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VOL. 50 APRIL 1967 No. 4

Representative Research Papers with Shortened Titles:

Detection of caeruloplasmin in bovine milk and blood serum

By L. Å. HANSON, E.-G. SAMUELSSON and JAN HOLMGREN

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(*Received* 14 *October* 1966)

Summary. The copper-binding protein, caeruloplasmin, has been detected in bovine blood serum, colostrum and normal milk using immuno-diffusion techniques by comparison with a precipitating oxidase-positive system of human caeruloplasmin and anti-caeruloplasmin. The possible importance of this copper-binding protein of milk in the oxidative deterioration of milk and dairy products is discussed.

Bovine milk as secreted contains about 0·040 ppm. of copper (natural copper; Mulder, Mengers & Meijers, 1964) but can, because of contact with copper utensils during processing, sometimes contain up to 1 ppm. of secondary copper (Samuelsson & Bresby, 1964). The concentration of natural copper in milk shows a considerable variation, but colostrum has a significantly higher concentration of about 0·1–0·2 ppm. (Mulder *et al.* 1964). Seven days after parturition the copper content of the secretion falls into the normal range for milk. According to Samuelsson (1966) and Astrup (1966) the copper content of milk from high-yielding cows is about 0·073 ppm. and for low-yielding cows about 0·052 ppm.

There is a good correlation between the copper content of cow's blood and that of milk as secreted (Richterich, Rossi, Stillhart & Gautier, 1961), but Mulder *et al.* (1964) could not find any relation between the natural copper content of the milk and that of the fodder. About 95% of the total plasma copper is bound to caeruloplasmin (Laurell, 1960), a special copper-binding protein in blood; the remaining 5% of the total plasma copper is present in a comparatively weak complex with albumin. The normal blood caeruloplasmin concentration range is 0·27–0·38 mg/ml (Cartwright, Markowitz, Shields & Wintroee, 1960) with a mean value of 0·030 mg/ml, but in late pregnancy the mean value increases to 0·84 mg/ml.

Copper plays an important part as a catalyst of oxidative changes in milk and dairy products and also in other fat-containing foods. To some degree the development of oxidative deterioration in milk and dairy products is independent of the amount of natural copper found in milk (Samuelsson, 1966). Therefore, it is probable that the chemical structures in which the copper exists in milk is of considerable importance. The distribution of copper in the different fractions of the milk has been investigated by Samuelsson (1966, 1967*a*, *b*, *c*).

Several investigators (Bell & Mucka, 1940; Chilson, 1935; Dahle, 1935; Dahle & Palmer, 1937; Dorn, 1953; Roberts, 1943; Sharp, Trout & Guthrie, 1936) have presented evidence that an enzyme is responsible for the oxidative deterioration of

dairy products and Aurand, Woods & Roberts (1959) suggest that xanthine oxidase is concerned. However, King & Dunkley (1959) and Smith & Dunkley (1960; 1962*a*, *b*) maintain that ascorbic acid and copper are essential compounds for the induction of spontaneous oxidized flavour and reject the xanthine oxidase hypothesis.

Hanson (1960) has shown that human milk contains caeruloplasmin. Because caeruloplasmin is an oxidase (Malmström & Vänngård, 1960) with a pronounced oxidative capacity (Broman, 1964; Holmberg & Laurell, 1951) it is of great interest to determine whether or not this enzyme is also present in bovine milk. This paper describes some experiments on the presence of caeruloplasmin in bovine colostrum and normal milk.

MATERIALS AND METHODS

Milk. Colostrum was taken 2 days after parturition from Friesian cows from the herd at the Alnarp Institute. Normal bulk milk was obtained from the same herd. The colostrum and normal milk were centrifuged at once in an Alfa Laval separator type 100–AE, in which all the parts which were in contact with milk were of stainless steel. Merthiolate (1 pt to 10000 pts) was added to the milk samples which were then stored at -22 °C.

Caeruloplasmin. Five litres of bovine blood were collected using citrate or heparin to prevent coagulation and the serum was obtained by centrifugation. Caeruloplasmin was isolated from the blood serum by the method of Broman (1964). This involves three steps: (1) adsorption on DEAE-cellulose; (2) desorption of the caeruloplasmin and filtration through Sephadex G–100; (3) adsorption on hydroxyapetite. In a typical experiment the ratio of adsorption at 610 and 280 mμ after the second step was 0·040, which corresponds to a purity of about 95 %. After the third step the ratio increased to 0·042 corresponding to almost 100 % pure caeruloplasmin.

Human caeruloplasmin, prepared according to Björling (1963), was kindly provided by AB Kabi, Stockholm, Sweden. For reference, a sample of human blood serum was employed.

Antisera. Antisera against bovine colostrum or blood serum were prepared from rabbits by weekly subcutaneous injections of 2 ml of colostrum or blood serum in Freund's incomplete adjuvant. Blood was sampled monthly 1 week after the last injection. Horse antiserum against human blood serum and rabbit antiserum against isolated human caeruloplasmin were obtained from Behringwerke AG (Germany).

Immuno-diffusion techniques. Immunoelectrophoresis was performed according to Wadsworth & Hanson (1960) and the double diffusion analyses were made by the very sensitive microtechnique described by Wadsworth (1957).

Staining techniques. The immuno-diffusion plates were stained after washing out unprecipitated material and drying the agar layer to a thin film on the glass plate (Uriel, 1958; Wadsworth, 1957; Wadsworth & Hanson, 1960). Caeruloplasmin was stained selectively after the immuno-diffusion experiments, by means of its oxidative activity on *p*-phenylenediamine. The staining reaction was performed as described by Uriel (1958).

RESULTS

Blood sera. When human blood serum was tested with anti-human serum immune serum using both double diffusion and immunoelectrophoresis, one of the precipitation lines formed was clearly stained with the *p*-phenylenediamine reagent. That this precipitate corresponded to human caeruloplasmin was further verified by its fusion with the precipitate formed by human serum or human caeruloplasmin with the specific anti-caeruloplasmin serum. This oxidase-positive caeruloplasmin precipitate was used as a reference in the following experiments.

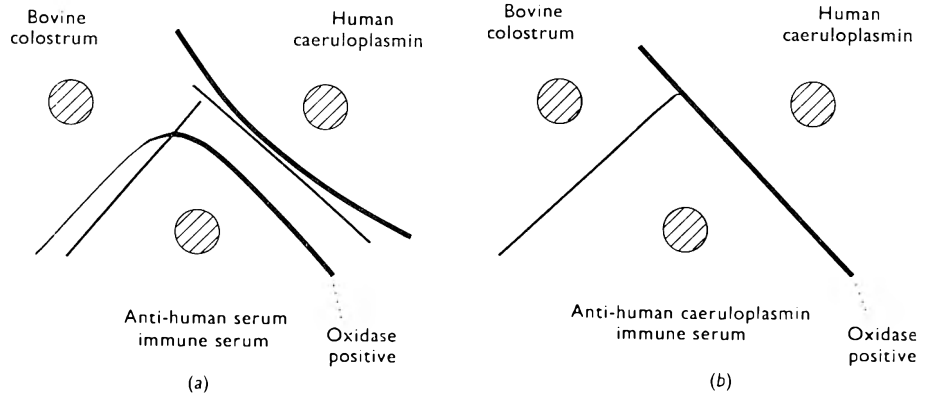

Fig. 1. (*a*) Bovine colostrum compared in a double diffusion analysis with a preparation of human caeruloplasmin by means of a horse antiserum to human blood serum. The oxidase-positive human caeruloplasmin precipitate gives a reaction of fusion with one of the precipitates formed with bovine colostrum. (*b*) Bovine colostrum compared in a double diffusion analysis with a preparation of human caeruloplasmin by means of a rabbit antiserum to human caeruloplasmin. The caeruloplasmin precipitate formed by bovine colostrum gives a reaction of partial fusion with the oxidase-positive human caeruloplasmin precipitate.

Diffusion analysis of bovine serum with anti-human serum immune serum gave several precipitates, one of which in comparative double diffusion plates gave a reaction of partial and in some cases complete fusion with the specifically staining human caeruloplasmin line. In contrast to the strongly staining human caeruloplasmin line, the much fainter bovine caeruloplasmin line stained only weakly or not at all. This might be due to the fact that in heterologous reactions such as this the precipitates are normally weaker than in homologous reactions. In agar electrophoresis of bovine serum an oxidase-positive fraction was seen in the $\alpha_2-\beta_1$-region.

Due to the high sensitivity of the immuno-diffusion techniques, several precipitates were obtained with the preparation of human as well as of bovine caeruloplasmin with their homologous antisera to blood serum. These extraneous precipitates were due to trace impurities, mainly of α-globulins, but in the bovine preparation albumin and γ-globulin were also involved according to the immunoelectrophoretic analysis. The caeruloplasmin in the bovine preparation was also identified by the reaction of complete or partial interference between the bovine caeruloplasmin precipitate and the oxidase-positive human caeruloplasmin precipitate.

Colostrum and normal milk. Double diffusion analyses of bovine colostrum with anti-human serum immune serum gave few precipitates, one of which showed a

reaction of partial or complete fusion with the specifically staining human caeruloplasmin (Fig. 1(*a*)). Comparing bovine colostrum with the preparation of human caeruloplasmin by means of the anti-human caeruloplasmin serum usually resulted in a reaction of partial interference. This heterologous reaction is illustrated in Fig 1 (*b*).

As in the experiments with the bovine serum, the bovine caeruloplasmin line formed by bovine colostrum and anti-human serum immune serum was very difficult to stain with the *p*-phenylenediamine reagent. The presence of caeruloplasmin in bovine colostrum was verified by comparative immunoelectrophoresis. The caeruloplasmin line formed by bovine colostrum had oxidase activity as judged from its staining reaction. After concentrating normal milk twice by ultrafiltration, caeruloplasmin could be demonstrated also in this secretion by double diffusion analysis.

DISCUSSION

The finding of caeruloplasmin in bovine colostrum and normal milk now makes it important to establish the concentrations involved.

The immuno-diffusion techniques used are only qualitative and the amounts of caeruloplasmin present in bovine colostrum cannot be estimated from these experiments. The methods are very sensitive, however, and in human milk they have detected several serum proteins in amounts less than 50 μg/ml (Hanson, 1960).

Because caeruloplasmin is an oxidase containing copper, its presence in milk may explain many problems of the oxidative deterioration of milk and dairy products. For example, the variability of the sensitivity of milk to oxidation may be due to a variability in the caeruloplasmin content; it is known that the caeruloplasmin content of the blood of an animal varies from time to time resulting in similar variations in the content of caeruloplasmin in milk.

The human and bovine caeruloplasmin would be expected to give reactions of partial interference in the comparative immuno-diffusion studies due to their biological relationship. That they in some cases gave reactions of complete fusion instead was probably due to the fact that some of our antisera to the human protein contained antibodies only to determinant groups common to the human and bovine proteins, and not to those present only in the human protein (cf. Fig. 1(*a*)).

Most of the protein in milk is synthesized in the mammary gland, but it is known that some of the whey proteins of the milk originate directly from the blood (Karlsson, 1966). Recent investigations of milk protein synthesis have shown that some transport of proteins from blood into the milk is detectable (Dixon, Weigle & Vasquez, 1961; Laurell & Morgan, 1965; Murphy, Aalund, Osebold & Carrol, 1964; Murphy, 1964; Pierce & Feinstein, 1965). Caeruloplasmin, which has a molecular weight of about 160000 could be one of the proteins entering the milk directly from the blood. This is also suggested by the quantitative correlation between copper content of milk and serum (Richterich *et al.* 1961).

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Acetaldehyde and diacetyl production by *Streptococcus thermophilus* and other lactic streptococci

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Summary. The quantities of acetaldehyde and diacetyl produced in skim-milk and MRS media are reported for strains of *Streptococcus lactis*, *Str. cremoris*, *Str. diacetilactis*, *Str. faecalis* and *Str. thermophilus* obtained from culture collections or freshly isolated from natural milk cultures used in cheese manufacture. The diacetyl:acetaldehyde ratio in skim-milk medium was found to be approximately 0·1:1 for *Str. lactis* and *Str. cremoris*, 0·5:1 for *Str. diacetilactis* (5:1 for *Str. diacetilactis* PIM 13 and K 2 strains) and for *Str. thermophilus* 0·3:1, in skim-milk medium, and 0·8:1, in MRS medium; in the latter species cultured in MRS medium the ratio varied from 3·5:1 to 0·04:1 and when cultured in milk from 1·2:1 to 0·03:1.

In both media all strains of *Str. thermophilus* formed more acetaldehyde and diacetyl than other homofermentative lactic streptococci.

The ratio diacetyl: acetaldehyde in 9 strains of *Str. diacetilactis* was found to be unsuitable for a good culture flavour.

It is well known that products other than lactic acid may be elaborated by mesophilic homofermentative lactic acid streptococci. In recent years attention has been given to small quantities of formic, acetic, propionic and butyric acid, ethanol, carbon dioxide, acetoin, acetaldehyde and diacetyl produced by these organisms. Of these components, however, acetaldehyde and diacetyl have been recognized as especially important flavour components of dairy products. Diacetyl has long been regarded as one of the more important compounds found in butter aroma, and acetaldehyde has been shown by Bading & Galesloot (1962) to be responsible for a flavour defect of lactic cultures.

Whereas low concentrations of acetaldehyde are desirable in mixed-strain butter cultures and in yoghurt cultures (Pette & Lolkema, 1950) excessive quantities of acetaldehyde have been shown to be correlated with 'green flavour' (Lindsay, Day & Sandine, 1965). Lindsay & Day (1965) found that the concentrations of diacetyl and acetaldehyde in lactic cultures determine the intensity of the flavour but that the relative amounts are of great importance for the flavour quality; the diacetyl: acetaldehyde ratio of 4:1 occurred in normal flavoured cultures and when the ratio was lower than 3:1 a 'green flavour' was observed.

The quantitative production of diacetyl and acetaldehyde in cultures of *Str. lactis*, *Str. lactis* var. *maltigenes*, *Str. cremoris*, *Str. diacetilactis* and *Leuconostoc citrovorum*

has already been studied (Lindsay *et al.* 1965; Lindsay & Day, 1965; Pack, Sandine, Elliker, Day & Lindsay, 1964; Keenan, Lindsay, Morgan & Day, 1966; Morgan, Lindsay, Libbey & Pereira, 1966; Vedamuthu, 1966). The present investigation was made to determine the diacetyl:acetaldehyde ratio in *Str. thermophilus* cultures in comparison with cultures of *Str. lactis*, *Str. cremoris* and *Str. diacetilactis* and some *Str. faecalis* strains in skim-milk and in medium (MRS) in which the growth of many strains was better than in sterilized milk.

EXPERIMENTAL PROCEDURES

Cultures

Authenticated strains of *Str. thermophilus* and strains of other streptococci are listed in Table 1.

Other strains of *Str. thermophilus* were isolated from natural milk cultures commonly used in the manufacture of many types of cheese in the north of Italy (Table 2).

Table 1. *Authenticated strains of streptococci examined*

Str. thermophilus	P 15[1], 23 A[1], T 4[1], 26[1]
	3203[2]
	Z 17[3]
Str. faecalis	NCDO 610, ATCC 11420
	B 65[1], F 1[4]
Str. lactis	NCDO 275, NCDO 605, NCDO 606
	M 87[1], T 2017[1]
Str. diacetilactis	NCDO 176, NCDO 188, NCDO 615
	2[1]
	DRC 1[3], DRC 3[3], DRC 4[3]
	PIM 13[5], PIM K2[5], PIM 704[5], PIM 710[5]
Str. cremoris	3141[2]
	C 3[3], C 13[3]
Streptococcus group N	NCDO 617

NCDO = National Collection of Dairy Organisms.
ATCC = American Type Culture Collection.
Other strains received from :

1. National Institute for Research in Dairying, Shinfield, Reading, England.
2. Nederlands Instituut voor Zuivelonderzoek, Ede, Holland.
3. Dairy Research Institute Laboratory, Department of Agriculture, Victoria, Australia.
4. Istituto di Microbiologia, Università Cattolica, Piacenza, Italy.
5. University of Lomonosov, Moscow, U.S.S.R.

The cultures were transferred every 2 days in MRS medium and incubated at 45 °C for 18 h (*Str. thermophilus*) or at 30 °C for 18 h (other species).

For determination of acetaldehyde and diacetyl production in milk, the bacterial strains were cultured in autoclaved skim-milk medium incubated at 30 °C or 45 °C depending on the type of micro-organism.

Determination of the acetaldehyde and diacetyl content

Each of the cultures after two 1-day transfers for activity restoration in the appropriate medium, were analysed for acetaldehyde, diacetyl and pH after 24- or 28-h incubation in skim-milk or MRS medium.

Acetaldehyde was determined by the 3-methyl-2-benzothiazolone hydrazone hydrochloride method described by Lindsay & Day (1965).

The Owades & Jakovac method, as modified by Pack *et al.* (1964), was used for diacetyl determination.

All analyses were made in duplicate.

Table 2. *Diacetyl and acetaldehyde production by freshly isolated strains of* Str. thermophilus *in skim-milk and in MRS media*

	Diacetyl (A) and acetaldehyde (B), μg/ml culture*						
Medium . . .	MRS			Skim-milk			
Strain	A	B	A/B	pH	A	B	A/B
S 215	9·60	2·80	3·40	4·72	0·90	2·90	0·30
S 112	6·20	4·60	1·40	4·50	1·50	6·50	0·20
S 127	6·10	2·70	2·50	4·60	3·80	6·20	0·60
S 152	3·60	3·50	1·00	4·57	1·20	6·50	0·20
S 61	5·20	2·60	2·00	4·60	1·10	5·60	0·20
S 221	10·00	3·70	2·70	4·60	1·90	6·30	0·30
S 222	4·30	4·20	1·00	4·65	0·70	4·80	0·20
S 120	2·10	5·70	0·30	4·40	0·50	8·30	0·06
S 164	3·80	6·10	0·60	4·45	0·10	7·40	0·01
S 188	6·40	6·50	1·00	4·40	0·40	7·50	0·05
S 18	3·00	2·60	1·10	5·02	0·10	1·60	0·06
S 26	1·10	4·30	0·30	4·50	0·30	1·80	0·15
S 82	4·30	4·60	0·90	4·52	1·70	6·50	0·25
S 128	1·80	4·80	0·30	4·45	1·70	5·90	0·25
S 132	0·85	3·70	0·20	4·50	1·85	4·60	0·40
S 53	6·40	4·00	1·60	4·60	2·30	6·70	0·35
S 88	10·00	4·60	2·20	4·45	1·65	7·10	0·25
S 97	8·50	3·10	2·80	4·70	1·45	6·70	0·20
S 130	8·20	3·80	2·20	4·50	2·45	8·30	0·30
S 133	5·70	5·00	1·10	4·60	1·75	7·70	0·25
S 142	6·30	2·70	2·30	4·50	2·00	4·00	0·50
S 155	8·80	2·70	3·00	4·26	3·10	2·60	1·20
S 190	2·00	3·30	0·70	5·39	1·20	2·80	0·40

* Average of duplicate analyses; blank values estimated on uninoculated media have been deducted.

RESULTS AND DISCUSSION

Table 3 shows the production of acetaldehyde and diacetyl in MRS and skim-milk media, by the authentic strains of *Str. cremoris*, *Str. lactis*, *Str. diacetilactis*, *Str. thermophilus* and *Str. faecalis;* whereas, in Table 2, figures are reported concerning the strains of *Str. thermophilus* isolated from natural milk cultures.

After 24-h incubation all the lactic homofermentative mesophilic streptococcal cultures were found to produce diacetyl and acetaldehyde in MRS and in autoclaved skim-milk media; some strains of *Str. cremoris*, and all strains of *Str. lactis*, produced very small amounts of diacetyl, and all cultures formed appreciably more acetalde-

Table 3. *Diacetyl and acetaldehyde production by lactic acid streptococci in skim-milk and in MRS media*

Medium . . .		Diacetyl (A) and acetaldehyde (B), μg/ml culture*						
		MRS			Skim-milk			
Species and strain		A	B	A/B	pH	A	B	A/B
Str. diacetilactis	DRC1	3·07	7·40	0·40	4·42	3·40	5·20	0·65
	No. 2	3·20	8·90	0·30	4·55	4·10	6·40	0·65
	PIM13	22·30	8·60	2·60	5·15	30·10	7·20	4·10
	614	3·45	5·40	0·65	4·50	6·00	8·30	0·70
	PIMK2	39·20	11·20	3·50	5·02	55·00	11·40	4·80
	PIM704	3·90	4·60	0·90	4·72	1·50	9·70	0·15
	PIM710	3·60	6·40	0·60	4·71	1·20	10·30	0·10
	DRC3	2·90	10·20	0·30	4·35	1·40	2·20	0·65
	DRC4	2·30	2·30	1·00	5·20	0·60	5·50	0·10
	188	2·05	9·70	0·20	5·10	1·30	4·10	0·30
	176	5·70	7·30	0·80	4·30	3·70	7·50	0·50
Str. lactis	M87	0·50	4·70	0·10	4·45	0·10	2·30	0·05
	275	0·60	4·70	0·15	4·40	0·05	0·70	0·07
	605	0·30	3·70	0·10	4·50	0·10	1·10	0·10
	606	0·30	3·50	0·10	4·40	0·10	0·80	0·10
	T2017	0·25	6·50	0·04	4·50	0·10	2·00	0·05
Str. cremoris	C3	0·25	8·00	0·03	4·90	0·05	1·10	0·05
	C13	0·20	2·90	0·07	4·80	0·10	0·30	0·30
	3141	1·90	7·20	0·25	4·80	2·30	3·70	0·60
Str. Group N (*lactis-cremoris* intermediate)	617	2·15	7·80	0·30	4·80	1·60	1·80	0·90
Str. thermophilus	P15	0·70	1·60	0·40	4·60	0·10	0·40	0·25
	Z17	1·20	2·50	0·50	5·10	1·00	1·30	0·75
	T4	2·80	4·55	0·60	4·15	0·50	6·20	0·08
	23A	3·20	2·65	1·30	4·35	1·20	4·50	0·25
	26	2·30	4·60	0·50	4·15	2·10	6·40	0·35
	3203	8·50	5·10	1·70	4·55	1·40	1·00	1·40
Str. faecalis	F1	3·90	3·90	1·00	4·95	0 00	0·00	—
	B65	1·20	11·20	0·10	5·20	0 00	3·50	—
	610	1·70	13·60	0·10	4·95	0 00	5·30	—
	11420	0·60	12·60	0·06	5·60	0 00	4·10	—

* Average of duplicate analyses; blank values estimated on uninoculated media have been deducted.

hyde; similar results were obtained by Harvey (1960), Lindsay & Day (1965), Lindsay *et al.* (1965) and Keenan *et al.* (1966).

In general, *Str. diacetilactis* formed considerably higher quantities of both compounds than did *Str. cremoris* or *Str. lactis* either in MRS medium or in autoclaved skim-milk medium; the acetaldehyde production, however, except for the strains PIM, was somewhat lower than those reported by Harvey (1960) (mean 7 ppm. as compared to 12 ppm. reported by Harvey). The acetaldehyde production by the PIM strains was similar to that found by Harvey (1960) but the diacetyl production reached exceptionally higher levels (30–50 ppm).

As indicated in Table 2, milk cultures of all our isolates of *Str. thermophilus* contained acetaldehyde and diacetyl: mean 3·0 and 1·0 ppm. in skim-milk of acetaldehyde and diacetyl, respectively, compared with 3·5 and 3·0 ppm. in MRS medium.

This is consistent with our observation (Bottazzi, 1966) that strains of *Str. thermophilus* give positive Voges-Proskauer reaction.

