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Observed ratios of $^{90}Sr/Ca$ and $^{137}Cs/K$ in the food of nursing mothers and in their milk

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Summary. The $^{90}Sr/Ca$ and $^{137}Cs/K$ 'observed ratios' (OR) in the food intake of nursing mothers and in their milk have been determined. Between 4 and 7 days after parturition, groups of mothers were put on a controlled diet for 4 days. Pooled samples of milk were then taken from each group. ^{90}Sr, Ca, ^{137}Cs and K were determined in the pooled samples and in all foods taken during the experimental period. $^{90}Sr/Ca$ ratios were determined in 4 experiments and $^{137}Cs/K$ ratios in 3. The OR for the 4th day's intake did not differ significantly from average ratios for the whole 4 days, so that a state of metabolic equilibrium appears to have been reached. The $^{90}Sr/Ca$ OR was fairly constant and considerably less than unity, but the $^{137}Cs/K$ OR was greater than unity and varied considerably between experiments.

Since potassium, which is metabolically analogous to caesium, showed a fairly constant ratio between intake and excretion in the milk, the physiological behaviour of ^{137}Cs must be quite different from that of ^{90}Sr. The maternal organism therefore provides the baby with efficient protection against ^{90}Sr but not against ^{137}Cs.

New measurements have been made of the observed ratios (OR) of ^{90}Sr and ^{137}Cs to their metabolic analogues Ca and K in the diet of nursing mothers and in their milk. A preliminary report has been published (Calapaj & Ongaro, 1967).

The OR of a radionuclide to its metabolic analogue reflects the metabolism of the two when introduced into a biological system. It is defined, for example, as the ratio

$$\frac{^{90}Sr/Ca \text{ in product}}{^{90}Sr/Ca \text{ in precursor}}.$$

Little information about the $^{90}Sr/Ca$ and $^{137}Cs/K$ OR can be found in the literature. Lough, Hamada & Comar (1960) have reported values ranging from 0·085 to 0·132 for the $^{90}Sr/Ca$ OR in 5 experiments with American mothers fed on a controlled diet for one month *post partum*; in Canada, Jarvis, Brown & Tiefenbach (1963) obtained values between 0·14 and 0·16, but the estimates were indirect. In this work, in fact, the $^{90}Sr/Ca$ OR was calculated on the assumption that 70–80 % of dietary Ca would be derived from milk and milk products, and 20–30 % from other foods for which a $^{90}Sr/Ca$ ratio 3 times that of cow's milk was assumed. For the $^{137}Cs/K$ OR, the only reported values were obtained by Aarkrog (1963) for one Danish mother; they ranged from 1·1 in September to 1·9 in March according to the composition of the diet and its content of ^{137}Cs.

MATERIALS AND METHODS

The experiments were performed in 1966 and 1967 with 4 groups—of 9, 16, 14 and 7— of Italian mothers aged between 19 and 25, admitted for normal delivery to public hospitals in Padua, Belluno and Dolo, in north-eastern Italy.

Samples of milk of equal volume were collected from each mother between the evening of the seventh and the morning of the eighth days *post partum*. The samples

Table 1. *Dietary intake of radionuclides and their secretion in the milk of 9 nursing mothers*

(Samples taken at the clinic of Gynaecology and Obstetrics of the University of Padua on 6–9 February 1966. Expt 1.)

(*a*) Secretion in mother's milk

^{90}Sr = 0·7 pC/l; Ca = 0·296 g/l; ^{90}Sr/Ca = 2·38 pC/g

(*b*) Measurements on the diet (uncooked weight)

Food	^{90}Sr, pC/kg	Ca, g/kg
Cow's milk	19·0	1·150
Bread	19·1	0·246
Italian pasta	40·6	1·399
Beef	2·6	0·072
Chicken	0·8	0·293
Soft cheese	57·7	7·096
Spinach	33·7	1·690
Chicory	22·2	1·101
Artichokes	17·3	0·736
Potatoes	8·9	0·134
Oranges	6·9	0·455
Apples	1·3	0·038

(*c*) Average diet (uncooked weight) and daily average intakes of ^{90}Sr and Ca

Food	Quantity, g	^{90}Sr, pC	Ca, g
Milk	1000	19·0	1·150
Bread	330	6·3	0·081
Italian pasta	240	6·9	0·236
Choice of meat			
Beef	145	0·3	0·032
Chicken	160		
Choice of raw vegetables			
Spinach	150	3·6	0·175
Chicory	150		
Artichokes	150		
Potatoes	110	0·9	0·013
Choice of supper foods			
Cheese	80	2·5	0·288
Beef	120		
Choice of fruit			
Apples	300	1·2	0·070
Oranges	300		
	Total mean intake	40·7	2·045

Ratio ^{90}Sr/Ca = 19·9 pC/g

(*d*) Calculation of the 'observed ratio' OR = $\frac{2{\cdot}38 \text{ pC/g}}{19{\cdot}9 \text{ pC/g}} = 0{\cdot}120.$

were pooled to form a single 1000 ml sample of the milk of the group. Samples of each food eaten by the mothers in the last 4 days before milk sampling were also collected. The average daily intake of each group was estimated from the composition and quantity of food eaten by all in the ward. Precise quantities consumed individually could not be measured except on the basis of the total amount served on a ward; for food 'chosen' (e.g. the choice of an apple as against a pear) a weighted mean was computed.

In human milk and food, ^{90}Sr and ^{137}Cs were separated chemically by the standard

Table 2. *Dietary intake of radionuclides and their secretion in the milk of* 16 *nursing mothers*

(Samples taken at the Civic Hospital of Belluno on 23–26 May 1966. Expt 2.)

(*a*) Secretion in mother's milk

^{90}Sr = 1·3 pC/l	Ca = 0·316 g/l	$^{90}Sr/Ca$ = 4·11 pC/g
^{137}Cs = 115 pC/l	K = 0·674 g/l	$^{137}Cs/K$ = 171 pC/g

(*b*) Measurements on the diet (uncooked weight)

Food	^{90}Sr, pC/kg	Ca, g/kg	^{137}Cs, pC/kg	K, g/kg
Milk	28·2	1·178	69·5	1·500
Bread	24·6	0·214	27·0	0·805
Italian pasta	14·4	0·231	69·0	0·670
Beef	2·45	0·786	200·9	2·430
Cheese	132·3	7·592	*	0·252
Ham	6·6	0·303	20·2	2·405
Beans	66·8	1·258	100·30	12·462
Apples	2·7	0·054	32·3	0·780
Beer	*	0·044	*	0·454
Wine	8·0	0·104	13·0	0·848

* Not detected.

(*c*) Average diet (uncooked weight) and daily average intakes of ^{90}Sr, Ca, ^{137}Cs and K

Food	Quantity, g	^{90}Sr, pC	Ca, g	^{137}Cs, pC	K, g
Milk	400	11·3	0·471	27·8	0·600
Bread	200	4·9	0·042	5·4	0·160
Italian pasta	250	3·6	0·057	17·2	0·267
Beef	150	0·4	0·117	30·1	0·364
Choice of foods					
Cheese	80	5·5	0·314	0·7	0·094
Ham	70				
Beans	150	10·0	0·188	15·0	1·869
Apples	200	0·5	0·010	6·5	0·156
Beer	750	—	0·033	—	0·341
	Mean intake	36·2	1·232	102·7	3·851

Ratios Sr/Ca = 29·4 pC/g $^{137}Cs/K$ = 26·7 pC/g

(*d*) Calculation of the 'observed ratio'

$$\text{OR} \quad ^{90}Sr/Ca = \frac{4{\cdot}11}{29{\cdot}4} = 0{\cdot}1397$$

$$^{137}Cs/K = \frac{171}{26{\cdot}7} = 6{\cdot}404$$

ion exchange technique (Ferraris, 1963) of the laboratory of the Italian Nuclear Energy National Commission (CNEN). ^{90}Sr was measured by beta counting at equilibrium of its daughter ^{90}Y, separated as oxalate, in a low background G.M.

Table 3. *Dietary intake of radionuclides and their secretion in the milk of* 14 *nursing mothers*

Samples taken at the Civic Hospital of Dolo (Venice) on 8–11 May 1967. Expt 3

(*a*) Secretion in mother's milk

^{90}Sr = 0·33 pC/l	Ca = 0·330 g/l	^{90}Sr/Ca = 1·0 pC/g
^{137}Cs = 5·8 pC/l	K = 0·751 g/l	^{137}Cs/K = 7·7 pC/g

(*b*) Measurements on the diet (uncooked weight)

Food	^{90}Sr, pC/kg	Ca, g/kg	^{137}Cs, pC/kg	K, g/kg
Milk	6·8	1·102	17·3	1·596
Bread	7·0	0·264	10·1	1·158
Italian pasta	6·9	0·177	48·4	2·151
Rice	*	0·909	13·6	0·394
Beef	11·0	0·126	81·9	3·450
Chicken	1·6	0·297	*	2·556
Fish	2·9	0·178	11·0	3·400
Cheese	129·7	7·442	*	0·744
Ham	6·6	0·303	20·2	2·405
Spinach	84·1	0·970	*	3·338
Oranges	7·2	0·474	14·2	1·457
Apples	0·7	0·362	9·6	0·728

* Not detected.

(*c*) Average diet (uncooked weight) and daily average intakes of ^{90}Sr, Ca, ^{137}Cs and K

Food	Quantity, g	^{90}Sr, pC	Ca, g	^{137}Cs, pC	K, g
Milk	200	1·4	0·220	3·5	0·319
Bread	330	2·1	0·087	3·3	0·382
Choice of foods					
Italian pasta	160	0·5	0·041	4·3	0·184
Rice	60				
Choice of foods*					
Beef	120	0·8	0·025	4·1	0·397
Chicken	120				
Fish	150				
Choice of foods†					
Cheese	70	7·1	0·394	0·4	0·081
Ham	70				
Spinach	600	50·4	0·582	—	2·002
Fruit	300	1·2	0·125	3·6	0·328
	Mean intake	63·5	1·474	19·2	3·693

* Three days beef or chicken at choice and one day fish.
† Three days cheese and one day ham (evening meal).
Ratios ^{90}Sr/Ca = 43·08 ^{137}Cs/K = 5·19

(*d*) Calculation of the 'observed ratio'

$$\text{OR} \quad {}^{90}\text{Sr/Ca} = \frac{1}{43{\cdot}08} = 0{\cdot}023$$

$$^{137}\text{Cs/K} = \frac{7{\cdot}7}{5{\cdot}19} = 1{\cdot}48$$

Table 4. *Dietary intake of radionuclides and their secretion in the milk of 7 nursing mothers*

Samples taken at the Civic Hospital of Belluno on 12–15 July 1967. Expt 4

(*a*) Secretion in mother's milk

^{90}Sr = 0·3 pC/l	Ca = 0·262 g/l	$^{90}Sr/Ca$ = 1·14 pC/Ca
^{137}Cs = 19·8 pC/l	K = 0·674 g/l	$^{137}Cs/K$ = 29·37 pC/g

(*b*) Measurements on the diet (uncooked weight)

Food	^{90}Sr, pC/kg	Ca, g/kg	^{137}Cs, pC/kg	K, g/kg
Milk	20·5	1·167	27·4	1·496
Bread	8·4	0·199	17·1	1·183
Soup of Italian pasta	18·7	0·994	29·5	1·738
Beef	5·4	0·081	51·4	3·770
Fish	3·0	0·182	9·0	3·200
Cheese	86·8	6·439	5·3	1·076
Ham	6·6	0·994	29·5	1·738
Onion	17·6	0·447	*	1·300
Beetroot	84·8	1·426	*	1·455
Lettuce	44·6	0·736	*	3·096
Squash	23·0	0·232	*	3·801
Carrots	38·0	0·430	*	1·792
Fruit	4·2	0·087	7·6	2·640
Wine	8·0	0·104	13·0	0·848
Beer	*	0·034	1·8	0·397

* Not detected.

(*c*) Average diet (uncooked weight) and daily average intakes of ^{90}Sr, Ca, ^{137}Cs and K

Food	Quantity, g	^{90}Sr, pC	Ca, g	^{137}Cs, pC	K, g
Milk	250	5·1	0·291	6·85	0·374
Bread	300	2·5	0·060	5·13	0·355
Soup of Italian pasta	92	1·72	0·092	2·72	0·161
Beef*	135	0·85	0·016	6·37	0·509
Fish	160				
Choice of supper foods					
Ham	70	3·7	0·218	0·91	0·127
Cheese	80				
Choice of raw vegetables					
Lettuce	114	2·62	0·043	—	0·222
Carrots	40				
Onions	114				
Squash	83				
Beetroot	106	16·70	0·280	—	0·286
Fruit	183	0·77	0·016	1·39	0·483
Wine	200	1·60	0·021	2·60	0·169
Beer	750	—	0·025	1·35	0·298
	Mean intake	35·56	1·062	27·32	2·984

* Three days beef, one day fish

$$\text{Ratio } ^{90}Sr/Ca = \frac{35{\cdot}56}{1{\cdot}062} = 33{\cdot}48 \qquad ^{137}Cs/K = \frac{27{\cdot}32}{2{\cdot}984} = 9{\cdot}15$$

(*d*) Calculation of the 'observed ratio'

$$\text{OR} \quad ^{90}Sr/Ca\,\frac{1{\cdot}14}{33{\cdot}48} = 0{\cdot}034$$

$$^{137}Cs/K = \frac{29{\cdot}37}{9{\cdot}15} = 3{\cdot}209$$

flow counter. ^{137}Cs was measured by scintillation counting of caesium phosphomolybdate dissolved in ammonia (Yamagata & Yamagata, 1958) in a thallium-activated well-type sodium iodide crystal (50 × 45 mm) connected to a 100 channels analyser. Radioactive standards were supplied by the Radiochemical Centre, Amersham, Bucks, England. For food samples, counting times were prolonged until statistical errors were limited to ± 3 %. This was unfortunately not possible with the milk from the mothers; the extremely low content of radionuclides and the necessarily small samples led to an uncertainty in the measurements of about ± 20 %.

