and C20 acids from different sources*

Acid	Source	Molecular rotation (ϕ)	Reference
3,7,11,15-Tetramethyl-hexadecanoic acid	Butterfat	233 mμ +70° 250 mμ +45° 300 mμ + 8° 400 mμ + 4° 500 mμ + 4°	Hansen *et al.* (1965)
3,7,11,15-Tetramethyl hexadecanoic acid	Ox perinephric fat	251 mμ +2·4° 300 mμ +3·6°	Hansen (1965*b*)
3,7,11,15-Tetramethyl-hexadecanoic acid	Refsum's syndrome kidney	286 mμ +44° 333 mμ +11°	Hansen (1965*a*)
3,7,11,15-Tetramethyl-hexadecanoic acid	Synthesized from phytol	312 mμ −4·1° 400 mμ −3·0° 500 mμ −2·2°	Hansen, Shorland & Prior (1966)
3,7,11,15-Tetramethyl-hexadecanoic acid	Ox plasma	$[\alpha]_{500}^{20}$ −3·8°	Lough (1964)
2,6,10,14-Tetramethyl pentadecanoic acid	Butterfat	238 mμ −120° 250 mμ − 55°	Hansen & Morrison (1964) Not previously reported)
2,6,10,14-Tetramethyl-pentadecanoic acid	Sheep perinephric fat	238 mμ − 80° 286 mμ − 45° 400 mμ − 15°	Not previously reported
Phytol	B.D.H. used in synthesis	Small negative rotation. Further penetration impossible due to unsaturation	Not previously reported

* These measurements were kindly made by Dr P. M. Scopes, Chemistry Department, Westfield College, University of London, England

Steinberg, Avigan, Mize & Baxter (1965*a*, *b*) demonstrated that when phytol was fed at high levels to rats it was absorbed and readily converted in part to phytanic acid. They also showed that phytol, and/or its metabolic products, were rapidly

oxidized to CO_2. Similarly, Steinberg, Avigan, Mize, Eldjarn, Try & Refsum (1965) and Stoffel & Kahlke (1965) independently illustrated that a corresponding conversion of phytol to phytanic acid occurred when phytol was fed to normal humans and to humans afflicted with Refsum's syndrome; but whereas in the former subjects, the phytanic acid was rapidly oxidized to CO_2, in the latter it accumulated in the body tissue lipids. The ease with which phytol is converted in the laboratory to phytanic acid by hydrogenation of the double bond followed by oxidation of the alcohol group (cf. Karrer & Epprecht, 1940), suggests that such changes may be brought about by enzyme systems associated with bacterial flora. That hydrogenation takes place in the rumen is well established (Reiser, 1951; Hartman *et al.* 1954; Shorland *et al.* 1957). Sörensen & Sörensen (1949), in discussing the origin of the hydrocarbon pristane from shark liver oil, postulated that enzymic processes of the bacterial flora of dietary zooplankton oxidize the primary alcohol group of phytol to a carboxyl group and also hydrogenate the allylic group to yield phytanic acid. Presumably, the phytanic acid now reported from rumen bacteria is synthesized within the tissues of the rumen micro-organisms from the preformed phytol moiety of chlorophyll, and is subsequently assimilated into the body and milk fats of the host ruminant. The singly branched iso- and anteiso-acids, on the other hand, are apparently synthesized by enzymic action from the lower volatile branched-chain acids by the addition of 2 carbon units (Allison *et al.* 1962). The investigation now reported provides further evidence that the branched-chain fatty acids of ruminant milk and body fats are derived mainly from rumen bacteria and not from pasture.

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Reviews of the progress of dairy science

Section B. Recent developments affecting the Cheddar cheese-making process

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CONTENTS

SCOPE OF REVIEW

Except for the book *Cheese* by J. G. Davis (1965*a*), no other review covering the whole field of Cheddar cheese-making has appeared in recent years. Reviews have appeared on some subjects related to the Cheddar-making process (Park, 1951; Storgårds, 1954; Harper & Kristoffersen, 1956; Kosikowski & Mocquot, 1958; Czulak, 1958; Kosikowski, 1959; Mabbitt, 1961; Michie & Fenton, 1963; Eisenreich, 1963, 1965; Reiter & Møller-Madsen, 1963; Robertson, 1965*a*), on processes for making other types of cheese which bear on Cheddar cheese manufacture (Zeiler, 1950; Anon. 1956; Eisenreich, 1958; Swartling, 1963; Anon. 1963), and patent developments in the cheese industry (Irvine & Price, 1952). Several thousand articles, papers and patents concerned with the Cheddar cheese-making process have been published in recent years and for review purposes a selection has been made. In many instances the references cited do not necessarily represent all the work on the topic with which they deal.

The review is concerned primarily with developments in the last 15 years with particular emphasis on the last decade. Simple mechanical inventions and innovations have with a few exceptions been excluded from consideration. Most attention is given to developments which have applied directly or indirectly to the manufacture of Cheddar cheese, but developments applied to other types of cheese are also reviewed where these appear to be of significance to Cheddar cheese-making. Apart from the introduction the survey is in two parts. The first is concerned with general developments and ideas which impinge on cheese-making; for example, the effect of heat treatment on the suitability of milk for cheese-making and the suitability of various proteolytic enzymes as coagulants in cheese-making. The second part considers developments in the cheese-making process proper.

INTRODUCTION

The main streams of activity concerning Cheddar cheese-making have, in the last few decades, been directed towards improving uniformity and quality, and to reducing manufacturing labour costs through mechanization of parts of the process. Another development of considerable importance has been the widespread replacement of the traditional rinded form of Cheddar cheese by a rindless, film-wrapped cheese of rectilinear shape. The Kraft Cheese Co. of the U.S.A. was, in large measure, responsible for the introduction of the rindless form of Cheddar cheese.

Outline of the basic making process

The following description of the Cheddar-making process as generally practised a decade or so ago will provide a background against which the more recent developments may be viewed; the basic principles have changed little since factory, as opposed to farmhouse, cheese-making originated a little over 100 years ago in North America (Anon. 1964*b*).

The raw cheesemilk was usually preheated or 'flashed' in a heat treatment less than that required for 'pasteurization', and then cooled to a setting temperature of about 88 °F before passing into jacketed tinned-steel rectangular vats of about 800 to 1000-gal capacity. Some 2–3 % of a coagulated starter culture (a pair of pure strains of *Streptococcus lactis* or *Str. cremoris* in New Zealand and Australia, and an unknown mixture of lactic streptococci in most other countries) was added to the vat during filling. Once full, the vat was 'ripened' for 20 min or longer before the addition of rennet. When the coagulum was of the desired firmness (some 30–40 min later) it was cut into small cubes by 'knives' with many blades, or a grid of wires. The vat was stirred to keep the cubes of curd in suspension and the temperature of the curds and whey was gradually raised to a 'cooking' temperature of 100–104 °F. This, along with the action of rennet and increasing acidity, assisted curd contraction, or syneresis, and the expulsion of moisture from the curd. The cooking temperature used frequently represented a compromise between inhibition of growth of starter bacteria and the attaining of maximum moisture expulsion by this agency.

About $2\frac{1}{2}$–3 h from renneting, acid development and the increase in plasticity of the curd was sufficient for the whey to be drained from the curd, drainage being assisted by dry-stirring to prevent matting together of the curd particles. When the

moisture and acidity of the whey were suitable, the curd was banked along the sides of the vat and allowed to fuse together. This part of the process was known as 'cheddaring' and was the most important difference between Cheddar cheese-making and the manufacture of other types of cheese. The matted curd was cut into 10 to 20-lb blocks and 'flow' induced within the cheddar blocks by turning and stacking them upon one another. The importance attached to flow varied markedly from factory to factory and from country to country. In Britain, it was common for each cheddar block to be made to spread into a thin, hide-like sheet covering an area of several square feet, whereas in New Zealand, only moderate flow was induced, the final cheddar block being little different in dimensions from when first cut.

Some 2–3½ h after draining the curd, the expressed whey reached an acidity equivalent to 0·70–0·90 % lactic acid, and the cheddared curd was milled into finger-sized pieces. About ½–1½ h after milling, salt was distributed evenly over the curd at a rate (about 3–5 %) which previous experience had shown to give a satisfactory 'body' in the maturing cheese. Once the salt had been incorporated in the curd, and some further moisture lost, the curd was packed in cylindrical hoops lined with cloth and pressed overnight in a gang-press. Hot water poured over the outside of the hoops assisted the curd to penetrate the bandage and form a rind. After pressing the cheese were transferred to cool, airy, curing rooms where they were held, with occasional turning, until of desired maturity. This often took 6 months or longer.