Str. faecalis did not produce diacetyl in milk but produced about 5 ppm. of acetaldehyde.

Of more interest is the ratio diacetyl:acetaldehyde and its variations under the different experimental conditions: the mean ratio was found in MRS medium to be approximately 0·10 for *Str. lactis*, 0·15 for *Str. cremoris*, 0·50 for *Str. diacetilactis* (5·3 for *Str. diacetilactis* PIM 13 and K 2 strains) and 0·80 for *Str. thermophilus*; in autoclaved skim-milk medium the corresponding values were 0·08, 0·8, 0·4 (5·2) and 0·3.

As shown in Table 2, *Str. thermophilus* produced diacetyl and acetaldehyde in the approximate ratios of 3·5 to 0·04 in MRS medium and 1·20 to 0·03 in skim-milk medium.

In view of the established significance of this ratio in the flavour of lactic cultures, our data suggest that in the group of mesophilic homofermentative streptococci all single-strain cultures produce these compounds in an unfavourable ratio. Lindsay *et al.* (1965) observed 'green flavour' when the ratio of the 2 compounds was lower than 3·0; on this basis only the *Str. diacetilactis* strains PIM 13 and K2 would be likely to produce a good culture flavour.

Many strains of *Str. thermophilus*, isolated from natural milk cultures, produced in autoclaved skim-milk medium significant quantities of diacetyl and for some strains the ratio with acetaldehyde reached 1·2. The quantities of diacetyl produced in MRS medium by *Str. thermophilus* were found to be from 0·30 to 10·00 ppm. and in skim-milk medium from 0·30 to 3·80 ppm.; the acetaldehyde concentration varied from 2·60 to 6·50 ppm. in MRS medium and from 1·60 to 8·30 ppm. in skim-milk medium. This production of diacetyl in MRS and skim-milk media from strains of *Str. thermophilus* was higher than that of all the other homofermentative lactic streptococci.

Further investigations are now in progress to determine the diacetyl:acetaldehyde ratio in mixed-strain cultures of *Str. thermophilus* and also to determine other carbonyl compounds with particular reference to the neutral monocarbonyl class.

The authors thank Dr C. Corradini for his assistance and helpful suggestions.

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Identification of fatty acids from butterfat using a combined gas chromatograph-mass spectrometer

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Summary. The identification and approximate quantitative determination of methyl esters of fatty acids from commercial butterfat was obtained with a combined gas-chromatograph-mass spectrometer instrument. Fifty-two components, straight chain saturated and unsaturated, as well as branched chain compounds, were identified. Seven monomethyl saturated fatty acid methyl ester isomers were identified for both C_{15} and C_{17}, i.e. with chain lengths of 14 and 16 carbon atoms, respectively. Multibranched fatty acids with molecular weights of 326 and 368 were found. The results were obtained in one day.

The fatty acid composition of butterfat has been studied for a long time and many papers have been written describing quantitative and qualitative determinations (e.g. Hansen & Shorland, 1951; Gerson, Hawke, Shorland & Melhuish, 1960). In almost all cases the compounds have been isolated and carefully studied by conventional methods, although mass spectrometers have been used as a complementary method of checking the results (Hansen & Morrison, 1964). Almost all of these previously used identification methods required a lot of material and the isolation of a single compound from a complex mixture was often difficult and time consuming.

In recent work, the value of using a combination of a gas chromatograph and a mass spectrometer in the separation of a complex mixture of methyl esters of fatty acid from butterfat was illustrated (Ryhage, 1964). A packed high temperature GLC column was used and 27 compounds were identified from the mass spectra alone. In the present work the same complex mixture of methyl esters of fatty acids from butterfat was studied by using a capillary column in a combined GLC-MS instrument. This method enabled the complete composition of a butterfat sample to be studied in one day. Systematic studies can, therefore, now be made of the effects of change in the cow's food and the age and breed of the cows on the day-to-day composition of the butterfat.

EXPERIMENTAL

The methyl esters of the fatty acids from commercial butterfat were obtained using the following procedure. Butter (2 g) was refluxed with 50 ml of 50 % ethanolic potassium hydroxide for 2 h. After dilution with water the non-saponifiable matter was extracted with petroleum ether. The aqueous phase was acidified with hydrochloric acid and extracted twice with ether. The combined ether extracts were

washed with water until they were neutral and then evaporated to dryness. A 20-mg sample of the residue was methylated with diazomethane and dissolved in 0·1 ml acetone (i.e., 20 μg/μl); to enable minor components to be identified up to 0·5 μl was injected on to the chromatogram column. An LKB 9000 GLC-MS instrument was used with a 25 m × 0·25 mm capillary column coated with Castorwax 2G29 (Perkin-Elmer Ltd.) and a helium flow rate of 5 ml/min. The temperature of the column was programmed at 1 deg C/min from an initial value of 110 °C. The temperature of the flash heater and the molecule separator was kept at about 225 °C. The mass spectra were obtained at a constant accelerating voltage of 3500 V with an electron energy of 70 eV. The magnetic current scan and the oscillograph recorder were started at about the same time and the scanning time was about 3 sec in the mass range m/e 12–300. The approximate calculated percentage of each compound is based on the area in mm^2 of the peak obtained from the total ion current recorder. For this calculation it has been assumed that the following conditions are fulfilled: (1) the area is proportional to the number of moles; (2) the ionization in the ion source is proportional to the pressure and is independent of the compound studies; (3) no mass discrimination occurs in the molecule separators.

For accurate quantitative work, these assumptions would require investigation and appropriate correction factors would have to be used. Some low molecular weight compounds were lost due to the initial temperature of the column and some high molecular weight compounds were also lost due to the experiment being stopped after methyl n-henieicosanoate (marked 66) had been recorded.

RESULTS AND DISCUSSION

The chromatographic record of the methyl esters obtained from butterfat is shown in Fig. 1. The 'spikes' indicate where the mass spectra were taken. In some cases, as a check on overlapping components, several mass spectra were taken for each peak. Sixty-six mass spectra were recorded and of these 52 were identified and are shown in Tables 1*a–d*.

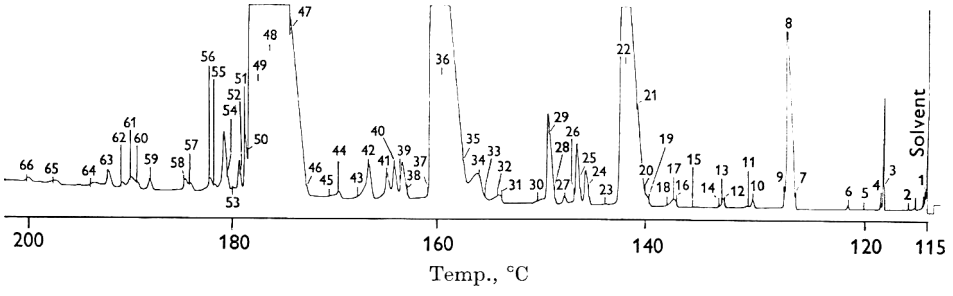

Fig. 1. Chromatographic separation of methyl esters of fatty acid from butterfat. Capillary column 25 m × 0·25 mm. Castorwax coated. Programmed 1 deg. C/min.

The identified methyl esters of normal fatty acids are given in Table 1*a*, and these are seen to be a homologous series of compounds from methyl n-decanoate (M = 186) up to methyl n-henieicosanoate (M = 340). These components were not completely separated from the unsaturated compounds, and for this reason it was difficult to

make direct quantitative determinations from the peak area. By using the heights of the parent molecular ion peaks, which are proportional to the gas concentration of sample in the ion source, an approximate quantitative composition could be obtained (Hallgren, Ryhage & Stenhagen, 1957). Fig 2 shows the heights of the parent molecule peaks for methyl stearate, methyl oleate, methyl linoleate and methyl linolenate, obtained from mass spectra taken at points 46–51 marked on the gas

Table 1*a*. *Methyl esters of straight chain saturated fatty acids*

Component	Identified compound	Mol. wt.	Relative quantity mol., %
3	Methyl n-decanoate	186	0·5
6	Methyl n-undecanoate	200	0·1
8	Methyl n-dodecanoate	214	3·8
12–13–14	Methyl n-tridecanoate	228	0·2
21–22–23	Methyl n-tetradecanoate	242	9·8
28–29	Methyl n-pentadecanoate	256	2·1
35–36–37	Methyl n-hexadecanoate	270	18·0
42–43	Methyl n-heptadecanoate	284	1·1
49–50	Methyl n-octadecanoate	298	10·6
57	Methyl n-nonadecanoate	312	0·2
63	Methyl n-eicosanoate	326	0·3
66	Methyl n-henieicosanoate	340	0·1
		Total	46·8

Table 1*b*. *Methyl esters of straight chain unsaturated fatty acids*

Component	Identified compound	Mol. wt.	Relative quantity mol., %
4	Methyl decenoate	184	0·1
9	Methyl dodeconoate	212	1·0
19–20	Methyl tetradecenoate	240	3·5
27	Methyl pentadecenoate	254	0·2
33–34	Methyl hexadecenoate	268	2·3
41	Methyl heptadecenoate	282	0·6
48–51	Methyl octadecenoate	296	28·3
48–51	Methyl octadecandienoate	294	4·5
48–51	Methyl octadecantrienoate	292	3·0
54	Methyl octadecandienoate	294	1·3
56	Methyl nonadecenoate	310	0·2
61–62	Methyl eicosenoate	324	0·6
65	Methyl henieicosenoate	338	0·2
		Total	45·8

chromatographic diagram Fig. 1. Since the heights of these peaks are not proportional to the relative quantity of each compound, correction factors must be used. These were found to be 2·60, 0·35, 1·00, 0·76 for stearate, oleate, linoleate and linolenate, respectively. From the area of the curves it was possible to calculate the approximate relative quantities of the 4 components. More exact quantitative figures are obtained by taking mass spectra more frequently (Sweeley, Elliott, Fries & Ryhage, 1966). Quantitative determination of other groups of overlapped components are calculated in the same way. Fig. 3 shows the high mass end of the mass spectrum identified as methyl n-eicosanoate (M = 326).

The unsaturated components identified are given in Table 1*b*. A homologous series

Table 1c. *Methyl esters of monomethyl fatty acids*

Component	Identified compound	Mol. wt.	Relative quantity mol., %
7	Methyl 9-methylundecanoate	214	0·1
10	Methyl 11-methyldodecanoate	228	0·15
11	Methyl 10-methyldodecanoate	228	0·05
16–17–18	Methyl 12-methyltridecanoate	242	0·26
25	Methyl 13-methyltetradecanoate	256	
25	Methyl 12-methyltetradecanoate	256	
25	Methyl 10-methyltetradecanoate	256	
25	Methyl 9-methyltetradecanoate	256	1·20
25	Methyl 8-methyltetradecanoate	256	
25	Methyl 7-methyltetradecanoate	256	
26	Almost identical to component 25	256	
30	Methyl 11-methyltetradecanoate	256	
31–32	Methyl 14-methylpentadecanoate	270	0·35
39	Almost identical to component 40	284	
40	Methyl 15-methylhexadecanoate	284	
40	Methyl 14-methylhexadecanoate	284	
40	Methyl 12-methylhexadecanoate	284	0·70
40	Methyl 11-methylhexadecanoate	284	
40	Methyl 10-methylhexadecanoate	284	
40	Methyl 9-methylhexadecanoate	284	
40	Methyl 8-methylhexadecanoate	284	
55	Methyl 16-methyloctadecanoate	312	0·3
		Total	3·75

Table 1d. *Miscellaneous types of compounds from butterfat*

Component	Identified compound	Mol. wt.	Relative quantity mol., %
1	Impurities from solvent and components from butterfat	—	—
2	Mixture of some low molecule weight compounds	—	—
5	Ethyl n-decanoate	200	0·02
15	Mass spectrum of background	—	—
24	Ethyl n-tetradecanoate	256	0·7
38	Ethyl n-hexadecanoate	284	0·45
44	Butyl phthalate (impurity)	278	0·2
45	$CH_3—O—\overset{\overset{O}{\|\|}}{C}—C—\overset{\overset{C}{\|}}{C}—C_3—\overset{\overset{C}{\|}}{C} \cdots C_{11}$	326	0·05
52–53	Ethyl octadecenoate	310	0·4
58	Spectra missed because the roll of recording paper ran out at m/e 200.		
59	Unsaturated methylester of fatty acids	318	0·3
60	Methyl cosantrienoate	320	0·4
64	$CH_3—O—\overset{\overset{O}{\|\|}}{C}—C_6—\overset{\overset{C}{\|}}{C} \cdots C_{13}$	368	0·3
		Total	2·82

is found for the unsaturated compounds as for the normal saturated compounds, i.e. from methyl decenoate (M = 184) to methyl henieicosandienoate (M = 338) except for methyl undecenoate (M = 198) and methyl tridecenoate (M = 224) which could not be found. The dominating component in the C_{18} group is methyl oleate with an approximate relative molar proportion of 28·3 %. The mass-spectra of unsaturated

compounds reveal the number of double bonds but not their positions. For this reason, the common names for the identified unsaturated components are only tentatively given for the C_{18} compounds.

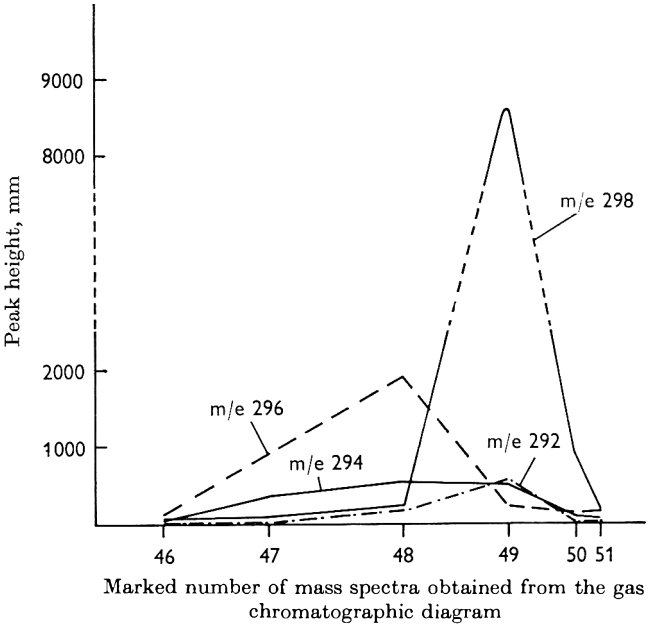

Fig. 2. Diagram showing the parent molecular ions obtained from mass spectra taken at points 46–51 marked on the gas chromatographic diagram, Fig. 1.

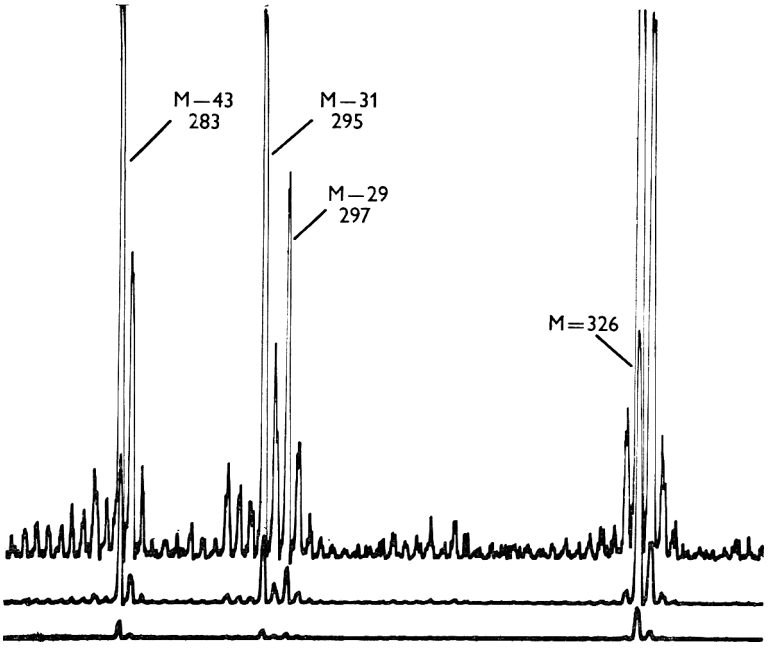

Fig. 3. The high mass end of a mass spectrum (taken at point 63, Fig. 1) identified as methyl n-eicosanoate.

Methyl esters of monomethyl branched fatty acids are found for almost any chain-length between C_{10} and C_{18}. Iso- and *ante*-iso components have shorter retention times than the normal straight-chain isomers. Isomers with the methyl substituent in the middle of the chain do not separate at all with the column used. A mass spectrum taken at point marked 25 in the gas chromatographic record (Fig. 1) is identified as a mixture of 6 monomethyl substituted isomers from methyl 7-methyltetradecanoate to methyl 13-methyltetradecanoate except methyl 11-methyltetradecanoate. It is surprising that this latter compound which corresponds to the component marked 30 in Fig. 1, is alone completely separated from its isomer and is the only *ante-ante*-iso compound identified in the total fraction. Fig. 4 shows a mass spectrum taken at point 40 and this component was identified as a mixture of monomethyl

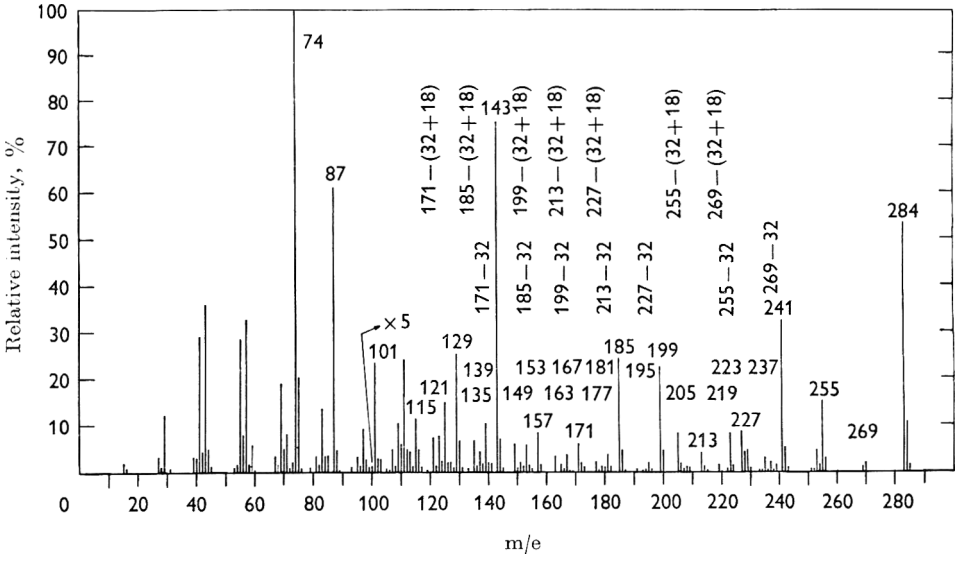

Fig. 4. A mass spectrum taken at point 40 marked on the gas chromatographic diagram Fig. 1. Compound was identified as methyl-eicosanoate.

substituted isomers from methyl 8-methyltetradecanoate to methyl 15-methylhexadecanoate. As mentioned above, the *anti-anti*-iso compound, i.e. the methyl 13-methyltetradecanoate was not found. The retention times for the monomethyl compounds are in agreement with the chain lengths of the compound.

Methyl branched fatty acids show characteristic peaks at M − R, M − (R + 28), M − (R + 32) and M − (R + 32 + 18) where R, 32, 18 are the molecular weights of alkyl part of the chain attached to the methyl, of methanol and of water, respectively (Ryhage & Stenhagen, 1960). For a single monomethyl compound the ion fragments M − 32 and M − (32 + 18) are greater than 3 % of the base peak m/e 74. In the case of spectrum 40, the typical ion fragments M − (R + 32) and M − (R + 32 + 18) are only about 1 % of m/e 74. For this reason the high mass end of the mass-spectrum has been enlarged 5 times to simplify reading of the spectrum. The mass spectrum indicates methyl groups at different positions and the gas-chromatographic diagram shows that the length of the chain must be identical for all components since the retention time is identical.

Table 1*c* shows that altogether 20 components were identified as monomethyl fatty acids but the approximate quantity present was only 3·75 %. Monomethyl substituted compounds were found for all chain lengths from C_{11} to C_{18} except for C_{17}. However, this could be a misinterpretation since the C_{18} components, which contain about 50 % of the total, overlap the C_{17} monomethyl substituted compounds on GLC Table 1*d* contains miscellaneous types of components which are not related to the 3 general types of compounds given in the Table 1*a–c*. It is somewhat difficult to identify components at points marked 1 and 2 in the gas chromatogram, since these are probably mixed with components from the solvent. Four components at points 5, 24, 38 and 52–53 are found to be ethyl esters of straight chain fatty acids. It is not known if they were present in the butter sample or artifacts resulting from the working of the sample. The estimated amount of ethyl decanoate is 30 ng.

Spectrum 45 is a very interesting compound with a molecular weight of M = 326. It is identified as having methyl substituents at carbon atoms 3 and 7 and there are some indications of another methyl group at carbon atom 11. It is possible that the structure of this compound is methyl 3,7,11,14-tetramethylhexadecanoate (Sonneveld, Haverkamp Bergemann, van Beers, Kemming & Schogt, 1962), or methyl 3,7,11,15-tetramethylhexadecanoate (Eglington, Douglas, Maxwell, Ramsey & Ställberg-Stenhagen, 1966). The retention time also indicates such a multibranched compound. Component 64 with a molecular weight of M = 368 with an identified methyl substituent on carbon 8 probably represents a similar compound. This compound must have several methyl groups to give approximately the same retention time as component 63 which is found to be methyl n-eicosanoate (M = 326).

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n-Pent-1-en-3-ol and n-pent-1-en-3-one in oxidized dairy products

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Summary. Pent-1-en-3-ol and pent-1-en-3-one were isolated in approximately equal amounts from buttermilk obtained from oxidized cream. The two vinyl compounds were identified by combined gas chromatography and mass spectrometry. The flavour of pent-1-en-3-ol is comparatively mild being detectable in water, skim-milk, butter oil and butter at 3 pts in 10^6, 3 pts in 10^6, 1 pt in 10^5 and 1 pt in 10^5, respectively. The flavour of pent-1-en-3-one is approximately 1000 times stronger being detectable in water, skim-milk, butter oil and butter at 1 pt in 10^9, 3 pts in 10^9, 5 pts in 10^9 and 5 pts in 10^9, respectively: these concentrations are of the same order of magnitude as for the compound responsible for metallic flavour, oct-1-en-3-one.

The formation of pent-1-en-3-ol and pent-1-en-3-one is discussed.

Two vinyl compounds have been shown to contribute to certain types of oxidized flavours in dairy products. Oct-1-en-3-one is responsible for a metallic flavour (Stark & Forss, 1962) and oct-1-en-3-ol is responsible for a mushroom flavour (Stark & Forss, 1964). The ketone can be detected organoleptically in butter oil at 1 pt in 10^9 and the alcohol at 1 pt in 10^7.

In an investigation of n-alkan-1-ols in oxidized butter (Stark & Forss, 1966), a compound occurring in large amount and with a gas chromatographic retention time on Apiezon M and Carbowax 20M columns similar to that of pent-1-en-3-ol was detected.

From theoretical considerations of the oxidative breakdown of arachidonic acid with the formation of oct-1-en-3-one, the presence of the corresponding vinyl ketone (pent-1-en-3-one) might be expected from linolenic acid. This paper reports the identification of pent-1-en-3-ol from oxidized butter, and pent-1-en-3-one and pent-1-en-3-ol from buttermilk prepared from oxidized cream.

The purification and identification of the 2 compounds was achieved mainly by a combination of gas chromatography and mass spectrometry.