Ca and K were measured in the ash of all foods by the usual analytical methods, i.e. precipitation as oxalate and titration with 0·1 M $KMnO_4$ for Ca, and precipitation with a saturated solution of sodium tetraphenylborate and gravimetric estimation for K. Errors were estimated to be approximately ± 4 %.

RESULTS AND DISCUSSION

Four experiments were performed. The ^{90}Sr/Ca OR was determined in each experiment, but that of ^{137}Cs/K only in the last 3. The data obtained together with values derived from them are given in Tables 1–4.

Table 5. *Summary of results from Tables* 1–4

Expt no.	OR ^{90}Sr/Ca	OR ^{137}Cs/K
1	0·120	—
2	0·140	6·404
3	0·023	1·480
4	0·034	3·209

Table 6. *Observed ratios ^{90}Sr/Ca and ^{137}Cs/K based on food intake on the sampling day only*

Expt no.	OR ^{90}Sr/Ca	OR ^{137}Cs/K
1	0·121	—
2	0·141	5·650
3	0·025	1·801
4	0·032	3·210

The results are summarized in Table 5. Daily intakes were calculated as weighted means of the intakes during the 4 days up to and including that on which the milk samples were taken. Intakes on the sampling day only are given in Table 6. The OR based on the average intake over 4 days are very close to those based on intakes on the sampling day only, indicating that metabolic equilibrium had been almost, if not entirely, reached.

The ^{90}Sr/Ca OR were always less than unity and showed small variation, but the OR for ^{137}Cs/K were always greater than unity and varied much more widely. These conclusions are not affected by the errors of up to 20 % in estimating ^{90}Sr concentrations in the milk, since only the numerators of the ratios are affected, and they are very much smaller than the denominators.

In Table 7, quantities of ^{90}Sr, Ca, ^{137}Cs and K secreted in 1 l milk are expressed as percentages of the average daily intake. Variations in the percentages for ^{90}Sr and Ca

are small, amounting to a few units for ^{90}Sr and about 20 percentage units for Ca. The same is true for K, but its metabolic analogue ^{137}Cs showed great variations, from 40 to 115%. In passing, therefore, from the food to the milk, the behaviour of ^{137}Cs differs greatly from that of ^{90}Sr. The maternal organism appears not to discriminate between ^{137}Cs and K in milk secretion.

Table 7. *^{90}Sr, Ca, ^{137}Cs and K secreted in 1 l milk, expressed as percentages of the average dietary intake*

Expt no.	^{90}Sr	Ca	^{137}Cs	K
1	1·72	14·47	—	—
2	3·59	25·64	114·69	17·92
3	0·52	22·38	40·10	20·33
4	0·83	24·78	72·47	22·59

The observations of Wasserman, Comar & Twardock (1962) on goat's milk are similar; the average $^{137}Cs/K$ OR in this case was 1·7. Moreover Wasserman & Comar (1967) studied in detail the influence of dietary K on the retention of ^{137}Cs in the organs and soft tissues of the rat, and concluded that 'potassium does not effectively compete with caesium for membrane transport sites or intracellular binding sites'.

Our conclusions, therefore, are the following: (1) the maternal organism exercises very effective protection for the suckling baby against radioactive contamination from ^{90}Sr, since the percentage of dietary Sr which is excreted in the milk is very much smaller than the percentage of dietary Ca so excreted; (2) no such discrimination is exerted against ^{137}Cs, since the quantity of Cs associated with a given quantity of K in the milk may be 6 times as great as that associated with an equal amount of dietary K.

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Utilization of citrate by lactobacilli isolated from dairy products

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Summary. Twenty-five strains of lactobacilli isolated from dairy products, including *Lactobacillus casei*, *Lactobacillus plantarum* and *Lactobacillus brevis* species, were grown in semi-defined media and examined for their ability to ferment citrate and produce formate. Of 7 strains of *L. casei*, all utilized citrate to varying extents, as did 9 of 10 strains of *L. plantarum* and 3 of 8 strains of *L. brevis*. *L. casei* produced 19–35 % of the theoretical yield of formate from the citrate utilized, *L. plantarum* 0–11 % and *L. brevis* 0–28 %. Of 2 strains of *L. casei* tested for their abilities to ferment citrate in the presence of 2 % lactose, strain C 5 was unaffected by lactose whereas strain C 2 showed a 45 % decrease in the citrate utilized. However, lactose used at 2 % concentration greatly reduced formate production by *L. casei* C 5, which produced 62 % of the theoretical yield of formate from citrate after 35 days in the absence of lactose and only 8 % in its presence. The yield of diacetyl produced by *L. casei* C 5 from citrate plus lactose (both at 2 % concentration) was 145 times that produced from citrate alone, and 800 times that from lactose alone.

The lactic acid bacteria *Streptococcus diacetilactis* and leuconostoc species ferment citrate and characteristically produce diacetyl and acetoin. Fermentation of citrate is also a fairly general property of strains of *Lactobacillus casei* isolated from silage (Keddie, 1959), faeces (Gasser, 1964) and Dutch cheese (de Man, 1956). However, for this species the pathway of citrate breakdown has not been worked out although it is generally assumed to be the same as that for *Str. diacetilactis* and leuconostoc species because there are a number of reports of the production of diacetyl by lactobacilli (Christensen & Pederson, 1958; Keenan & Lindsay, 1968). In addition, evidence presented by Campbell & Gunsalus (1942) and Van den Hamer (1960) indicates that lactobacilli may utilize citrate in the absence of carbohydrate as an energy source, phosphoroclastic cleavage of pyruvate resulting in formic acid and energy-rich acetyl CoA.

The present paper describes an investigation of the ability of lactobacilli isolated from dairy sources to ferment citrate, and of the pathways involved.

Materials and methods

Organisms

The lactobacilli studied were from the collection of Dr M. E. Sharpe of the National Institute for Research in Dairying, Shinfield, Reading, and were as follows: *L. casei* C 2, C 5, C 6, C 9, A 121/C, A 148/C, B 142/C; *Lactobacillus plantarum* P 5, P 10, P 12,

P 16, A 164/C, AB 41/C, Cα 106/C, H 10/C, K 50/C, L 50/C; *Lactobacillus brevis* A 160/C, B 146/C, K 46/C, T 21/C, V 7/C, X 1, X 2, C 10.

Bacteriological methods

Culture media

The organisms were grown in a basal medium (BM) containing per litre: Oxoid peptone, 10 g; Oxoid Lemco, 10 g; Oxoid yeast extract, 5 g; Tween 80, 1 ml; K_2HPO_4, 2 g; and 5 ml of salts solution 'A', which contained 11·5 g $MgSO_4$, 7 H_2O and 2·28 g $MnSO_4$, 4 H_2O/100 ml. Additions to the BM were made as required. When 2% sodium citrate was added to BM, 5% (v/v) salts solution 'A' was also added to provide an excess of Mg^{2+} and Mn^{2+} ions. The pH values of all the test media were adjusted to 6·0.

All the cultures were incubated at 30 °C and cultured in MRS broth (de Man, Rogosa & Sharpe, 1960) before being subcultured into the test medium. The organisms were subcultured twice in 10 ml portions of the test medium and sufficient organisms were then added to 10 ml portions of test medium to give initial counts of about 50/ml. Tubes were gassed with carbon dioxide and stoppered with sterile rubber bungs using the method of Hungate (1950).

Chemical methods

Determination of citrate

The method of Marier & Boulet (1958) was used. To cold 1 ml samples of broth (diluted as necessary) were added 1·3 ml pyridine and 5·7 ml acetic anhydride. After thorough mixing, followed by incubation for 30 min in a constant temperature water bath at 32 °C, the solutions were centrifuged as necessary to clarify them, and their absorbance at 420 nm was determined with a Unicam SP 500 Spectrophotometer (Unicam Instruments Ltd, Arbury Works, Cambridge). The citric acid contents of the samples were estimated by reference to standards.

Determination of formate

The method of Wood & Gest (1957) was used, in which formate is reduced to formaldehyde which is then made to react with acetylacetone to produce a coloured compound.

Cold 0·5 ml samples of broth, diluted as necessary, were added to 80 mg coils of magnesium ribbon followed by the slow addition, with shaking, of 0·5 ml cold concentrated HCl. One min after the addition of the HCl, 3 ml cold N-NaOH were added to each tube and the contents shaken and centrifuged to sediment the precipitate of $Mg(OH)_2$. To 2 ml portions of the supernatant liquid were added 2 ml Nash's reagent B (which contains 2 ml redistilled acetylacetone, 3 ml glacial acetic acid, 150 g ammonium acetate and water to 1 l). After incubation for 45 min at 37 °C the absorbance at 412 nm was determined.

Determination of carbohydrate

The method of Dubois, Gilles, Hamilton, Rebers & Smith (1956) was used. To 2 ml of sample (diluted as necessary) were added 1 ml 5% phenol and 5 ml concentrated sulphuric acid. The mixture was allowed to stand for 10 min at room temperature. It was then well shaken and placed for 15 min in a water bath at 23–30 °C. The absorbance was determined at 490 nm.

Determination of diacetyl/acetoin

The method of Eggleton, Elsden & Gough (1943) was used. To each 5 ml sample were added 1 ml 0·5 % creatine and 1 ml freshly prepared α-naphthol solution, containing 1 g α-naphthol in 20 ml 2·5 N-NaOH. The tubes were shaken and after 1 h at room temperature the absorbance at 530 nm was determined.

RESULTS

Utilization of citrate and production of formate by lactobacilli

The 25 strains of lactobacilli were examined for their ability to ferment citrate and produce formate. The organisms were grown in BM containing 2 % sodium citrate to which 0·25 % lactose was added to initiate growth. They were also grown in BM containing 0·25 % lactose only. The inoculated tubes of media were gassed and incubated for 4–10 days.

Table 1. *Production of formate from citrate and lactose by lactobacilli grown in media with and without citrate*

		Basal medium plus 2 % sodium citrate and 0·25 % lactose			Basal medium plus 0·25 % lactose	
		Citrate utilized*, g/100 ml medium	Formate produced, mg/100 ml medium	Moles formate produced/ mole citrate utilized	Formate produced, mg/100 ml medium	Moles formate ($\times 10^{-2}$) produced/ mole lactose utilized
L. casei	C2	1·217	73·14	0·25	1·84	5·47
	C5†	1·217	89·25	0·31	16·1	47·9
	C6	1·217	56·13	0·19	1·84	5·47
	C9	1·218	64·86	0·22	2·3	6·84
	A148/C	1·218	69·92	0·24	3·22	9·58
	A121/C†	1·220	101·2	0·35	1·38	4·10
	B142/C†	1·218	74·52	0·26	2·3	6·84
L. plantarum	P5	1·211	31·74	0·11	1·38	4·10
	P10	0·91	0·92	0·004	1·38	4·10
	P12†	0	0	0	0	0
	P16	0·95	3·35	0·059	4·14	12·3
	A164/C	1·208	26·68	0·092	ND	ND
	AB41/C	0·63	1·841	0·012	5·06	15·1
	Cα106/C	0·94	0	0	ND	ND
	H10/C	1·147	23·92	0·087	5·06	15·1
	K50/C	1·143	15·64	0·057	ND	ND
	L50/C	1·020	19·78	0·081	4·6	13·7
L. brevis	A160/C†	0	0	0	ND	ND
	B146/C	0·87	13·80	0·066	1·84	5·47
	K46/C†	1·213	ND	ND	ND	ND
	T21/C†	0	0	0	ND	ND
	V7/C†	0	0	0	ND	ND
	X1†	0	0	0	ND	ND
	X2†	0	0	0	ND	ND
	C10†	1·218	80·5	0·276	0·92	2·74

* Uninoculated medium contained 1·22 g citrate/100 ml.
† Cultures examined after 10 days, remainder examined after 4 days.
ND, Not determined.

Of 7 strains of *L. casei*, all utilized citrate to varying extents, as did 9 of 10 strains of *L. plantarum* and 3 of 8 strains of *L. brevis* (Table 1). Formate was produced by 17 of the 19 citrate fermenting strains. Theoretically, 1 mole of citrate could give rise to 1 mole of formate. On this basis the amounts of formate formed by the different strains expressed as percentages of the theoretical yield were: *L. casei*, 19–35 %; *L. plantarum*, 0–11 %; *L. brevis*, 0–28 %. Of the organisms tested, all those which produced formate from citrate also produced small quantities from lactose. Assuming complete utilization of the lactose and a theoretical yield of 4 moles of formate for each mole of lactose utilized, the amounts of formate formed by the different strains expressed as percentages of the theoretical yield were: *L. casei*, 1–12 %; *L. plantarum*, 0–4 %; *L. brevis*, 0·7–1·4 %.

Table 2. *Effect of lactose upon the utilization of citrate by* Lactobacillus casei *C* 2 *and* L. casei *C* 5

	Basal medium* plus	Citrate utilized, g/100 ml medium	Citrate utilized, %
L. casei C 2	2 % sodium citrate	0·562	98·6
C 5		0·565	99·1
L. casei C 2	2 % sodium citrate and 0·25 % lactose	0·565	99·1
C 5		0·554	97·2
L. casei C 2	2 % sodium citrate and 2 % lactose	0·35	54·7
C 5		0·634	99·1

* Media contained 0·57, 0·57 and 0·64 g citrate/100 ml medium, respectively.

Table 3. *Utilization of citrate and lactose by* Lactobacillus casei *C* 5

	Citrate* g substrate utilized/100 ml medium		Lactose† g substrate utilized/100 ml medium	
Days	6	35	6	35
Basal medium plus				
2 % sodium citrate	1·2	1·2	—	—
2 % sodium citrate and 2 % lactose	0·967	0·97	2·12	2·18
2 % lactose	—	—	< 1·65	1·67

* Media contained 1·2, 0·97, 0 g citrate/100 ml medium, respectively.
† Media contained 0, 2·25, 2·02 g lactose/100 ml medium, respectively.
— No substrate added.