Broad changes in recent years

Much of the process has remained as described above. Only the more general changes which have become widely adopted in recent years are noted here; the more detailed changes are described later.

Tinned-steel vats have generally been replaced by stainless steel vats, and hand stirring or forking of the curd has been replaced by electrically powered stirring with stirrers of various designs.

High-temperature short-time pasteurized milk has become widely used for cheese-making.

Starter cultures composed of unknown mixtures of lactic streptococci have been widely replaced by single strains, or defined mixtures of pure strains. Associated with the use of these more active strains is a reduction in the proportion of starter used and the time taken for the whole cheese-making process. The acidities used as the criteria for running the vats, milling, and salting the curd have decreased considerably.

The importance of cheddaring and the need for extensive flow has been widely questioned.

Cheeses are now most often pressed in rectilinear, stainless steel, hoops in pneumatically operated presses, and brief vacuum pressing before or after dressing is being adopted as a method of improving the texture of the finished cheese.

The traditional cylindrical rinded cheese with its inherent loss in weight during maturation and tendency to rind faults has been extensively displaced by a rindless form enclosed in an oxygen impermeable film or bag. The rind of the traditional cheese is now commonly improved by a heavy coating of a flexible wax applied as soon as the surface of the cheese is sufficiently dry.

Important new trends

Many recent investigations have had as their goal a reduction in manufacturing costs either by speeding up the making process or by reducing the high labour content of the normal process through extensive mechanization.

The approach of speeding up the making process was initiated, albeit unwittingly, some 30 years ago by the studies of Whitehead and his associates into the use of pure cultures of lactic acid bacteria as starter. The relatively vigorous acid production by many of these single strain starters appreciably reduced the length of the cheese-making day. More recent modifications to the making process *per se* have included the use of more starter, the use of special thermoduric and salt-tolerant starters and the elimination of cheddaring.

Attempts to reduce manufacturing costs through mechanization have been of two types. On the one hand there has been piecemeal mechanization of individual manually performed tasks and, on the other, efforts to develop flow-line cheese-making processes amenable to integrated mechanical handling or automation. For the study of mechanization the cheese-making process may be conveniently divided into 4 stages:

(1) Pasteurization of milk, to curd cooked and ready for draining.
(2) Draining the curd, to salted curd ready for hooping.
(3) Hooping, to the pressed cheese wrapped and ready for curing.
(4) Curing or maturing.

By far the greatest effort has been devoted to the development of continuous or semi-continuous mechanization of stage (2). Moderate attention has been given to stage (1), rather less to stage (3) and almost none to stage (4). The distribution of effort is somewhat illogical for about half the labour involved in the cheese-making process is concerned with stage (3) whilst stage (2), which has received much attention in the main Cheddar cheese-producing countries, is concerned with only about a quarter of the total manhours expended.

These new developments are considered in detail later.

GENERAL DEVELOPMENTS EFFECTING CHEESE-MAKING

Milk for cheese-making

Consideration of the influence of milk composition and the bacteriological quality of milk on cheese-making is outside the scope of the present review. The desirability of bulking milk supplies as much as possible before cheese-making has been recognized for many years, and Chapman & Harrison (1963) have recently pointed out that the effect of antibiotics and other inhibitors can be reduced by bulking of milk.

The influence of the natural inhibitors of milk on starter growth has been reviewed by Reiter & Møller-Madsen (1963). The discussion in this section is, therefore, confined to other inhibitory substances and the influence of various pre-treatments on the suitability of milk for cheese-making.

Antibiotics and similar inhibitory substances

In the early years of antibiotic therapy, Hunter (1949*a*, *b*) demonstrated that the lactic streptococci employed as starters for the manufacture of Cheddar cheese were sensitive to the action of penicillin. In subsequent years the range of antibiotics and antibacterial compounds has increased widely and many workers have attested to the resultant difficulties associated with the production of cheese (Whitehead & Lane, 1956; Thomas, Panes & Lewis, 1956; Ridgeway, 1960). Surveys conducted in various countries (Kosikowski, Henningson & Silverman, 1952; Berridge, 1956; McGillivray, Robertson & Liddell, 1966) revealed a high incidence of antibiotics at levels likely to interfere with cheese-starter growth, and routine methods of testing directly (Crawford & Galloway, 1964; McGillivray *et al.* 1966; Anon. 1966; Robertson & Martley, 1966) and indirectly (Dalgaard-Mikkelsen & Rasmussen, 1957; Dawson & Feagan, 1960; Feagan & Griffin, 1965) for the presence of inhibitory substances have been introduced. Feagan (1966) has recently reviewed the main methods for the detection of antibiotic residues in milk.

McGillivray *et al.* (1966) have provided evidence for a marked decrease in the incidence of antibiotic residues in milk during the 3 years following the introduction of routine testing in New Zealand. Accompanying this decrease was a definite reduction in the incidence of cheese-making difficulties associated with slow vats and irregular rates of acid production from vat to vat.

In an attempt to overcome starter failure induced artificially by the addition of penicillin Kieseker & Faulkner (1964) investigated the effect of using abnormally high cooking temperatures and the addition of hydrochloric and lactic acids. The results of these trials were not encouraging. In a similar series of trials, but using citric acid, Harkness (personal communication) has succeeded in producing cheese of fair quality from vats in which the starter had failed through the action of bacteriophage.

Heat and other bacteria-removing pre-treatments of cheesemilk

Pasteurization. Cheddar cheese is traditionally manufactured from raw milk. In recent years there has been a considerable increase in the importance attached to the exclusion of pathogenic bacteria from foodstuffs. Bacteria of the types which present a public health risk do not normally proliferate appreciably during the manufacture of hard cheese, and because of the unfavourable acid and saline environment in Cheddar cheese most pathogens die out during the maturation of this type of cheese (Jezeski, Morris, Zottola, George & Busta, 1961; Roughley & McLeod, 1961; Zottola & Jezeski, 1963; Tuckey, Stiles, Ordal & Witter, 1964).

However, in unusual circumstances such as failure of acid production by the starter, the conditions during making and storage of Cheddar cheese can be sufficiently abnormal for potentially pathogenic bacteria to survive in appreciable numbers (McLeod, Roughley & Richards, 1962; Reiter, Fewins, Fryer & Sharpe, 1964), and outbreaks of food poisoning have occasionally been traced to the consumption of Cheddar cheese (Anon. 1961, 1962*a*; Donnelly, Black & Lewis, 1962; Epsom, 1964). In a survey of the incidence of coagulase-positive staphylococci in market milk and cheese in England and Wales, Sharpe, Fewins, Reiter & Cuthbert (1965) found that

9% of the cheese examined contained over 500000 viable staphylococci/g. These cheeses were all made from raw milk. They found that all but the mildest of heat treatments of cheesemilk were effective in virtually eliminating staphylococci from the resultant cheese. This latter observation is supported by the work of Zottola, Al-Dulaimi & Jezeski (1965) who found that only 3 of 236 of the more heat-resistant strains found in raw milk survived 152 °F for 21 sec.

During the period under review the use of raw milk or flash pasteurized milk for cheese-making was widely replaced by the use of milk which had been subjected to a combination of temperature and time, usually 162 °F for 15 sec (H.T.S.T. pasteurization), sufficient to destroy all milk-borne bacteria which present a hazard to health. It had previously been established that, provided care was taken to avoid overheating, H.T.S.T. pasteurization has little effect on cheese-making or cheese quality (Park, 1951; Birkkjaer, Sørensen, Jørgensen & Sigersted, 1961; Pimblett, 1962). The syneresis of curd from cow's milk has been shown to be only slightly reduced by pasteurization at temperatures up to 185 °F (Dimov & Mineva, 1962). Nevertheless, cheese-makers were often reluctant to use pasteurized milk for Cheddar cheese-making and there was a widely held belief that the best, full-mature Cheddar flavours could only be produced from raw milk. However, with the improvement in milking hygiene and in the handling of milk, particularly farm refrigeration, the natural flora of raw milk too has undoubtedly changed considerably in recent years.

Hydrogen peroxide. The bacteriological quality of milk can be improved by agencies other than heat. Treatment of cheesemilk with hydrogen peroxide under various conditions, followed by destruction of excess H_2O_2 by catalase, was studied in several countries (Wassermann, 1959; Kristoffersen & Cole, 1960; Doležálek & Závodský, 1962; Fox & Kosikowski, 1962; Keogh, 1964). The use of the hydrogen peroxide-catalase system has been approved by the U.S. Food and Drug Administration for use under defined conditions for the treatment of milk intended for Cheddar production (Anon. 1962*b*). The hydrogen peroxide-catalase treatment of cheesemilk has not been widely adopted as an alternative to pasteurization. Several factors have contributed to this; a tendency to need more starter and more rennet, a tendency for the curd to lack firmness, and most important, the fact that hydrogen peroxide does not destroy all pathogenic bacteria (Keogh, 1964).