EXPERIMENTAL

Control (normal) and oxidized (copper and ascorbic acid) butters were prepared, and the volatile compounds isolated as previously described (Stark & Forss, 1966). Control and oxidized buttermilks were obtained from the churning of normal and oxidized (copper and ascorbic acid) cream held for 5 days at 2 °C. By vacuum sub-

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limation through a trap at $-55\,^{\circ}C$ most of the pent-1-en-3-ol and pent-1-en-3-one in a 2–4 ml concentrated distillate from 6 kg buttermilk was isolated in less than 100 μl water. One to 10 μl of this solution was sufficient for analysis by gas chromatography-mass spectrometry.

A Perkin-Elmer F11 Gas Chromatograph was modified to allow continuous sampling of the effluent from the gas chromatographic column into a mass spectrometer and a flame ionization detector (Fig. 1).

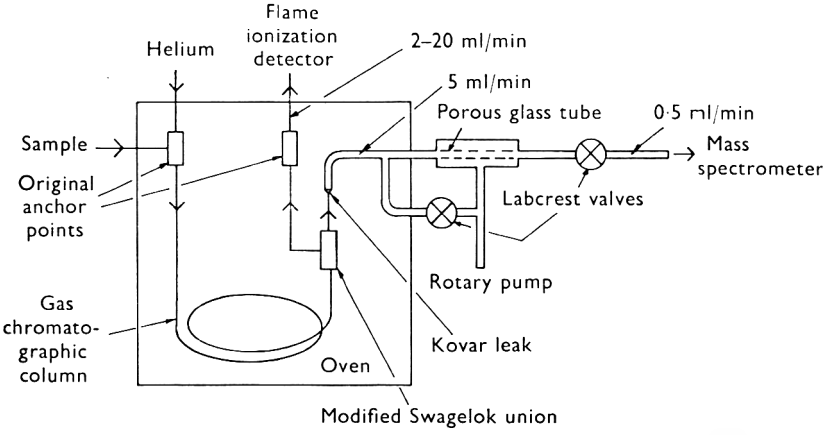


Fig. 1. Diagram of gas chromatography-mass spectrometer combination.

In the gas chromatographic oven, a new column outlet anchor point was constructed of a Swagelok $\frac{1}{16}$ in. $\times \frac{1}{16}$ in. union silver-soldered to a fixed bracket. The body of this union was drilled to accept a piece of $\frac{1}{16}$ in. O.D. stainless steel tubing which was then silver-soldered into place and the other end was connected to the normal anchor point leading to the flame detector. A short length (approx. 3 cm) of $\frac{1}{16}$ in. Kovar tube was sealed to a 5 mm O.D. Pyrex tube and inserted into the new anchor point. The other end of the Pyrex tube was joined to a porous glass tube (20 cm long, 8 mm O.D., 2 mm wall thickness, average pore diam. 1 μ; Watson & Biemann, 1965). The outlet end of the porous tube was taken to the mass spectrometer via a Labcrest 4 mm quick opening Teflon valve (Catalogue no. 7951200004) so that, apart from the Swagelok union and the short length of Kovar tubing, the connexion between the column outlet and the mass spectrometer was constructed entirely from Pyrex. The line was maintained at $140\,^{\circ}C$.

The porous tube was sealed into a 2·5-cm glass tube connected to a rotary oil pump so that the helium carrier-gas could be preferentially removed from the flow to the mass spectrometer. The Kovar tube was crimped to allow a flow of 5 ml/min of helium into the mass spectrometer inlet line.

In use, the Labcrest valve is adjusted until the pressure indicated on an ionization gauge connected to the ion source region rises to approximately 5×10^{-5} torr corresponding to an inlet flow of approximately 0·5 ml/min helium. Since the analyser section of the mass spectrometer is differentially pumped, the pressure in the analyser does not rise above 1×10^{-6} torr.

The rest of the 5 ml/min passing through the crimped Kovar tube is drawn off by the rotary pump via the porous tube. The remainder of the column effluent (2–20 ml/min) is allowed to flow through the flame ionization detector.

It is sometimes necessary to inject large amounts of water into the gas chromatographic column, up to 40 μl being commonly required. To prevent this large quantity of water passing into the mass spectrometer a by-pass line was placed between the mass spectrometer inlet and the rotary pump line through another Labcrest valve. During the time when the water was being eluted this valve could be opened to allow some of the water to be pumped away. This naturally affects the sensitivity of the instrument and since the two C_5 vinyl compounds discussed in this paper were eluted at approximately the same time as water, it was an advantage to be able to concentrate them, thereby minimizing the quantity of water injected onto the column.

The volume between the Kovar leak and the flame ionization detector was kept as small as possible to reduce hold-up time. It has been found that even for high boiling compounds such as 5-dodecalactone, the difference in time between the signals from the flame ionization detector and mass spectrometer is less than 1 sec.

Mass spectra were measured on a 60°, 30-cm radius single focusing instrument of the Inghram type constructed by the C.S.I.R.O., Division of Chemical Physics. Spectra were recorded on a Philips 4-channel 'Oszilloscript' galvanometer type recorder. The use of a ratio recording device made the matching of the mass spectra considerably easier. This is a simple system in which the deflexion of the gas chromatographic recorder is made to continuously adjust the gain of the mass spectrometer electrometer amplifier so that the spectrum recorded is substantially the same over a tenfold change of sample concentration.

In the present investigation one gas chromatographic column was used. This was glass (2·5 m × 3 mm ($\frac{1}{8}$ in.) O.D.) and packed with 5 % Carbowax 20M on 60–70 mesh, acid-washed 'Chromosorb G'. The column was operated isothermally at 60 °C for 10 min then programmed to 200 °C, at 2 °C/min.

Synthetic pent-1-en-3-ol and pent-1-en-3-one were suspended in water at similar concentrations to those isolated and examined under identical conditions.

RESULTS

Fig. 2 shows typical gas chromatograms of the lower-boiling compounds isolated from control and oxidized buttermilk. It is interesting to observe the presence of n-butan-1-ol and n-pentan-1-ol in this control (normal) buttermilk where the cream was held for 5 days, whereas in a previous experiment (Stark & Forss, 1966) when the cream was only held overnight, methanol and ethanol were the only alcohols observed. Only 3 kg of control buttermilk was used and so a larger volume (10 instead of 2 μl) of sample was used. Fig. 3 shows all the volatile compounds isolated from the concentrated distillate of oxidized buttermilk. Since this was not further concentrated (compare Fig. 2) 40 μl samples were used and the gas chromatographic resolution was inferior. The small quantities of the higher boiling compounds reflect the inefficiency of the isolation procedure. It is difficult to detect small quantities of oct-1-en-3-one eluted just after the n-pentanol since both have large m/e = 70 mass spectral peaks. Oct-1-en-3-ol is eluted in a position coinciding with that for n-heptan-

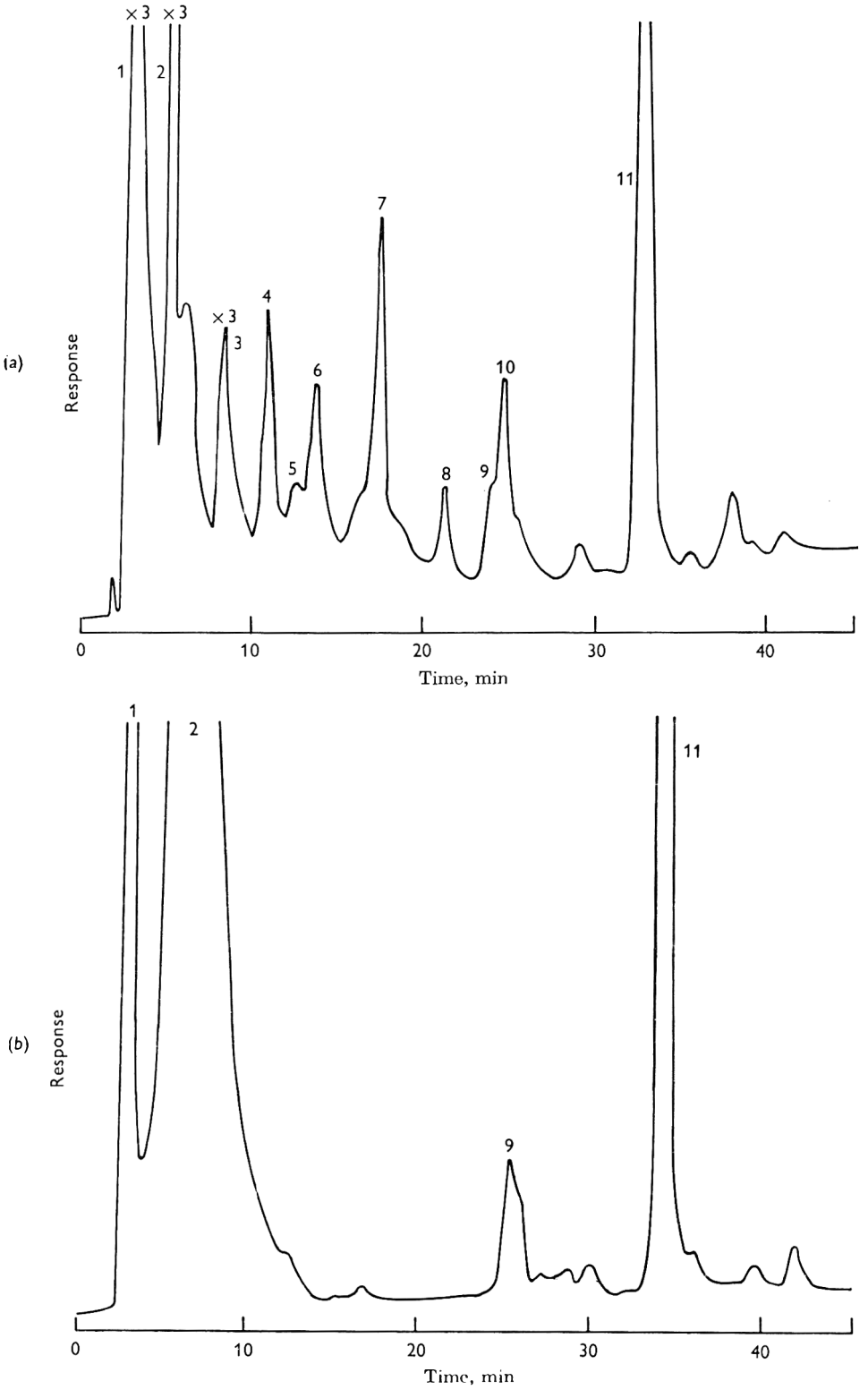


Fig. 2. Gas chromatograms of samples of lower boiling fraction of concentrated distillates of (*a*) oxidized and (*b*) control buttermilk—1, propanone; 2, methanol, ethanol; 3, diacetyl and n-pentanal; 4, pent-1-en-3-one; 5, propanol and toluene; 6, penta-2,3-dione; 7, n-hexanal; 8, pent-2-enal; 9, n-butan-1-ol; 10, pent-1-en-3-ol; 11, n-pentan-1-ol. Sample volume: (*a*), 2μl; (*b*), 10μl.

1-ol (peak 13). No oct-1-en-3-ol could, however, be detected in the fraction represented by peak 13.

The retention times and mass spectra of isolated and synthetic pent-1-en-3-ol were identical; similar results were obtained for pent-1-en-3-one. The amounts of pent-1-en-3-ol and pent-1-en-3-one in buttermilk were approximately the same and about 1 μM/kg. Pent-1-en-3-ol was also identified from oxidized butter.

The flavour threshold values in water, skim-milk, butter oil and bland unsalted butter were measured by 6 colleagues. The values obtained were 3 pts in 10^6, 3 pts in 10^6, 1 pt in 10^5 and 1 pt in 10^5, respectively, for the pent-1-en-3-ol, and 1 pt in 10^9, 3 pts in 10^9, 5 pts in 10^9 and 5 pts in 10^9, respectively, for the pent-1-en-3-one.

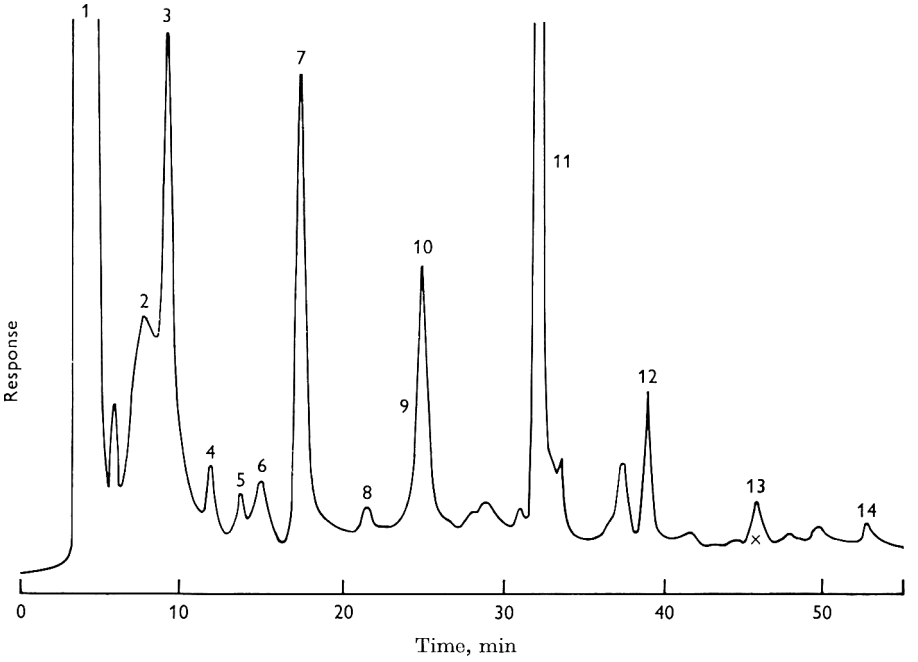


Fig. 3. Gas chromatogram of 40-μl sample of concentrated distillate of oxidized buttermilk. Nos. 1–11 as for Fig. 2; 12, n-hexan-1-ol; 13, n-heptan-1-ol; 14, n-octan-1-ol; ×, position for oct-1-en-3-ol.

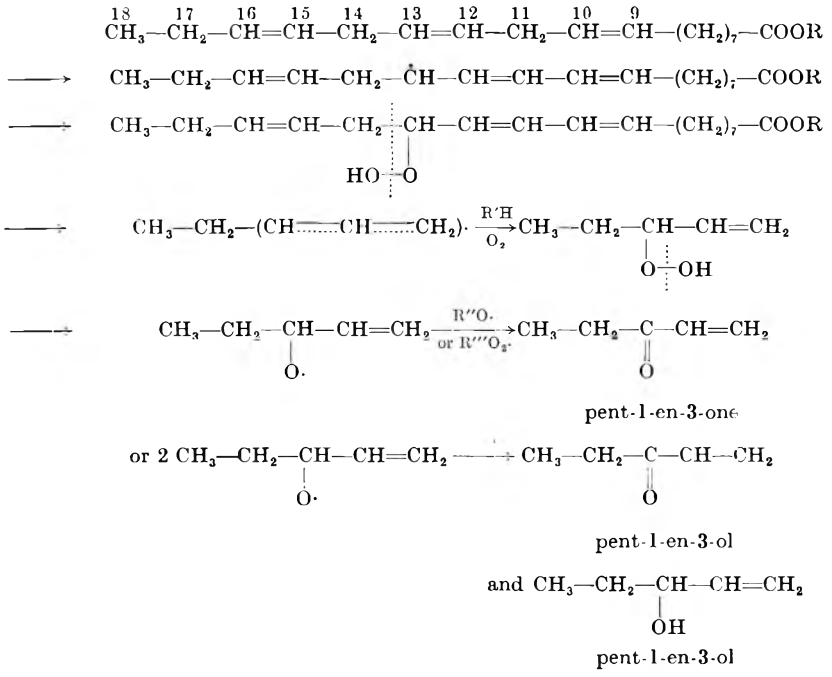
The alcohol and ketone in butter were examined by Dairy Produce Inspectors of the Commonwealth Department of Primary Industry who detected 1 pt in 10^6 and 1 pt in 10^9, respectively. They described pent-1-en-3-ol at 1 pt in 10^6 as slightly oily, and at 1 pt in 10^5 as grassy or like linoleum. They described the flavour of pent-1-en-3-one at 1 pt in 10^8 as ant-like, and at 1 pt in 10^7 as painty or like linseed oil.

DISCUSSION

Pent-1-en-3-ol has been isolated from soyabean oil oxidized at 110°C (Iwata, Morita & Ota, 1965), fresh strawberries (Winter & Willhalm, 1964), black tea (Kobayashi *et al.* 1966), and black currants (Andersson & von Sydow, 1966), and pent-1-en-3-one as the 2,4-dinitrophenylhydrazone from autoxidized soyabean oil (Hill &

Hammond, 1965). The flavour of pent-1-en-3-ol is relatively mild with a threshold value in butter oil of 1 pt in 10^5 compared with 1 pt in 10^7 for its mushroom-flavoured homologue, oct-1-en-3-ol. It is unlikely that pent-1-en-3-ol as such contributes directly to oxidized flavour, but it may be oxidized to some extent to pent-1-en-3-one and hence contribute indirectly.

Scheme 1



Pent-1-en-3-one is considerably more potent than pent-1-en-3-ol, with a flavour threshold value in butter oil of 5 pts in 10^9 which is of the same order as its metallic-flavoured homologue, oct-1-en-3-one. Hill & Hammond (1965) found that a mixture of equal quantities of pent-1-en-3-one and n-pentanal dissolved in deodorized vegetable oil had a flavour similar to that of autoxidized soya bean oil. The optimum amount of each compound was 1 pt in 10^8. If the amount of n-pentanal was 1·5 times that of the pent-1-en-3-one, the flavour intensity decreased and became less like that of autoxidized soya bean oil. Blends of pent-1-en-3-one with various combinations of n-hexanal, hex-*cis*-3-enal and n-pentanal were no closer to the autoxidized flavour than pent-1-en-3-one and n-pentanal alone. Forss, Dunstone & Stark (1960) observed that a painty flavoured fraction from oxidized butter, separated on a gas chromatographic column coated with silicone oil, could not be duplicated by a synthetic mixture of the 2 major constituents, n-pentanal and pent-2-enal. Any pent-1-en-3-one present would be included in this fraction, and its addition to the synthetic mixture might permit duplication of the painty flavour.

Our concentrated distillate from oxidized buttermilk contained a relatively large amount of pent-1-en-3-one. In view of the high flavour potency of pent-1-en-3-one,

higher than most of the other compounds in this distillate, the flavour of oxidized buttermilk must be partly due to the compound.

Stark & Forss (1964) suggested oct-1-en-3-ol might be formed from arachidonic acid ester, and in a similar manner pent-1-en-3-ol and pent-1-en-3-one might be formed from linolenic acid ester (see scheme 1 on p. 128).

The presence of the alcohol (Iwata, Morita & Ota, 1965) and of the ketone (Hill & Hammond, 1965) in soyabean oil, a material rich in linolenic acid, is consistent with their formation from linolenic acid.

The authors thank Mr M. E. McShane and Dairy Produce Inspectors of the Department of Primary Industry, Melbourne, for grading butters; and Dr J. D. Morrison of the Division of Chemical Physics, C.S.I.R.O. for helpful discussion on the gas chromatography-mass spectrometer combination.

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Volatile compounds in butter oil

I. Lower boiling compounds

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(*Received* 28 *December* 1966)

Summary. The following compounds were isolated from best-quality Australian butter oil by high vacuum degassing, and identified by the combination of gas chromatography and mass spectrometry: the $C_{3,5,7,9}$ alkan-2-ones, the $C_{2,4,6,8,10,12}$ n-alkanoic acids, the $C_{6,8,10}$ 5-lactones (or their hydroxyacids), dimethyl sulphone, all isolated in amounts above 1 pt in 10^8 of butter oil, and butanone, undecan-2-one, 5-dodecalactone, diacetyl, n-nonanoic acid, a decenoic acid, methyl n-decanoate, toluene, o-methoxyphenol, a cresol, phenol, benzaldehyde, methyl benzoate and benzothiazole, all isolated in amounts between 1 pt in 10^{11} and 1 pt in 10^8 of butter oil.

No compound was isolated in an amount greater than flavour threshold, in fact most compounds were below one-tenth threshold level. However, the distillate obtained by degassing produced a very attractive creamy flavour when added to bland non-cultured butter.

In Australia, butter for export is made only from sweet or neutralized farm-ripened cream. Its flavour is therefore not a predominantly diacetyl-type derived from fermentation, but is due to the flavours inherent in the cream or derived from processing. Butter is graded according to flavour and aroma (maximum 50 points), texture (maximum 30 points) and condition (maximum 20 points). Most butters are awarded the full 50 points for texture and condition, the main variation being in the flavour score. Butter graded as 93 points and over is called choicest quality. A small proportion may receive 94 points and such terms as 'a good 93', '93+' are used to indicate that the butter has some desirable characteristic over and above the bland 93-point butter which merely lacks undesirable flavours.

The Dairy Produce Inspectors of the Commonwealth Department of Primary Industry grade butter for export and complete reliance is placed on their judgement. The aim of the present series of investigations was to relate grade scores on butters to qualitative and quantitative chemical data on volatile compounds isolated from butter oil.

Moncrieff (1965) reviewed the literature on butter flavour up to the end of 1963. Since then Parliment, Nawar & Fagerson (1965) have identified 5-hexalactone in heated milk fat and Jurriens & Oele (1965) have reported the presence of 4- and 5-lactones from C_{10} to C_{16} inclusive in butter. Haverkamp Begemann & Koster (1964) claimed that hept-*cis*-4-enal is responsible for a creamy flavour in milk fat and occurs

in fresh milk fat at concentrations of 1·5 pts in 10^9. Badings' (1965) observations were similar; hept-*cis*-4-enal added to high vacuum degassed milk fat at a concentration of 1 pt in 10^9 conferred a creamy flavour as judged by experienced Dutch butter graders. Kawanishi & Saito (1965) isolated from Japanese sweet cream butter a series of normal ($C_{3-3,8}$) and iso ($C_{4,5,8}$) fatty acids, and aliphatic carbonyl compounds having 3–8 carbon atoms and they claimed that this mixture reproduced the flavour of sweet cream butter.

It became obvious in our investigation of butter flavour that neither reduced pressure steam distillates nor high vacuum distillates (pressure below 10^{-3} torr) of butter or butter oil held at 45 °C had typical buttery flavours. However, cold finger molecular distillates of highest quality butter oil had a strong butter odour.

This paper describes the identification of the compounds collected during the degassing stage prior to molecular distillation. Subsequent papers will describe the identification of the higher boiling compounds which collected on the cold finger and which are mainly responsible for the characteristic flavour of butter. The presence of 4-dodecalactone and other lactones deposited on the cold finger has already been reported (Forss, Urbach & Stark, 1966).

EXPERIMENTAL

The butter oil was prepared commercially from a mixture of farm and factory separated cream (35% fat) by the Alfa-Laval process (1963). After vacreation the cream was concentrated to 45% fat and fed into a 'Clarifixator' where it was homogenized and concentrated to 80% fat. After further centrifugal concentration to 97–98% fat, the product was passed through a plate heat exchanger (94 °C; 9 s) into a vacuum vessel where the water content was reduced to below 0·1%. In the manufacture of butter oil more than in the manufacture of butter, a mild-flavoured product free of taints is sought so that due to the more severe processing some of the most volatile compounds would be lost. All butter oil investigated came from the same factory and was graded highest quality. Distillations were performed on autumn and spring samples less than 3 weeks old which had been stored at −12 °C.