Influence of lactose on the utilization of citrate by L. casei *C* 2 *and* L. casei *C* 5

Both *L. casei* C 2 and C 5 utilized > 98 % of the citrate in 7 days when grown in the absence of lactose or in the presence of 0·25 % lactose (Table 2). In the presence of 2 % lactose, however, *L. casei* C 5 utilized > 99 % of the citrate in 7 days whereas *L. casei* C 2 utilized only about 55 % in this time.

Influence of lactose on the metabolism of citrate by L. casei *C* 5

L. casei C 5 was grown in nutrient broth to which had been added 2 % sodium citrate, 2 % lactose, 2 % sodium pyruvate, and 2 % sodium citrate plus 2 % lactose. Table 3 shows the utilization of citrate and lactose after 6 days and 5 weeks and Tables 4 and 5 show the production of formate and diacetyl/acetoin.

L. casei C 5 utilized all the citrate within 6 days whether or not lactose was present. Between 82 and 97 % of the lactose was utilized within 6 days and prolonged incubation did not result in any further utilization. Large amounts of formate were produced from citrate alone and significant, although much smaller, quantities from lactose and citrate + lactose. A significant quantity of formate was also produced from sodium pyruvate after 35 days incubation. Table 5 shows that for *L. casei* C 5 62 % of the citrate utilized was converted to formate.

The presence of lactose also considerably influenced the production of diacetyl/acetoin by *L. casei* C 5 (Table 4). The yield of diacetyl produced from citrate + lactose (both at 2 % concentration) was 145 times greater than that produced from citrate alone, and 800 times greater than that from lactose.

Table 4. *Production of formate and diacetyl/acetoin from citrate and lactose by* Lactobacillus casei *C* 5

	mg produced/100 ml medium:		
	Formate		Diacetyl/acetoin
Days	6	35	35
Basal medium plus:			
2 % sodium citrate	6	25	35
2 % sodium citrate and 2 % lactose	7·82	18·87	80
2 % lactose	12·43	10·12	0·1
2 % sodium pyruvate	0	42·79	ND

ND, not determined.

Table 5. *Number of moles formate and diacetyl/acetoin produced by* Lactobacillus casei *C* 5*/mole citrate utilized*

	No. moles/mole citrate utilized		
	Formate		Diacetyl/acetoin
Days	6	35	35
Basal medium plus:			
2 % sodium citrate	0·40	0·62	0·00102
2 % sodium citrate and 2 % lactose	0·03	0·08	ND
2 % lactose	—	—	0·00022

— No substrate added.
ND, not determined.

DISCUSSION

Lactic acid is the principal end product in the fermentation of carbohydrate and citrate by lactic acid bacteria, and pyruvate is an intermediate product in both pathways. However, whereas fermentation of glucose via the Embden–Meyerhof pathway results in the production of sufficient NADH to reduce pyruvate to lactate, the fermentation of citrate results in the production of pyruvate without the simultaneous supply of reduced NAD so that products other than lactic acid are formed (Harvey & Collins, 1961). As yet the NAD-independent dehydrogenases have not been shown to produce lactate from pyruvate.

Of the 25 strains of lactobacilli examined, 19 utilized 50–100 % of the available citrate within 10 days. All strains of *L. casei* examined consistently utilized all the citrate present. Of the citrate-utilizing strains of lactobacilli 17 produced formate; *L. casei* produced between 19 and 35 % of the theoretical yield from the citrate utilized, *L. plantarum* between 0 and 11 % and *L. brevis* between 0 and 28 %. Small quantities of formate were also produced from lactose by those organisms which produced formate from citrate.

The presence of fermentable carbohydrate may influence the extent of citrate utilization by lactobacilli (Gibson & Abdel-Malek, 1945; Gasser, 1964). In the present work *L. casei* C 5 utilized all the citrate within 7 days regardless of the presence of up to 2 % lactose whereas *L. casei* C 2 utilized only 55 % of the citrate when 2 % lactose was present. Gasser (1964) found 3 strains of *L. plantarum* which also showed reduced ability to ferment citrate in the presence of fermentable sugar.

Besides influencing the extent of citrate fermentation, the presence of fermentable carbohydrate also influenced the products formed. *L. casei* C 5 produced 40 and 62 % of the theoretical yields of formate after 6 and 35 days, respectively, from the citrate utilized when citrate was the only fermentable material, whereas when citrate and lactose were supplied together both at 2 % concentrations only 3 and 8 % of the theoretical yields were produced, assuming that the formate was produced from citrate. In both cases all the citrate was utilized within 6 days. Since pyruvate is the only common intermediate in the breakdown of citrate and of lactose it appears that the source of the pyruvate is the factor which governs the extent to which the formate pathway is utilized. When an abundant supply of fermentable carbohydrate is present energy will be available to the organisms before pyruvate is produced, and thus there will be no necessity for the pyruvate-formate pathway to be invoked. However, the production of pyruvate from citrate is not accompanied by a release of energy and thus in the absence of a carbohydrate source the pyruvate-formate system may become important. The evidence suggests (Tables 3 and 4) that the pyruvate-formate step is a slow reaction since, although citrate was utilized very quickly by *L. casei* C 5, formate was produced more slowly and continued to be produced for some considerable time after the cessation of citrate breakdown. Addition of sodium pyruvate to BM also resulted in formate production only after a lag of several days. It is concluded, therefore, that the mechanism for the pyruvate-formate step, although present in most lactobacilli, is a slow reaction and is only invoked under conditions of carbohydrate starvation, such as those found in cheese.

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Utilization of milk citrate by lactic acid bacteria and 'blowing' of film-wrapped cheese

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Summary. A study was made of the utilization of citrate in milk by some lactic acid bacteria. When *Streptococcus diacetilactis* 1007 was grown alone or with either *Streptococcus cremoris* 924 or *Lactobacillus casei* B 142/C or with both these latter organisms, > 99% of the milk citrate was utilized within 5 days. *L. casei* B 142/C and *L. casei*/*Str. cremoris* utilized 57 and 14% of the citrate, respectively. When *L. casei* C 2 and *L. casei* C 5 were grown in milk in which *Str. cremoris* 924 had been previously grown, 94 and 64%, respectively, of the citrate was utilized after 7 days at 30 °C.

Cheeses were made using a citrate-fermenting and a non-citrate-fermenting starter and citrate concentrations of the milks, wheys and curds were determined during cheese-making. With *Str. cremoris* 924, citrate was preferentially retained in the curd at pressing, the concentration in the curd moisture being 2·9 times that in the whey. With the mixed starter *Str. cremoris* 924/*Str. diacetilactis* 1007, the curd at pressing and from the press contained only 27 and 5%, respectively, of the citrate present in the *Str. cremoris* curd at these times.

Cheeses were made using *Str. cremoris* 924, combinations of *Str. cremoris*/*Str. diacetilactis* 1007, or with δ-gluconic acid lactone instead of starter, with and without the addition of *L. casei* C 5, in order to examine the ability of the latter organism to produce 'blowing' in the sense of distension of the Cryovac wrapping of film-wrapped cheeses. *L. casei* C 5 neither accelerated the decrease in cheese citrate nor produced blowing of the film-wrapping. Possible reasons for this behaviour are discussed.

Introduction

Following reports (unpublished) that film-wrapped territorial cheese blocks, stored at 21 °C for 31 weeks before being cut into portions and rewrapped, showed considerable distension ('blowing') of the wrapping, blown and 'unblown' cheeses were examined bacteriologically to identify the causative organisms. The non-starter floras of the cheeses were practically identical, and it was concluded that the problem had probably arisen due to the use of the heterofermentative starter organism *Streptococcus diacetilactis*, which is known to produce gas defects in cheese (Vedamuthu, Sandine & Elliker, 1964; Stadhouders, Galesloot, Hassing & Waals, 1968). In a subsequent commercial cheese trial, in which 34 cheeses were made with either single-strain starter *Streptococcus cremoris* 924 or a mixture of *Str. cremoris* 924 and *Str. diacetilactis* 1007 and carefully sealed in film, only the cheese made with the

single-strain starter showed gas formation. From results obtained for these cheeses, and in the light of the findings of Fryer (1970) that the *Lactobacillus casei-plantarum* group of organisms isolated from dairy products was able to utilize citrate, it was concluded that the blowing of the film-wrapped cheeses might have been produced by lactobacilli. Milk contains about 0·2 % (w/v) citrate, mostly as soluble calcium citrate, which if not fermented during cheese-making is present in the curd at about 0·2 % (w/w). In addition to producing carbon dioxide from citrate, lactobacilli would also produce flavourful substances such as acetic acid and diacetyl.

Investigations were therefore carried out to determine the ability of strains of lactobacilli to utilize citrate when grown in milk in association with starter organisms. The utilization of citrate and production of acetate by *Str. diacetilactis* and *Str. cremoris* during cheese-making, and the metabolism of citrate in cheeses by a strain of *L. casei*, with special reference to the production of gas, were also investigated.

METHODS

Organisms

The starters employed in this study were *Str. diacetilactis* NCDO 1007 and *Str. cremoris* NCDO 924. The strains of *L. casei*, C 2, C 5 and B 142/C were obtained from the collection of Dr M. E. Sharpe.

Utilization of citrate by lactic acid bacteria grown in association in milk

Production of carbon dioxide by a non-lactose fermenting lactobacillus

L. casei B 142/C, *Str. cremoris* 924 and *Str. diacetilactis* 1007 were grown alone and in all the 4 possible combinations with each other in separated milk. After 5 days the cultures were examined for CO_2, free fatty acids (FFA) and citrate. Uninoculated milk was also examined as a control.

Utilization of citrate in milk by lactobacilli after the growth of starter

Str. cremoris 924 was grown in 10 ml quantities of separated milk for 5 days at 30 °C, and the cultures were then inoculated with about 500 organisms of *L. casei* C 2 and *L. casei* C 5. The tubes were gassed with CO_2 after inoculation with lactobacilli and stoppered with rubber bungs. After a further 7 days of incubation with daily shaking, the milks were examined for citrate content, final pH and numbers of lactobacilli.

Utilization of citrate by Str. diacetilactis *and* Str. cremoris *during cheese-making*

Cheeses were made using *Str. cremoris* 924 alone and together with an equal proportion of *Str. diacetilactis* 1007. Samples of milk, whey and curd were taken during the cheese-making and analysed for citrate, acetate, moisture and numbers of starter organisms. The results for citrate and acetate were expressed as μg/g water and provided an indication of whether citrate and acetate were retained within the curd, assuming that at any point in the cheese-making their concentrations would be the same in the water phases of both whey and curd.

Utilization of citrate by L. casei *C* 5 *in film-wrapped cheeses*

Cheeses were made on 10 occasions from 80 gal of milk that had been heated at 71·7 °C for 17 s. From each batch of cheese curd two 40-lb rectangular cheeses were produced measuring 6 in. × $10\frac{3}{4}$ in. × $13\frac{1}{2}$ in. One was waxed and the other wrapped in airtight Cryovac film. The cheeses were matured for 2 months at 12·7 °C and for a further 4 months at 18·3 °C. Bacteriological and chemical analyses were performed on the curd after salting and on the waxed cheeses after 1, 3 and 6 months and the Cryovac wrapped cheeses were observed for blowing.

Microflora of cheese

Starter

Cheeses were made using the single-strain starter *Str. cremoris* 924 and the mixed starter *Str. cremoris* 924/*Str. diacetilactis* 1007 used in the proportions 50:50, 90:10, 99:1. All starter cultures were used at the rate of 12 pints/100 gal milk. On 2 occasions cheese was made aseptically by the method of Mabbitt, Chapman & Berridge (1955), using δ-gluconic acid lactone (GAL) instead of starter. Where it was necessary to prevent bacterial growth in these two GAL cheeses, Streptopen (Glaxo, Ltd, England) and Nisin (Aplin & Barrett, Yeovil, England) were added to give concentrations/ml milk of 100 units Nisin, 1·2 units procaine penicillin G and 1·2 μg dihydrostreptomycin sulphate. Sorbic acid was added at the rate of 0·05 % (w/v) to prevent the growth of moulds.

L. casei *C* 5

A cheese was made with and without the addition of *L. casei* C 5 for each starter used and from δ-gluconic acid lactone. Sufficient numbers of actively growing cells from an 18 h MRS broth culture (de Man, Rogosa & Sharpe, 1960) were added to cheese-milk to give a count of about 10^5/g curd. No antibiotics were added to the GAL cheese with *L. casei* C 5.

BACTERIOLOGICAL ANALYSES

Enumeration of the cheese microflora

The curd and cheeses were sampled and homogenized using the method of Naylor & Sharpe (1958). The citrate agar of Nickels & Leesment (1964) was used to enumerate the starter organisms and to differentiate citrate fermenting (*Str. diacetilactis*) and non-citrate fermenting (*Str. cremoris*) species. The plates were incubated for 4 days at 25 °C. Lactobacilli were enumerated on the trypticase acetate agar (TAcA) of Rogosa, Mitchell & Wiseman (1951).

CHEMICAL ANALYSES

Determination of CO_2

Two 100-ml conical filter flasks were joined by a short length of autoclavable polythene tubing connecting the 2 side arms and sterilized at 121 °C for 15 min. Where determinations of FFA were also required, Quickfit flasks and stoppers were used; otherwise the flasks were stoppered with rubber bungs.

Milk cultures

To one flask 100 ml of sterile separated milk were added using a sterile pipette and to the other flask 60 ml of sterile 0·1 N-NaOH. The milk was inoculated to give about 50 organisms/ml and the apparatus incubated at 30 °C with daily shaking. After the required incubation period 25 ml quantities of the NaOH solution were titrated with 0·1 N-HCl, before and after treatment with a slight excess of 10% $BaCl_2$ solution (2 ml) to precipitate the carbonate. The volume of CO_2 (NTP) produced by the culture was calculated (1 ml CO_2 at NTP weighs 1·96 mg). Control uninoculated flasks were also examined.