Ultracentrifugation. High-temperature short-time pasteurization, while destroying potential pathogens, does not destroy heat-resistant bacteria, especially bacterial spores. In recent years attention has been given to the possibility of removing spores and other bacteria from cheesemilk by subjecting the milk to high gravitational fields in the so-called ultracentrifuges and 'Bactofuges' (Gantz, 1959; Surkov, Mizeretskiĭ & Gurova, 1961; Simonart, Poffé & Weckx, 1962; Syrjänen, 1963). This method of removing bacteria is a refinement of the process of centrifugal removal of fine particulate matter from cheesemilk known as 'clarification' (Bain & Gillan, 1955).

The technical difficulties of achieving efficient bacterial removal at the high processing rate essential for large-scale cheese-making are likely to limit the commercial use of ultracentrifugation for cheddar cheesemilk. However, for some types of cheese such as Emmental, the importance of removing bacterial spores, which are not destroyed by pasteurization, could provide an incentive to use high-gravity centri-

fuges (Peltola & Syrjänen, 1965). This method of removing bacteria will undoubtedly play an important part in research into some aspects of cheese-making, particularly attempts to elucidate the influence of added bacteria, and starters, on cheese flavour.

Homogenization of cheesemilk

The influence of homogenization on the suitability of milk for cheese-making has recently been reviewed by Peters (1964). In addition to reducing fat losses in whey, and fat leakage from mature cheese, there is some evidence of improved body and decreased flavour defects in Cheddar cheese. These advantages are at least partially offset by increased manufacturing costs. Eisele & Budny (1964) have reported that homogenization reduces coagulation time and increases curd 'viscosity'.

Vacuum treatment of cheesemilk

Volatile tainting substances and dissolved gases may be partially removed from milk by passing the hot milk from the holding section of a pasteurizer into a vacuum vessel. Associated with the near-instantaneous cooling of the milk is a loss of water vapour and some concentration of the milk. In recent years vacuum treatment of cheesemilk has been adopted to some extent in the U.S.A. and it is claimed that this treatment can improve flavour and texture (McGillivray, personal communication). The improvement in texture can probably be ascribed to a lowering of the carbon dioxide content of the milk. However, in limited trials conducted in New Zealand the texture and flavour of cheese was not improved by vacuum cooling of the cheesemilk (McGillivray & Robertson, unpublished).

Influence of added micro-organisms on cheese flavour

Research aimed at the elucidation of the chemical nature of the compound or compounds responsible for the characteristic flavour of mature Cheddar cheese has been actively pursued in recent years. Despite a number of claims to have solved the riddle, the complete answer remains elusive.

It has often been suggested that the characteristic flavour of cheese results to a large extent from the action of bacteria or fungi during the ripening process. Robertson & Perry (1961) have, however, pointed out it is not necessarily the strains of micro-organisms that are numerically important in mature cheese which are responsible for flavour development. They produced evidence for distinct flavour enhancement as a result of the addition of a very small proportion of a culture of a lipolytic micrococcus to cheesemilk. This strain was not only unable to proliferate in cheese but also rapidly decreased in numerical importance. More extensive trials involving several thousand cheeses (Robertson & Lawrence, 1963; Robertson, McGillivray & Harkness, 1964) have confirmed the flavour-enhancing attributes of this micrococcus when used alone or in combination with a flavour enhancing lactobacillus isolated earlier by Sherwood (1939).

Although the influence of the aroma-producing species in traditional mixed strain starter cultures on the flavour of young Cheddar cheese is well recognized, it is only recently that serious attention has been given to the effect on flavour of the essentially

homo-fermentative *Str. lactis* and *Str. cremoris* strains used as single strain starters. Emmons, McGugan & Elliott (1960) and Czulak & Shimmin (1961) have noted that bitterness in cheese is associated with certain of these strains while Perry (1961) was the first to report the existence of characteristic, slightly abnormal fruity or 'lactis' flavour in cheese made using *Str. lactis* strains as starter. He found that *Str. cremoris* used alone gave cheese of normal flavour. Perry & McGillivray (1964) reaffirmed the existence of a lactis flavour in cheese made under aseptic conditions from very low count milk.

The results of recent studies suggest that the flavour of Cheddar cheese may be attributed in part to the action of the starter strains used (Reiter, Fryer, Sharpe & Lawrence; personal communication). From time to time freshly isolated strains of *Str. cremoris* and *Str. lactis* with good activity for Cheddar cheese-making have had to be abandoned for use as cheese starters because they consistently led to abnormal flavours in the mature cheese (Robertson & Gilles, unpublished).

Lawrence (1964) has followed up the suggestion of Robertson & Perry (1961) that lipolytic species which thrive in milk and on dairy utensils, but do not grow actively in cheese, may contribute to Cheddar flavour. His study of the metabolism of fatty acids and synthetic triglycerides by spores and mycelium of *Penicillium roqueforti* has shown that triglycerides are oxidized to highly flavourful methyl ketones, whereas equivalent concentration of free acid inhibited methyl ketone formation.

Chebotarev & Alekseev (1964) have reported acceleration of ripening and improvement in cheese quality by the addition of a strain of Torulopsis yeast to cheesemilk.

Starter cultures and bacteriophage control

The subjects of starter characteristics and the control of bacteriophage have been ably reviewed by Reiter & Møller-Madsen (1963). Their review did not, however, discuss the use of thermoduric and thermophilic starters (Walter, Sadler, Malkames & Mitchell, 1953; Czulak, Hammond & Meharry, 1954) and this will be considered later under 'fast making processes'.

Perhaps the most important development in the interval since the review by Reiter & Møller-Madsen has been the experiments directed to the protection of starters from bacteriophage infection by the use of milk containing antibodies for phage. Erskine (1964) has reported the induction of high concentrations of antibody in blood serum and milk by direct intramuscular injection of high-titre phage into cows. When 1 % of immune whey obtained from such milk is added to normal milk containing homologous phage a few minutes before the addition of starter, the starter growth was unhindered. Erskine has also established that closely related bacteriophages are neutralized to some extent by the antibody milk and that the whey or milk may be freeze dried or low heat dried without undue loss in potency.

Active investigation of methods of reducing cheese-making difficulties as a result of the action of bacteriophage are continuing. Sing, Elliker & Sandine (1964*a*, *b*) have studied the effect of various germicides applied as aerosols on air-borne bacteriophage destruction. Keen (1966) has compared rates of bacteriophage destruction by solutions of idophor and hypochlorite. New methods of protecting starter cultures and bulk starters from phage infection and methods of detection of infection have been reported (Boelter, 1964; Robertson & Gilles, 1964; Robertson, 1966*b*).

Milk coagulating enzymes

Rennin, or some similar milk coagulating enzyme, is essential for Cheddar cheese-making and the properties and use of these enzymes have been reviewed by Davis (1965*a*). Although all proteolytic enzymes will, under suitable conditions, coagulate milk (Berridge, 1954) most are unsuitable for use in cheese-making because they give rise to bitter flavours or pasty body. However, because of the high price of calf vells, and in some instances because of religious objections to the slaughter of calves there has, in recent years, been an intense search for a satisfactory alternative to the traditional rennet. Sources examined have included the higher plants, products of micro-organisms, and materials of animal origin. The suitability of these rennet substitutes has been reviewed by Veringa (1961).

In recent years there has been renewed interest in the properties of pepsin, an enzyme normally obtained from the stomach of the pig, following the introduction of 'Metroclot', a commercial pepsin preparation. There is some conflict in the literature as to the suitability of pepsin preparations as alternatives for rennet. Maragoudakis, Young & Stein (1961) found, in a comparison of rennet and Metroclot, that rennet cheeses were of better body, texture, flavour, and flavour intensity until the cheeses were 3 months old. However, when 5 months old the flavour was better in the Metroclot cheese. Bitterness was more frequently detected in the rennet cheese at all ages. Melachouris & Tuckey (1963) have reported that the rate and order of appearance of free amino acids and flavour scores of 9-month-old cheese were similar when made with rennet and with Metroclot. They found that bitter flavours did not develop in cheese made with either coagulant. Raadsveld (1964) compared 3 commercial pepsin preparations with rennet. He found that the proportion of soluble nitrogen was lower in all the cheeses made with pepsin and that 2 of the pepsin preparations gave cheese with a comparable or better flavour score than rennet while the other pepsin gave cheese with a significantly lower flavour score. Ernstrom (1961) has drawn attention to the importance of milk pH when comparing the strength of rennin, crystalline swine pepsin and Metroclot.