The distillation apparatus is shown in Plate 1. Butter oil (3·0 kg) was allowed to flow downwards through 2 Davies condensers (the first filled with Fenske helices) at 55 °C into a reservoir at such a rate that the pressure was less than 5×10^{-2} torr. Most of the water (1–2 ml) collected in trap 1. Very little water or flavour passed into trap 2. Partially degassed butter oil was allowed to flow into the 10 l. flask at such a rate that the pressure measured on a Penning gauge after trap 4 did not exceed 2×10^{-3} torr. The flask was immersed in water at 50 °C and the butter oil was magnetically stirred. The complete degassing took 4–5 h. Trap 3 was removed and replaced by a similar trap (no. 5). All stopcocks were closed overnight. On the following day the butter oil was degassed to a pressure of less than 5×10^{-5} torr on the Penning gauge (2 h) after which liquid nitrogen was added to the cold finger. After 5 h the cold finger was removed, the liquid nitrogen replaced by water at 60 °C and the material on the cold finger (1–2 g) was allowed to flow into a flask or was washed off with chloroform. Control experiments on the distillation apparatus established that the technique did not yield any contaminants apart from benzothiazole which came

from the milking machine rubber tubing initially used to connect the Edwards diffusion pump to the first stage of the degassing section. This rubber tubing was replaced by a glass tube.

The volatile compounds isolated during the degassing (traps 1, 3 and 5) are reported in this paper. The contents of traps 1, 3 and 5 were combined to give the total distillate.

The distillate was separated into 'acid' and non-acid fractions. The distillate, which was acid to litmus, was made alkaline with 50 μl of N-NaOH and the volatiles removed by vacuum sublimation of the frozen distillate followed by distillation at 50 °C and 1×10^{-5} torr. Under these conditions synthetic C_6 and C_8 5-lactones stayed behind with the 'acid' fraction.

'Free acids' were liberated from the 'acid' residue by treatment with water and Dowex 50W–H^+ form (Bio-Rad). The methyl esters were prepared by suspending the 'acid' residue in methanol and adding Dowex 50W–H^+ form resin previously washed with methanol. The reaction was carried out in a sealed glass tube heated at 70 °C for 10 min.

The total distillate and the 'acid' fraction (free and esterified) were analysed by combined gas chromatography and mass spectrometry (Stark, Smith & Forss, 1967) on a glass column 8 ft $\times \frac{1}{8}$ in. O.D. packed with 5 % Carbowax 20M on 60–70 mesh, acid-washed 'Chromosorb G', held at 60 °C for 10 min and then programmed to 220 °C at 2 °C/min. Mass spectra were recorded in 4–15 s.

The Carbowax 20M column was suitable for separating most of the isolated compounds but acids were strongly adsorbed, and desorbed very slowly. This was first shown by the mass spectra which contained peaks at m/e = 60, 73, 87 even when there was no gas chromatographic peak. No indication of the C_{12} acid was given from the unesterified sample but this was shown to be a major component after esterification.

Authentic compounds were treated in the same way to obtain retention data and mass spectra, and compounds were regarded as identified if retention times and mass spectra of known and isolated compounds agreed.

Trap 1 was also analysed by 2-dimensional gas chromatography/thin-layer chromatography (Janák, 1964) with manual movement of the thin-layer chromatographic plate. This confirmed the presence of the $C_{6,8,10}$ 5-lactones.

Bland, unsalted 93-point butter was used as the vehicle for flavour evaluations by the Dairy Produce Inspectors (Graders). In this way the total distillate and the 'acid' and non-acid fractions were examined. The following synthetic compounds were also examined for flavour: gas chromatographically purified $C_{6,8,10}$ 5-lactones (the decalactone from Price's (Bromborough) Ltd., Cheshire, England), $C_{2,6,8}$ n-alkanoic acids and 5-hydroxyhexanoic acid. The hydroxyacid was not gas chromatographically purified.

RESULTS

Identifications

The following compounds were isolated from all samples of butter oil in amounts above 1 pt in 10^8: the $C_{3,5,7,9}$ alkan-2-ones, the $C_{2,4,6,8,10,12}$ n-alkanoic acids, the $C_{6,8,10}$ 5-lactones (or their hydroxyacids) and dimethyl sulphone. Butanone, undecan-

2-one, 5-dodecalactone, methyl n-decanoate, diacetyl, n-nonanoic acid, a decenoic acid, toluene, o-methoxyphenol, a cresol, phenol, benzaldehyde, methyl benzoate and benzothiazole were isolated in amounts between 1 pt in 10^{11} and 1 in 10^{8} of butter oil. In different experiments these compounds were differently distributed in traps 1, 3 and 5 both qualitatively and quantitatively. Most of the compounds were found to some extent in all the traps and there is indication that they were also collected on the cold finger.

Thin-layer chromatography and mass spectrometry established that the compounds reported as 5-lactones were substituted in the 5-position.

The total distillate consisted of about 80% n-alkanoic acids and about 10% 5-lactones.

Flavour evaluations

The Graders' comments clearly indicated that the distillate contained some very desirable elements described by them as 'sweet' and 'creamy'. These desirable elements were associated with the 'acid' fraction. Experiments showed that the flavour of this fraction was not appreciably affected by gas chromatography. The non-acid fraction contained undesirable flavours. Compounds identified in the 'acid' fraction were the $C_{2,4,6,8,9,10,12}$ n-alkanoic acids, a decenoic acid, the $C_{6,8,10,12}$ 5-lactones, phenol, o-methoxyphenol, and a cresol. It is not clear whether the lactones were originally present as such or as their hydroxyacids because in the separation of 'acid' and non-acid fractions the lactones remain with the 'acid' fraction and during gas chromatography on a polar phase (Ansell & Palmer, 1963) 4- and 5-hydroxy-acids are converted to the corresponding lactones.

Table 1. *Flavour of synthetic compounds added to 93-point butter*

Compound	Added concentration in butter pt/pts butter	Comments
5-Lactone C_6	1 in 10^4	Scarcely perceptible, undesirable
5-Lactone C_8	1 in 10^6	Strong coconut oil, undesirable
5-Lactone C_8	1 in 10^7	Just perceptible, undesirable
5-Lactone C_{10}	1 in 10^5	Coconut, rancid, anty, undesirable
n-Alkanoic acid C_2	2·5 in 10^4	Fragrant, desirable
n-Alkanoic acid C_6	1 in 10^6	Desirable
n-Alkanoic acid C_8	1 in 10^6	Desirable
5-Hydroxyhexanoic acid	1 in 10^4	Astringent, flat

No compound was isolated in amount greater than flavour threshold, in fact most compounds were below one-tenth flavour threshold level. Yet examination by the Graders clearly showed that a creamy element was contained in this lower boiling fraction and that some components in it improved 93-point to 94+ butter.

Table 1 lists the comments of the Graders on various synthetics added to 93-point butter. The concentration given is additional to the amount naturally present in 93-point butter. None of the $C_{6,8,10}$ 5-lactones, $C_{2,6,8}$ n-alkanoic acids or 5-hydroxyhexanoic acid produced a creamy flavour.

DISCUSSION

In Australia, the very top quality butters are those completely free of off-flavours and containing one or more desirable flavour elements such as those usually described as creamy or nutty.

In the present investigation we have shown that 93-point butter can be changed to 94+ butter by the addition of compounds trapped during the degassing of butter oil. The elements responsible for this improvement are in the 'acid' fraction but we have not been able to determine as yet whether any of the compounds identified are actually responsible for the creamy flavour or whether the creamy flavour is due to a single compound or a mixture. We were not able to detect any hept-*cis*-4-enal at a concentration of 1 pt in 10^{11} in butter oil (Haverkamp Begemann & Koster, 1964).

The presence of dimethyl sulphone is interesting in relation to the presence of dimethyl sulphide in butter (Day, Lindsay & Forss, 1964) although the sulphone itself is odourless. Dimethyl sulphone was recently isolated from pasteurized milk (Williams, Burstein & Layne, 1966*a*) but no dimethyl sulphoxide was detected. We also did not detect any dimethyl sulphoxide in butter oil. Williams *et al.* (1966*b*) observed that in humans and rabbits, dimethyl sulphide is oxidized to the sulphone and eliminated in the urine. The sulphone might therefore be a normal metabolite of the cow.

Since we showed that benzothiazole is a volatile constituent of some rubber, it is possible that the benzothiazole detected in the butter oil may have been absorbed during milking. Alternatively, since Arnold, Libbey & Day (1966) observed benzothiazole in stale but not in fresh sterilized concentrated milk, it may be a product of ageing. Several other aromatic compounds from butter oil were identified, but as no anti-oxidants were used in the preparation of this butter oil, the aromatic compounds could not have been derived from them.

The Firmenich group (Winter, Stoll, Warnhoff, Greuter & Büchi, 1963), Japanese investigators (Kawanishi & Saito, 1965) and the Unilever group (Boldingh & Taylor, 1962) claim that the compounds which they have isolated together give a complete butter flavour. In view of the difference between their findings—carbonyl compounds by the Firmenich group, a mixture of fatty acids and carbonyl compounds by the Japanese workers and $C_{8,10,12}$ 5-lactones by the Unilever group, one wonders whether the term 'butter flavour' means very different things to people in different countries. Certainly, the 'degassing fraction' reported in this paper, although highly odorous and on the whole pleasant, does not represent 'butter flavour' but only a part of it. Examination of the cold finger distillate showed that a fraction containing esters, acids and indole-type compounds conferred a very desirable nutty flavour when added to butter.

From the point of view of the dairy industry, the most important finding emerging from the present investigation is the fact that 93-point butter can be changed to 94+-point butter by compounds trapped during the degassing of butter oil. It will, therefore, be necessary to direct future work towards the origin and mode of formation of these compounds.

The authors' thanks are due to their colleague, Mr J. F. Smith, for help with the mass spectrometry, and to Mr M. E. McShane and Dairy Produce Inspectors of the Department of Primary Industry, Melbourne, for grading butters.

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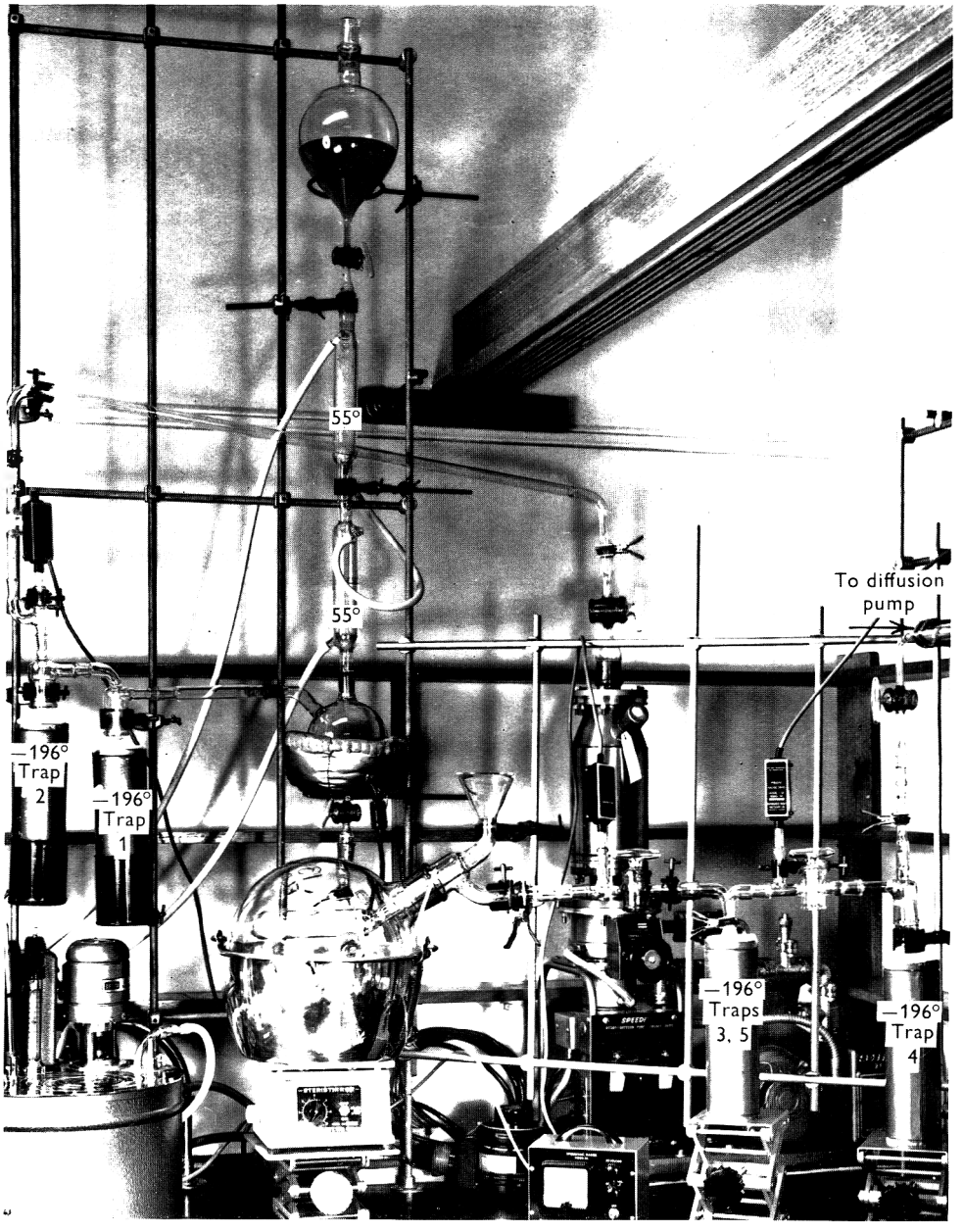
EXPLANATION OF PLATE

PLATE 1

Apparatus for the degassing and cold-finger distillation of butter oil.

Printed in Great Britain



D. A. FORSS, W. STARK AND GERDA URBACH

(*Facing p.* 136)

Seasonal variation in deposit formation from whole milk on a heated surface

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(*Received* 3 *February* 1967)

Summary. The variation in deposit formation from milk of 2 herds was measured over a period of more than 12 months using a hot-wire laboratory apparatus. A marked minimum was found in May–June, with high deposit levels between September and April. There was a difference of 2 to 1 between maximum and minimum levels. The amount of deposit was not positively correlated with the mineral or protein contents of the milk, but was strongly positively correlated with fat content. It is suggested that the controlling factor is closely associated with fat content and varies with it.

In a previous paper (Burton, 1966*a*), it was shown that at different times of the year bulk milk from the same herd produced different amounts of deposit on a heated surface, whether the milk was heated in a small ultra-high-temperature heat exchanger or in a laboratory apparatus. The purpose of the present work was to examine the amount and form of the seasonal variation in more detail, using the hot-wire laboratory apparatus described previously (Burton, 1965).

METHODS

Milk supplies and analyses. Samples were obtained from the bulk whole milk of 2 herds. Herd A was a commercial herd in the neighbourhood of the Institute with approximately 60 milking cows, predominantly Shorthorn, and producing 110–240 gal/day of milk during the experiment. Herd B was one of the Institute's own herds and during the experiment it consisted of 40–70 Friesian cows producing 120–260 gal/day.

The samples were of the refrigerated mixed evening's and morning's milk, and were taken at about 9.30 a.m. The tests were performed on the day of sampling, with the milk refrigerated until the time of test. Normally both milks were tested in duplicate on the same day, at intervals of approximately 1 month, but additional tests were made in duplicate on the commercial herd milk (A) to give approximately fortnightly intervals.

From September 1965 until the end of the experiment in September 1966 chemical analyses were made whenever both herd milks were tested on the same day. Similar analyses were available for some earlier samples of the milk from herd A.

The samples were analysed for fat by the Gerber method (British Standards Institution, 1955) and for total solids gravimetrically (British Standards Institution, 1951), the SNF being obtained by difference. Lactose was determined by the chloramine-T method (British Standards Institution, 1963), and chloride by an adaptation

of the Volhard method (British Standards Institution, 1963). Total N, non-casein N and non-protein N were determined by the method of Rowland (1938), and casein N and soluble protein N obtained by difference. The factor 6·38 was used to convert the values for total N, casein N and soluble protein N into values for protein, casein and soluble protein. Ash was determined by incineration of the milk at 520–540°C, and calcium by precipitation as oxalate from a solution of the ash and titration with permanganate. pH values were measured with a glass electrode.

Deposit determination. The laboratory apparatus was as described previously (Burton, 1965), but modified to take a 4·5 cm length of 38 s.w.g. (0·006 in., 0·15 mm diam.) pure platinum wire to increase the wire surface temperature (Burton, 1966*a*).

The milk to be tested was well mixed and 350 ml was preheated to 55–65°C in a glass beaker over a gas flame with vigorous stirring. It was then immediately homogenized in a laboratory single-piston homogenizer (Ormerod Engineers Ltd., Rochdale) to prevent rising of fat during an experiment with consequent change in milk composition around the hot wire. The homogenizer was preheated with almost boiling water. It was not fitted with a pressure gauge, but the homogenizing valve setting remained unaltered throughout the experiments. Other tests have shown that the effect of homogenizing pressure variation on deposit formation is slight within a moderate range of pressures. Variation of homogenizing temperature in the range 50–70°C has also been found not to affect deposit formation but, nevertheless, on general grounds it was felt desirable to keep the homogenizing temperature as low and constant as possible in this range.

The milk was transferred after homogenization to the preheating vessel of the hot-wire apparatus, and was preheated with stirring to 67°C. It was then run into the test vessel, and the test performed with an air pressure of 40 lb/in^2 (2·7 atm) and a wire current of 4·8 A (Burton, 1966*a*).

The slope, K, of the line relating wire-milk differential temperature, $\Delta\theta$, to the square root of elapsed time, $\sqrt{t}$, was obtained. The value K^2 was used as a measure of the amount of deposit formation, since it had previously been shown that K^2 is correlated linearly with the amount of deposit formed in a plate heat exchanger (Burton, 1966*a*).

When the experiments were well advanced, it was realized that the principle of testing the milk as soon as possible after production was unsatisfactory. The effect of storing the milk for some time following production in reducing the amount of deposit was recognized in these experiments as well as in those reported previously (Burton, 1966*a*). When 2 determinations are made on the same sample, the amount of deposit is lower in the second determination. The amount of deposit formation reaches a minimum 15–20 h after milking and is then almost constant for a further day (Report, 1965). Duplicate measurements after this time would therefore be strictly comparable. However, it was inconvenient at this stage to change the basis of the experiments and make the measurements more than 20 h after production. An attempt was, therefore, made to minimize the effect of ageing on the results by always performing the experiments in the order A, B, B, A. When the duplicate results were averaged, a result was obtained for each herd corresponding approximately to the result of a single experiment performed about midday on the day the milk was collected.

RESULTS AND DISCUSSION

Seasonal variation. The variation of K^2 over a period of more than 12 months is shown for both herds in Fig. 1. The results of duplicate experiments are shown separately, but the variation shown is based on the average results for each herd.

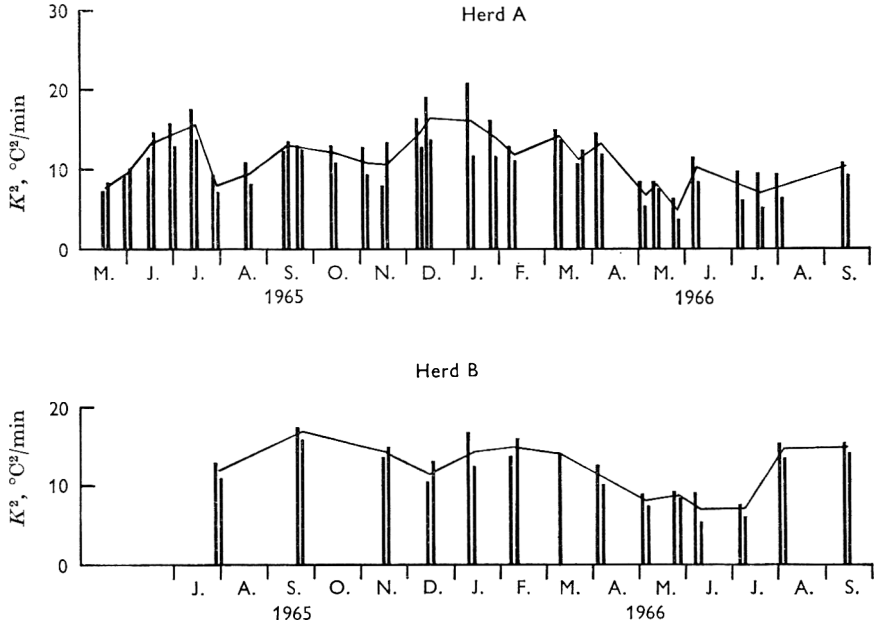

Fig. 1. Seasonal variation in amount of deposit formation from milk of 2 herds as measured by K^2 (see p. 138 for definition of K).

The duplicate result was normally lower than the original because of the ageing effect: where the reverse occurred, experimental error was presumably responsible. The difference between duplicates for herd B was frequently less than for herd A. This was presumably again an effect of ageing since, because of the experimental order A, B, B, A, the time between the duplicates was less for herd B than for herd A.

A seasonal variation existed for both herds. For herd B it was clearly defined, but with herd A the general seasonal variation was modified by short-term fluctuations. The amount of deposit was highest in the autumn and winter months, between September and March, but there were restricted periods of lower deposit as in December 1965 for herd B, and in November 1965 and February 1966 for herd A.

The amount of deposit fell sharply during April, reaching a minimum in May which was less than half the winter value. In herd B, this minimum was maintained throughout May and June 1966, but a sharp rise at the end of July led to August and September values which agreed well with those for the previous year. In herd A, however, the behaviour of the milk at this time of the year was less clear-cut. In 1955, the May minimum was followed by a sharp rise and an even sharper fall in late July. A rise through August then occurred as with herd B, but less markedly. In May 1966, a minimum again occurred, but there was little sign of the rise in

June and July. Overall, there was a slight rise from May to September, with the August and September 1966 values rather lower than for the same 2 months in 1965. During the last months of the experiment, herd A was experiencing troubles with mastitis and the low values may have arisen from this cause.

In this geographical area, therefore, there was a period of relatively low deposit between late April and early July, and a period of relatively high deposit between September and March. However, within this general picture the 2 herds show differences which suggest that it would be unwise to attempt general conclusions about the country as a whole from these local results. The short-term variations found with herd A suggest that factors peculiar to an individual herd, perhaps in feeding policy, may cause quite large changes to occur relatively rapidly. The absence of short-term fluctuations in the deposit variation from month to month in herd B may have resulted from its being maintained largely on fixed diets for considerable periods. The low deposit period for herd B in 1966 coincided with the period when only grass was fed. The increase in August coincided with the introduction of concentrates in the diet, and the onset of autumn calving.

Chemical composition and seasonal variation. As the tests were being made on fresh milk, pH variation was not expected to be a factor. pH values were therefore measured for only a third of all the samples. For herd A, the maximum and minimum values obtained were pH 6·81 and 6·67. For herd B, rather lower values were found, from pH 6·80 to 6·55. If the pH of the milk was above pH 6·65 there was no apparent relation between pH and amount of deposit. The only lower values were found with herd B (pH 6·63 on 22 September 1965) and pH 6·55 on 14 September 1966, and they were both associated with relatively high deposit values ($K^2 = 17$ and 15, respectively).

The available analyses for herd A are collected in Table 1, together with the corresponding K^2 values. The results for herd B are given in Table 2. The coefficients of correlation between K^2 and all components for both herds are summarized in Table 3, with the corresponding significance levels.

There was a positive correlation between K^2 and total solids content, but it was not as good as might have been expected. For herd A, $r = 0{\cdot}567$, which was significant at $P = 0{\cdot}05$. For herd B, $r = 0{\cdot}218$, which was not significant at $P = 0{\cdot}1$.