Cheese

A screw-clip was placed around the tubing joining the flasks, each of which contained a 1-in. plastic-coated magnet. With the screw-clip closed, 50 ml 0·5 N-NaOH were added to one flask and the apparatus weighed. A cheese plug $1\frac{1}{2}$–2 in. long was taken from 2–4 in. below the top surface of the cheese, quickly transferred into the flask containing the NaOH and the apparatus reweighed. The cheese was agitated for 2 h at 37 °C with a magnetic stirrer, the flask cooled in iced water for 15 min and 20 ml 0·1 N-NaOH added to the empty flask. To the cheese homogenate, stirred quickly on a magnetic stirrer at room temperature, 5 ml ice-cold 10 N-H_2SO_4 were quickly added using a Propipette (Hospital and Laboratory Supplies Ltd, London). The stopper was then replaced and the homogenate allowed to mix for 1 min. The screw-clip was released and both flasks stirred for 20 h at 37 °C, after which the polythene tubing was cut and the NaOH treated with 2 ml 1·5 N-$BaCl_2$ to precipitate the carbonate as barium carbonate, and titrated with 0·1 N-HCl to the phenolphthalein endpoint. The values obtained were subtracted from that obtained after titrating 0·1 N-NaOH, that had previously been treated with $BaCl_2$, with standard 0·1 N-HCl. The volume of CO_2 at NTP/g cheese was calculated. The method was tested by adding 1 ml of a standard solution of Na_2CO_3 to cheese homogenate after complete removal of CO_2 (20 h) and determining the recovery of CO_2 after a further 20 h using fresh 0·1 N-NaOH. Complete recovery was obtained.

Determination of FFA

The silicic acid column method of Harper, Schwartz & El-Hagarawy (1956) was used for determining C_2, C_3, C_4 and $> C_4$ FFA. Samples of separated milk cultures and wheys were adjusted to neutrality, shell-frozen and freeze-dried before analysis. The quantity of fatty acid in mg/g was obtained using a weighed amount of the freeze-dried sample, a correction for residual moisture being made. Results expressed in μmoles/ml were obtained by freeze-drying measured amounts of milk and whey, thus eliminating any need for determining moisture content and adding a correction factor. In order that the FFA results for milk, curd and cheese should be comparable, freeze-dried milk equivalent to 50 ml fresh milk, 5·4 g of curd and 5·0 g of cheese were used for analysis.

Determination of citrate, carbohydrate and diacetyl/acetoin in milk, whey, curd and cheese

Reference to the methods used is given by Fryer (1969).

Preparation of milk, whey, curd and cheese for chemical analysis

Determination of citrate and carbohydrate

Suitable dilutions of milk and whey were made and examined.

To 1 g grated cheese or curd in a 150 × 25 mm test tube were added 9 ml distilled water at 50 °C and the mixture boiled for 15 min, with thorough mixing every min by means of a Whirlimixer (Scientific Industries International Inc. Ltd, London). The resulting homogenate was placed in iced water until an upper fat layer formed and solidified, and the middle liquid layer was then removed with a Propipette and centrifuged for 10 min. The middle layer was again removed and filtered through a Whatman No. 1 filter paper and examined for citrate and lactose. Water samples passed through filter paper were used as controls.

Determination of diacetyl/acetoin

To 9 g grated cheese or curd were added 90 ml iced cool water. The mixture was homogenized in an MSE homogenizer (Measuring & Scientific Equipment, Ltd, London), slowly for 15 min and then at top speed for 2 min, a jacket of iced water being used to keep the mixture cold. The foam so produced was allowed to settle and the middle liquid layer removed with a Propipette and treated with 0·3 ml N-HCl/15 ml sample to precipitate the casein. The liquid was centrifuged for 5 min and the supernatant liquid removed and centrifuged again if necessary. Portions of the supernatant liquid (5 ml) were added to each of 2 tubes and the diacetyl concentration was determined. To one tube, the control, the addition of creatine and α-naphthol were omitted and only 1 ml 2·5 N-NaOH was added.

RESULTS

Utilization of citrate in milk by a non-lactose fermenting lactobacillus grown in association with starter organisms

Table 1 shows the citrate utilized and carbon dioxide and acetate produced by *Str. cremoris* 924, *Str. diacetilactis* 1007 and the non-lactose fermenting strain *L. casei* B 142/C when grown separately and together. Table 1 also shows the molar ratios found for citrate, carbon dioxide and acetic acid assuming that all the carbon dioxide and acetate were derived from citrate.

All combinations containing *Str. diacetilactis* 1007 utilized more than 99 % of the citrate, each mole of citrate yielding between 1·15–1·26 moles of CO_2. Except in the combination *Str. diacetilactis*/*Str. cremoris* when 0·68 moles of acetic acid were produced/mole of citrate utilized, combinations containing *Str. diacetilactis* 1007 produced 1·49 moles. *L. casei* B 142/C, *L. casei* B 142/C/*Str. cremoris* 924 and *Str. cremoris* utilized 57 %, 14 % and 4 % (not significant) of the citrate, respectively. When grown alone, *L. casei* produced 0·78 moles CO_2/mole of citrate utilized, whereas for *L. casei* grown in combination with *Str. cremoris* 924 a negative value was obtained,

the CO_2 dissolved in the milk being utilized. *L. casei* B 142/C and *L. casei* B 142/C/*Str. cremoris* produced 2 moles of acetic acid/mole of citrate utilized, but only a very small quantity was produced by *Str. cremoris* when grown alone.

Table 1. *Production of carbon dioxide and acetate from milk citrate by lactic acid bacteria grown for 5 days at 30 °C separately and in combination*

mg utilized or produced/g dry weight separated milk

	Organism*						
	B142/C	B142/C +924	B142/C +1007	B142 C + 924 +1007	924	1007	1007 + 924
Citrate†	11·2	2·8	19·8	19·8	0·8	19·8	19·8
CO_2	2·0	−0·067	5·2	5·7	−0·15	5·7	5·7
CO_2/citrate‡	(0·78)	(.)	(1·15)	(1·26)	(.)	(1·26)	(1·26)
Acetic acid	6·9	1·5	9·2	8·6	0·64	9·2	4·2
Acetic acid/citrate‡	(1·97)	(1·72)	(1·49)	(1·39)	(2·5)	(1·49)	(0·68)

* B142/C, *Lactobacillus casei*; 924, *Streptococcus cremoris*; 1007, *Str. diacetilactis*.
† Uninoculated milk contained 19·8 mg citrate/g dry weight separated milk.
‡ Expressed as moles CO_2 and acetic acid produced/mole of citrate utilized.

Table 2. *Utilization of milk citrate by* Lactobacillus casei *C 2 and C 5 after the growth of the starter*

Organism*	Citrate, mg/100 ml medium†	Utilized, %	Final pH
Streptococcus cremoris 924	1220	0	5·0
Str. cremoris 924/*L. casei* C2	70	94·26	3·95
Str. cremoris 924/*L. casei* C5	440	63·93	4·0

* *Str. cremoris* 924 was grown for 5 days at 30 °C in tubes of separated milk before being inoculated with *L. casei* C2 and C5, gassed with CO_2 and stoppered with rubber bungs. Incubation was continued for a further 7 days.
† Separated milk contained 1220 mg citrate/100 ml medium.

Utilization of citrate by L. casei *C 2 and* L. casei *C 5 after growth of starter*

Table 2 shows the utilization of milk citrate by *L. casei* C 2 and C 5 inoculated into separated milk following 5 days growth of *Str. cremoris* 924. After a further 7 days (12 days in all) *L. casei* C 2 and *L. casei* C 5 had utilized 94 and 64 % of the citrate, respectively. The pH value of the milks containing *Str. cremoris* 924 was 5·0 at 5 and 12 days whereas that of both milks containing the lactobacilli was 4·0 at 12 days. Both strains of lactobacilli reached counts of 5×10^8/ml.

Utilization of citrate during cheese-making

Fig. 1 shows the counts of *Str. cremoris* and *Str. diacetilactis* during cheese-making. When used together *Str. diacetilactis* grew much better than *Str. cremoris*, reaching 2×10^9/g curd as against 5×10^8/g. Used alone *Str. cremoris* reached 6×10^8/g curd.

Fig. 2 shows that when the non-citrate-fermenting *Str. cremoris* 924 was used, citrate and acetate were preferentially retained in the curd at pressing, 2·9 and 4·7 times more citrate and acetate, respectively, being present/g water in the curd than

in the whey. Using the mixed starter *Str. cremoris*/*Str. diacetilactis* the citrate in the whey and curd decreased rapidly from the beginning of cheddaring, the curd at pressing containing only 27 % of the amount of citrate present in the *Str. cremoris* curd. For both starters the acetate concentrations in the wheys and curds increased after cutting, the increase in the whey being much greater for the mixed starter than for *Str. cremoris* alone. The levels of acetate reached in the curds at pressing for both starters were very similar although after pressing a much greater increase was produced by the mixed starter than by *Str. cremoris* alone.

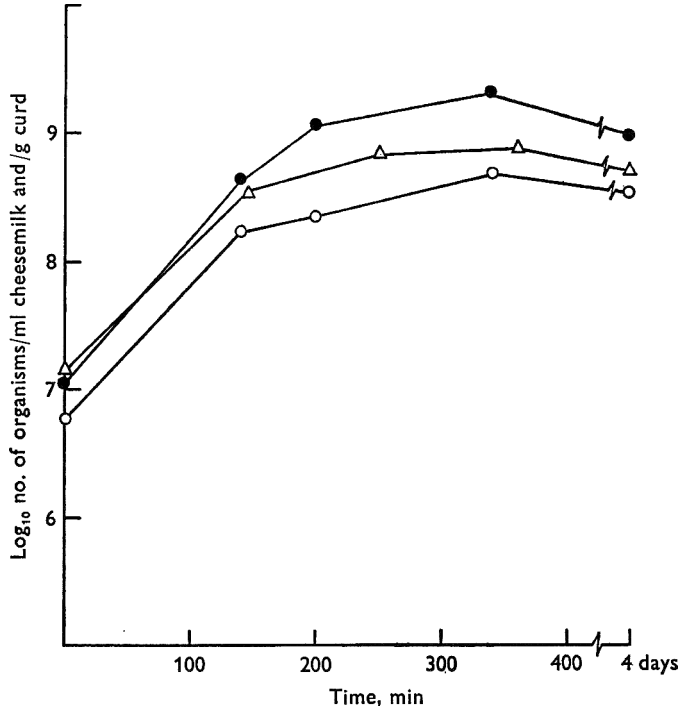

Fig. 1. Growth of starter organisms during cheese-making. ○—○, ●—●, Growth of *Streptococcus cremoris* 924 and *Str. diacetilactis* 1007, respectively, used together; △—△, growth of *Str. cremoris* 924 used as single-strain starter.

Table 3. *Levels of carbon dioxide (ml/g) in waxed cheeses during maturation*

Time, months	0	1	3	6
Starter*				
S/924†	—	0·26	0·24	0·46
S/924 + *Lactobacillus casei* C5	0·23	0·25	0·16	0·38
S/924 + 1007 (50/50)†	0·14	0·49	0·46	0·56
S/924 + 1007 (50/50) + *L. casei* C5	0·17	0·49	0·44	0·23
S/924 + 1007 (90/10)†	0·19	0·62	0·42	0·48
S/924 + 1007 (90/10) + *L. casei* C5†	0·27	0·53	0·42	0·63
S/924 + 1007 (99/1)†	0·15	0·57	0·43	0·65
S/924 + 1007 (99/1) + *L. casei* C5	—	0·52	0·43	0·52
GAL	0·20	0·23	0·35	0·29
GAL + *L. casei* C5	0·37	0·62	0·85	0·46

* S, Single-strain starter. † Cheeses developed blowing.

Bacteriological analyses of cheese

Non-starter lactic acid bacteria reached counts of about 2×10^7/g in the 'starter-only' cheeses and about 5×10^7/g in the cheeses containing *L. casei* C 5. *Str. diacetilactis* maintained higher numbers during 6 months than did *Str. cremoris* regardless of the proportions of *Str. diacetilactis*/*Str. cremoris* used in the starter.

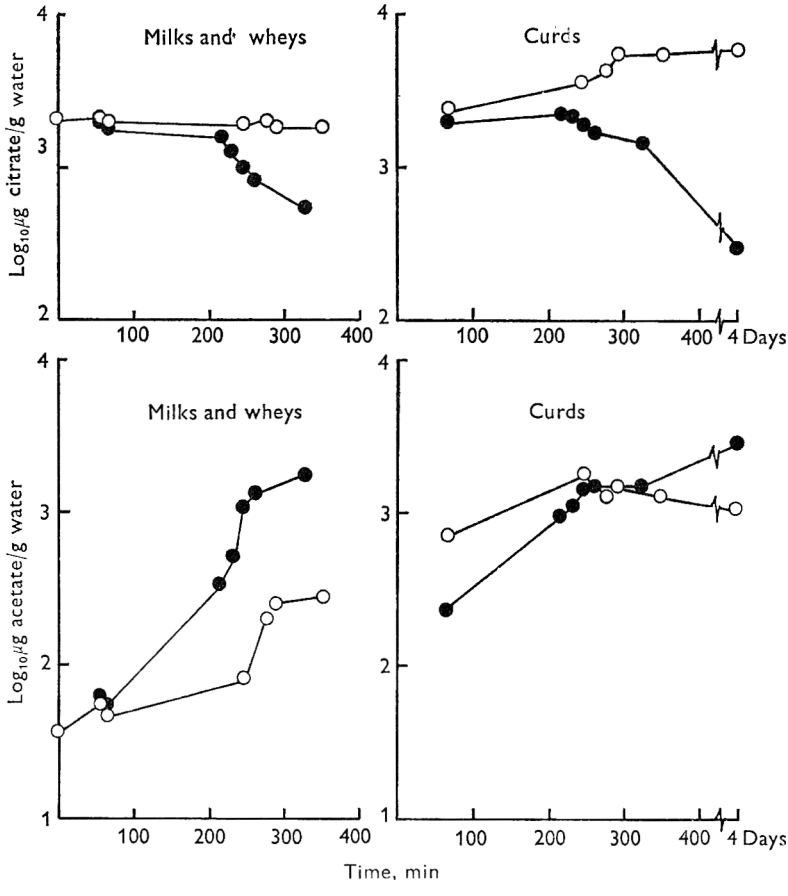

Fig. 2. Concentrations of citrate and acetate (expressed as µg/g water) in the aqueous phase of milks, wheys and curds during cheese-making with *Streptococcus cremoris* 924 (○—○) and *Str. cremoris* 924/*Str. diacetilactis* 1007 (●—●). Top row: concentration of citrate in milks and wheys (left) and in curds (right). Bottom row: concentration of acetate in milks and wheys (left) and in curds (right).