Krishnaswamy, Johar, Subrahmanyan & Thomas (1961) have given a detailed account of trials in which a vegetable rennet, obtained from *Ficus carica*, and calf vell rennet were compared for Cheddar cheese-making. No significant differences in analytical composition were noted but yield and flavour scores were lower when vegetable rennet was used.

Cheese-making trials have recently been conducted in Australia (Czulak, personal communication), the U.S.A. (Ernstrom, personal communication) and New Zealand (Robertson, Harkness & Gilles, unpublished) using a microbial rennet produced by Meito Sangyo Co. Ltd., Japan. The initial New Zealand trials indicted that the flavours of the cheese made with the rennet substitute were comparable with, or slightly superior to those of the control cheese. Recent commercial scale trials of this Japanese microbial rennet in New Zealand show a slight tendency to lower yield.

At the present time a number of North American companies are developing alternatives to calf vell rennet.

An important development in recent years has been a tendency to combat the high price of rennet by a reduction in the proportion of rennet used. This is often

quite practical provided it is not carried to the stage where cheese body and yield are impaired (Ernstrom, Price & Swanson, 1958; Pimblett, 1962; Raadsveld, 1964). The effect of the use of a reduced proportion of rennet may be partially offset by the addition of calcium chloride to the cheesemilk and raising the setting temperature.

Cheese-making under aseptic conditions

The manufacture and ripening of cheese is an extremely complex process and the effect on cheese quality and flavour of starter bacteria, the adventitious flora of cheesemilk, microbiological contamination during cheese-making, and the enzymes of milk, are difficult to disentangle. The concept of cheese-making from bacteria-free milk under aseptic conditions, as developed by Mabbitt, Chapman & Sharpe (1959), is of the utmost importance in providing a tool for the elucidation of the contribution of starter bacteria and other micro-organisms to cheese characteristics.

Mabbitt *et al.* (1959) developed a special 40-gal cheese vat with a close-fitting cover to prevent bacterial contamination during the cheese-making process. After pasteurization of the milk a satisfactory degree of asepsis was attained in 3 out of 6 cheeses. An improved version of the vat which has been described recently (Chapman, Mabbitt & Sharpe, 1966) has a sealed lid and is sterilized by autoclaving.

Perry & McGillivray (1964) have further developed the aseptic cheese-making technique. The main changes introduced by these workers were a reduction in the size of vat to 18 gal in order to eliminate some of the manipulative difficulties when working through glove ports; maintenance of the vat at a positive pressure with sterile air to prevent ingress of non-sterile air through the water-seal of the lid; and the use of a 'fast make' (Whitehead & Harkness, 1959) to minimize opportunities for contaminant bacteria to proliferate. 'Non-aseptic', control cheeses were made in the same vat but with the lid removed and without aseptic precautions. Two types of aseptic and control cheeses were made: (*a*) with single strain starters and in many instances, the addition of other species thought to influence cheese flavour; and (*b*) without the addition of starter, gluconic acid lactone being used as a source of acid (Mabbitt, Chapman & Berridge, 1955). Bacteriological and other changes were studied as the 46 cheeses produced in these experiments matured. The flavour of the aseptic cheeses varied but were generally 'very mild' or 'bland', those with the best flavour resulted from the addition of both '*Lactobacillus plantarum-casei* 25·2' (Sherwood, 1939) and micrococcus L_1 (Robertson & Perry, 1961). 'Lactis' flavour (Perry, 1961) was present in most of the cheeses for which cultures of *Str. lactis* strains were used as starter. The flavours of the gluconic acid lactone cheese, both controls and 'aseptic', were generally unpleasant.

Whatever the effect of adventitious organisms on Cheddar flavour they appear to play no major part in protein breakdown, since the body and texture of the 'starter-only' cheese of Perry & McGillivray (1964) compared well with the appropriate control cheese.

Starterless cheese-making—direct acidification

The cheese-making problems associated with bacteriophage, antibiotics, and natural inhibitors of bacterial growth, would be eliminated if acid production by cheese starters could be replaced by alternative methods of acidification such as the

direct addition of lactic or hydrochloric acid. Mabbitt *et al.* (1955), who have briefly reviewed such methods, were the first workers to develop a reasonably satisfactory method of starterless cheese-making. After a number of experiments involving the addition of lactic and hydrochloric acid to cheesemilk and curd, the possibilities of gluconic acid lactone were investigated. This lactone hydrolyses to the free acid at a convenient rate, is non-toxic, and has no other objectionable properties. In the preferred method the lactone was added at renneting and again after milling. The flavour of the resultant cheese, although acceptable, did not have the characteristics associated with normal Cheddar nor was it improved by the inoculation of the cheesemilk with a strain of *L. casei* and *L. brevis*.

The principle developed by Mabbitt *et al.* (1955) has been used by other workers. Deane & Hammond (1959) obtained normal yields of cottage cheese of good keeping quality by the addition to skim-milk, of gluconic acid lactone or the racemic lactide of lactic acid at the rate of 9% (w/w) of the solids-not-fat. Hammond & Deane (1961) have patented a similar process. As described earlier, Perry & McGillivray (1964) have used gluconic acid lactone in experiments in Cheddar cheese-making under aseptic conditions.

The most successful use of gluconic acid lactone has been reported by Dodson, Hammond & Reinbold (1965). They found that it was difficult to distinguish between normal curd and curd resulting from the use of the lactone instead of starter for acidification. Bitter and fermented flavours developed in the lactone cheese during curing. However, when selected strains of lactobacilli and 10 ppm. manganese were added to cheesemilk the lactone cheeses were of good flavour and matured more rapidly than conventional Cheddar. On the other hand, when lactone-acidified cheese was used to evaluate the contribution of attenuated lactic starters, and some strains of *L. casei* and *Str. durans* on cheese ripening, flavour development was not comparable to that of conventional Cheddar cheese.

The possibilities of starterless cheese-making by the direct addition of free acids have been studied further in recent years. In studies of cold renneting and continuous curd production Berridge (1961, 1963*a*, *b*, *c*) has adjusted the pH of cheesemilk and curd by the addition of hydrochloric acid and phosphoric acid. Ernstrom (1963) has patented a method of making cottage cheese curd which involves lowering the pH of milk to pH 4·6 with hydrochloric, phosphoric, lactic or acetic acids. This process is akin to the use of hydrochloric and sulphuric acids in the process for making casein. Breene, Price & Ernstrom (1964*b*) have made a detailed study of the effect of direct acidification with lactic acid on the composition and quality of Cheddar cheese. They found that the bitterness associated with cheese made from milk adjusted to pH 5·40 could be avoided by adjusting to pH 5·60 and adding a 1–2% of starter. Breene *et al.* (1964*a*) have also investigated the manufacture of Pizza cheese from milk adjusted to pH 5·6 with hydrochloric, lactic and acetic acids. As noted earlier, Harkness (unpublished) and Kieseker & Faulkner (1964) have investigated the possibility of overcoming the effect of starter failure by treatment of Cheddar cheese curd with acids.

DEVELOPMENTS IN CHEESE-MAKING PROPER

Fast making processes

As noted earlier, the introduction of the use of single strain starters for cheese-making by Whitehead of New Zealand in the mid-1930's, and the use of defined mixtures of strains, has resulted in a definite reduction in the length of the cheese-making day, and hence in manufacturing costs, as a consequence of an overall increase in the consistency and rate of acid production by the better starters. Since that time several ways of reducing the manufacturing time have been introduced or investigated but not widely adopted.

Modified traditional process

Whitehead & Harkness (1959) compared the thermoduric and salt-tolerant strain of *Str. durans* used by Walter *et al.* (1953) (see below) with a *Str. cremoris* starter but detected no difference in the cheese vat. Their method of accelerating the making process was therefore subsequently based on modifications of the traditional methods without the use of special cultures or apparatus. The changes introduced by Whitehead & Harkness led to an increased rate of acid production and moisture expulsion. This was achieved by increasing the proportion of starter, raising the setting temperature, and increasing the proportion of rennet. The cheddared curd was milled and salted at a lower acidity. In the 23 cheese-making experiments reported the making times from adding rennet to salting were consistently $3\frac{1}{2}$ h or less—a reduction of about $1\frac{1}{2}$ h on the normal New Zealand making time. The fast-make cheeses were judged by experienced graders to be of good quality.