The solids-not-fat (SNF) portion of the milk was not responsible for this positive correlation. Although the SNF contains protein and mineral constituents which might be precipitated by heat, the SNF results showed no positive correlation with deposit ($r = 0{\cdot}017$ for herd A, $r = -0{\cdot}452$ for herd B). Surprisingly, the fat content showed a strong positive correlation with K^2, and for both herds the correlation coefficients of K^2 with fat (herd A, $r = 0{\cdot}716$; herd B, $r = 0{\cdot}746$) were significant at the 1 % level. It appears that the relatively low positive correlation coefficients for total solids content were due to the effect of fat content, made less significant by lack of correlation with the SNF content. Fat content was the only value which was strongly correlated with K^2.

There is a negative correlation for all the protein components in the milk from both herds, but the correlations were not significant at the 10 % level except for the soluble protein content in herd A. These negative correlations were in accordance with an observation that the addition of crystalline β-lactoglobulin to separated milk tends to reduce the amount of deposit formed (Report, 1964).

Table 1. K^2 *values and analyses for milks from herd A*

Date	K^2	TS, %	Fat, %	SNF, %	Lactose, %	Protein, %	Casein, %	Soluble protein, %	NPN, mg/100 g	Ash, %	Ca, mg/100 g	Cl, mg/100 g
16. vi. 65	13·10	11·88	3·37	8·51	4·40	3·29	2·48	0·64	27	0·73	116	112
14. vii. 65	15·52	11·95	3·51	8·44	4·39	3·19	2·39	0·59	33	0·74	116	108
22. ix. 65	12·82	12·40	3·77	8·63	4·43	3·43	2·60	0·61	35	0·76	122	105
17. xi. 65	10·50	12·70	3·89	8·81	4·41	3·55	2·69	0·66	30	0·76	126	103
15. xii. 65	16·32	12·87	4·11	8·76	4·46	3·45	2·59	0·66	32	0·76	134	101
12. i. 66	15·84	12·68	3·80	8·88	4·54	3·38	2·60	0·60	28	0·77	124	104
9. ii. 66	11·90	12·08	3·58	8·50	4·52	3·14	2·37	0·59	28	0·74	116	104
9. iii. 66	14·36	11·96	3·51	8·45	4·48	3·09	2·35	0·57	27	0·72	116	107
5. iv. 66	13·32	12·19	3·65	8·54	4·39	3·29	2·49	0·59	33	0·74	116	105
4. v. 66	6·71	11·78	3·08	8·70	4·47	3·40	2·59	0·61	31	0·74	116	105
25. v. 66	4·84	11·76	3·09	8·67	4·41	3·46	2·58	0·66	34	0·76	116	107
8. vi. 66	9·92	12·21	3·52	8·69	4·44	3·38	2·53	0·64	33	0·74	118	113
6. vii. 66	7·67	11·99	3·41	8·58	4·40	3·39	2·49	0·66	38	0·73	118	109
3. viii. 66	7·78	11·86	3·44	8·42	4·19	3·34	2·42	0·67	40	0·76	118	122

Table 2. K^2 *values and analyses for milks from herd B*

Date	K^2	TS, %	Fat, %	SNF, %	Lactose, %	Protein, %	Casein, %	Soluble protein, %	NPN, mg/100 g	Ash, %	Ca, mg/100 g	Cl, mg/100 g
22. ix. 65	16·81	12·93	4·09	8·84	4·66	3·34	2·56	0·58	31	0·73	118	87
17. xi. 65	14·36	12·68	4·04	8·64	4·67	3·09	2·37	0·54	28	0·73	116	89
15. xii. 65	11·70	12·35	3·88	8·47	4·61	2·99	2·29	0·53	27	0·72	113	90
12. i. 66	14·44	12·33	3·78	8·55	4·66	3·04	2·35	0·49	30	0·72	112	94
9. ii. 66	14·82	12·13	3·66	8·47	4·68	3·00	2·30	0·50	32	0·73	112	92
9. iii. 66	13·91	12·23	3·69	8·54	4·60	3·05	2·33	0·52	32	0·71	114	98
5. iv. 66	11·42	12·29	3·76	8·53	4·54	3·11	2·39	0·53	30	0·74	114	102
4. v. 66	8·12	12·28	3·47	8·81	4·58	3·40	2·65	0·58	26	0·72	112	95
25. v. 66	8·94	12·49	3·64	8·85	4·64	3·34	2·63	0·57	22	0·74	116	99
8. vi. 66	7·29	12·62	3·68	8·94	4·64	3·45	2·66	0·56	36	0·71	118	96
6. vii. 66	6·96	12·49	3·54	8·95	4·67	3·46	2·66	0·60	32	0·72	116	99
3. viii. 66	14·60	12·91	3·97	8·94	4·46	3·56	2·78	0·61	27	0·75	122	106

The only other component for which a common effect appeared in both herds was chloride content, where there was a non-significant ($P > 0{\cdot}1$) negative correlation (herd A, $r = -0{\cdot}381$; herd B, $r = -0{\cdot}330$).

Table 3. *Correlations between K^2 and concentrations of different milk constituents*

	Correlation coefficient, r	
Herd	A	B
No. of samples	14	12
Constituent: Total solids, %	0·567*	0·218
Fat, %	0·716**	0·746**
SNF, %	0·017	−0·452
Lactose, %	0·376	−0·034
Protein, %	−0·374	−0·446
Casein, %	−0·172	−0·455
Soluble protein, %	−0·514†	−0·368
NPN, mg/100 g	−0·492†	0·029
Ash, %	−0·010	0·300
Ca, mg/100 g	0·404	0·071
Cl, mg/100 g	−0·381	−0·330

Significance levels: ** $0{\cdot}01 > P > 0{\cdot}001$;
* $0{\cdot}05 > P > 0{\cdot}01$;
† $0{\cdot}1 > P > 0{\cdot}05$.

The overriding effect of fat is unexpected. Fat is normally present in heat exchanger deposits together with protein and mineral constituents (Ito, Sato & Suzuki, 1962*a*, *b*; Lyster, 1965), but because of the effect of forewarming processes in reducing deposit formation, it has generally been believed that the controlling factor is either a soluble protein or mineral constituent (Burton, 1966*b*). If this is true, and the fat acts merely as an inert filler trapped in a deposit which is controlled by a protein or mineral component, then that controlling component has not been identified in the analyses used in these experiments. Also, if this were true, the amount of deposit would be expected to vary approximately directly as the fat content. This is not so, since in herd A an increase in fat content over the range 3·1–4·1 % was associated with an increase of more than 3 times in the K^2 value, from 4·8 to 16·3. In herd B, an increase in fat content from 3·5 to 4·1 % was associated with an increase in K^2 from 7·0 to 16·8.

It seems more probable that the controlling factor is some constituent which varies with the fat content and was not included in the analyses. It is also probably some constituent which, although found in close association with the fat, also occurs in the separated milk fraction, since it is well recognized that the deposit problem exists with separated as well as with whole milk. A phospholipid is one possibility, since the phospholipid content is likely to vary with the total fat content, and phospholipids are present both in the fat and non-fat parts of the milk. Moreover, there is probably a seasonal variation in the phospholipid content of milk (Holden, Aceto, Dellamonica & Calhoun, 1966), somewhat similar to that found here for deposit formation. Phospholipids are strongly surface active, and might be expected to influence a surface effect such as deposition. Holden *et al.* (1966) have suggested that the seasonal variations in phospholipid concentration are responsible for seasonal variations in the foaming properties of milk, while Ito, Sato & Suzuki (1963) have

suggested a relation between surface tension, among other factors, and deposition. The variation in the foaming properties of milk is much greater than the apparent change in the phospholipid content: this is a similar situation to the relation between fat and amount of deposit formation noted above.

The factor in milk composition which controls deposit formation is not yet identified, but it appears not to be one of the major constituents. Closer study will be needed to identify it precisely.

The author thanks Miss H. R. Chapman and the staff of the Institute's Experimental Dairy for their help throughout, particularly in the collection of milk samples and for the supply of certain analytical results. The chemical analyses were performed by Mr A. Wagstaff and the statistical calculations by Mrs A. Ford, and I am grateful to them for their skilled cooperation.

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The effect of milking throughout pregnancy on milk secretion in the succeeding lactation

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Summary. In each of 5 cows, 2 quarters which had been regularly milked throughout the whole of pregnancy produced less milk in the next lactation than the other 2 quarters which had a normal dry period. The concentrations of the individual constituents in the milk of all 4 quarters of each cow were similar. The effect appears to be due to factors located within the individual quarter of the mammary gland rather than to factors such as the hormonal background or plane of nutrition which affect the whole animal.

In milk secretion the role of the dry period between consecutive lactations of dairy cattle is not clearly understood. But the generally accepted views have been summarized by Smith (1959); that it is needed to replenish the body with nutrients that were depleted during lactation, to repair and regenerate the alveolar system, and to gain new hormonal stimulation for lactation as a result of parturition. There is evidence from the analysis of milk records that the yield of milk in any one lactation is related to the length of the preceding dry period, and that for maximum yield a dry period should be about 50 days when the calving interval is 365 days (Sanders, 1928; Johansson & Hansson, 1940; Klein & Woodward, 1943).

Smith, Wheelock & Dodd (1967) have shown that there is little secretory activity of the mammary gland throughout the greater part of the dry period, except for a time immediately before parturition. Recently Swanson (1965), using identical twins, demonstrated that cows milked throughout pregnancy produced in their next lactation only about 70 % of the milk yield of their mates which had a normal dry period. The cows, milked throughout pregnancy, were on a high plane of nutrition which eliminated nutrition as the main limiting factor.

The investigation reported here is one of a series on the importance of the dry period between successive lactations and is concerned with the effects of treatments similar to those used by Swanson except that we have made the treatment comparison between quarters of the same mammary gland, rather than between individual cows. A preliminary account of this work has already been published (Smith, Wheelock & Dodd, 1966).

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EXPERIMENTAL

Some 8–10 weeks before the expected date of parturition 2 diagonally opposed quarters (control) of 5 Friesian cows were dried off by suspension of milking, whereas milking was continued twice daily throughout the whole of pregnancy in the other 2 quarters (experimental). After parturition, all 4 quarters were milked twice daily. At regular intervals in both of the lactations the cows were milked with a milking machine designed for the separate collection of milk from individual quarters, the milk yields recorded, and a sample taken.

Weighted composite samples representing 1-day periods were prepared for lactating quarters and analysed for total solids, fat, lactose, potassium, sodium, chloride and total N (Wheelock, Rook & Dodd, 1965*a*).

The cows grazed with the Institute herd and were fed according to milk production, but received a minimum of 10 lb concentrates/day during the experimental period.

Regular bacteriological examinations were made on the milk of all the cows and with the exception of the left-hind quarter of cow 288, which was infected at the beginning of the second lactation, all the udders were free of bacterial infections throughout the period of the experiment.

RESULTS

Table 1 shows that, with the exception of the infected quarter in cow 288, similar results were obtained for both the control and for both the experimental quarters of each cow. Detailed results are therefore shown for only 1 quarter pair of each cow.

Yield of milk (*Table* 1, *Figs.* 1, 2)

Before the control quarters were dried off the milk yields of contralateral quarters were equal for all cows except cow 287 (Table 1). After drying off the 2 control quarters of each cow, the experimental quarters continued to lactate normally and the rate of decline in milk yield was apparently not affected by drying off the adjacent control quarters. In the last 1–2 weeks before parturition the decline was more rapid. No direct measurements were made on the control quarters after milking was suspended, but palpation of the mammary gland indicated that drying off progressed normally and the quarters became distinctly smaller.

After parturition, when all 4 quarters were again milked twice daily, the yield of milk from individual quarters increased rapidly, but within 1 day of parturition the control quarters produced more milk than the experimental quarters (Fig. 1). With cows 251, 252 and 297 the differences in milk yield between the experimental and control quarters were considerable. On the other hand, the comparable differences for cows 287 and 288 were small and may be related to differences between the yield of the quarters before the start of the experiment (Table 1). It is also evident that for cows 287 and 288 the yield obtained from the experimental quarters in the period just before parturition was < 100 g/day, and with a lactose content similar to dry-period secretions, while the quarter yields of cows 251, 252 and 297 exceeded 1 kg/day and the composition was similar to late lactation milk. The differences between

Table 1. *Average daily milk yields and the concentration of lactose in the milk of individual quarters before the start of the experiment and in the succeeding lactation*

		Milk yield, g		Concentration of lactose, g/100 g	
Cow	Quarter	When 2 quarters were dried off	One month after parturition	When 2 quarters were dried off	One month after parturition
251	Right-fore	2035	4660	4·73	4·62
	Left-fore	1915	2800*	4·72	4·62*
	Right-hind	4325	3570*	4·70	4·63*
	Left-hind	4350	6700	4·78	4·72
252	Right-fore	2335	4360	4·69	4·59
	Left-fore	2245	2355*	4·64	4·64*
	Right-hind	3650	4250*	4·67	4·41*
	Left-hind	3570	6270	4·68	4·59
297	Right-fore	2080	2595	4·60	4·77
	Left-fore	2155	1450*	4·51	4·70*
	Right-hind	2325	2220*	4·63	4·61*
	Left-hind	2325	2900	4·61	4·75
288	Right-fore	1420	4485	4·41	4·53
	Left-fore	1365	4205*	4·40	4·77*
	Right-hind	1320	5550*	4·47	4·70*
	Left-hind	1295	5340†	4·47	4·61†
287	Right-fore	1920	5530	4·59	4·55
	Left-fore	1665	5450*	4·21	4·60*
	Right-hind	2080	6460*	4·54	4·50*
	Left-hind	2520	6750	4·61	4·57

* Quarters milked throughout pregnancy.
† Intra-mammary infection.

the yields of the control and experimental quarters were maintained throughout the succeeding lactation (Fig. 2).

Concentration of individual milk constituents (*Table* 1, *Fig.* 3)

Both before the start of the experiment and after parturition, the concentrations of the individual constituents in the milk obtained from the 4 quarters of each cow were similar. Values for lactose are shown in Table 1 and for lactose and other constituents after parturition for 2 cows in Fig. 3.

The composition of milk from the 2 quarters of each cow milked throughout pregnancy followed the trends previously reported (Wheelock, Rook & Dodd, 1965*b*) though there were considerable differences in the degree of changes between animals. The lactose content of milk obtained from cows 251, 252 and 297 did not fall below 3·5 %, while values of < 2 % were obtained for cows 287 and 288. The latter are similar to values obtained in the early part of the dry period (Wheelock, Smith, Dodd & Lyster, 1967).

DISCUSSION

This experiment shows that if mammary gland quarters are milked throughout pregnancy and lactation is maintained, the yields of these quarters, relative to the quarters which had a normal dry period, are depressed after parturition. The results

confirm the conclusions of Swanson (1965) that the effect of milking throughout pregnancy cannot be due to nutritional factors because, in the experiment described here, the comparison was made between quarters within cows. Hormonal factors are not primarily involved, since these are common to all quarters. It appears that the removal of the secretion twice daily from a quarter throughout pregnancy directly affects the quarter and probably prevents the regeneration of mammary tissue so that yield after parturition is depressed. This could be due to the continued removal of secretion maintaining lactation and preventing involution, or to a direct effect of the

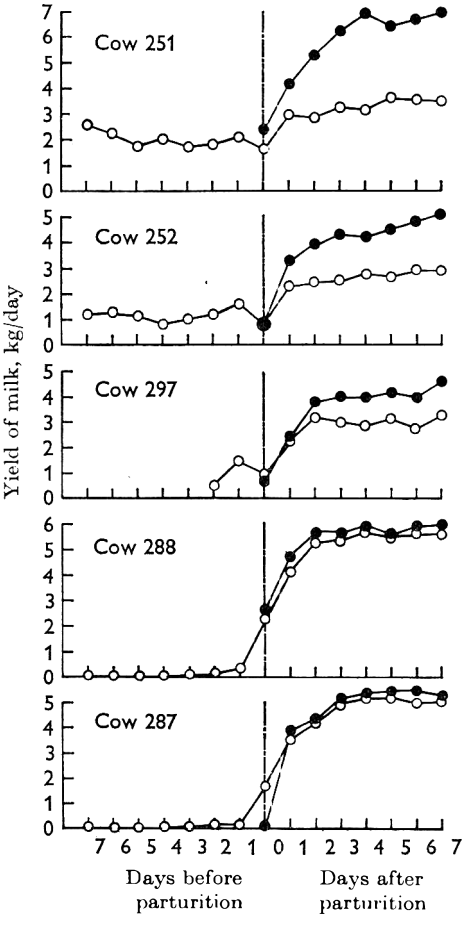

Fig. 1

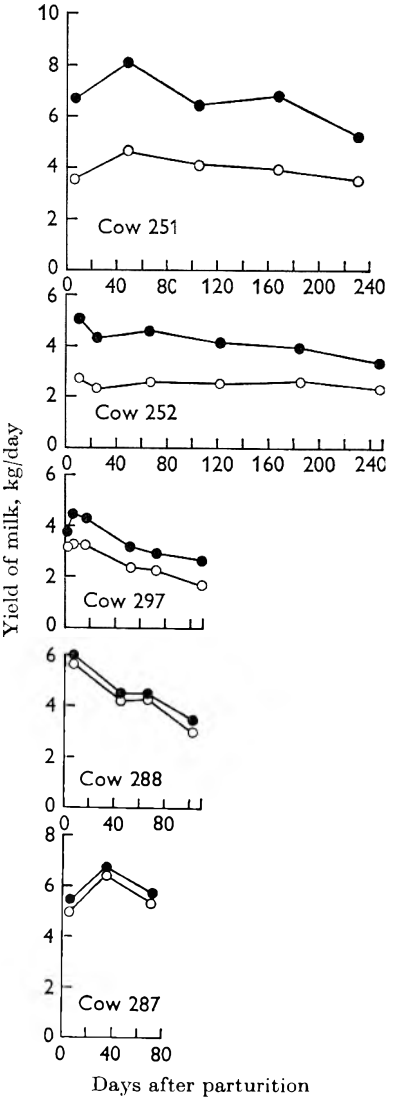

Fig. 2

Fig. 1. The changes in the yield of milk at parturition. ●. Control quarter; ○, experimental quarter.

Fig. 2. The changes in the yield of milk throughout the second lactation. ●, Control quarter; ○, experimental quarter.

act of milking on the quarter independent of the removal of secretion. The former explanation appears to be the most likely since experimental response after parturition appears to be related to the milk yields before parturition of the quarters milked throughout pregnancy. Thus, cows 251, 252 and 297, in which there was a marked difference between yields from the control quarters and those from the experimental quarters after parturition, were also the animals that maintained the highest milk yields of normal milk in the quarters that were milked throughout pregnancy.

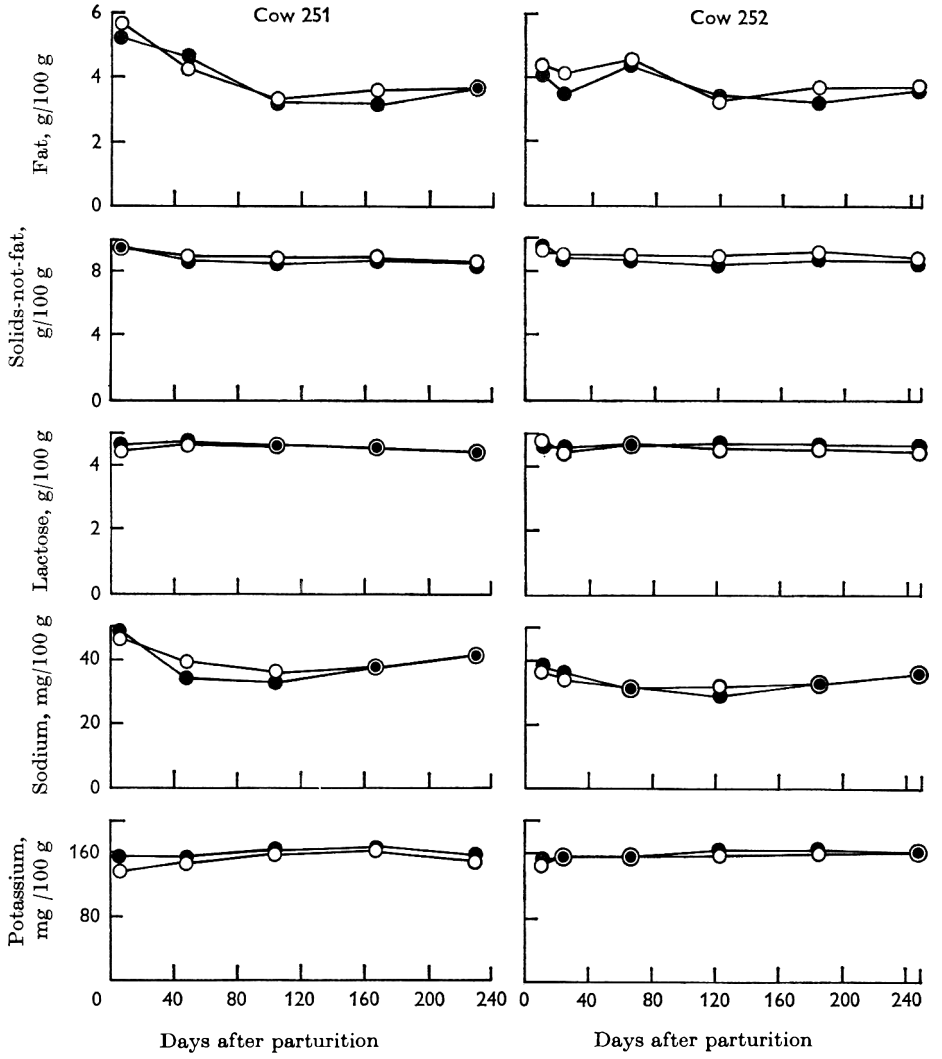

Fig. 3. The concentrations of fat, solids-not-fat, lactose, sodium and potassium in the milk of cows 251 and 252 during the second lactation. ●, Control quarter; ○, experimental quarter.

Assuming that for particular cows milk yield is roughly proportional to the amount of active secretory tissue (Linzell, 1963), it appears that for these cows more than 25% of the secretory tissue of the milked quarters was maintained throughout pregnancy. Milk yield after parturition was also depressed by about this proportion.

Conversely, for cows 287 and 288, less than 3% of secretory tissue was maintained and the secretion obtained was similar to that of the non-lactating udder. No claim can be made that any experimental response was obtained after parturition in these 2 animals.

It is of interest to note that Altman (1945) observed that the frequency of mitosis was considerably greater in secretion from the mammary gland of non-lactating cows 8–9 months pregnant than in those from lactating cows. He concluded that a dry period is important to allow for the rapid regeneration of secretory epithelium before the next lactation.

It has previously been suggested that a function of the dry period is to allow the cow to replenish body nutrients, The current study does not exclude this as an important aspect of the dry period. However, it does demonstrate that regeneration of mammary tissue occurs even in lactating glands, and that the degree of regeneration is inversely related to the secretory activity of the gland in the period before parturition. The main physiological function of the dry period is probably to induce full involution in the mammary gland, which then will allow full regeneration of mammary tissue so that there is maximum milk production after parturition.

We are grateful to Mr N. Jackson, Miss M. Weston, Miss S. Futcher, Miss E. Jenkins and Mrs P. Athey for skilled technical assistance.