Blowing

Gas production, as indicated by blowing, was not evident in any of the cheeses at 2 months. After 4 months at 18·3 °C all the starter-only cheeses were blown whereas of those cheeses containing *L. casei*, only that made with *Str. cremoris*:*Str. diacetilactis* (90:10) was blown.

Carbon dioxide

Table 3 shows the content of CO_2 in the cheeses during maturation. At 6 months blown starter-only cheeses contained higher levels of CO_2 than their unblown counterparts containing *L. casei* in addition to the starter, whereas in the blown starter cheese containing *L. casei* a higher level was present than in the blown starter-only counterpart. Between 0·44 and 0·66 ml/g were recorded in the blown cheeses at 6 months and 0·23 and 0·52 ml/g in the unblown cheeses. Levels in the curd were between 0·13 and 0·27 ml/g. The greatest fluctuations were produced in the GAL cheese containing *L. casei* (0·85 ml/g at 3 months, 0·46 ml/g at 6 months) and the least in the GAL-only cheese (0·35 ml/g at 3 months, 0·29 ml/g at 6 months).

Table 4. *Citrate concentrations* (%) *in waxed cheeses during maturation*

Time, months	0	1	3	6
Starter				
S/924	0·2	0·24	0·24	0·1
S/924 + *Lactobacillus casei* C5	0·17	0·19	0·14	0·13
S/924 + 1007 (50/50)	0·02	0·03	0·003	0
S/924 + 1007 (50/50) + *L. casei* C5	0·02	0	0	0
S/924 + 1007 (90/10)	0·14	0·05	0	0
S/924 + 1007 (90/10) + *L. casei* C5	0·11	0·006	0	0
S/924 + 1007 (99/1)	0·18	0·10	0	0
S/924 + 1007 (99/1) + *L. casei* C5	0·15	0·06	0	0
GAL*	0·14	0·23	0·14	0·13
GAL + *L. casei* C5	0·14	0·23	0·11	0·04

* δ-Gluconic acid lactone.

Table 5. *Acetic acid in waxed cheese* (*μmoles*/5 *g*) *during maturation*

Time, months	0	3	6	Increase
Starter				
S/924	25·8	35·7	89·5	62·7
S/924 + *Lactobacillus casei* C5	20·8	38·0	39·4	18·6
S/924 + 1007 (50/50)	100·8	95·6	77·0	22·2
S/924 + 1007 (50/50) + *L. casei* C5	90·4	76·2	97·1	6·7
S/924 + 1007 (90/10)	53·9	93·6	149·6	95·7
S/924 + 1007 (90/10) + *L. casei* C5	70·7	113·5	215·8	145·1
S/924 + 1007 (99/1)	20·7	80·9	97·7	77·0
S/924 + 1007 (99/1) + *L. casei* C5	26·0	61·8	118·0	92·0
GAL	9·7	10·6	10·6	0·9
GAL + *L. casei* C5	10·0	227·7	307·9	297·9

Citrate

Table 4 shows the citrate concentrations in the cheeses during maturation.

With increased proportion of *Str. diacetilactis* in the starter there was a decreased concentration of citrate in the curd. At 3 months there was no citrate in any of the cheeses containing *Str. diacetilactis*. The pressure of *L. casei* in *Str. cremoris*/*diacetilactis* cheeses resulted in slightly lower concentrations of citrate in the curd and the cheese at 1 month. In the cheese made with *Str. cremoris* only, the citrate concentration was constant at 0·24 % up to 3 months and dropped to 0·1 % by 6 months, whereas the citrate concentration in the *Str. cremoris*/*L. casei* cheese fell from 0·17% in the curd to 0·13 % at 6 months.

Acetic acid

Table 5 shows the acetic acid concentration in the cheeses during maturation.

During 6 months maturation the GAL-only cheese had a constant level of C_2 acid whereas the GAL cheese containing *L. casei* showed a very large increase (298 μmoles/5 g). Levels in the curd decreased as the proportion of *Str. cremoris*/*Str. diacetilactis* increased (about 100 μmoles/5 g for the proportion 50:50; about 20 μmoles/5 g for 99:1). Increase of up to 145 μmoles over 6 months was found using the starter containing 90 % *Str. cremoris* + 10 % *Str. diacetilactis*. When used alone *Str. cremoris* produced an increase of 63 μmoles/5 g whereas when used together with *L. casei* only 19 μmoles/5 g were produced.

Carbohydrate

Using the phenol-sulphuric acid method of Dubois, Gilles, Hamilton, Rebers & Smith (1956), concentrations of about 0·1 % were found in all the starter cheeses at 6 months (Table 6).

Table 6. *Carbohydrate concentration* (%) *in waxed cheeses during maturation*

Time, months	0	1	3	6
Starter				
S/924	0·31	0·22	0·20	0·11
S/924 + *Lactobacillus casei* C5	0·63	0·14	0·11	0·10
S/924 + 1007 (50/50)	1·25	0·78	0·16	0·11
S/924 + 1007 (50/50) + *L. casei* C5	0·75	0·13	.	0·10
S/924 + 1007 (90/10)	0·39	0·1	0·13	0·12
S/924 + 1007 (90/10) + *L. casei* C5	1·03	0·15	0·11	0·10
S/924 + 1007 (99/1)	1·0	0·11	0·13	0·13
S/924 + 1007 (99/1) + *L. casei* C5	0·65	0·11	0·16	0·13
GAL	1·15	1·5	1·7	0·8
GAL + *L. casei* C5	2·1	1·75	1·2	0·36

Table 7. *Diacetyl/acetoin* (%) *concentrations in waxed cheeses during maturation*

Time, months	0	3	6
Starter			
S/924	0·0023	0·0013	0
S/924 + *Lactobacillus casei* C5	0·034	0	0
S/924 + 1007 (50/50)	0·056	0·0013	0·0005
S/924 + 1007 (50/50) + *L. casei* C5	0·052	0·0014	0·0008
S924 + 1007/(90/10)	—	0·0053	0·0009
S924 + 1007 (90/10) + *L. casei* C5	0·035	0	0
S/924 + 1007 (99/1)	0·028	0·008	0·0012
S/924 + 1007 (99/1) + *L. casei* C5	0·016	0·004	0·0022
GAL	0	0	0
GAL + *L. casei* C5	0	0	0

Diacetyl/acetoin

Table 7 shows the diacetyl/acetoin concentrations found in the cheeses throughout maturation.

Starter cheeses contained 0·0023–0·056 % diacetyl in the curd, the level being highest when the proportion of *Str. diacetilactis* in the starter was highest, and lowest when *Str. cremoris* was used alone. The diacetyl concentrations decreased in all cheeses during maturation, between 0 and 0·0014 % being present at 6 months. No diacetyl was present at any time in the GAL cheeses.

DISCUSSION

All lactobacillus/starter milk cultures containing *Str. diacetilactis* 1007 utilized all the milk citrate, indicating that when starters containing strains of *Str. diacetilactis* that actively ferment citrate are used for cheese-making, as in many commercial starters, it is likely that all or most of the citrate will be utilized before film wrapping. Blowing after film-wrapping due to citrate fermenting lactobacilli should not result, therefore, unless, as pointed out by Crawford (1962), a strain of *Str. diacetilactis* was used which did not ferment citrate during cheese-making but only after wrapping.

Under conditions of pH and carbohydrate concentration simulating those in cheese, *L. casei* C 2 and *L. casei* C 5 utilized 94 and 64 % of the citrate, indicating that these organisms could possibly utilize citrate present in high-acid cheese. When cheese was made using *Str. cremoris* 924 and *Str. cremoris* 924/*Str. diacetilactis* 1007 it was found that at pressing and ex-press the *Str. cremoris*/*Str. diacetilactis* curd contained only 27 and 5 %, respectively, of the citrate present in the *Str. cremoris* curd. If a high percentage of this particular strain of *Str. diacetilactis* is used in the starter it can be expected therefore that most of the citrate will be utilized during making or at least by the time the cheeses leave the presses. Thus, from the *in vitro* experiments and the cheese-making experiment it would appear that when a non-citrate-fermenting starter is used for cheese-making, blowing of film-wrapped cheeses might be caused by citrate-fermenting lactobacilli.

In the cheese experiment in which lactobacilli were introduced into some of the cheesemilks, the failure of *L. casei* C 5 to utilize cheese citrate and produce blowing may have been due to its inability to utilize citrate under the conditions of cheese maturation or because, being introduced into the cheesemilk in large numbers from young actively growing cultures, it reached maximum counts early in the maturation period (probably 1–2 weeks) and utilized lactose in preference to citrate. The latter suggestion would appear to be the more likely since the curds had counts of about 10^5 lactobacilli/g and carbohydrate concentrations of up to 1 %. Even at 6 months, however, carbohydrate was present at about 0·1 % (Table 6), and this concentration may satisfy the energy requirements of the lactobacilli which grow only slowly under the conditions in cheese. The amount of carbohydrate remaining in the cheese may exert a considerable influence on citrate utilization within cheese and therefore on the subsequent blowing. With increased proportion of *Str. diacetilactis* in the starter there was decreased citrate and increased acetic acid and diacetyl/acetoin in the curd. However, although most of the acetate produced by *Str. diacetilactis* during cheese-making was undoubtedly derived from citrate, some acetate may also have been produced from lactose (Platt & Foster, 1958). A comparison of the *Str. cremoris* 924 and GAL cheese curd showed that *Str. cremoris* also produced acetate which, since the organism is non-citrate-fermenting, was also possibly derived from lactose.

Thus, although no blowing of film-wrapped cheeses was produced by the strain of *L. casei* used in this series of cheese experiments, the evidence gained from studies on milk and the findings observed in commerce strongly suggest that given suitable conditions, and strains of lactobacilli which will ferment citrate under these conditions, blowing of film-wrapped cheeses will occur.

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Observations on the use of 2,4-dinitrophenylhydrazine and of 2,6-dichlorophenolindophenol for the determination of vitamin C in raw and in heat-treated milk

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Summary. A study has been made of methods using 2,4-dinitrophenylhydrazine (DNPH) or 2,6-dichlorophenolindophenol (DCP) for the determination of vitamin C (ascorbic acid + dehydroascorbic acid) in raw, UHT processed, evaporated and sterilized milk.

Interfering substances were not detected in milk that had received a heat treatment no more severe than 145 °C for 4 s (UHT process), so that either reagent could be used.

With more drastic heat treatment, interfering substances were formed and only the DNPH method with column and thin layer chromatography of the DNPH derivatives was specific for vitamin C. With in-bottle sterilized milk, the values for ascorbic acid were (in mg/100 ml) 1·16 (DCP method with H_2S reduction); 0·58 (DCP method with *Escherichia coli* reduction); 0·64 (DNPH method); 0·33 (DNPH method combined with chromatography).

In our experience the DNPH method combined with chromatography of the derivatives is highly specific for vitamin C and should be used to check the results obtained by other and simpler methods.

The determination of vitamin C by chemical methods requires the measurement of ascorbic acid (AA) and dehydroascorbic acid (DHA), both of which may occur in foodstuffs and both of which are biologically active.

The reducing properties of AA allow its determination by titration against suitable oxidizing agents, 2,6-dichlorophenolindophenol (DCP), iodine, ferricyanide, methylene blue and others; of these DCP has been widely used, but its value is limited when other reducing substances are present. Such substances include those with free sulphydryl groups and the group of compounds collectively termed reductones, which includes condensation products of sugars and amines, reductic acid and reductones. Fortunately none of these are present in cow's milk, whether raw or after mild heat treatment, but the more intensive heat treatment used in the preparation of evaporated and, until recently, in-bottle sterilized milks leads to the formation both of free sulphydryl groups and of reductones. Storage may also lead to the formation of reductones. One method for minimizing interference by these compounds is based on

the different rates of condensation with formaldehyde of sulphydryl compounds, AA and reductones at different pH values (Lugg, 1942; Mapson, 1943), but until the exact nature of the interfering substances is known, it cannot be claimed that this method is specific (Snow & Zilva, 1944). DHA can be estimated with DCP only after it has been reduced to AA, and hydrogen sulphide is generally used for this purpose. This reducing agent can however cause the formation of other reducing compounds that may interfere with the titration (Kon & Watson, 1936; Doan & Josephson, 1943). Another approach was made by Stewart & Sharp (1945), who used cucumber juice for the non-selective enzymic oxidation of reducing substances, followed by the specific reduction of DHA to AA by a suspension of *Escherichia coli*. Our experience with this method several years ago suggested that it was the most specific of those we had tried.

A completely different approach is that based on the transformation of DHA into 2,3-dioxogulonic acid (DOA) and subsequent coupling with 2, 4-dinitrophenylhydrazine (DNPH) to form a characteristic dinitrophenylosazone (Roe & Kuether, 1943; Roe & Oesterling, 1944). AA reacts only after oxidation to DHA. Treatment of the osazone with sulphuric acid gives a red coloured solution with a characteristic absorption spectrum. In processed and in stored foods, DOA may be found together with AA and DHA but it has no antiscorbutic activity and its presence must be taken into account when DNPH is used for the determination of vitamin C. A correction for DOA can be made by reducing any DHA present in the extract to AA with hydrogen sulphide and then coupling the DOA with DNPH (Roe, Mills, Oesterling & Damron, 1948). The method is not entirely specific but it has the advantage that the DNPH derivatives, either hydrazones or osazones, of interfering substances can be removed by chromatography (Gordon & Noble, 1959; Mapson, 1961; Vuilleumier & Nobile, 1962; Strohecker & Henning, 1965).

In the present paper we report the results of our studies on the use of DNPH and DCP for the determination of AA and DHA. The methods used are described fully since we have found it necessary to combine the procedures given in numerous papers with our own modifications.