Whitehead & Harkness (1959) pointed out that such a major acceleration of the process makes considerable demands on the skill of the cheese-maker and they expressed doubt whether a manufacturing time of $3\frac{1}{2}$ h would be practical for regular commercial cheese-making.

High-temperature makes: thermoduric starters

No cheddaring—hooping under whey. A revolutionary process for the production of cheese with Cheddar characteristics was introduced by Walter *et al.* in 1953. The new process differed in several important respects from the traditional process and, although the original method has not been widely adopted, the principles used are outlined because of their effect on subsequent developments. Two starters are used, one a conventional mixture of lactic streptococci, and the other a heat- and salt-tolerant strain of *Str. durans*. The milk is ripened for 1 h and a normal making process is followed until after cooking to 100 °F. The curd and whey is pumped into a revolving perforated drum where one-half of the whey is removed. The remaining curd and whey is heated to a temperature within the range 115–120 °F and salt added. The salted curd and whey is pumped into a special hoop completely immersed in a tank containing whey which has been preheated to the same temperature. After settling under whey the curd is consolidated into a cheese by pressing. The overall make from adding starter to the completion of hooping is $3\frac{1}{2}$ h although the process has been referred to as a $2\frac{1}{2}$ h method which is the time from setting to hooping.

Walter *et al.* (1953) claimed that the body, texture and flavour of cheese made by this method are equal or superior to those of high-grade Cheddar cheese made by conventional methods.

The new method has been studied by several groups, and has been pronounced commercially unacceptable on several grounds, but particularly because of the need for special equipment and the fact that half the whey was unusable because it contained 4 % added salt. The authors of the original process introduced modifications (Walter *et al.* 1956) to enable more conventional cheese-making equipment to be used and to reduce salt contamination of the whey.

Irvine & Price (1958) have patented a process for the production of quick ripening cheese of high moisture content. The method shows some resemblance to that of Walter *et al.* (1953, 1956) as does the 'automatic' cheese-making process outlined by Saal (1958) and McCadam & Leber (1958).

Further changes introduced by Walter, Sadler & Mitchell (1958) involved the use of conventional procedures except between draining and milling when the curd and whey was pumped into a cloth-lined, perforated curd-retention device within a tank. The curd was allowed to mat for 2 h without turning. This paper basically describes a simple method of mechanized draining and cheddaring and represents the abandonment of the main principles of the original short-time make.

Short cheddaring—normal hooping. The Australians, Czulak *et al.* (1954), introduced a short-time method for making Cheddar cheese based on that of Walter *et al.* (1953), but differing from it in some important aspects. After cooking the curd at 100 °F half the whey is run off and the cooking temperature raised to 108–110 °F. The remaining whey is then run off and the curd cheddared for $\frac{1}{2}$ h at 108–115 °F. Milling, salting, hooping and pressing are carried out as in the normal process. Overall making time from adding starter to completion of hooping is 3–3$\frac{1}{2}$ h.

Morris (1955) has reported that in experimental trials of the new method of Czulak *et al.* (1954), conducted in Queensland, persistence of curdy body in the cheese was a problem. He suggested that variations in pH were responsible for this defect and modified the method.

Czulak & Hammond (1956*a*) reported on the results of commercial use of the process in 6 dairy factories during 1954–55 when about 2500 tons of cheese were produced. In general, the quality of the 'short-time' Cheddar compared favourably with cheese made by the traditional process. It was found that overheating during the second cooking led to slow maturation.

Czulak & Hammond (1956*b*) found that fast-make cheese fresh from the press should have a pH of 5·1–5·3, i.e. appreciably higher than usual for Australian cheese at that time, for satisfactory flavour development. This can be achieved by using 2 % normal starter and 2 % of vigorous thermoduric starter. The use of strains of *Str. durans*, *Str. thermophilus* and *L. bulgaricus*, as thermoduric starters are described by Czulak & Hammond (1956*a*, *c*), Feagan (1956, 1959*a*, *b*) and Crawford (1956). The latter found that the use of *L. bulgaricus* in place of *Str. durans* resulted in a marked improvement in the rate and extent of flavour development.

Feagan (1956) modified the method of Czulak *et al.* (1954) by omitting the conventional starter and using only a slightly increased proportion of thermoduric starter. Good quality cheese was produced.

In a comparison of the rapid methods of Walter *et al.* (1953) and Czulak *et al.* (1954), with the normal Cheddar cheese-making process, Szabć (1959) experienced difficulty with slow acid development. Cheese of good flavour was, however, produced after the addition of *L. helveticus* and *L. casei* to the starter and some modification of the manufacturing process.

Bevan, Dawson, Feagan, Howey & Park (1959), in a study of the texture of cheese made by the short-time method of Czulak & Hammond (1956*a*, *b*), found that the texture of the 3- and 6-month-old cheese was more open than that of cheese produced by orthodox methods. In the absence of measures to reduce this openness the use of the high temperature method was not recommended.

During a 4 year comparison of the orthodox and Australian short-time method of cheese-making, Downs & Nilson (1961) found that flavour developed more rapidly in the short-time cheese made from raw milk than in their pasteurized milk counterparts. They have reported that in subsequent commercial scale trials in 1959–60 both raw and pasteurized milk were made into satisfactory cheese by the rapid method.

As a result of the better and more rapid flavour development in cheese made with starters consisting of strains of serological group-D streptococci, as opposed to normal lactic cultures, Zhitkova (1964) has, like Dahlberg & Kosikowski (1948, 1949), recommended the use of such heat-tolerant strains. Zhitkova particularly recommended the use of a strain of *Str. faecalis*, for cheese-making.

High temperature makes: normal, mesophilic, starters

As was noted above, cheese made by the high-temperature short-time method of Czulak & Hammond (1956*a*, *b*) is more open than orthodox Cheddar cheese (Bevan *et al.* 1959). Dawson & Feagan (1960) have suggested that this openness may be attributed to the heterofermentative nature of the thermoduric starters, although the validity of this surmise may be questioned since the greatest openness was noted when a strain of *Str. thermophilus*, a species considered homofermentative by bacteriologists, was used as thermoduric starter. Dawson & Feagan (1960) undertook a series of high-temperature cheese-making trials in which normal cheese-making strains of *Str. lactis* and *Str. cremoris* were used as starter. Cheddar cheese made using these homofermentative starters and cooking temperatures of 110 °F were also of open texture. They concluded that the openness associated with high-temperature makes results from gas production by components of the non-starter flora, which develops actively at the relatively high pH of the cheese produced by high-temperature methods.

Elimination of cheddaring: stirred curd

In the traditional process the cheddaring stage represents a substantial fraction of the cheese-making day, and is one of the more laborious phases of the making process. No part of the making process is sacrosanct and in recent years rising labour costs, and a shortage of labour in rural areas, have directed attention to the possibility of the elimination of cheddaring or at least its replacement by more easily mechanized procedures. The validity of the use of the term 'Cheddar cheese' if the cheddaring stage is omitted, has been questioned (Downs & Nilson, 1961) but many realists

adopt the view that it is the character of the final cheese which is important—provided a cheese has the somewhat idealized body, texture and flavour associated with Cheddar cheese the method of attaining the goal is thought to be immaterial.

One method (Walter *et al.* 1953, 1956) of Cheddar cheese-making which eliminates the cheddaring step has been described, and mechanized processes which drastically modify cheddaring or omit the step are referred to later. Only the stirred curd alternative to cheddaring will be referred to at this point.

During the period when the drained curd is allowed to mat or cheddar on the floor of the traditional cheese vat one of the most obvious changes is the transition of the curd from a multiplicity of the small particles into an almost homogeneous mass which is essentially free of small air-holes and whey pockets. When the drained curd is continuously or intermittently stirred (Van Slyke & Price, 1932) at this stage moisture expulsion is undoubtedly faster than during cheddaring but the curd particles remain as more-or-less discrete entities. It has been found (Czulak & Hammond, 1956*d*) that granular, stirred curd gives rise to open textured cheese as a result of air being entrapped within the cheese. This was confirmed by the elimination of openness when stirred curd was hooped under whey. Following the development of methods of pressing cheese under vacuum (Smith, Roberts & Wagner, 1959) the problem of open texture was eliminated and good quality Cheddar cheese was produced without cheddaring by vacuum pressing the dry-stirred curd produced by the use of normal starters (Czulak, 1962) and by direct acidification (Breene *et al.* 1964*b*). It thus appears that the main purpose of cheddaring is to achieve a reduction in trapped air, and that this effect may be achieved in other ways which are commercially practical.