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The effect of a temporary suspension of milking in mid-lactation on milk secretion after the resumption of milking and in the following lactation

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Summary. After the suspension of milking for 2 weeks in 2 'experimental' quarters of each of 4 Friesian cows, there was a reduction in the yield of milk from these quarters. Although subsequently there was a marked recovery, for the remainder of the lactation the yield was lower than that of the corresponding control quarters in which milking had not been suspended. Throughout the next lactation, in all 4 cows, the experimental quarters produced more milk than the corresponding control quarters. At the first milking after the suspension, there were marked increases in the concentrations of sodium and chloride and decreases in those of lactose and potassium in the milk obtained. Within 8 weeks, the composition recovered, and for the remainder of the experiment the composition of the milk from the control quarters was similar to that from the experimental quarters of each cow.

Milk secretion is inhibited by the incomplete removal of the secreted milk (Wheelock, Rook & Dodd, 1965*a*), and by the suspension of milking for periods of up to 60 h (Wheelock, Rook, Dodd & Griffin, 1966; Wheelock *et al.* 1965*a*). In these experiments, on the resumption of normal milking the yield and composition recovered completely within a few days. The present paper describes an experiment designed to determine how similar but more severe treatments affect milk secretion both in the lactation in which they are applied and also in the following lactation.

EXPERIMENTAL

Animals and management

The relevant details of the 4 Friesian cows (68, 162, 67 and 165) used in the experiment are shown in Table 1. Throughout the experiment the cows were milked twice daily. After an initial control period of 3 weeks, milking was suspended in the right-fore and left-hind quarters of cows 68 and 162 for an experimental period of 14 days. For the final control period milking was resumed and continued twice daily

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throughout the whole of pregnancy into the next lactation without a dry period. The experiment was similar for cows 67 and 165 except that milking was suspended in the right-hind and left-fore quarters for 16 days before regular milking was resumed, and also these cows were allowed a dry period between lactations. In fact the dry

Table 1. *Details of cows on experiment*

Cow	Lactation no. at the beginning of experiment	Stage of lactation at the beginning of experimental period, days since parturition	Length of dry period before next parturition, days	Calving interval, days
68	3	111	Milked throughout the whole of pregnancy	325
162	2	180		326
67	3	190	344	599
165	2	155	327	547

periods were abnormally long because of infertility. At intervals throughout the experiment, the cows were milked with a machine designed for the separate collection of milk from the individual quarters, the yield was recorded and a sample taken

Regular bacteriological and Whiteside tests were made to determine the presence of pathogenic udder infections.

Analysis

Milk samples were analysed for fat, total solids, lactose, potassium, sodium, chloride, total N and non-casein N by methods previously described (Wheelock, Rook & Dodd, 1965*b*).

RESULTS

During the initial control period, all the cows were free of bacterial infections of the udder. The compositions of the milk from all the quarters of each individual animal were similar. The yields of milk and individual constituents for each quarter during the initial control period are shown in Table 2. During the experimental period the right-hind quarter of cow 165 became infected with pathogenic bacteria. The infection was eliminated by intra-mammary antibiotic therapy but the quarter failed to secrete milk for the remainder of the lactation. An infection also developed in the left-hind quarter of cow 162 in the final control period.

In cows 67 and 68 the fore- and hind-quarter pairs responded similarly to the experimental treatments. Detailed results are therefore presented for only one quarter pair of each of these 2 animals as well as for the single pair of quarters of cows 165 and 162, that remained free of infections.

Yield of milk (*Figs.* 1, 3 *and* 4)

The milk yields for the whole of the experiment are shown in Fig. 1 and the detailed changes when milking was resumed at the beginning of the final control period in Fig. 3.

When milking was first suspended in the experimental quarters there was a slight increase in the milk yield of the control quarters in cows 68, 162 and 67.

At the first milking of the final control period, the yield of the experimental quarters was considerably less than their daily yield in the initial control period. The highest

yield obtained was only about 35 % of the average daily yield in the initial control period. After the removal of this small quantity of secretion, the yield gradually increased. The extent of the recovery ranged from 70 % of the yield in the initial control period in cow 162 to 10 % in cow 67.

Table 2. *The yield (g/day) of milk and its constituents produced by the individual quarters of each cow during the initial control period*

Cow	Right-fore	Right-hind	Left-fore	Left-hind
		Milk		
68	2180	5305	2100	4878
162	3347	6463	3228	6183
67	2585	5405	2703	5180
165	2730	5165	2853	5182
		Fat		
68	67	169	63	151
162	82	169	88	161
67	83	137	88	136
165	99	184	105	187
		SNF		
68	193	467	185	431
162	287	546	275	524
67	229	487	236	461
165	238	452	249	451
		Total N		
68	10·5	25·4	10·0	23·3
162	14·6	28·3	14·2	26·8
67	12·7	27·0	13·2	26·0
165	13·0	24·9	13·7	24·5
		Lactose		
68	102	244	98	227
162	154	299	150	285
67	118	253	122	239
165	121	231	129	232
		Potassium		
68	3·15	7·69	3·02	7·04
162	5·71	10·9	5·49	10·4
67	4·12	8·56	4·20	8·16
165	4·48	8·47	4·66	8·52
		Sodium		
68	0·74	1·70	0·71	1·70
162	0·94	1·88	0·91	1·69
67	0·87	1·75	1·05	1·86
165	0·98	1·78	0·93	1·69
		Chloride		
68	1·79	4·26	1·70	3·95
162	3·05	5·99	2·94	5·46
67	2·48	4·99	2·67	4·99
165	2·53	4·69	2·52	4·52

For most of the first lactation after the experimental period, the control quarters consistently produced more milk than the corresponding experimental quarters, but in the next lactation the experimental quarters produced more milk than the control quarters. The detailed changes in milk yield immediately before and after parturition are shown in Fig. 4 for cows 68 and 162.

The right-hind quarter of cow 165, which failed to produce milk in the first lactation following a severe bacterial infection, produced substantial quantities of milk in the next lactation. In comparison with the milk of the other quarters, there were only slight changes in composition.

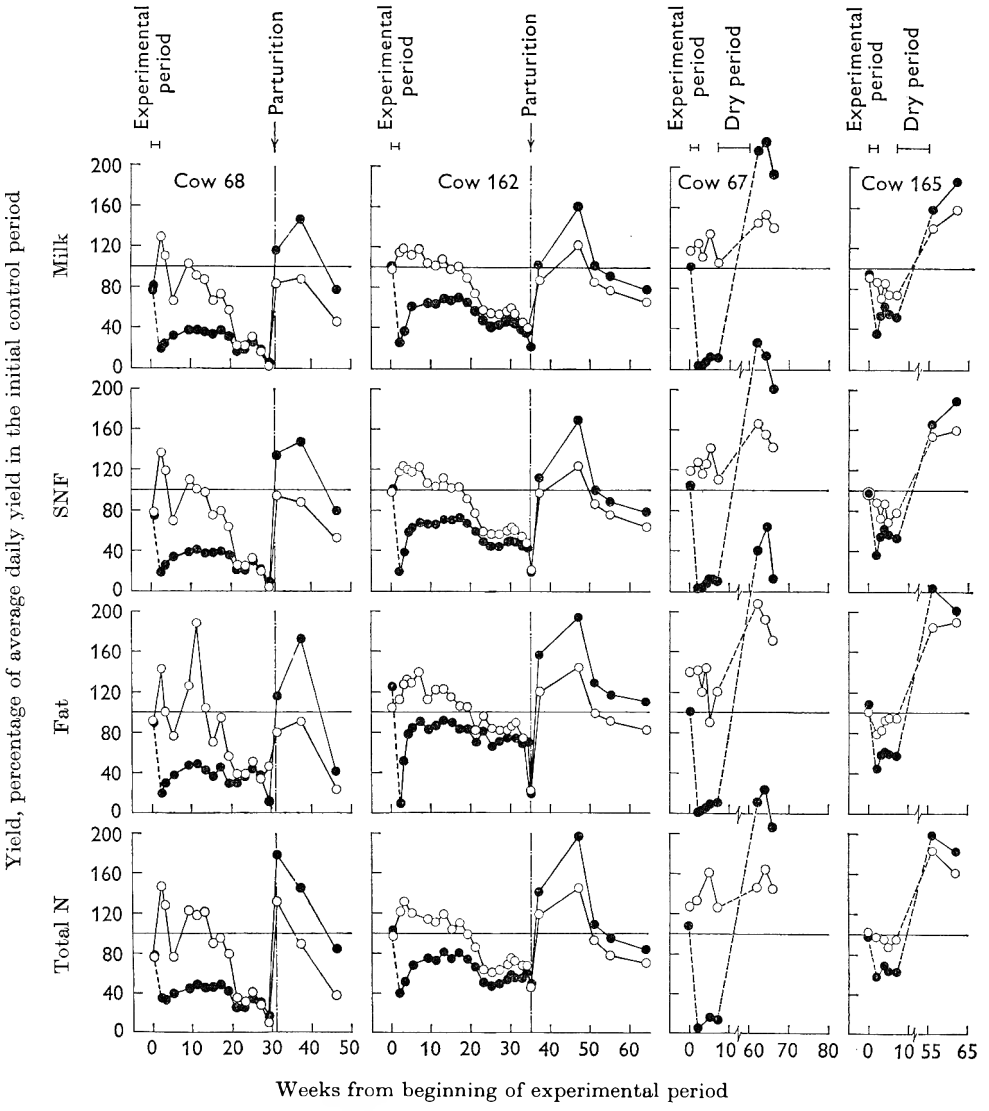

Fig. 1. The changes in the yield of milk, solids-not-fat, fat and total N throughout the experiment. ○, Control quarter; ●, experimental quarter; - - - -, milk not removed from quarter.

Yields of the individual milk constituents (*Figs.* 1, 2)

During the initial control period the yields of the individual constituents were similar for the control and experimental quarters of each cow, but at the beginning of the final control period the yields of all the constituents from the experimental quarters were considerably depressed, with the single exception of sodium for cow 68.

On the resumption of milking, the yields of the experimental quarters increased progressively but the recovery was not complete in any of the cows. In general, the changes in the yields of the constituents were similar to those for milk yield except that at the beginning of the final control period the effect on lactose and potassium was more marked, and on sodium, chloride and non-casein N less marked. In the second lactation, the yields of all the constituents were greater for the experimental quarters than for the control quarters.

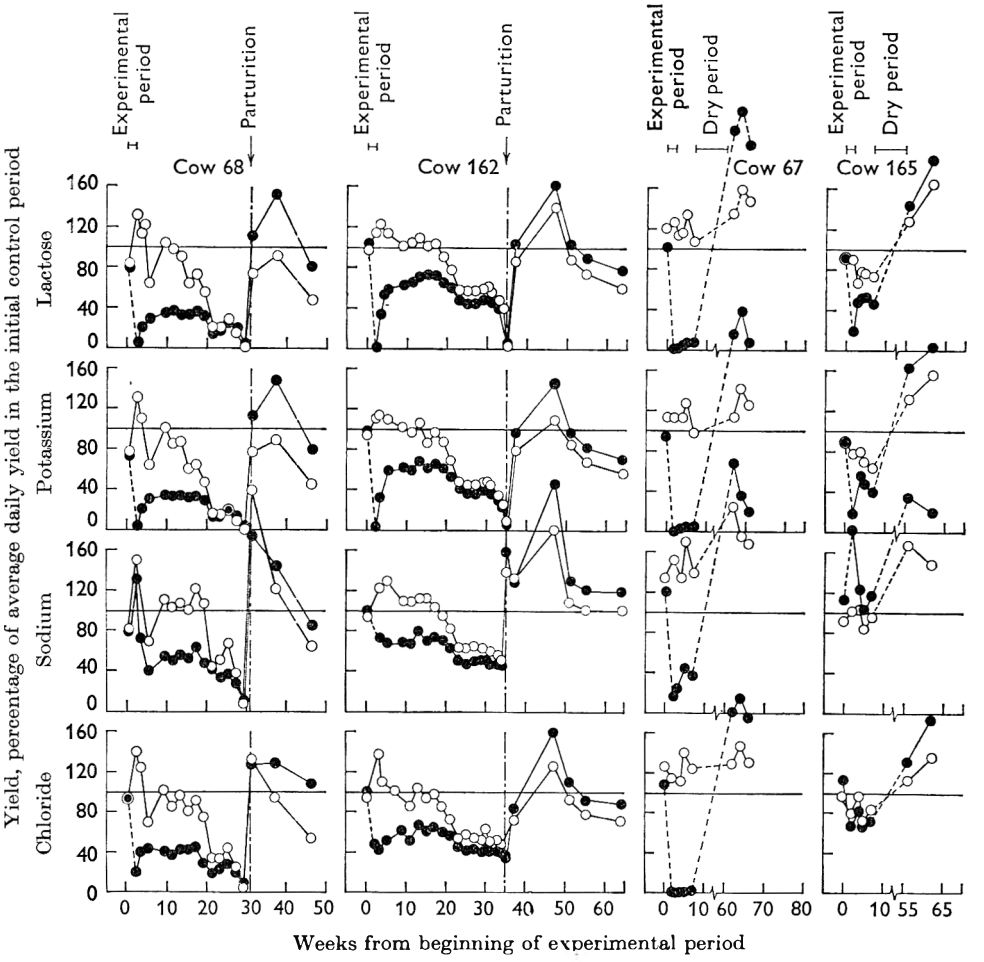

Fig. 2. The changes in the yield of lactose, potassium, sodium and chloride throughout the experiment. ○, Control quarter; ●, experimental quarter; ----, milk not removed from quarter.

Concentrations of the individual milk constituents (*Figs.* 3, 5 *and* 6)

During the experimental period when milking was suspended in 2 quarters, the composition of the milk from the control quarters was unchanged, but in the experimental quarters, marked changes in the composition of the milk were observed when milking was resumed at the beginning of the final control period. The concentrations of solids-not-fat (SNF), lactose and potassium were decreased and those of sodium,

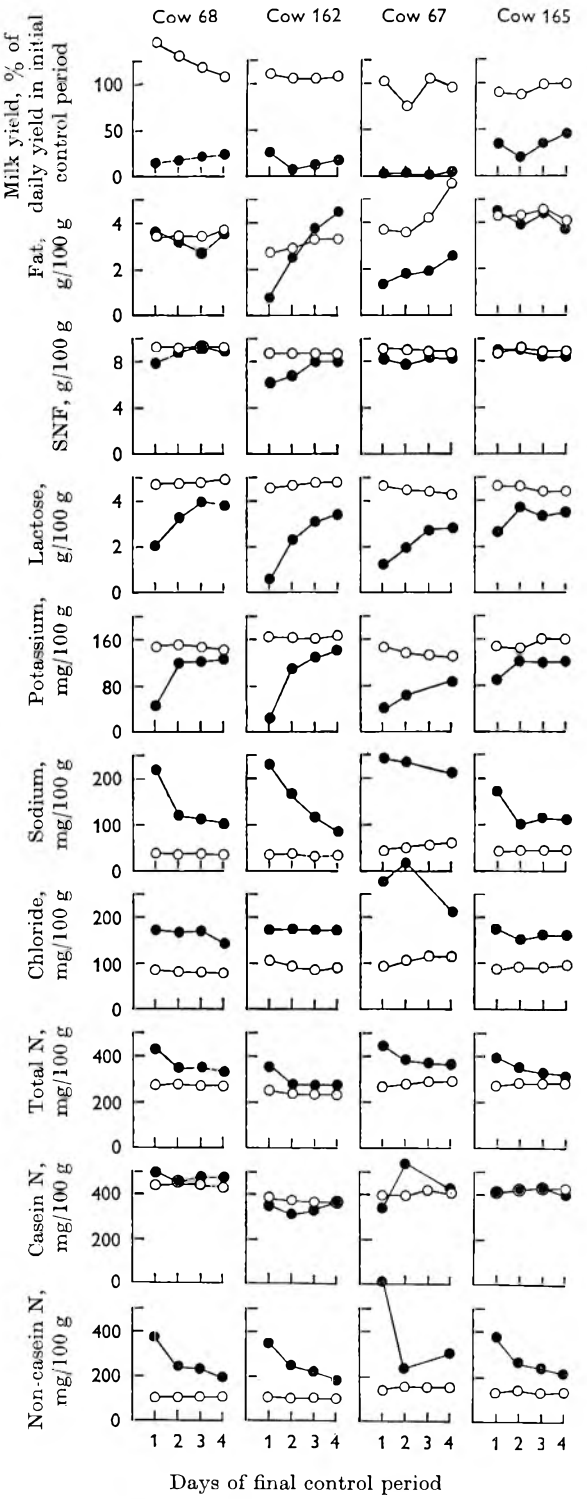

Fig. 3. The changes in the yield of milk and in the concentrations of the individual milk constituents at the beginning of the final control period. ○, Control quarter; ●, experimental quarter.

chloride, non-casein N and total N increased. Casein concentration was not greatly affected by the treatment and there was no consistent pattern of changes for fat concentration. Subsequently there was a progressive recovery in the concentration of all the constituents which was most marked during the first days of the final control period (Fig. 3), and in cows 68 and 162 recovery was virtually complete 8 weeks later. In the second lactation, the compositions of the milk from control and experimental quarters of each animal were similar.

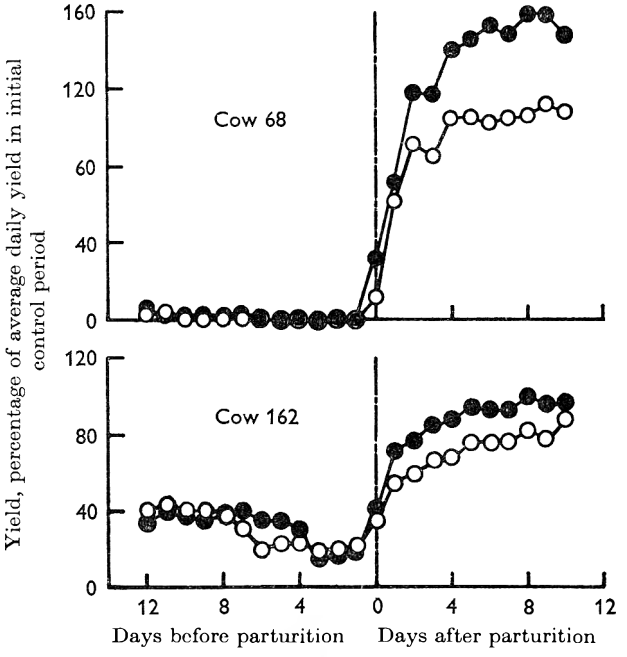

Fig. 4. The changes in the yield of milk at parturition in cows 68 and 162. ○, Control quarter; ●, experimental quarter.

DISCUSSION

The results of this experiment show that when milking of 2 quarters of a cow is suspended for a period of 2 weeks, the secretory activity is considerably depressed and probably ceases completely. On the resumption of normal milking, the yields, relative to those of the control quarters, are initially very small with a composition approaching that of secretion obtained from the mammary gland during the dry period. However, within 8 weeks, the composition of the milk recovers completely and there is a partial recovery in milk yield, though it remains depressed until the end of lactation or until the milk yield falls to a very low level. After the next parturition, the milk yields of the quarters that had the temporary suspension of milking in the previous lactation were considerably greater than those of the control quarters, and there were no differences in the composition of the milk. These trends in the secretory activity occurred both for the 2 cows milked throughout pregnancy (68 and 162), and for the 2 cows given a dry period (67 and 165). The results of the latter are particularly interesting because the dry periods lasted for about 1 year.

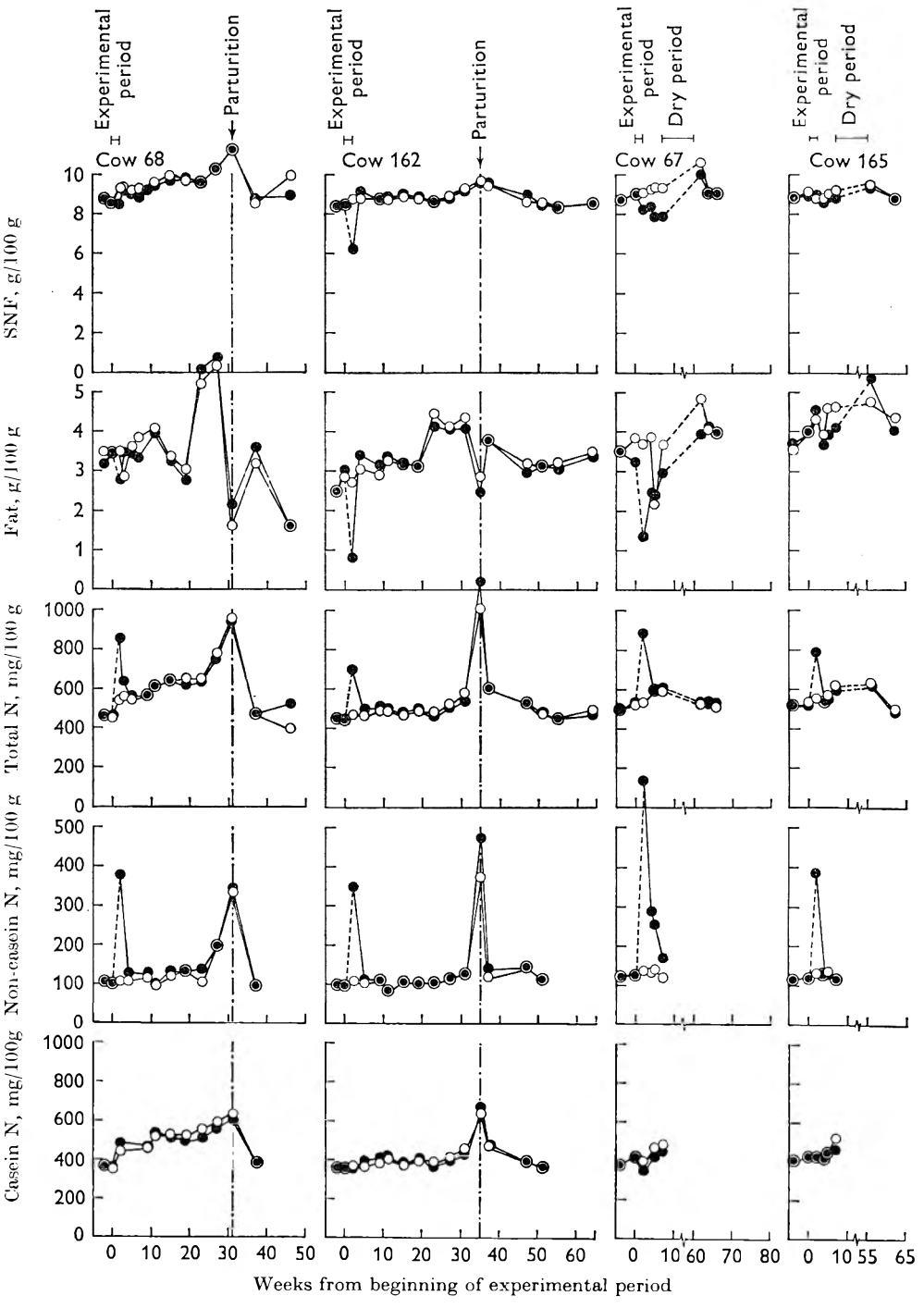

Fig. 5. The changes in the concentrations of SNF, fat, total N, non-casein N and casein N throughout the experiment. ○, Control quarter; ●, experimental quarter; ----, milk not removed from quarter.