PART I. DINITROPHENYLHYDRAZINE METHODS

For brevity the DNPH derivatives are referred to as hydrazones.

EXPERIMENTAL

Milks

Details of the milks studied are given in Table 1. They are grouped in order of increasing severity of heat treatment.

Method of analysis

The method was based on that of Roe *et al.* (1948) but included some of the procedure used by Roe & Kuether (1943). The modifications that we introduced are discussed in a later section (p. 33).

Reagents

The following reagents were used (Analar grade when available):

Sodium hexametaphosphate (flake) solution: 10 % (w/v), aqueous; stored at about 3 °C; prepared weekly.

Trichloroacetic acid (TCA) solution: 8 % (w/v), aqueous; prepared shortly before use.

Table 1. *Milks studied, grouped in order of increasing severity of heat treatment*

Milk	Heat treatment, and period of storage*	
Raw	Samples taken from dairy bulk tank	—
Pasteurized	Heated at 72 °C for about 15 s	Stored at about 3 °C in waxed cartons for 2 days after processing
Homogenized	Heated at 75 °C for about 15 s	Not stored
Ultra-high-temperature (UHT) processed		
UHT A_{1-90}	Directly heated by steam injection to about 145 °C for 3–4 s. Cooled by evaporation	Stored in Tetra Paks for up to 90 days after processing
UHT B	Deaerated, then indirectly heated to about 137 °C for 4 s. Indirectly cooled	Stored in Tetra Paks for 2 days after processing
UHT C (a, b, c, d)	Indirectly heated to about 138 °C for 2 s. Cooled by partial evaporation	Batches a, b, c, d stored for 0, 1, 90 and 180 days, respectively, after processing
Sterilized (2-stage process)		
Ster. A	Indirectly heated to about 135 °C for 1½ s. Cooled to 70 °C, bottled and capped. Heated to 110 °C within 15 min, cooled rapidly	Stored for 21 days after processing
Ster. B (a, b, c)	Indirectly heated to about 131 °C for 20 s. Cooled to 70 °C, bottled and capped. Heated to 112 °C within 15 min, cooled rapidly	Batches a, b, c, stored for 1, 30 and 42 days, respectively, after processing
Evaporated		
Evap. A (a, b) Evap. B (a, b) Evap. C	Processing conditions not known. Usual commercial treatment involves heating at 115 °C for 15 min	Brands A, B and C purchased locally on occasions a and b
Sterilized (1-stage process)		
Ster. C	Sterilized in bottle at 121 °C for 15 min and placed immediately in a cold store at 3 °C	Stored at about 3 °C for up to 47 days after processing
Ster. D	Sterilized in bottle at 121 °C for 20 min and left to cool gradually to about 18 °C	Stored for 2 days after processing

* All the milks were stored at room temperature (about 18 °C) unless otherwise stated.

Buffer solution (pH 5·9): 30·65 g KH_2PO_4 and 8·95 g Na_2HPO_4, $12H_2O$ dissolved in 1 l water and stored at about 3 °C.

Precipitating Solution: equal volumes 10 % sodium hexametaphosphate solution and 8 % TCA solution.

Diluting Solution: equal volumes Precipitating Solution and buffer solution; it had a pH of about 1·9, which was similar to that of the milk extracts.

Thiourea solution: 10 % (w/v) in 50 % (v/v) aqueous ethanol.

2,6-Dichlorophenolindophenol (DCP) solution: 0·125 g DCP sodium salt (Puriss grade, Koch-Light Laboratories, Ltd) dissolved in warm water, filtered and made to 50 ml with water; stored at about 3 °C; freshly prepared each week.

2,4-Dinitrophenylhydrazine (DNPH) solution: 2 g DNPH dissolved in 100 ml warm 9 N-sulphuric acid, left at 3 °C for about 24 h, passed through a sintered glass filter (no. 4 porosity), and stored at about 3 °C; freshly prepared each week.

85 % sulphuric acid solution: 900 ml sulphuric acid (sp. gr. 1·84) added to 100 ml water, with cooling.

Stannous chloride solution: 1·8 g recently manufactured stannous chloride ($SnCl_2$, $2H_2O$) dissolved in 1 ml water containing a drop of concentrated HCl shortly before use; old batches gave very cloudy solutions and were discarded.

Standard AA solution:

Solution A: 40 mg L-ascorbic acid (British Drug Houses, Ltd) dissolved in 200 ml Diluting Solution in an amber glass flask and stored at about 3 °C; freshly prepared each week.

Solution B: 25 ml solution A diluted to 100 ml with Diluting Solution shortly before use.

Antifoaming agent: Silicone MS Antifoam emulsion RD (Hopkin & Williams, Ltd).

Hydrogen sulphide: cylinder of liquid H_2S (Cambrian Chemicals, Ltd).

Procedure

The milk extracts and dilute AA solution (solution B) were prepared in amber-glass flasks. All manipulations, except for the development of the colour and the determination of the absorbance, were carried out in subdued light.

Preparation of DHA standards and DCP reagent blank. 0, 1, 3, 6 or 10 ml solution B were placed in 50 ml volumetric flasks containing about 25 ml of Diluting Solution and oxidized with 0·75 ml of DCP solution. After 15–20 min, about 5 drops of thiourea solution were added to each flask so as just to decolorize the excess of DCP. The solutions were then made to volume with Diluting Solution to give a series of standards covering the range 0–10 μg DHA/ml.

Preparation of untreated milk extract for determination of DHA + DOA + interfering substances (IS). 100 ml of milk were added to 100 ml of Precipitating Solution and mixed well. The mixture was shaken occasionally, and after 1 h at room temperature it was filtered through a fluted Whatman No. 42 filter paper, the first few ml of filtrate being discarded.

Preparation of oxidized milk extract for the determination of AA + DHA + DOA + IS. 40 ml of untreated milk extract were treated with DCP and thiourea solutions as described above and made to 50 ml in a volumetric flask with Diluting Solution.

Preparation of reduced milk extract for the determination of DOA + IS. About 30 ml of untreated milk extract were taken, 0·1 ml of stannous chloride solution added and the pH adjusted to 1·4–1·5 with concentrated hydrochloric acid. One drop of antifoaming agent was then added and water-saturated hydrogen sulphide bubbled through for 20 min. After the addition of 15 drops of thiourea solution, the solution was filtered, one drop of antifoaming agent was added to the filtrate and the hydrogen sulphide was removed with nitrogen. Finally the pH of the solution was adjusted to that of the original milk extract by the addition of anhydrous sodium carbonate and the solution was filtered.

Coupling with DNPH. Three 4-ml aliquots were taken from each of the solutions and extracts prepared as described above and placed in test-tubes. Two drops of thiourea solution were then added to each of the tubes except those containing reduced extract, as this already contained sufficient thiourea. One tube from each set of 3 was set aside at room temperature until required for the blank determination. DNPH (1 ml) was added to each of the other 2 tubes and these were kept in a water-bath at 37 °C for 3 h.

Development of colour and measurement of absorbance. The tubes, together with the corresponding tubes for the blank determinations, were then cooled in ice for at least 10 min. Keeping the tubes in ice, 1 ml of DNPH solution was added to the blank tubes and 5 ml of 85 % sulphuric acid to all the tubes; the acid was added slowly to avoid any significant increase in temperature. For routine analyses the solutions were then left at room temperature for 40 min and the absorbance determined in a 1 cm cell at 520 or 540 nm, with water in the reference cell, using a Beckman spectrophotometer, model DU. Absorption spectra were determined using an Optica recording spectrophotometer, model CF4DR.

Factors studied on the development of the method

In addition to our modifications, the following factors were studied: the stability of AA, DHA and DOA in the milk extract; the recovery of AA added to milk; the effect of stannous chloride on the reduction of DHA; the effect of lactose; the stability of the colour of the hydrazones at 520 and 540 nm during the 40-min period after addition of 85 % sulphuric acid.

The absorption spectra of the hydrazones

The hydrazones of the milk extracts and standards were prepared and treated with sulphuric acid as described above. The absorption spectra (400–600 nm) were determined at 10 and 40 min after addition of the acid, first with 42 % sulphuric acid solution and then with the appropriate blank (see above) in the reference cell.

In addition, difference spectra (400–600 nm) were obtained 40 min after the addition of 85 % sulphuric acid to give curves for DHA, DHA + AA, and AA directly. These curves were obtained by comparing: untreated extract with reduced extract (DHA); oxidized extract with reduced extract (DHA + AA); oxidized extract with untreated extract (AA). To avoid the dilution of the oxidized extract that occurred in the usual procedure, the DCP was replaced by a few drops of bromine water and the excess of bromine removed with nitrogen.

Chromatography of the hydrazones

Duplicate 10 ml volumes of reduced milk extract, each with 5 drops of thiourea solution, and of standard DHA solutions, untreated and oxidized milk extracts, each with 10 drops of thiourea solution, were all treated with 2·5 ml DNPH solution. For convenience, they were left to react for about 17 h at room temperature rather than for 3 h at 37 °C. The hydrazones were then extracted with ethyl acetate and subjected to column and thin layer chromatography on silica gel following the procedure devised by Vuilleumier & Nobile (1962) and described in detail in Association of Vitamin Chemists (1966). Since only half to three-quarters of the material from each column was used for thin layer chromatography, the amount of sulphuric acid solution used to develop the colour was reduced from 10 to 5 ml. If at this stage the solutions became slightly opalescent they were centrifuged, before determining the absorption spectra over the range 300–600 nm, 30 min after addition of the sulphuric acid.

RESULTS AND DISCUSSION

Modifications

The following modifications to the methods of Roe & Kuether (1943) and Roe *et al.* (1948) were found to be either more convenient or necessary.

Preparation of milk extract. Metaphosphoric acid solution (5 %) which was used by Roe *et al.* (1948) for the preparation of extracts of plant and animal tissues did not give satisfactory extracts, especially with raw milk. Gradual charring occurred after addition of the 85 % sulphuric acid to the solution containing the hydrazones and this caused a marked increase in absorbance. From our experience, it would appear that the difficulties reported by Hansson & Håkansson (1953) and Tobias & Herreid (1959) with the DNPH method when applied to milk were due to the use of unsatisfactory extracts. In the present work a mixture of TCA and sodium hexametaphosphate (Mattick *et al.* 1945) gave satisfactory extracts provided sufficient time was allowed for precipitation and the filtrates were clear. Although TCA alone is a protein precipitant, metaphosphate was included to prevent the catalytic oxidation of AA by copper (Fujita & Iwatake, 1935; Musulin & King, 1936; Lyman, Schultze & King, 1937). The analysis of milk extracts prepared by our procedure showed AA, DHA and DOA to be stable for at least 3 h. Furthermore, the mean recovery of AA added to milk was 98 %.

Oxidation of AA to DHA. DCP was used in preference to Norit (Roe & Kuether, 1943) or bromine (Roe *et al.* 1948). The excess of DCP was removed with thiourea (Bolin & Book, 1947). The DCP reagent blank was small provided that the DCP was sufficiently pure. Schwartz & Williams (1955) reported high DCP blanks but these may have been due to their use of a mixture of hydrochloric and phosphoric acids, instead of sulphuric acid, for colour development.

Length of time of coupling with DNPH. Roe *et al.* (1948) showed that DHA and DOA couple with DNPH at different rates because DHA must first be converted into DOA. They chose to stop the reaction after 6 h, when the coupling of DHA was 95 % that

of DOA. In the present work, in order to reduce the time taken for the analysis, the reaction was stopped after 3 h when, according to Roe *et al.* (1948), coupling of DHA is about 90 % that of DOA. Since DHA is used for the standard curve, the use of a 3-h coupling period leads to an over-estimate of DOA by about 10 %. This error is eliminated in the determination of AA and DHA, as these values are calculated by difference.

Reduction of DHA to AA

Roe *et al.* (1948) found that at a pH value of 3·5 the reduction of DHA by hydrogen sulphide in the presence of stannous chloride was practically complete in 5 min but recommended that, in order to protect DOA, the pH should be lowered to 1·25–1·3. At this lower pH the reduction of DHA was virtually complete in 15 min but below pH 1·2 the reduction of DHA was sharply diminished. In the present work, a fall of about 0·1 pH units generally occurred during the hydrogen sulphide treatment, and in order to prevent the pH falling below the critical value of 1·2, the initial pH value was adjusted to 1·4–1·5.

Stannous chloride was used by Roe *et al.* (1948) as an antioxidant during the extraction of tissues containing oxyhaemoglobin, and consequently it was present at the reduction step with hydrogen sulphide. Although milk contains no strong oxidant, we found that stannous chloride increased the efficiency of the reduction both of milk extracts and of standard DHA solutions. Even when stannous chloride was present, reduction of DHA standard solutions was only 93–95 % complete after 20-min treatment with hydrogen sulphide, but 99 % complete after 16 h. A 20-min reduction period was preferred, as prolonged treatment with hydrogen sulphide could lead to the formation of artifacts in milk extracts. Other workers have also found the reduction of DHA to be incomplete or very slow at low pH values and Levenson, Rosen & Hitchings (1951) showed that the reaction was influenced by the type of buffer used.

Effect of lactose

Certain sugars at high concentrations interfere with the DNPH method (Roe & Kuether, 1943), but an aqueous solution containing 4·8 % lactose, the concentration typically present in cow's milk, gave negligible readings at 520 and 540 nm 40 min after the addition of the sulphuric acid.

Stability of absorbance at 520 nm and 540 nm during the 40-min period after the addition of sulphuric acid

Although our most recent work has shown that the absorption maximum of the standards lies between 522 and 526 nm (Fig. 1) in close agreement with the range 520–525 nm reported by Strohecker & Henning (1965), earlier workers determined the absorbance at 520 or at 540 nm (Lowry, Lopez & Bessey, 1945; Mills & Roe, 1947; Roe & Kuether, 1943; Roe *et al.* 1948) and these wavelengths were used in the present investigation. The milks studied (Table 1) were raw, pasteurized, ultra high temperature processed (UHT A_{1-90} and B), sterilized (Ster. A, Bb, C and D) and evaporated (Evap. Aa and Ba).