Mechanized processes

The word mechanized may be used to mean the simple replacement of manual effort, replacement of manual control by automatic mechanical control, or replacement of a sequence of manual operations by integrated mechanical operations. Appropriate examples are: (*a*) replacement of hand stirring of cheesemilk by mechanical stirring; (*b*) replacement of manual control of pasteurizing temperature by automatic, thermostatic, control; and (*c*) replacement of the manual filling of vats, addition of starter, setting, cutting, stirring and cooking of the curd by the automatic mechanical performance of these operations. Clearly examples (*a*) and (*b*) are likely to be performed more consistently and reliably by well-known mechanical or electro-mechanical methods. On the other hand, the complex sequences inherent in example (*c*) raises doubts as to whether the mechanical approach could, at justifiable cost, be superior to a manual-mechanical combination. A complex batch process is often difficult to adapt to integrated mechanical operation, and conversely a continuous process is often difficult to adapt to manual control. For this type of reason existing manual-cum-mechanical batch processes must often be drastically revised before they are amenable to automatic mechanical control and with biological systems like cheese-making the transition from batch to continuous processes can pose formidable problems.

Curd-making and cooking

The mechanization of cheese-curd-making and cooking has followed 2 courses. On the one hand there has been the development of improved vats (Zeiler, 1953; Dumbleton, 1957; Nessler & Hartman, 1959; Duconlombier, 1963; Schwarzhaupt, 1964), improved methods of cutting and stirring the vats (Zeiler, 1953; Schwarte, 1954; Dumbleton, 1957; Croucher & Crowder, 1957; Schwarzhaupt, 1960; Duconlombier, 1963), and programmers to control the pH, temperature, and temperature rise during cooking (Buhrgard, 1957; Holland & White, 1960; Birkkjaer & Sigersted, 1962; Kosikowski, 1963; Czulak & Lawrence, 1964). On the other hand, there have been attempts to develop methods of continuously forming and cutting curd and methods of continuously cooking the curd. It is these latter developments which will be considered here for these or their derivatives will ultimately surplant the batch processes. Some of these new developments have been reviewed elsewhere (Montoure, 1962; Arentzen, 1962).

Continuous renneting at normal temperatures. Under normal cheese-making conditions the quantity of rennet is adjusted so that the curd formed from milk at 86–90 °F is firm enough to cut in 30–40 min. Setting can be considerably accelerated by increasing the temperature and proportion of rennet and by lowering the pH of the milk. For the production of curd of satisfactory gel strength, it is essential that the milk–rennet mixture be completely free of turbulence during coagulation. The main problems facing those concerned with continuous renneting and cooking have been those associated with attaining the necessary quiescence in the mixture, the achievement of plug-flow, and the avoidance of curd adhesion to the surfaces of the coagulation chamber or tube. Patented approaches to these problems are numerous. For example, Harper & Seiberling (1957) have described a process in which cheese-milk containing added starter and rennet passes through a mixer and then passes up a vertical tube in a non-turbulent column. The coagulum is cut by a cutting element at the top of the column and passes into a horizontal cylinder where it is moved along by a screw conveyor. The curd is scalded or cooked in a 2-stage process by the circulation of hot whey. A similar principle is used in the patent of Spiess & Hollis (1959) except that a flat velocity profile in the milk stream during coagulation is achieved by the use of a special design of radial distributor at the entry to the coagulation tube. In this instance the coagulum is divided into ribbons by a wire screen and then subdivided into cubes by a rotating blade. Lankford (1959) has patented a descending system in which the rennetted and partially acidified milk flows over a cone-shaped distributor as it enters a coagulation cylinder. The coagulum passes through a wire mesh screen at the bottom of the cylinder under the influence of gravity. A different, semi-batch, system is described by the Imperial Cold Storage and Supply Co. (1952). The milk–starter–rennet mixture is delivered automatically to containers on an endless conveyor, the curd is cut mechanically and is discharged via a hopper into a perforated cylinder for cooking.

Montoure (1962), after a brief review of methods of mechanized cheese-making, has described a new continuous process for semi-hard cheese manufacture. Starter and rennet are added to pasteurized milk at 65 °F and the mixture is pumped to a tubular preheater where it is rapidly heated to 86 °F before passing into a coagula-

tion chamber which consists of a spiral of 'Tygon' tubing in a water bath. The coagulum is cut by a wire grid and is heated to a cooking temperature of 100 °F in an electrode chamber before the curd-whey mixture passes to a vat lined with perforated stainless steel. The curd is pressed in this vat before being milled, hooped and salted in the usual manner.

Thus far none of the processes described appear to have been widely adopted as a commercial method of making Cheddar cheese curd.

Cold renneting. It is long established (Effront, 1917) that a milk–rennet mixture does not coagulate if the temperature is low, and Berridge (1942) has shown that milk treated with rennet at 5 °C coagulates very rapidly when heated to 37 °C. The necessity of a relatively long period of quiescence during normal coagulation has been referred to above. The very rapid coagulation on warming of milk pre-treated with rennet at a low temperature has been used to reduce the problems of turbulence during coagulation (Arentzen, 1962; Ubbels & Linde, 1962; Berridge, 1963*b*, *c*; Ernstrom, 1963). Development of the cold renneting approach to curd formation appears to have proceeded independently at different centres and has been the subject of several patents in recent years (Berridge, 1961, 1964; National Research and Development Corpn. 1961; Ernstrom, 1963).

In early experiments Berridge (1963*a*) studied the possibility that rapid cooking could be tolerated if the difference in acidity between the whey and centre of curd particles could be maintained. By a system of direct acidification of milk before rennet coagulation and the addition of alkali to whey he was able, on a very small scale, to reduce manufacturing time from renneting to pressing to about 30 min. The cheese produced was abnormal. His subsequent investigations (Berridge, 1961, 1963*b*, *c*; National Research and Development Corpn. 1961) involved cold renneting. Milk and starter at 10 °C was acidified to pH 5·8 and rennet (1 in 5000) was allowed to act on the milk for at least 1 h in a stirred, segmented, delay-vessel. The rennet-treated milk was then heated to at least 30 °C when coagulation took place in a few seconds. Several methods of heating were tried, including electrical conduction heating, passing the milk into a hot immiscible liquid, and spreading in a thin layer on a hot surface. The coagulating system most thoroughly investigated involved the spreading of the milk on a hot metal surface and the scraping off of the resultant ribbon of scalded curd into a bath of hot whey with a doctor-blade. The curd was allowed to drain for several hours before milling, salting and pressing (Berridge, 1963*d*). The quality and flavour of the cheese produced did not consistently resemble Cheddar cheese, and it appears that the process requires further development before it could be adopted for commercial cheese-making.

The method of continuous curd formation developed in the Netherlands (Arentzen, 1962; Ubbels & Linde, 1962; Radema, Ubbels, Arentzen & Linde, 1964) for the manufacture of Edam cheese involves a combination of cold renneting with the continuous coagulating chamber approach described earlier. A mixture of milk, rennet, starter and potassium nitrate is held at 2–6 °C for 2–6 h. Sufficient calcium chloride is added to adjust the coagulation time to about 1 min and the milk is pumped to a plate heat exchanger where it is heated gradually to a temperature in the range 29–32 °C with special care to avoid an appreciable temperature gradient between the potentially coagulable milk and the plates. The warm milk then passes

to the bottom of a vertical coagulation tank. The coagulum is divided into cubes as it leaves the top of the tank and enters a slightly inclined rotating cylinder equipped with a worm conveyor and working bars. The curd is heated with warm water before passing into the top of a vertical tube where it is washed with warm water and consolidated. The curd is then ready for moulding and pressing. The perfection of this process is well advanced and although it was developed for Edam cheese-making many of the principles could be used for Cheddar cheese-making. The replacement of the washing and consolidation tube by cheddaring equipment such as the tube of Budd & Chapman (1961) is an interesting possibility.

The cottage cheese curd-making process patented by Ernstrom (1963) resembles in some respects both the Berridge and Ubbels & Linde cold renneting methods. However, Ernstrom (1963, 1965) added sufficient hydrochloric, or other acids, to acidify the milk to pH 4·6 before renneting.