It has been suggested (Smith, Wheelock & Dodd, 1967) that complete regeneration of secretory tissue occurs only when the secretory tissue of the preceding lactation has completely involuted, a hypothesis based on the observation that milking throughout pregnancy without a dry period reduces lactation yields after parturition. The present results are not completely explained by this hypothesis. The partial

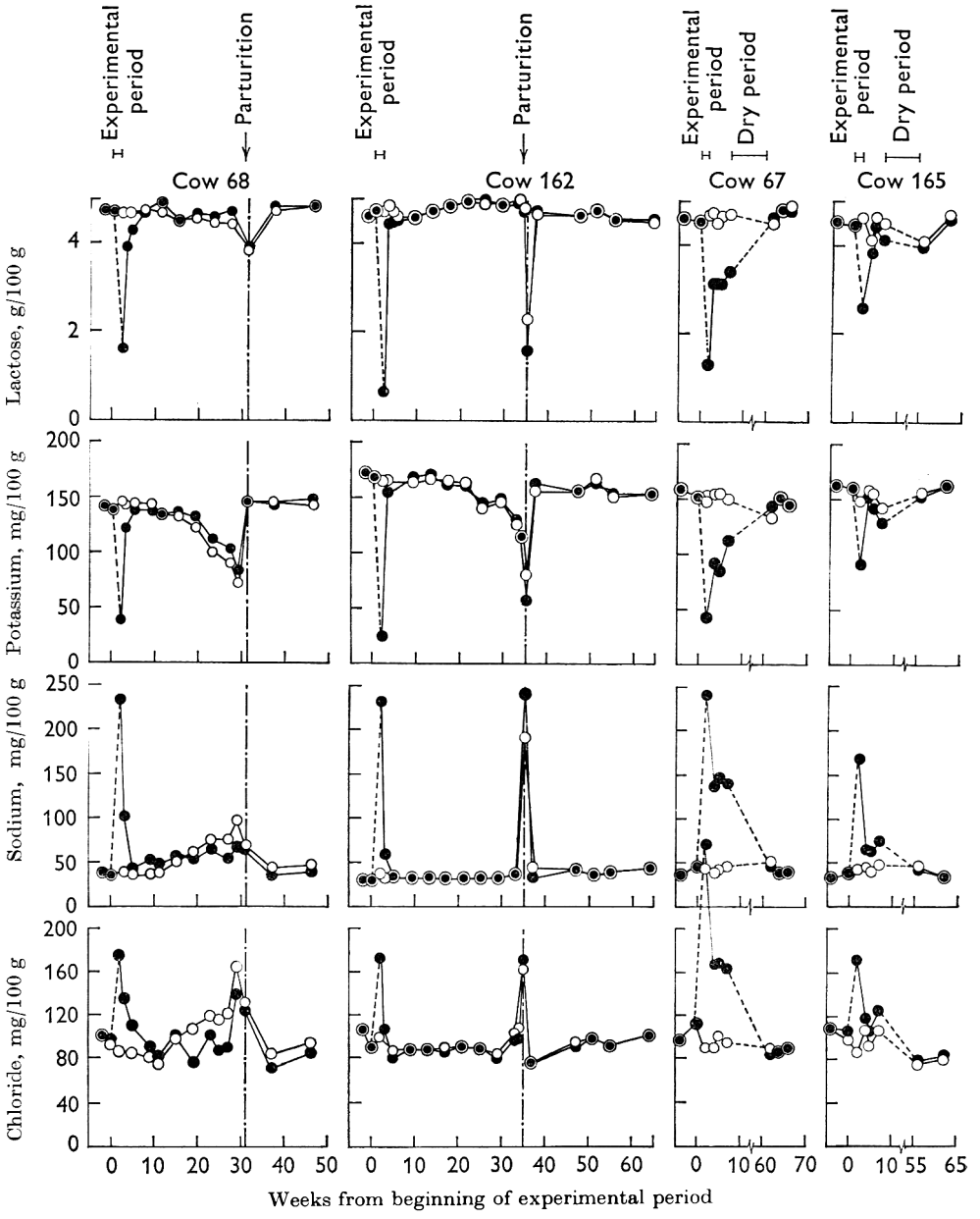

Fig. 6. The changes in the concentrations of lactose, potassium, sodium and chloride throughout the experiment. ○, Control quarter; ●, experimental quarter; ----, milk not removed from quarter.

involution in mid-lactation indicated by the permanent reduction in milk yield resulted in an increase in milk yield, relative to that of the control quarter, in the next lactation. As these findings were obtained even for cows which also had an adequate dry period, it seems that the extent of involution is not the only factor in determining the degree of regeneration of mammary tissue.

When milking is suspended in mid-lactation, the quarters become engorged with milk but within a day resorption begins and on the resumption of milking, the yield is initially very low. This process is similar to that described by Wheelock, Smith, Dodd & Lyster (1967), when milking is suspended at the end of lactation. Thus, it appears that quarters can be dried off rapidly and normally while other quarters of the same gland are milked twice daily. Furthermore, the inhibition of secretion is not due to increased intramammary pressure since the amount of secretion obtained when milking begins again is so small. Neither can the inhibition of secretion be due to any change in the hormonal background of the animal since the milked quarters of the gland continue to lactate normally and it is, therefore, apparently due directly to the presence of certain constituents in the gland, and as soon as these are removed milk secretion rapidly recovers.

This view is in agreement with the work of Levy (1964) who showed that certain fatty acids, which are normally present in milk, inhibit the synthesis of fat by rat mammary gland tissue preparations, and he concluded from his results that the inhibition of fat synthesis at the end of lactation was caused by the increase of these constituents within the gland. It is quite possible that the synthesis of other milk constituents can be inhibited by a similar type of mechanism.

The increase in the milk yield of the control quarters of cows 67, 68 and 162, shortly after the experimental treatment commenced, is similar to that observed previously in one control quarter when the other 3 quarters were subjected to varying degrees of incomplete removal of milk, (Wheelock *et al.* 1965*a*). This result may be due to an increase in the rate of blood flow to the control quarters, or to an increase in concentration of precursors in the blood.

The gradual recovery in the yield and composition of the milk of the experimental quarters when milking was resumed is similar to that observed with shorter periods of suspended milking (Wheelock *et al.* 1965*a*, 1966). In the present experiment, the point at which complete recovery in milk yield would be achieved cannot be established precisely because of the decline in yield that occurred as lactation advanced, and it is not possible to make an unbiased comparison with the control quarter because of the compensatory increase in yield which occurred at the beginning of the experimental period. Nevertheless, in contrast to the results for shorter intervals, the recovery was not complete.

The maximum recovery was observed with cow 162, which was lactating for only 80 days when milking was suspended, and the least recovery was made by cow 67, which had been lactating for 190 days. Therefore, the effect of the experimental treatment was increasingly severe as the lactation advanced. Mizuno (1961), using mice, showed that the accumulation of milk for one day on the 14th day of lactation caused no change in the nucleic acid content of the gland, but reduced the ratio RNA:DNA on the 20th day of lactation, and suggested that the mammary function might be more susceptible to inhibition in late lactation than at peak lactation.

We are grateful to Mr N. Jackson, Miss M. Weston, Miss S. Futcher, Miss E. Jenkins and Mrs P. Athey for skilled technical assistance.

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The effect of equal and unequal intervals in twice-daily milking on the milk yield of ewes

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Summary. The effect of equal and unequal milking intervals (12 + 12, 10 + 14 and 8 + 16 h) was investigated in milch ewes. The treatments were applied to 9 ewes using a double 3 × 3 Latin square. The daily production of milk and fat was unaffected by the changes in milking interval.

It was traditionally believed that in order to obtain maximum milk yield from cows which were milked twice daily, it was necessary to milk at equal (12 h) intervals. Justification for this husbandry practice was found in the measurement of milk secretion rate made by Ragsdale, Turner & Brody (1924) who reported a decrease in the rate of milk secretion with the lengthening of the milking interval. Further support was given by the survey data presented by Bartlett (1929) and by Edwards (1950). During recent years, however, there have been many reports showing that the daily production of cows was unaffected by a change from equal (12 h) to unequal (10 + 14 h and even 8 + 16 h) milking intervals. Although the reports were based on different experimental techniques (Koshi & Petersen, 1954; Turner, 1955*c*; Hansson, Claesson & Brannang, 1956; MacMeekan & Brumby, 1956; Linnerud & Williams, 1962; Johansson, 1938; Poijarvi, 1953, 1954; Schmidt & Trimberger, 1963; Himmel, 1964) in all cases the change in milking routine from equal to unequal intervals had no significant effect on the daily production of milk and fat.

No reports on the effect of equal and unequal milking intervals on the yield of milch ewes have been published. Sheep dairy farmers endure a prolonged working day because husbandry milking practice in ewe flocks is still based on a belief that maximum yield is obtained by milking after equal intervals (Becker, 1958). The experiment reported below was to investigate the effect of equal and unequal milking intervals on the daily yield of milch ewes.

MATERIALS AND METHODS

A small number of 3-year-old recently lambed cross-bred ewes [Dorset Horn × (Border Leicester × Cheviot)] were available for this investigation. Previous unpublished studies have shown that such ewes, when separated from their lambs at birth, and machine milked, have very variable lactation curves. For this reason the experiment was done using a double 3 × 3 Latin square, in which the second square was a mirror image of the first. It was believed that with the small number of treatments this was the most satisfactory method of overcoming the difficulties caused by the

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decline in milk yield with the advance of lactation. The Latin squares and treatment definitions are set out in Table 1. It should be noted that the long intervals occurred overnight. Each treatment was applied for 6 days and the records of the first day of each period were discarded in order to eliminate any carry-over effects of residual milk from the last interval of the previous treatment.

Table 1. *Experimental design*

	Periods					
Ewe no.	1	2	3	4	5	6
1, 11, 21	A	B	C	C	B	A
2, 12, 22	B	C	A	A	C	B
3, 13, 22	C	A	B	B	A	C

Treatment	Day interval		Night interval
A	12 h	followed by	12 h
B	10 h	followed by	14 h
C	8 h	followed by	16 h

Table 2. *Details of experimental animals**

Ewe no.	Mean daily yield in 10 days prior to experiment, ml	Days after lambing	Yield group
1	1018	20	High
2	946	19	
3	956	23	
11	854	18	Medium
12	849	23	
13	775	22	
21	609	20	Low
22	526	19	
23	458	22	

* The ewes were cross-breds, i.e. Dorset Horn × (Border Leicester × Cheviot). They were all 3 years old and in their 2nd lactation.

Nine ewes were ranked according to their yields over the previous 10 days into 3 yield blocks. They were then allocated at random to treatment sequences (see Table 1).

The ewes were milked in an adapted herring-bone type parlour using an Alfa Laval 4-unit machine (pulsation ratio, 1:1; pulsation rate, 180 c/min; vacuum, 33 cmHg). All the animals were milked at 19.00 h and, in accordance with treatment, at 07.00, 09.00 or 11.00 h, respectively. The actual interval never varied from that planned by more than 2 min. The udders were not washed or stimulated prior to the application of the teat cups and foremilking was not practised. Milking was accompanied by vigorous udder massage and was carried out by the same operator on all occasions.

The ewes were kept in treatment groups in 3 m × 3 m pens on slats in a walled barn, brightly illuminated day and night throughout the experiment. Grassnuts and water were provided *ad lib.* in the pens, and the ewes had access for 2 h each day to good-quality hay. At every milking the ewes were offered 1 kg of concentrates prepared

from a mixture of soya 20 %, fish meal 20 %, rolled barley 60 %, to which bone flour and $A+D_3$ premix were added at the rate of 3 and 5 kg/ton, respectively. No ewe showed any signs of illness or of loss of appetite throughout the experiment.

Composite period samples of milk were taken for morning and evening milking during the last 3 periods and analysed for fat using a modified Gerber test (Macdonald, 1959).

The daily morning and evening secretion rates (mean milk yield/h) were analysed using the following model:

$$Y_{ijkl} = \mu + B_i + S_{ij} + P_k + T_l + (BT)_{il} + \epsilon_{ijkl},$$

when μ = mean secretion rate of a sheep in a period;

B_i = the effect due to ith yield block when $i = 1, 2, 3$;
S_{ij} = the effect due to jth sheep in the ith yield block when $j = 1, 2, 3$;
P_k = the effect due to kth period when $k = 1, 2, \ldots, 6$;
T_l = the effect due to lth treatment when $l = 1, 2, 3$;
$(BT)_{il}$ = the effect due to the combination of ith yield block and the lth treatment.

Fat percentage was analysed using a similar model when $k = 4, 5, 6$.

The experiment was done at the University of Reading, from 24 April to 29 May 1965.

RESULTS AND DISCUSSION

The effects of treatments for the compared milking schedules on the mean secretion rate and on the mean fat percentage over 24 h are set out in Table 3. It is clear that the daily production, both of milk and fat, was quite unaffected by changes in the milking schedule.

The interaction between yield blocks and treatments was very small and non-significant in all the investigated measurements, which indicates that the ability to maintain a constant daily production is a characteristic which is independent of yield. This is in general accordance with the cow studies referred to above. Schmidt & Trimberger (1963) reported some indication (non-significant) that unequal intervals may have affected the higher yielding cows more than the rest of the animals in their trials. In this connexion, however, it is noted that Schmidt (1960) has reported a secretion rate experiment in which it was the high yielding cows whose yields were least reduced by long intervals. Similarly, Claesson, Hansson, Gustafsson & Brannang (1959) found that once-a-day, as compared with twice-a-day, milking appeared to have a less adverse effect on the milk yield of higher yielding animals.

The relatively low coefficients of variation indicate the satisfactory nature of the design and the sensitivity of the measurement of treatment effects.

The milk yield data after the longer night intervals shown in Table 3 appear to indicate that the secretion rate declined progressively with the lengthening of the interval. These measured secretion rates are, however, heavily biased by the carry-over of variable amounts of residual milk from one interval to another (Turner, 1955*a*, *b*). Elimination of this bias and an estimation of the true secretion rate was attempted. Semjan (1962) published data on the amounts of residual milk after milking intervals of 8–24 h in Improved Moravian sheep, whose daily yields were comparable to those of the ewes in the present experiment. A linear regression relating

the amount of residual milk to interval length has been fitted to their data and has been used to estimate the initial and final amounts of milk present in the udder of the ewes in the examined intervals of our experiment, and hence to estimate the true

Table 3. *Effect of milking interval on mean apparent secretion rates and mean fat percentages of milk*

Treatments	Mean apparent secretion rate, ml/h			Mean fat content, %		
	Over 24 h period	P.m. milking	A.m. milking	Over 24 h period	P.m. milking	A.m. milking
A, 12+12 h	24·85	23·48	25·76	4·42	4·99	4·03
B, 10+14 h	24·67	25·95	23·33	4·44	4·98	4·11
C, 8+16 h	24·38	27·86	22·26	4·32	4·74	4·13
Standard error	1·23	1·03	1·14	0·11	0·23	0·33
Coefficient of variation	10·60	11·92	13·14	10·48	18·78	8·56
Degrees of freedom	34	34	34	10	10	10

Table 4. *Estimated true milk secretion rates during various milking intervals (estimates calculated from the relationship: estimated milk secreted in interval = residual milk* from preceding interval + observed milk yield − residual milk* from preceding interval*

Interval, h	Observed milk yield, ml		Added final residue ml	Estimated		Apparent† secretion rate ml/h
	Uncorrected	Corrected for initial residue		Milk secretion, ml	Milk secretion rate, ml/h	
Day 8	222·88	119·06	67·70	171·52	21·44	27·86
10	259·50	106·22	80·54	233·82	23·38	25·95
12	281·76	93·38	93·38	281·76	23·48	23·48
Night 12	309·12	93·38	93·38	309·12	25·76	25·76
14	326·62	80·54	106·22	352·30	25·16	23·33
16	356·16	67·70	119·06	407·52	25·47	22·26

* The residual values are derived from a linear regression fitted to the quantities of residual milk published by Semjan (1962) in which $y = 16{\cdot}34 + 6{\cdot}42x$; when y = amount of residual milk in ml after x h of secretion.

† Apparent secretion rate = observed milk yield divided by length of milking interval.

secretion rates. A check on the relevance of Semjan's data to the reported results was made on the day following the experiment when the mean percentage of residual milk in our ewes was found to be 24·1 (range from 17·4 to 32·2). This observation was after a 16 h interval and is similar to the figure 23·0 calculated from Semjan's original report.

Table 4 sets out the estimated true secretion rates and the method of their derivation. The mean rates of secretion for all night milking intervals were similar, and with the exception of the 8-h interval, the rates for the day milkings were also uniform. However, the results indicate a higher secretion rate for the night milkings despite the fact that good illumination was provided at all times. A difference between daytime and night-time secretion rates in the ewe has been noted by Semjan (1962). He observed that ewes milked twice daily at equal intervals yielded similar quantities

of milk at the morning and the evening milkings, but that the evening milk was 15 % richer in fat. (This report was based on 628 lactation weeks). In a further investigation he showed that the retention of residual milk and fat was 25 % greater at the morning milking and he concluded that the rate of milk and fat synthesis was higher during the night interval. The reasons for this apparent diurnal rhythm in secretion are obscure, as, indeed, are the reasons for the apparent differences in the degree of ejection at the morning and evening milkings.

The results presented for ewes are consistent with those reported in studies with cows.

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Reviews of the progress of dairy science

Section D. Nutritive value of milk and milk products. Water-soluble vitamins in milk and milk products

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CONTENTS

INTRODUCTION

This review covers publications that have appeared during the 5-year period from the beginning of 1962 to the end of 1966. In presenting it, the author has attempted to include as many results as possible from countries overseas. The problem of scanning world literature has been largely eliminated with the help of *Dairy Science Abstracts*, and where the author has been unable to refer to the original publication (particularly with Russian and Japanese publications) the relevant reference to *Dairy Science Abstracts* is included in the list of references.

WATER SOLUBLE VITAMINS IN COW'S MILK AND MILK PRODUCTS

The recent excellent review by Hartman & Dryden (1965) on *Vitamins in Milk and Milk Products* gives a concise yet comprehensive account, not only of vitamin levels in milk, but also of factors affecting these levels. It also includes some historical notes on the discoveries of the different vitamins and outlines the part they play in animal nutrition. The reader is referred to this review to fill in earlier findings which the present review does not cover.

Milk

The levels of the water-soluble vitamins in cow's milk are now well established, but there have been some recent estimations which are worth mentioning. These are summarized in Table 1.

With the exception of two low values for ascorbic acid in cow's milk reported by Nakanishi & Yamaji (1964) and Barakat & Abdel-Wahab (1961) all these values for vitamins in milk, reported from different countries, fall within the expected range of values (see Hartman & Dryden, 1965).

Pantothenic acid

Černá (1963) has studied different methods for extracting pantothenic acid from biological materials. She found that heating in a weakly acid solution, such as acetate at pH 4·5, was sufficient to extract the vitamin from cow's milk. Autolysis or enzyme hydrolysis did not give a higher result. She reports that market milk in Czechoslovakia contains 1·7–2·5 μg pantothenic acid/g. This low value may be due to the use of *Saccharomyces carlsbergensis* as test organism instead of the more conventional *Lactobacillus arabinosus*. Ishiguro (1963) measured both free and bound pantothenic acid in raw milk. Free pantothenic acid was 3·8 μg/g and bound pantothenate 0·4 μg/g. It is interesting that Hibbitt (1964) has also measured bound pantothenate in milk, using a new microbiological method. He found appreciable amounts of co-enzyme A (0·2–1·72 μg/ml) to be present in early lactation milk. By the 12th–16th week the level had dropped to 0·1–0·3 μg/ml.

Riboflavin

Nagasawa, Kuzuya & Shigeta (1961*a*) have studied the distribution of the bound forms of riboflavin in milk. They found that mid-lactation milk contained 1·70 μg total riboflavin/ml. This was made up of 53 % free riboflavin, 29 % flavinmononucleotide (F.M.N.) and 18 % flavinadeninedinucleotide (F.A.D.). Colostrum, which is high in riboflavin activity, contained a higher proportion of F.M.N. and F.A.D. than the mid-lactation milk. Hotta, Ishiguro, Naito & Tanaka (1960) have also studied the distribution of bound forms of riboflavin in the milks of different species. They showed that the milk of both the cow and goat had a higher total riboflavin content and alkaline phosphatase activity than that of the human, pig or dog. A high proportion of free riboflavin was present in cow's and goat's milks, whereas F.A.D. was the predominant form in the milks of the other 3 species. Gupte, Karmarkar, Modi & Tate (1963) showed that the F.A.D. present in human milk is bound to one or more of the milk proteins. On heating the milk these proteins are denatured and free F.A.D. is liberated. Swope, Brunner & Vadehra (1965) found that 92–96 % of the total riboflavin present in the fat globule membrane exists as F.A.D., probably as the coenzyme of xanthine oxidase. Milk from quarters showing subclinical mastitis had slightly lower riboflavin levels than that from uninfected quarters. At the same time, an increase in the proportion of F.A.D. present in the milk from the infected quarters was noted (Nagasawa, Kuzuya & Shigeta, 1961*b*).

Vitamin B_{12}

The work of Schweigert and his colleagues on the bound form of vitamin B_{12} in milk has produced some interesting results. Their original studies seemed to indicate that all the milk proteins contained some vitamin B_{12} and could combine with the added vitamin (Kim, Gizis, Brunner & Schweigert, 1965; Gizis, Kim, Brunner & Schweigert, 1965). However, careful study of the different fractions and a technique of electro-dialysis have shown that peptide-bound vitamin B_{12} accounts for at least 70 % of the total bound vitamin (Gizis, Brunner & Schweigert, 1965) and it may well turn out that one specific peptide is responsible for the binding of vitamin B_{12} in milk.

Table 1. *Water-soluble vitamins in cow's milk*

Type of milk	Country of origin	Ascorbic acid, μg/ml	Nicotinic acid, μg/ml	Pantothenic acid, μg/g	Riboflavin, μg/ml	Thiamine, μg/ml	Vitamin B_{12}, mμg/ml	Reference
Raw	Chile	11·0	0·99	—	1·41	0·71	—	Santibanez (1959)
Market	Poland	—	—	—	1·49–1·69	0·30–0·40	—	Łuczak, Barska & Kwinta (1961)
Market	Japan	5	—	—	—	0·28	—	Nakanishi & Yamaji (1964)
Market	Japan	—	—	—	—	—	2·9 (1·0–6·0)	Kawashima, Uesaka & Uchiyama (1961)
Raw	Japan	—	—	—	—	—	3·3 (1·0–13·0)	Kawashima, Uesaka & Mitsuhashi (1961)
Colostrum	Japan	—	—	—	—	—	16.2 (1·3–62·0)	Kawashima, Uesaka & Kita (1962)
Pasteurized, homogenized	Canada	—	—	—	—	—	3·03–3·38	St-Pierre, Blais & Beaudoin (1963)
Raw	Egypt	7·1–7·8	—	—	—	—	—	Barakat & Abdel-Wahab (1961)
Raw	Yugoslavia	17·2–23·0	—	—	—	—	—	Mihelić & Mikić (1961)
Pasteurized	Yugoslavia	8·6–14·3	—	—	—	—	—	Mihelić & Mikić (1961)
Sofia Brown cows	Bulgaria	15·6 (7·7–24·4)	—	—	—	—	—	Tsvetkova, Ikonomov & Todorov (1962)
			μg/g		μg/g	μg/g	—	
Pasteurized	Poland	—	0·6–1·0	1·86–2·40	1·41–2·05	0·40–0·49		Myszkowska (1960)

Folic acid

With the discovery by Herbert (1961) that ascorbic acid protects labile forms of folic acid in blood serum from destruction during treatment of the samples for assay, it has become possible to re-evaluate the levels of folic acid in milk. Previously milk was thought to be a poor source of folic acid (see Hartman & Dryden, 1965) and difficulty was encountered in obtaining reliable results using microbiological methods. The application of Herbert's method to the measurement of folic acid in milk has shown that cow's milk contains appreciable amounts of this vitamin: 62–100 mμg/ml (Naiman & Oski, 1964), 36–43 mμg/ml (Ghitis & Canosa, 1965), 17–63 mμg/ml (Matoth, Pinkas & Sroka, 1965) and 27–55 mμg/ml (Sullivan, Luhby & Streiff, 1966). Matoth *et al.* (1965) found that human milk contains 7–61 (mean 24) mμg folic acid activity/ml, and since boiling or sterilizing cows' milk caused considerable loss of folic acid activity, they concluded that artificially fed infants had a lower dietary intake of folic acid than breast-fed infants. However, Ramasastri (1965), using the same assay method, found only 7·5–23·7 (mean 16·5) mμg folic acid activity/ml of milk from Indian women of the lower socio-economic group. He also reports that human colostrum contains less folic acid (2·4–8·4 mμg/ml) than the mature milk. Goat's milk is considered an inadequate source of folic acid for infants since it contains low levels of the vitamin (7–13 mμg/ml, Naiman & Oski, 1964; 2–20 mμg/ml, Sullivan *et al.* 1966).