In contrast to the absorbance values for the standards, which were virtually stable, the values for the various milk extracts changed with time, indicating the presence

of other DNPH derivatives. At 520 nm the absorbance of the milk extracts generally decreased with time, and with samples Ster. Bb, C and D, Evap. Aa and Ba this decrease was very large and continued beyond the 40-min period. At 540 nm, how-

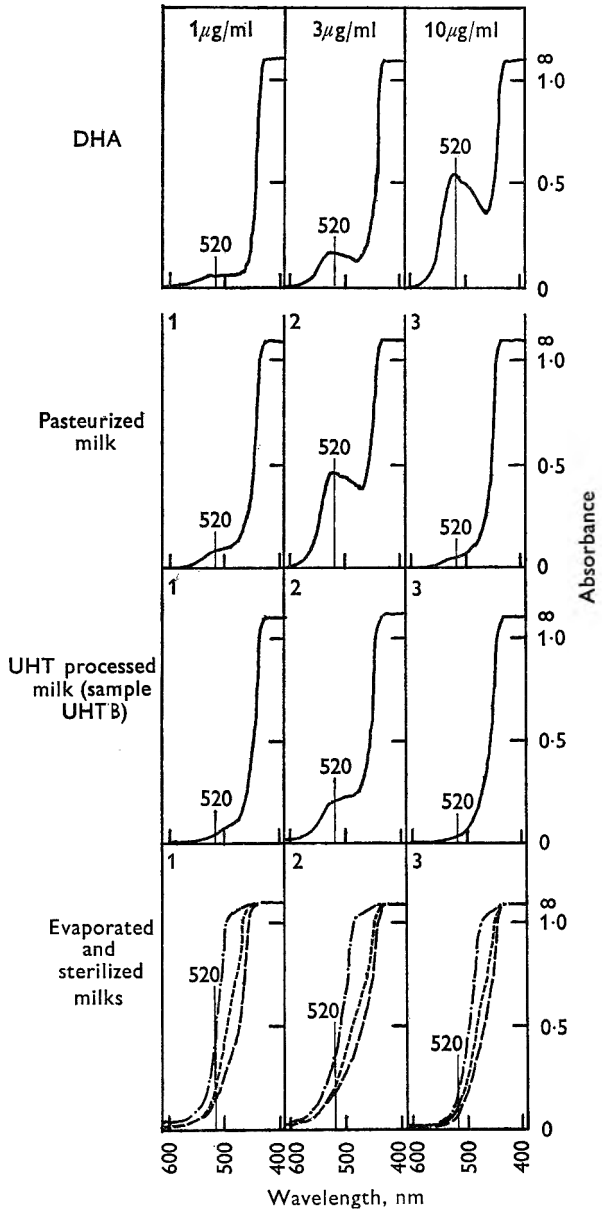

Fig. 1. The absorption spectra of the 2,4-dinitrophenylhydrazones of dehydroascorbic acid (DHA) and of pasteurized, ultra-high-temperature (UHT) processed, evaporated and sterilized milks, 40 min after development of the colour with 85 % sulphuric acid. 42 % Sulphuric acid in the reference cell. 1, Untreated extracts (DHA + dioxogulonic acid (DOA) + interfering substances); 2, oxidized extracts (ascorbic acid + DHA + DOA + interfering substances); and 3, reduced extracts (DOA + interfering substances). ----, Evaporated milk, sample Evap. Ab; – – –, sterilized milk, sample Ster. Ba; —·—·—, sterilized milk, sample Ster. C. Details of the milks are given in Table 1.

and with 2,6-dichlorophenolindophenol

Milk	Code name†	Storage, days	2,4-dinitrophenylhydrazine procedure*: Ascorbic acid, mg/100 ml‡ 520 nm	540 nm	Dehydroascorbic acid, mg/100 ml‡ 520 nm	540 nm	2,6-dichlorophenolindophenol procedure (bromine-hydrogen sulphide): Ascorbic acid, mg/100 ml‡	Dehydroascorbic acid, mg/100 ml‡
Raw		1 In dark	1·61	1·60	0·25	0·23	1·71	0·22
Pasteurized		2 In dark	1·57	1·57	0·18	0·18	—	—
Ultra-high-temperature (UHT) processed	UHT A	1 In dark	1·65	1·65	0·10	0·07	1·78	0·07
		2 In dark	1·61	1·59	0·09	0·07	1·68	0·07
		7 In dark	1·64	1·67	0·06	0·05	1·56	0·08
		14 In dark	1·69	1·69	0·05	0·04	1·64	0·09
		30 In dark	1·51	1·52	0·08	0·05	1·56	0·07
		60 In dark	1·44	1·45	0·14	0·10	1·48	0·02
		90 In dark	1·50	1·53	0·24	0·18	1·53	0·04
Evaporated	Evap. Aa	Not known In dark	0·59	0·57	0·04	0·07	0·65	0·24
	Evap. Ba	Not known In dark	0·89	0·86	0·06	0·09	—	—
	Evap. C	Not known In dark	—	—	—	—	0·64	0·17
Sterilized	Ster. A	21 In light	0·01	0·00	0·05	0·03	0·05	0·03
	Ster. Bc	42 In dark	0·14	0·14	0·08	0·08	0·25	0·09
	Ster. Bc	42 In light	0·01	0·00	0·05	0·06	0·07	0·00
	Ster. C	0 In dark	—	—	—	—	1·20	0·02
	Ster. C	13 In dark	0·60	0·55	0·00	0·06	0·93	0·04
	Ster. C	47 In dark	0·72	0·64	0·13	0·12	1·16	0·14
	Ster. C	47 In light	0·33§	—	0·01§	—	—	—
	Ster. D	2 In dark	—	—	—	—	1·70	0·10
	Ster. D	2 In light	0·58	0·61	0·36	0·28	1·63	0·08

* Absorbance determined 30 min after addition of the 85 % sulphuric acid for the raw and UHT samples, and after 40 min for the evaporated and sterilized samples.

† Code names and details concerning the preparation of the milks are given in Table 1.

ever, the absorbance values for all the milks were more stable, as there was less effect of interfering substances.

The AA and DHA content of the various milks studied was calculated from absorbance values at 520 and 540 nm and Table 2 shows that the results at the 2 wavelengths were similar even for some of the more strongly heated milks. We found, however, a serious effect on the results for DOA content of the more strongly heated milks, since these contained more interfering substances, and values for DOA are not calculated by difference.

The ratio (absorbance at 520 nm)/(absorbance at 540 nm) for the oxidized extracts was found to be a guide to the validity of the determination. This ratio was 1·32 for standards, and similar values (1·32–1·40) were found for raw, pasteurized and UHT processed milks 40 min after the addition of the sulphuric acid. The sterilized and evaporated milks contained large amounts of substances that coupled with DNPH to give compounds that increased absorption at 520 nm to a much greater extent than at 540 nm and this caused the ratio to increase to 1·75–2·03. Ratios as large as this threw doubt on the specificity of the method for such milks and this doubt was confirmed by studies of absorption spectra and the use of chromatography.

Absorption spectra of the hydrazones

Spectra obtained with 42 % *sulphuric acid or the blank in the reference cell.* With acid in the reference cell any peaks or shoulders between 522 and 526 nm were more clearly defined at 40 min than at 10 min because of a decrease in absorption, particularly at shorter wavelengths. This was due to the breakdown, in the strongly acidic solution, of the hydrazones of interfering substances (Mills & Roe, 1947).

The spectra for raw and pasteurized milks were very similar and those for the oxidized extracts resembled the standard and showed a clearly defined maximum at 522–523 nm (Fig. 1). In contrast, the oxidized extract for UHT B (Fig. 1) and also for UHT Cb showed only a shoulder in this region. The shape of the curve was considerably worse for Evap. Ab, Ster. Ba and C and showed no evidence of the presence of AA although the calculated results indicated that these milks contained about 0·5 mg AA/100 ml. For comparison, the spectra of the untreated and reduced milk extracts are shown in Fig. 1.

With the corresponding blank (see p. 32) in the reference cell, there was no improvement in the shape of the curves in the region 522–526 nm.

Difference spectra. The effect of interfering substances should be largely eliminated when absorption curves are obtained by difference. The spectra for DHA, DHA + AA and AA for raw milk (Fig. 2) were identical with those obtained for standard solutions (Fig. 1) at all wavelengths above 460 nm and each showed a peak at 524–525 nm. Those for sterilized milk (Ster. C) were not entirely satisfactory but were a marked improvement on the curves for the same milk shown in Fig. 1. Although the curve for AA in Fig. 2 showed a peak at 521 nm, chromatography showed that only about half the absorbance at 521 nm was actually due to AA.

Chromatography of the hydrazones

After column and thin layer chromatography of the hydrazones obtained from extracts of Ster. C, the absorption spectrum shown in Fig. 3 was obtained for the

oxidized extract. Those for the untreated and reduced extracts showed less well defined peaks because of the very low content of DHA and DOA, and are not shown. It can be seen that the absorption spectrum for the sterilized milk differed from that of a similarly treated standard only in the slightly higher absorbance at 364–367 nm. When the solvent system described by Strohecker & Henning (1965) for thin layer

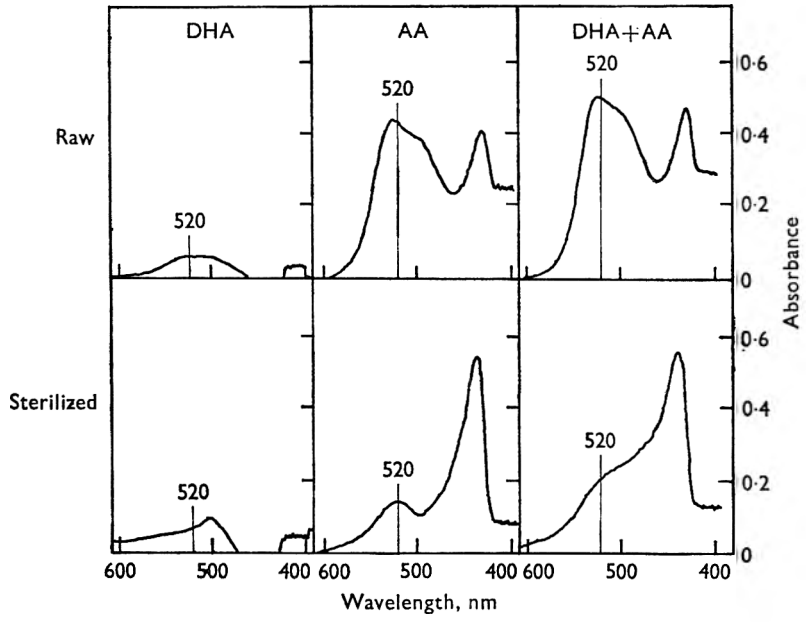

Fig. 2. Difference curves of the absorption spectra of the 2,4-dinitrophenylhydrazones of untreated, oxidized and reduced extracts of raw and in-bottle sterilized milks, 40 min after development of the colour with 85% sulphuric acid. Dehydroascorbic acid (DHA), untreated versus reduced extract. Ascorbic acid (AA), oxidized versus untreated extract. DHA + AA, oxidized versus reduced extract.

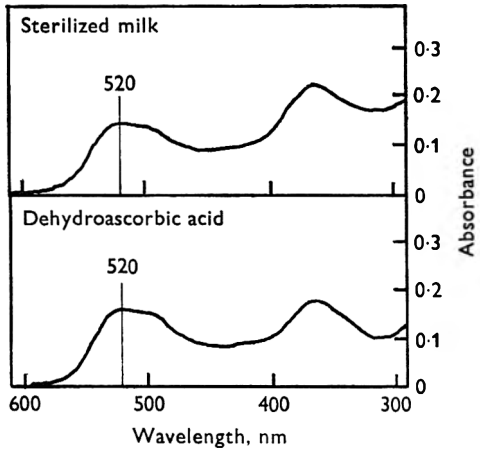

Fig. 3. The absorption spectra of the 2,4-dinitrophenylhydrazones of the oxidized extract from in-bottle sterilized milk, and of dehydroascorbic acid after column and thin layer chromatography and development of the colour with 85% sulphuric acid. 42% Sulphuric acid in the reference cell.

chromatography was used the separation from interfering compounds was less satisfactory.

The chromatographic method was applied to Ster. C on 4 occasions and the results for the oxidized extract (AA + DHA + DOA) ranged from 0·37 to 0·42 mg/100 ml milk. On 2 of these occasions the untreated and reduced extracts were also analysed, the results for AA being 0·33 and 0·34, and for DHA 0·01 and 0·02 mg/100 ml. These were considerably lower than the values of 0·55 and 0·64 for AA and of 0·06 and 0·12 mg/100 ml for DHA, obtained without chromatography.

Vitamin C content of milks processed by various methods

Results obtained with the DNPH methods are given in Table 2 and are discussed later.

PART II. 2,6-DICHLOROPHENOLINDOPHENOL (DCP) VISUAL TITRATION METHODS

EXPERIMENTAL

Milks

Details of the milks studied are given in Table 1.

Methods of Analysis

The procedures described are the method of Stewart & Sharp (1945) in which cucumber juice is used for oxidation and a suspension of *E. coli* for reduction, and 2 variations of this method. In the first variation cucumber juice was replaced by bromine at the oxidation step, and in the second variation oxidation with bromine was followed by reduction with hydrogen sulphide instead of with *E. coli*.

Reagents

Since most of the reagents used are the same in the method of Stewart & Sharp (1945) as in our modified procedures they are listed together.

Metaphosphoric acid (MPA) solution: sticks dissolved to give a 5% (w/v) aqueous solution; filtered and stored at about 3 °C; fresh solution prepared every 3 days.

DCP solution: for titration, 5 ml of the solution prepared as in Part I, diluted to 100 ml with distilled water; prepared daily.

Ascorbic acid (AA) solution for recovery tests (solution A): 200 mg L-ascorbic acid dissolved in 10 ml MPA solution and made to 100 ml with distilled water; stored at about 3 °C; prepared weekly.

Ascorbic acid solution for standardization of DCP solution (solution B): 1 ml solution A diluted to 100 ml with distilled water; prepared daily.