Cold renneting of concentrated milk. The so called 'Hutin' or 'Hutin-Stenne' process developed in France by Stenne of the Hutin company has been described in a number of anonymous articles (Anon. 1964*a*, 1965*a*, *b*) and by Stenne (1965). This new method of continuous curd formation developed originally for soft cheese manufacture closely resembles some aspects of the Berridge cold renneting process but differs from it in other respects. The most important difference is that the starting material for the process is milk concentrated in a plate evaporator to about 36% total solids. This use of concentrated milk in turn effects the pH, the time for rennet to act (Alais & Stenne, 1965), the growth of the starter, and of course, the physical dimensions of the curd handling equipment. The costs of concentration and chilling of the concentrate, and the necessity of providing relatively large storage capacity for the maturing of the concentrate, are disadvantages of the process.

In essence, the procedure for making curd for semi-hard and hard varieties of cheese involves the adjustment of the cold concentrated milk to a predetermined pH with lactic starter, maturing for several hours, renneting and further holding, instantaneous coagulation by discharging into hot water, syneresis in a holding tube, and primary separation of curd and whey in a revolving drum. Subsequent treatment of the curd depends on the type of cheese to be produced. During maturation of the acidified concentrate the phospho-caseinate particles aggregate and provide a matrix which is claimed to reduce the loss of fine particles of casein and fat from the curd. Substantial increases in yield have been reported for soft cheese (Ardebili, Camus & Jouzier, 1964). A semi-commercial unit with a capacity of 200 gal/h has been tested and a larger version with an input of 1100 gal milk/h is projected. The feasibility of the process for making soft cheese is well established and the results of trials of its suitability for producing Cheddar cheese are awaited with interest.

Draining, cheddaring, milling and salting

As in the preceding section the mechanization of the cheese-making process from draining to hooping has developed in 2 ways. On the one hand, there has been the introduction of improved draining devices, dry-stirrers and cheddaring vats and, on the other hand, there have been attempts, of varied sophistication, to evolve continuous or semi-continuous processes to overcome the problems inherent in the traditional batch process. Again, only the latter developments are considered in

detail and it is interesting to note that the main developments appear to stem from the dramatic modification of the cheese-making process brought forward by Walter *et al.* (1953).

A relatively small number of groups, usually working in different countries, have been responsible for the main developments in this section. Since the approach of individual groups has been distinctive, the work of each group is considered separately.

American 'Ched-o-matic' system. The Ched-o-matic method of cheese-making developed in the U.S.A. was the first extensively mechanized system to be used commercially and has been operated in a cheese factory since 1956 after large-scale testing for several years previously. The method, which is essentially a batch process, has been well described and illustrated in a number of publications (McCadam & Leber, 1958, 1959; Saal, 1958; Gemmill & Leber, 1958; Leber, 1959). The traditional cheese-making process is followed, using normal lactic starter, until the vat is ready to run. The vat contents are then pumped to a large revolving horizontal drum made from finely perforated stainless steel in which the curd and whey are separated with appropriate measures to recover fine particles of curd. The curd is discharged into a rectangular bin in which 1200-lb curd are hydraulically pressed into a single block. A system of top and bottom screens facilitates the loss of moisture during cheddaring. After about 100 min milling acidity is reached and the block is automatically divided into small slabs by a somewhat complex cutting system at the bottom of the bin. The rather spongy blocks of curd are milled and the shredded curd is immediately washed free of excess whey, and salted in a screw conveyor by a combination of warm water sprays and warm brine sprays. Salt absorption by the porous curd is remarkably fast, cheese of 1·5% salt resulting from curd exposed to a series of 4 saturated brine sprays for a total of 30 sec (McCadam & Leber, 1958).

Cheddar cheese produced by the Ched-o-matic system has been reported to be true to type and of good-to-excellent quality, although the occurrence of slight whey taint and some mechanical openness has also been noted (McCadam & Leber, 1958). The Ched-o-matic system has been used commercially in North America and in England but this mechanized system has not been widely adopted. The reasons for this are not clear.

Modifications to the process which make allowance for dry salting and salting in brine have been patented (McCadam, 1963; Leber, 1963).

Australian 'Cheesemaker' systems. Czulak and his Australian associates have divided their work on the draining to salting part of the cheese-making process into 2 sections: 'Cheesemaker II' which is concerned with draining and cheddaring, and 'Cheesemaker III' which involves flow-line milling, salting and hooping.

The difficulty of producing close-textured cheese by a stirred curd process and the relative success of their short-time Cheddar cheese-making process led Czulak & Hammond (1956*d*) to a study of the mechanical behaviour of curd during cheddaring. Their conclusion that extensive deformation and flow are essential to cheese-making led to the design and construction of a curd fusing or cheddaring machine which specifically made provision for these changes in the curd (Czulak, 1958). The curd-fusing machine was subsequently modified so that the partially cheddared curd was divided into smaller blocks which underwent further cheddaring on a slow moving conveyor (Czulak, Freeman & Hammond, 1960). In subsequent development of the

cheddaring system Czulak (1962) omitted the curd-fusing machine in favour of an improved cheddaring conveyor. The Cheesemaker II system tested early in 1965 consisted of a high-level draining vat, equipped for dry-stirring, mounted above the cheddaring conveyor. When the curd is sufficiently dry it is discharged from the draining vat into an endless belt of L-shaped shelves. The trough formed between successive shelves divides the curd into portions such that, as the curd particles coalesce, cheddar blocks of about normal dimensions are formed. The path of the conveyor is such that the blocks are turned automatically and provision is made for limited flow during cheddaring. It is understood that modifications to the Cheesemaker II during the last year have included the re-introduction of a curd fusing stage. Cheesemaker II as at present envisaged is somewhat complex and expensive but the results of the forthcoming testing of the modified system are awaited with interest in several countries.

Cheesemaker III, an integrated series of mechanical and electro-mechanical devices for milling, salting and hooping blocks of cheddared curd was described by Czulak, Freeman & Chapman (1958). The cheddar blocks are milled in a hydraulically operated reciprocating mill and the milled curd is transferred to an inclined belt conveyor by a vibrating conveyor. At the top of the incline the curd falls on to a floating conveyor which senses the weight of curd upon it and regulates the quantity of salt added to the curd as it enters a small inclined mixing drum. An electrically controlled gate at the end of the drum prevents discharge as an automatic hoop weighing and indexing device positions a fresh hoop beneath the end of the drum. The time lapse between milling and hooping is very short, less than 1 min, and to provide for post-salting drainage it is necessary to allow hoops to stand for $\frac{1}{2}$–1 h before pressing. Cheese, processed with this milling, salting and hooping machine, would appear to have been of satisfactory quality although seamy texture has posed difficulties especially in coloured cheese made in the 2 U.S.A. factories equipped with Cheesemaker III (Czulak, 1963; Czulak, Conochie & Hammond, 1964). The problem was mainly overcome by spraying the curd with warm water immediately after milling (cf. McCadam & Leber, 1958). An interesting by-product of the investigation was the demonstration by Conochie & Sutherland (1965*a*) that seaminess is caused by deposits of $CaHPO_4.2H_2O$ along the curd particle interfaces.

The commercial version of Cheesemaker III has a throughput of 8000 lb curd/h and is capable of accurate salting. It is claimed to reduce fat and casein losses as a result of the very clean cut of the mill (Czulak & Freeman, 1961; Czulak, Freeman & Hammond, 1961). A number of these machines are installed in Australian cheese factories and several are operating in other countries.

British system. Of the various systems which have been developed, that of Budd & Chapman (1960, 1961, 1962) is the most fully integrated, the operations of draining, cheddaring, and milling being performed in a single unit. The equipment consists of a tall, lagged cylinder which increases in diameter from top to bottom in the ratio 1:4–6. The bottom of the tapered cylinder is essentially closed by a cutter blade. In operation, curd and whey are pumped to the top of the tube and the whey escapes through the perforations in the upper section of the column. Rotation of the cutter blade at the bottom of the column mills the curd and its speed of rotation controls the residence time of the curd within the column. Deformation and flow of the curd

are induced as a result of the increasing weight of curd above and the increasing diameter of the column. The quality of the cheddard curd, and the body, texture, and flavour of the mature Cheddar cheese produced in the prototype column, have been comparable with curd and cheese produced in the traditional manner.

This continuous method of cheddaring holds much promise for the future but, as Budd & Chapman (1961) have pointed out, the problems of scaling up the process are formidable. One of the main difficulties is that when starting up a large column, a considerable quantity of inadequately cheddared curd must be re-cycled through the system.

New Zealand 'Cheddarmaster' system. Work on the mechanization of the cheese-making operations from draining to hooping was initiated relatively late in New Zealand but commercial acceptance of the system has been rapid. The Cheddar-master system differs in several important respects from those described above. It is less radical than the British system, more nearly continuous than the Ched-o-matic approach and more flexible than the Cheesemaker systems.