Vitamin B_6

As part of a collaborative study of methods for measuring vitamin B_6, a sample of dried skim-milk was analysed in 13 different laboratories, using *Saccharomyces carlsbergensis* as the test micro-organism (Edwards, Benson & Storvick, 1963). The overall average value found (expressed in terms of pyridoxine HCl) was 4·63 μg/g. The range of average values from the different laboratories was from 3·02 to 5·5 μg/g. Similar wide variations were found with the other test samples (wheat flour, brewer's yeast and liver powder), and it was concluded that further tests were necessary to achieve a more satisfactory method for measuring vitamin B_6 in biological materials.

Ascorbic acid

Two new methods for measuring ascorbic acid in milk have been described. Deschacht & Hendrickx (1962) claim that their modification of the colorimetric method of Moor (1956) is more reproducible than the 2,6-dichlorphenolindophenol method for measuring ascorbic acid in milk, and Deutsch & Weeks (1965) recommend a microfluorimetric method for measuring the vitamin in fortified milk. The stability of ascorbic acid in milk has been studied by Herrmann & Grossmann (1963). They found that ascorbic acid was oxidized more slowly in milk than in aqueous solution and that even at 100°C the L-ascorbic acid content of milk diminished only slowly.

Cheese

The B-vitamin content of 23 varieties of cheeses and of 8 cheese spreads has been measured by Shahani, Hathaway & Kelly (1962). They found that, in general, cheeses such as Camembert, in which proteolysis was extensive, had the highest

vitamin contents and the soft unripened types had lower contents. The average values (expressed as μg/100 g) for all the cheeses tested, together with the highest and lowest values, were: nicotinic acid, 277 (62–1247); vitamin B_6, 94 (53–248); pantothenic acid, 691 (144–2946); biotin, 1·79 (0·84–5·70); folic acid, 22·3 (6·4–120). These results are in agreement with those reported by others and summarized in a review by Karlin (1961*a*). The folic acid values may, however, be low since the improved assay method mentioned earlier in this section was not used. The same group in America have recently made a more thorough examination of the B-vitamin content of Cheddar cheese, including changes that occur during the ripening period (Nilson, Vakil & Shahani, 1965). Both temperature and length of ripening period have a pronounced effect on the vitamin content of cheese.

Paolis (1965) has analysed the B-vitamin content of Mozzarella cheese during the winter and spring cheese-making periods. The average winter and summer contents of the cheese were: vitamin B_6, 18 and 17 μg/100 g; pantothenic acid, 103 and 102 μg/100 g; riboflavin, 425 and 435 μg/100 g; nicotinic acid, 155 and 145 μg/100 g; thiamine, 45 and 65 μg/100 g, and vitamin B_{12}, 220 and 230 mμg/100 g. Samples of Trappist cheese contained 414–955 μg riboflavin/100 g and 262–513 μg nicotinic acid/100 g (Berger-Grüner, 1966) and 1·82–4·25 μg vitamin B_{12}/100 g (Rašič & Panič, 1963).

During the preparation of Cheddar cheese curd about 80 % of the nicotinic acid and vitamin B_6, 70 % of the pantothenic acid, 90 % of the biotin and vitamin B_{12} and 60 % of the folic acid present in the original milk was lost in the whey (Nilson *et al.* 1965). Paolis (1965) also noted that a high proportion of the nicotinic acid, pantothenic acid, thiamine and vitamins B_6 and B_{12}, but only a small proportion of the riboflavin, present in the milk was lost in the whey during the manufacture of Mozzarella cheese. In the preparation of Trappist cheese about 10 % of the nicotinic acid and 12–26 % of the riboflavin of the milk passed into the cheese (Berger-Grüner, 1966). Under normal industrial conditions, no losses of thiamine, riboflavin, nicotinic acid or pantothenic acid were observed during the manufacture of processed cheese in Poland (Dłuzewska, Dłuzewski, Pacholczyk, Pijanowski & Zmarlicki, 1964).

Fermented milks

Aithal & Sirsi (1964) showed that curdling cow's or buffalo's milk with a culture of household curds brought about a loss of about 30 % of the vitamin B_{12} and that therefore curds cannot be considered as a source of vitamin B_{12} for vegetarians. Similarly, Panič, Rašič & Hristič (1963) found that the vitamin B_{12} content of the milk decreased during the making and ripening of kefir, the loss being especially pronounced during the 18-hr incubation at 22 °C with the kefir culture.

FACTORS AFFECTING THE VITAMIN CONTENT OF MILK

Cow's milk

Ritter (1966) has reviewed the effect of feeding and management on the vitamin content of cow's milk. Thompson & Kon (1964) studied the effect of breed, season and geographical location on the riboflavin content of milk. They confirmed the higher concentration of riboflavin in the milk of Guernsey cows as compared with that of Shorthorns. In milk collected from 10 different areas of England and Wales there

Table 2. *Water-soluble vitamin contents of the milks of different species*

	Ascorbic acid, mg/100 g	Biotin	Nicotinic acid, μg/100 g	Pantothenic acid, μg/100 g	Riboflavin, μg/100 g	Thiamine, μg/100 g	Vitamin B_6	Vitamin B_{12}	Reference
Human	—	—	72–100	115–130	36–52	6–10	—	—	Myszkowska (1960)
Human	3·9	—	—	—	—	—	—	—	Lembrych & Lika (1965)
Human	6·6	—	—	—	36	9	—	—	Saito *et al.* (1963)
	mg/100 ml	μg/100 ml	μg/100 ml	μg/100 ml	μg/100 ml	μg/100 ml	μg/100 ml	μg/100 ml	
Human	2·6	—	—	—	17	15	—	—	Belavady & Gopalan (1959)
Human	1·3–6·3	—	—	—	8–14	15–18	—	—	Belavady, Pasricha & Shankar (1959)
Human	2·5	—	132	—	60	27	—	—	Santibanez (1959)
Buffalo	1·4	—	—	—	—	—	—	—	Grigorov, Shalichev & Goranov (1962)
Buffalo	—	7·9	82	202	167	81	25	0·43	Paolis & Gregory (1963)
Buffalo	—	—	—	—	235–248	—	—	—	Sirry & El-Said Saleh (1962)
Goat	1·7	—	57	—	97	21	—	—	Santibanez (1959)
Ass	1·2	—	371	—	178	61	—	—	Santibanez (1959)
Mare	3·1	—	61	—	81	47	—	—	Santibanez (1959)
Camel	6·1	—	—	—	—	—	—	0·22	Bestuzheva (1964)
Reindeer	—	14	156	720	1003	218	80	1·2	Aschaffenburg, Gregory, Kon, Rowland & Thompson (1962)
Giraffe	—	0·9	210	218	153	43	54	1·1	Aschaffenburg, Gregory, Rowland, Thompson & Kon (1962)
Rabbit	—	17–45	490–870	620–2210	230–490	60–170	210–360	5–11	Coates, Gregory & Thompson (1964)
Okapi	—	3	80	2100	1000	120	530	9	Gregory, Kon, Rowland & Thompson (1965)
Rhinoceros	—	0·3	< 100	340	30	25	22	0·1	Gregory, Rowland, Thompson & Kon (1965)

were no variations in riboflavin content that could be associated with geographical location or with the change from stall to pasture feeding. However, average values for the 6 months August–January were higher (175 μg/100 ml) than those from February to July (150 μg/100 ml). In contrast, Smorodina (1962), in the U.S.S.R., reports 139 μg riboflavin/100 ml in winter milk and 239 μg/100 ml in summer milk. Similarly, Sirry & El-Said Saleh (1962) found higher levels of riboflavin in milk during the grazing season (198 μg/100 ml) than in the dry feeding season (164 μg/100 ml). In Brazil, the riboflavin content of milk, sampled twice monthly on arrival at processing plants in São Paulo, was highest in autumn and winter, and a positive correlation was found between the vitamin content of the milk and the average amount of rain that had fallen 2–3 months earlier (Barbuto, 1963).

Raw milk, collected from farms in the Belgrade area, had a higher average vitamin B_{12} content of 5·3 mμg/ml (range, 3·9–8·1) than market milk which contained 3·4 mμg/ml (range, 2·4–4·1) (Rašič & Panič, 1963). The authors suggest that this may be due to the farms being located on soil with a plentiful supply of cobalt, whereas the market milk was collected from a wider and more varied region. Or, the lower vitamin B_{12} level could be due to the way in which the milk was handled at the dairies. Considerable day-to-day variations in the vitamin B_{12} content of milk from individual cows was noted. Miller, Wentworth & McCullough (1966) have attempted to find out why there should be such variations in the vitamin B_{12} content of milk. They studied individual cows of different breeds at 3 distinct geographical locations in the U.S.A., and were able to show that breed and season had a significant effect on the vitamin B_{12} content of the milk at one location and that type of feed had a significant effect at all locations. Administration of cobalt bullets had no effect on the vitamin B_{12} content of the milk. However, in Japan, a commercial mineral mixture providing 2 mg cobalt per head per day significantly increased the vitamin B_{12} content of the milk of 29 cows from 3·98 to 5·8 mμg/ml (Kawashima & Uesaka, 1962), and in the U.S.S.R. the vitamin B_{12} in milk was increased to 6·5–7·9 mμg/ml by supplementing the ration with $CoCl_3$ (Laganovskiĭ, 1960).

Human milk

The effect of diet on the vitamin composition of human milk has also been studied and some of the findings are mentioned here.

Contreras, Arroyave & Guzman (1962) studied milk from 43 women of high socio-economic level living in Guatemala City and from 69 women of low socio-economic level living in a country village. During the first 3 months of lactation, the total riboflavin content was similar in milk from women in the city and in the country, but from the 3rd to 6th month it was significantly lower in the milk from the country women. The levels of pantothenic acid, riboflavin, nicotinic acid, ascorbic acid and thiamine in the milk of Indian women were significantly correlated with the diet (Deodhar & Ramakrishnan, 1960; Rao & Subrahmanyam, 1964) and a supplement of these vitamins plus vitamins B_6 and B_{12}, biotin, and folic acid, given to women of low nutritional status significantly increased the vitamin content of the milk and appeared to have a beneficial effect on the milk yield (Deodhar, Rajalakshmi & Ramakrishnan, 1963). Similarly, supplementing the diet of poor Indian women with 10-25 mg thiamine and 50–500 mg ascorbic acid/day increased the level of these vitamins in the milk (Belavady & Gopalan, 1960).

In Russia, it was found that feeding 350 mg ascorbic acid/day (Kopylova, 1962) or 850 μg vitamin B_{12} (Bogdanova, 1962) increased the levels of these vitamins in human milk. In Hungary, Tarjan, Kramer, Szöke & Lindner (1963) also demonstrated that foodstuffs rich in riboflavin and ascorbic acid caused increases in these vitamins in milk, and from Japan, Saito, Furuichi, Noguchi, Takezaki & Imamura (1963) report that milk from mothers with a high standard of living contained more ascorbic acid, thiamine and riboflavin than that from mothers living in a poor-class district of Tokyo.

ENRICHMENT OF MILK AND MILK PRODUCTS

In the previous review (McGillivray & Gregory, 1962) the potentialities of the use of micro-organisms for enriching dairy products with vitamins were discussed. In the 5 years since then, the emphasis appears to have been mainly directed towards producing milk products with a high vitamin B_{12} content.

Karlin (1961*b*) studied the vitamin B_{12} content of fermented milks and showed that the micro-organisms associated with yoghurt (*Lactobacillus bulgaricus* and *L. thermophilus*) and bioghurt (*L. acidophilus* and *Str. lactis*) utilized, after 7 days incubation at 30 °C, about 80 % of the vitamin B_{12} present in milk. The addition of *Propionibacterium shermannii* to the yoghurt or bioghurt caused a smaller overall loss of only about 33%; this was because *P. shermannii* is able to synthesize vitamin B_{12}. Furthermore, the addition of *P. shermannii* to kefir (which contains yeast in addition to lactic acid bacteria) resulted in an increase in the vitamin B_{12} content. This increase was even greater if 0·5 % peptone was also added to the kefir. Further studies (Karlin, 1966) with kefir, made from dried skim-milk, showed that during the first 24 h of incubation, when the lactic acid bacteria in the kefir culture were multiplying rapidly, the levels of pantothenic acid, riboflavin, biotin and vitamin B_{12} decreased, and those of nicotinic acid, thiamine, vitamin B_6 and folic and folinic acids increased. After 48 h incubation, the synthesis of vitamins by the yeasts in the kefir culture was greater than their utilization by the lactic acid bacteria, and consequently the levels of pantothenic acid, riboflavin, nicotinic acid and vitamin B_6 increased. Only vitamin B_{12} continued to decrease. When *P. shermannii* was also present in the kefir culture, increases in riboflavin, nicotinic acid, thiamine, vitamin B_6, vitamin B_{12} and folic and folinic acids were noted after 24 h incubation at 30 °C. After 48 h incubation a marked increase in pantothenic acid was also noted. At the same time the vitamin B_{12} content had increased from 1·1 to 68 μg/l. in 48 h. These results led Karlin to suggest that it would be easy to prepare kefir from dried skim-milk in countries which are not milk producers and the product (made with the kefir culture grown in symbiosis with *P. shermannii*) would be rich in proteins and vitamins and, above all, be a good source of vitamin B_{12}.

Kruglova (1962) has suggested a method for enriching evaporated skim-milk and dried skim-milk with vitamin B_{12}. Prior to the evaporation or spray drying, 2 μg 5,6-dimethylbenzimidazole/l. and an inoculum of *P. shermannii* are added and the milk incubated at 30 °C for 2–3 days. In this manner evaporated milk and spray-dried milk with the extremely high vitamin B_{12} contents of 2600 and 5000 μg/kg were prepared.

Another approach has been to increase the vitamin B_{12} content of yoghurt and kefir by adding a vitamin concentrate—prepared by incubating propionic acid bacteria in whey with added hydrolysed milk and then drying (Grudzinskaya, 1965). However, this gives a product containing about 5 μg vitamin B_{12}/l. which is very much less than when the propionic acid bacteria are grown in symbiosis with the yoghurt or kefir culture.

The addition of propionic acid bacteria to starters used in the manufacture of Edam and Tilsit cheeses has also been proposed (Janicki, Pedziwilk & Kisza, 1963). The presence of *P. freudenreichii* increased the vitamin B_{12} content of Edam cheese by about 50 % compared with the control cheese without propionic acid bacteria.

In Poland, the enrichment of processed Tilsit cheese with ascorbic acid is said to improve its shelf life and flavour (Kudella & Wnuk, 1962), and butter containing added ascorbic acid kept better and had a better flavour, aroma and colour and lower peroxide values than control samples (Budsławski & Zakrzewska, 1962). The addition of ascorbic acid is also a potential alternative to de-aeration in stabilizing the flavour of concentrated sweetened cream over a 3-month period (Bell, Anderson & Tittsler, 1962).

PROCESSING AND STORAGE

It is well known that H.T. S.T. pasteurization (15 s at 71–73 °C) causes only slight losses in the vitamin content of milk but that in-bottle sterilization (110–120 °C for up to 1 h) destroys up to half of the thiamine, vitamin C and vitamin B_6 and 90 % of the vitamin B_{12} contents. In the newer ultra-high-temperature (U.H.T.) methods of sterilization now coming into commercial use, milk is directly or indirectly heated in a continuous process to temperatures of 130–150 °C for only a few seconds (Report, 1963). This subject is also discussed in an F.A.O. publication on Milk Sterilization (Burton, Pien & Thieulin, 1965) which includes a chapter by van Eckelen & Heijne on the nutritive value of sterilized milk.

Gregory & Burton (1965) took milk samples from 6 different indirectly heated plants and 3 directly heated plants and examined the losses of the heat-labile vitamins (thiamine, vitamin B_6 and vitamin B_{12}) that took place during the heat treatment. Losses of thiamine were negligible, while those of vitamins B_6 and B_{12} varied from 0 to 35 %.

Lhuissier, Hugot & Biette (1962) found no loss of riboflavin or vitamin B_6 but a 20 % loss of thiamine in uperized (UHT, directly heated) milk. These results and others described by Hostettler (1965) show that UHT treatment has less destructive effect on the vitamins of milk than in-bottle sterilization. Lhuissier *et al.* (1962) examined the vitamin content of uperized milk in Tetrapak cartons stored for a month at 7 °C or 20–22 °C in the dark, and at 20–22 °C in a sunny window. The only loss measured was of 15 % of the vitamin B_6 when the cartons were stored at 20 °C. The authors, therefore, conclude that uperized milk in Tetrapak cartons (which have a black lining) is an excellent source of vitamins even if kept at ordinary room temperatures in the daylight for a month.

The Preservatives in Food Regulations (Great Britain, Parliament, 1962) now make it permissible for certain foods to contain nisin. Gregory, Henry & Kon (1964) therefore compared the nutritive properties of evaporated milks prepared by a normal

commercial procedure and by 2 other processes using less intense heat treatment in the presence of nisin. In the normal evaporated milk, with the cans heated at 113 °C for 15 min, 83 % of the vitamin B_{12}, 38 % of the vitamin B_6 and 20 % of the thiamine was destroyed during processing. When nisin was present and the cans were heated at 105 °C for 15 min, only the loss of vitamin B_{12} was reduced, whereas heating for the shorter time of 3 min at the higher temperature of 113 °C caused less destruction of each of the vitamins. On storage for 12 months there was no further loss of thiamine at 4°C, but at room temperature and particularly at 37 °C, the thiamine content decreased as the length of storage increased. Similarly, the vitamin B_6 content of the evaporated milk decreased during the 12-month storage, the loss being greatest at 37 °C, but also detectable at 4 °C. No change in vitamin B_{12} content occurred on storage, and no losses of biotin, nicotinic acid, pantothenic acid or riboflavin were detected during manufacture or storage of the evaporated milks. The less intense heat treatments in the presence of nisin did not affect the keeping quality of the milks.

Davidov, Gul'ko & Bekhova (1962) found that during the preparation of condensed milk, 31 % of the ascorbic acid, 21 % of the riboflavin, 14 % of the thiamine and 10 % of the nicotinamide were lost. When the product was stored for 2 years at 7–12 °C, losses were 73 % for ascorbic acid, 47 % for thiamine and 28 % for riboflavin. During the manufacture of dried milk, 26 % of the thiamine, 7 % of the riboflavin, 41 % of the ascorbic acid and 7 % of the nicotinamide was lost. On storage for 2 years at 8–12 °C, the dried milk lost a further 10 % of the thiamine and ascorbic acid. Enrichment of dried milk with these 2 vitamins was recommended (Davidov, Gul'ko & Bekhova, 1963).

In evaporated milk stored for 4 years at 32 °F, there was no loss of thiamine or vitamin B_6, but a loss of about 25 % of the riboflavin and 23 % of the vitamin B_{12} (Adams *et al.* 1965). The controls for comparison were freshly prepared cans of evaporated milk, and therefore these values can only be taken as very approximate.

Lhuissier & Biette (1962) found that about 20 % of the thiamine and vitamin B_6 present in raw milk was destroyed by in-bottle sterilization. On storage of the sterilized milk, the remaining thiamine was stable at normal ambient temperatures if the bottle was kept in the dark. Vitamin B_6 was stable only if the bottles were kept at refrigerator temperatures; at 20 and 39 °C in the dark, losses of 20 and 30 % were observed after 6 months storage. Exposure to light at these temperatures completely destroyed vitamin B_6 in 6 months. The riboflavin content of in-bottle sterilized milk is stable at 4, 20 or 38 °C providing that the bottles are kept in the dark (Causeret, Hugot, Goulas-Scholler & Mocquot, 1961). Exposure of the bottles to diffuse light for 2 weeks at 20 °C destroyed 80 % of the riboflavin originally present. The use of amber glass bottles helps to protect the riboflavin present in milk (Hugot, Lhuissier & Causeret, 1962). In intense light, the amber glass gave satisfactory protection for 6 weeks during which time only 20 % of the riboflavin was lost.

After exposure to an incandescent lamp for 3–9 min, riboflavin losses were similar in milk that had been (*a*) pasteurized, (*b*) pasteurized and U.H.T. treated or (*c*) pasteurized, U.H.T. treated and homogenized (Lhuissier & Biette, 1964). In milk (*c*) which had then been sterilized in-bottle, there was less loss of riboflavin on exposure to light. The authors suggest that the high heat-treatment might change the ribo-

flavin into a form less sensitive to light or perhaps the factors favouring photolysis are destroyed or protecting factors are produced.

After exposure for 6 h to a white light, 84 % of the ascorbic acid present in milk (contained in clear glass bottles) was destroyed (Somogyi & Ott, 1962). In brown bottles the loss was about 18 % and in Tetrapak containers only about 10 %. Riboflavin losses under the same conditions were 40 % in clear glass bottles, 24 % in brown bottles and 14 % in Tetrapak containers. Losses were much lower when the milk was illuminated by red or yellow light. Similar results were obtained by Hendrickx & Moor (1962), who found that the rate of decomposition of ascorbic acid by light depended on the type of fluorescent lamp used and on the colour and transparency of the container.

Radema (1962), in Holland, and Dunkeley, Franklin & Pangborn (1962) in the U.S.A. have studied the effect of fluorescent light on milk in refrigerated display cabinets. The ascorbic acid content of the milk was shown to decrease more rapidly as the light intensity and exposure times increased. They also confirmed that different types of fluorescent lights destroyed ascorbic acid more rapidly or more slowly than others. Therefore, in order that the minimal amount of damage should be done to milk on display for sale, it is important to consider the type of lamp used for illumination, to reduce the intensity of the light as much as possible, to choose appropriate containers and to ensure a rapid turn-over in the display cabinet.

When milk was kept at refrigerator temperatures with exclusion of direct light and oxygen, the loss of ascorbic acid was only 35 % in 48 h, but it reached 80 % in 96 h (Dimov, 1965). Exposure to light greatly accelerated the losses of ascorbic acid when the milk was subsequently stored in darkness.

Wodsak (1965) found no detectable losses of riboflavin, vitamin B_6 or ascorbic acid in milk irradiated with ultraviolet light to enrich it with vitamin D.

Treatment of milk with ion-exchange resins to remove radiostrontium may also result in losses of certain vitamins (Aarkrog & Rosenbaum, 1962). A loss of 30 % of the thiamine activity during ion-exchange treatment of milk was demonstrated by Perfilov (1963) who recommended that such milk should be fortified with 0·2 mg thiamine/l.

In a review concerned with the effect of storage and commercial processing on the vitamin content of milk, Blanc (1966) suggests the following ways of avoiding losses of vitamins: (1) reduce the delay in refrigerating and utilizing milk after it is produced; (2) protect the milk from light; (3) eliminate dissolved oxygen before the milk is processed and avoid contact with oxidizing agents; (4) obtain the correct balance of temperature and time during heat treatment; (5) use substances such as nisin to reduce the heat treatment necessary for satisfactory keeping quality.

MILKS OF OTHER SPECIES

Values for water-soluble vitamins in the milks of other species (some of them non-domestic animals) are summarized in Table 2.

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