The original system (King & McGillivray, 1962) has been somewhat modified (McGillivray & King, 1966) in order that it may ultimately be directly coupled with the 'Pressmaster' system of pressing and packing cheese (Robertson, 1964*a*, 1965*c*). Curd and whey produced in normal, or considerably deepened vats (McGillivray, personal communication), is pumped onto a slow, variable speed, draining conveyor constructed of perforated stainless steel slats. As the curd moves along the conveyor it passes under 4 sets of peg stirrers which prevent matting and assist drainage. It is finally discharged into a series of narrow stainless steel cheddaring boxes which hold about 400-lb curd each. According to the size of the factory the cheddaring boxes are handled manually or automatically. To assist curd flow and cheddaring the boxes are rotated through quarter turns at intervals of 15–20 min until acid development is suitable for milling—usually about 2 h from the start of cheddaring. The large cheddar blocks are transferred to the hopper of a special rotary-drum mill. The milled curd is discharged on to the original draining belt or, in a large factory processing over 22,000 gal milk/day, on to a second draining conveyor. Pockets of whey trapped within the cheddar block drain quickly from the milled curd and, as the curd moves along the conveyer, salt is automatically distributed over it at an adjustable rate proportional to the thickness of the bed of curd on the conveyor. The salt is incorporated with the curd as it passes through the peg stirrers. Brine-whey drainage takes place before the salted curd is discharged from the draining conveyor at the hoop filling point. Special features of the Cheddarmaster system include: provision for variable draining time to cope with curd abnormalities, widely variable cheddaring time, and ability to mill individual or groups of cheddar blocks out of sequence if acid development should suggest this course. Another feature is that equipment cost is to some extent proportional to the throughput of the factory. For example, the number of cheddaring boxes and cheddar box handling units is proportional to factory size and, according to factory size, one, two or more draining conveyors are required.

For the past year all the cheese produced in a medium size New Zealand factory has been processed on a Cheddarmaster unit. The average grade and yield of cheese has increased compared with the previous year and compared with the neighbouring

factories. This, combined with the fact that the reduction in labour requirements will pay for a Cheddarmaster installation in a few years, has led to strong interest in the system both in New Zealand, America and Australia. The second and third Cheddarmaster systems are about to be installed (Robertson & McGillivray, 1966).

Miscellaneous systems. The system described by Walter *et al.* has been referred to earlier as has the possibility of a marriage of the Dutch cold renneting method of producing curd with the British cheddaring system. Other interesting systems have been described in the patent literature (Green & Zink, 1955; Hensgen, 1956; Hensgen & Beland, 1958) but do not appear to have enjoyed widespread commercial success.

Hooping, pressing and packing

In the interval since the introduction of rindless Cheddar cheese-making an almost bewildering array of designs for hoops, filling chutes, and even presses have been patented. These will not be discussed here beyond a comment that the designs which obviate the need for cheese cloths (Yvon, 1954; Robertson & Charles, 1957; Rossen, 1961, 1964; Ječmen & Kněz, 1963; Ziegler, 1965) could reduce manufacturing costs and problems of bandage adhesion (Conochie & Sutherland, 1961).

Hooping. The automatic hoop filling unit of the Australian Cheesemaker III has been referred to above. Although a uniform weight of curd enters each hoop the weight of the overnight pressed cheese shows much of the usual variation in weight from vat to vat and from day to day as a result of variations in moisture losses during pressing.

Pressing. In recent years a number of batch and semi-automatic presses have been introduced for the pre-pressing of hoops of curd before 'dressing' and putting down in the main press. A continuous, and fully automatic pre-press for use in conjunction with the Cheddarmaster system of cheese-making or with vacuum pressing installations has been described by Robertson (1966*a*).

A method of measuring the pressure applied to cheese in a conventional press has been described by Lambert (1963).

Vacuum pressing. The idea of vacuum pressing Cheddar cheese was first proposed by Udy (1935) but it was not until recently, following the re-discovery of the principle (Smith *et al.* 1959), that the effectiveness of vacuum pressing in eliminating mechanical openness was confirmed by thorough investigation. Work on this topic has been reviewed by Robertson (1965*a*). In the interim further detailed studies of vacuum pressing have been published (Robertson, 1965*b*; Tabachnikov, 1965; Tabachnikov, Kudryashova & Lebedeva, 1965).

Film wrapping of rindless cheese. Many films of low oxygen and moisture permeability have been tested for suitability for enclosing rindless cheese (Davis, 1965*b*). Methods of film wrapping cheese have been clearly described in a well illustrated booklet (Conochie, 1964).

Bulk-packed cheese. Very large cheeses have from time to time been produced for publicity purposes. A relatively recent development, with the object of reducing manufacturing and packaging costs, has been the use of bulk containers in place of a multiplicity of smaller packs for the short-term storage of cheese intended primarily for processed cheese manufacture. Film-lined sheet-steel drums and cardboard containers have been used. Methods of pressing, preforming and sealing have been

adequately described in the patent literature (Kraft Foods Co. 1957; Drösler, 1959; Erekson, Steinke & Mohr, 1961; Meeker & Tate, 1963; Snow, 1964; Herlache, 1965).

Mechanized pressing and cheese sizing. A new system of producing 40- and 60-lb cheese of uniform weight, which will go into commercial use in the near future, has been described by Robertson (1963*a*, *b*, 1964*b*). This method involves the vacuum pressing of 1000 lb and more of salted cheese curd into a single block within a single large hoop. After overnight pressing the consolidated cheese is extruded into a cutter unit which divides the large block into a number of 40- or 60-lb blocks of normal rindless cheese dimensions.

'Pressmaster': automatic cheese pressing and packing

The first integrated system of cheese forming and packing is under development at present in New Zealand and has been briefly described by Robertson (1964*a*, 1965*c*). In this Pressmaster system, the moisture content of salted curd is adjusted to that required in the finished cheese in a special semi-continuous pre-press. An exact weight of this curd is then placed in a heated cavity and vacuum-pressed under high pressure for about 5 min. The resultant fully consolidated cheese which is formed without further loss of moisture or weight is then sealed in a rigid, impermeable export container which serves as the outer protection during subsequent maturation. Two variations on the system are being investigated; in one the cheese is formed within the export container and in the other the freshly formed cheese is ejected directly into the container.

The high-pressure short-time system of pressing holds promise of substantial labour savings and eliminates the need for hoops, cloths, trimming, sizing and wrapping.

Maturing of cheese

In recent years, a number of patents have been concerned with mechanical methods of turning cheese and coating cheese with surface protecting materials but as rinded Cheddar cheese is becoming progressively less important as an item of commerce these developments will not be discussed.

Relatively few studies of the influence of factors such as temperature and time on cheese ripening have been reported. Riddet, Whitehead, Robertson & Harkness (1961) have studied the influence of a number of variables on the development of oxidative defects in cheese during ripening. They found that properly wrapped rindless cheese did not develop tallowy discoloration and that other measures such as the waxing of traditional cheese reduced the incidence of this type of defect. Very little fat oxidation occurred in cheese ripened at 58 °F but was progressively increased when cheese was held at 42 and 33 °F. It was suggested that lower bacterial activity at lower temperatures and hence lower production of easily oxidizable bacterial metabolites meant that oxygen which gained access to the interior of cheese remained free to attack the fat. Cheeses ripened at 33 °F for 11 months were 'flat' in flavour, whereas those ripened at 58 °F had full, somewhat 'sulphide', flavours.

The stowage and storage of Australian rindless cheese has been briefly described by Conochie (1964). Conochie & Sutherland (1965*b*) have drawn attention to the importance of allowing adequate air circulation between rindless cheese in the first few weeks of cooling. They have suggested that temperature differences between the

outer and inner cheeses in tight-block-stacked cheese may account for differences in maturing rate and quality of different cheeses from the same vat of cheesemilk.

Robertson (1957), in a study of factors influencing the carbon dioxide content of cheese, found that carbon dioxide accumulation within cheese stored at 35 °F for 1 month was appreciably less than in cheese stored at 45 °F and that the effect persisted for several months after the temperature was raised to 45 °F, the normal New Zealand long-term storage temperature. In recent studies using commercially made cheese, Robertson & Gilles (unpublished) have found that the texture of cheese stored for one month at 35 °F followed by storage at 45 °F for 9 months was substantially closer than that of the control cheese stored continuously at 45 °F. The bodies of the control cheese were better than those of the cheese held at 35 °F for 1 month. It has been suggested (Robertson, 1957) that the numbers and proportion of different bacterial types are modified by the period at a lower temperature.

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