Buffer solution (pH 5·9): as in Part I.

Antifoaming agent: as in Part I.

Hydrogen sulphide: as in Part I.

Saturated bromine water: distilled water saturated with bromine (Analar, B.D.H. Ltd) at room temperature.

Cucumber juice: cucumbers (variety Telegraph) from the Canary Islands or Holland were frozen and retained their enzymic activity for at least 1 year in deep freeze and after use could be refrozen: cucumber allowed to thaw out and drip until enough juice collected for a day's analyses; unused juice discarded at the end of the day.

Bacterial suspension: *E. coli* ATCC 8739 used as recommended by Stewart & Sharp (1945); activity checked by recovery of added AA after its conversion to DHA.

Procedure

The milk and milk extracts were protected from light by the use of amber-glass flasks or black cloth, except at the titration step where good illumination was needed. The milk was cooled before analysis and the vitamin C determined by one or other of the procedures described in Table 3. The DHA content of the milk was calculated from the difference between the values obtained for steps II and I. The AA + DHA content of the milk was calculated from the values in step III. The AA content was obtained by deducting the DHA content from the value for AA + DHA.

Standardization of DCP solution. Solution B (25 ml) was diluted with an equal volume of MPA solution and a 15-ml aliquot taken for titration against the DCP solution. A titration blank was obtained by using 25 ml water in place of solution B.

Recovery of added AA. To two 200-ml portions of milk were added 2 ml solution A or 2 ml water. The 2 solutions were analysed by one or other of the procedures described in Table 3.

RESULTS AND DISCUSSION

Choice of protein precipitant. Stewart & Sharp (1945) used sulphuric acid as a protein precipitant, but since it does not protect AA against copper catalysed oxidation and oxidative enzymes it was replaced by MPA. When traces of MPA, insufficient to affect the pH, were added to a solution at pH 5·9 containing 4 mg AA/100 ml, the loss of AA in 4 h at room temperature was only 5 %, whereas in the absence of MPA it was 88 %.

Oxidation by cucumber juice or bromine. The oxidation of AA and other reducing substances by cucumber juice depends on obtaining cucumbers which contain ascorbic acid oxidase. This is not always possible as we found no activity in cucumbers available here in late winter. This is no problem if a laboratory is using this method routinely, since suitable cucumbers can be collected in the summer and stored in deep freeze for at least a year without loss of enzymic activity. The juice lost its activity in 14 days at 3 °C. Two ml of juice generally ensured that sufficient oxidase was present but it is advisable to check the activity of the juice. A disadvantage to the use of cucumber juice was its vitamin C content which could be sufficient to cause an error of about 0·2 mg AA in 100 ml of milk.

Bromine was found to be just as effective as cucumber juice, and had none of its disadvantages. Two ml was sufficient for raw, pasteurized, UHT or sterilized (2-stage process, Table 1) milks. Three ml was required for evaporated and sterilized (1-stage process, Table 1) milks. The use of bromine did not affect the reduction by *E. coli* as the recovery of added AA was 87 %, compared with that of 89 % when cucumber juice was used.

Reduction by hydrogen sulphide. Since in the buffered milk (pH 6·2) the reduction of DHA by *E. coli* proceeded rapidly, the reduction with hydrogen sulphide was done at this pH. There is ample evidence in the literature to support the use of this pH and in the present work 93 % added AA was recovered.

Kon & Watson (1936) showed that 16-h reduction at the natural pH of milk (6·7) caused the formation of artifacts and that these could be prevented if the milk was deproteinized before the hydrogen sulphide treatment. In our experience, 5 min was sufficient for complete reduction of DHA and did not produce artifacts in raw, pasteurized and UHT processed milk.

Comparison of the DCP methods. The results obtained by the method of Stewart &

Table 3. *Summary of procedures used for the determination of vitamin C by the 2,6-dichlorophenolindophenol (DCP) method**

Step I Determination of AA + interfering substances (IS)

25 ml milk + 25 ml MPA solution†

Step II Determination of AA + DHA + IS

Reduction

by *Escherichia coli*

25 ml milk, 5 ml buffer solution, 1 ml *E. coli* suspension. Incubate at 37 °C for 35 min. Add 19 ml MPA solution†

by hydrogen sulphide (H_2S)

25 ml milk, 5 ml buffer solution, 1 drop antifoaming agent. Treat with H_2S, 5 min. Remove H_2S with nitrogen. Add 20 ml MPA solution†

Step III Determination of blank and of AA + DHA + blank

Oxidation

by cucumber juice

60 ml milk, 18 ml buffer solution, 2 ml cucumber juice. Leave 10 min at 3 °C

To 25 ml add 25 ml MPA solution† (blank)

by bromine

60 ml milk, 2 ml saturated bromine water, 1 drop antifoaming agent. Mix. Immediately remove bromine with nitrogen. Add 18 ml buffer solution

To 25 ml add 25 ml MPA solution† (blank)

Reduction

by *E. coli*

To 25 ml add 2 ml *E. coli* suspension. Incubate at 37° C for 35 min. Add 23 ml MPA solution† (AA + DHA + blank)

by *E. coli*

(see opposite)

by H_2S

Treat 25 ml with H_2S for 5 min. Remove H_2S with nitrogen. Add 25 ml MPA solution†(AA + DHA + blank)

* Details of reagents given on page 39.

† After addition of MPA solution, leave for 20 min at room temperature, filter through a Whatman No. 1 filter paper. Titrate 15 ml of filtrate against DCP solution until a pink colour lasting 15 s is obtained.

Sharp (1945), and 2 modifications which increased the convenience of the method are shown in Table 4.

Oxidation with cucumber juice gave higher results than oxidation with bromine. When a correction was made for the DHA content of the juice, the results agreed (expt 4).

Table 4. *The determination of vitamin C in homogenized, ultra-high-temperature (UHT) processed, evaporated and sterilized milks by modifications of the 2,6-dichlorophenol-indophenol method*

Expt no.	Type of milk*	Method		Vitamin C, mg/100 ml	
		Oxidation	Reduction	Ascorbic acid	Dehydro-ascorbic acid
1	UHT Ca	Cucumber	*Escherichia coli*	1·15	0·12
		Bromine	*E. coli*	0·77	0·12
2	UHT Cc	Cucumber	*E. coli*	0·28	0·03
		Bromine	*E. coli*	0·01	0·03
		Bromine	H_2S	0·12	0·03
3	Evap. Bb†	Bromine	*E. coli*	0·68	0·09
		Bromine	H_2S	0·65	0·30
4	Homogenized	Cucumber	*E. coli*	1·67	0·08
		Bromine	H_2S	1·61	0·08
	Ster. C	Cucumber	*E. coli*	0·88	0·11
		Cucumber	*E. coli*	0·58‡	0·11
		Bromine	*E. coli*	0·58	0·14
		Bromine	H_2S	1·16	0·14

* Details of milks are given in Table 1.
† Reconstituted basis.
‡ Corrected for ascorbic acid content of cucumber.

Reduction with hydrogen sulphide satisfactorily replaced *E. coli* in the determination of vitamin C in UHT processed milk (Table 4) and this was true also for raw, homogenized, and pasteurized milks. However, in evaporated milk, although the results for AA agreed by both methods, DHA was over-estimated when hydrogen sulphide was used. Henry, Houston, Kon & Osborne (1939) and Doan & Josephson (1943) both found DHA in evaporated milk, an unexpected result since DHA is destroyed even by the mild heat treatment used for pasteurization (Hartman & Dryden, 1965). Table 4 shows that when *E. coli* reduction was used little DHA was detected in evaporated milk, a finding in agreement with that of Doan & Josephson (1943) when they used the bacterial reduction method of Gunsalus & Hand (1941).

In sterilized milk prepared by the 1-stage process (Ster. C) involving drastic heat treatment, small amounts of DHA were found when either method of reduction was used, and the AA content appeared to be over-estimated when hydrogen sulphide was used, since reduction with *E. coli* gave a markedly lower value (Table 4). This discrepancy in AA values suggests that substances are formed by the bromine treatment that are reduced by hydrogen sulphide but not by *E. coli*. However, even the *E. coli* method over-estimated the AA content of this milk since chromatography of the hydrazones (Table 2) reduced the value to 0·33 mg/100 ml.

GENERAL DISCUSSION

Raw and UHT processed milk

Table 2 shows that there was close agreement between the DNPH and DCP methods and that it was immaterial whether the measurements of the hydrazones were made at 520 or 540 nm. Agreement between the 2 methods was not proof of specificity but virtually zero values found for AA in UHT Cd milk stored for 180 days gave confidence in the results. A low value for AA was expected since this milk initially contained oxygen which destroyed the AA on storage (Ford, Porter, Thompson, Toothill & Edwards-Webb, 1969).

Values for DHA in UHT A milks apparently increased towards the end of storage due to the formation of interfering substances that caused the absorbance of the untreated extracts to increase over the 40-min period, but not the absorbance of the reduced extracts. This was in contrast to the stable or slightly decreasing absorbance observed for samples stored for less than 30 days and for raw and pasteurized milks.

Evaporated milk

Table 2 shows that similar results were obtained for AA by both methods, but the DCP method gave high results for DHA. This discrepancy was eliminated when *E. coli* replaced hydrogen sulphide (Table 4). Although the DNPH and DCP methods agreed, the lack of a shoulder in the absorption curves at 522–526 nm (Fig. 1) and the high value for the ratio (absorbance at 520 nm)/(absorbance at 540 nm) suggested that neither method was specific.

Sterilized milk

Sterilized milk used to be made by a 1-stage process with severe heat treatment, but the 2-stage process currently in use involves milder heating.

Table 2 shows that the samples prepared by the 2-stage process (Ster. A and Ster. Bc) contained little vitamin C by either method. Absorption curves of the hydrazones (Fig. 1) obtained for a 1-day-old sterilized milk (Ster. Ba) from the same plant as Ster. Bc, were similar to those obtained for evaporated milk and once more suggested that the methods were not specific. However, the low values found after illumination of the milk showed that the heat treatment used in the 2-stage process did not, in fact, form interfering substances.

For milks prepared by the 1-stage process (Ster. C and D) there was considerable lack of agreement between the results obtained by the 2 methods (Table 2), particularly for the more strongly heated sample (Ster. D), in which it was nearly impossible by the DCP method to determine the end point of the titration. It is interesting to note that exposure of Ster. D to light did not reduce the value for AA. The values obtained for AA by the DNPH method were about one-third of those by the DCP method, but the considerable instability of the absorbance readings suggested that these values also were unreliable. Although Ster. C was prepared by less severe heat treatment than Ster. D the AA content obtained by the DCP method was still considerably higher than that obtained by the DNPH method. When the more specific *E. coli* reduction was used (Table 4) the value obtained was 0·58 mg AA/100

ml, in close agreement with the value of 0·64 obtained by the DNPH method (Table 2), but even the latter value was suspect since the absorbance readings were very unstable. When interfering substances were removed from Ster. C by chromatography of the hydrazones, values of 0·33 mg AA and of 0·01 mg DHA/100 ml were obtained. The remarkable similarity in the shape of the absorption curves (Fig. 3) for the hydrazones from the DHA standard solution and Ster. C was proof of the specificity of this chromatographic method for the determination of vitamin C in sterilized milk.

CONCLUSIONS

A re-evaluation of some of the methods used for the determination of vitamin C in heat-treated milk seemed timely, in view of the improvements in specificity achieved by new techniques using chromatography.

It is evident from the results presented here that for raw and mildly heated milks both the DCP method, with hydrogen sulphide as the reducing agent, and the DNPH method, without chromatography, were satisfactory.

With strongly heated milks both these methods were unsatisfactory, but some improvement in the DCP method was obtained by reduction with *E. coli* instead of with hydrogen sulphide. Even *E. coli* reduction was not entirely specific, as shown by the lower results obtained by chromatography of the hydrazones. Mapson & Ingram (1951) have also cast doubt on the specificity of the reducing action of *E. coli*. In the DCP method it was generally not possible to detect the presence of interfering substances, but in the DNPH method their presence was indicated by the instability of the absorbance readings, particularly at 520 nm. Values for vitamin C were also suspect if the absorption curves of the hydrazones fell steeply between 520 and 540 nm. The main advantage of the DNPH method is that interfering substances can be removed by chromatography. Moreover, chromatography of the stable DNPH derivative, which can be concentrated readily at various stages in the procedure, is preferable to chromatography of the very labile AA and DHA.

The DNPH method with chromatography was found to be highly specific and although it is time consuming its use is essential as a means of checking the specificity of simpler methods.

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Association of lipases with micellar and soluble casein complexes

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SUMMARY. The association of lipase with casein micelles and soluble casein complexes was investigated by gel-filtration on Sephadex G-200 and Sepharose 2B columns which were equilibrated with synthetic milk serum. Gel-filtration indicated that the molecular weight of casein micelles in milk is $> 10^8$ whereas the casein in colloidal phosphate-free milk is present as soluble complexes of molecular weight *ca.* 2×10^6 containing α_s-, β- and κ-casein. The soluble complexes appear to be stabilized in the micelle by colloidal calcium phosphate linkages. On addition of pancreatic lipase to milk, activity was impaired due to binding of the enzyme both to micellar and to soluble casein complexes. The enzyme dissociated from the latter during gel-filtration on Sepharose 2B columns. The binding of lipase to casein was not dependent on the presence of colloidal phosphate and consequently complete micellar structure is not essential for association of lipase with casein. Binding of the lipase to phosvitin did not result in a loss of enzyme activity. Lipases in milk appear to be involved in the equilibrium between micellar and soluble casein. The activity of lipases in milk is apparently influenced by this equilibrium. Some problems encountered in the use of gel-filtration to study the interactions of lipases with caseins are described.

It has been well documented that the lipase activity in milk is mainly associated with the casein (Harper, Gould & Badami, 1956; Tarassuk & Frankel, 1957; Saito, 1963; Gaffney, Harper & Gould, 1962). Aside from the hypothesis (Downey & Andrews, 1965*b*) that lipase will bind only to micellar casein, the nature of the binding has not been investigated nor has it been unequivocally established whether the lipase activity in milk is specifically associated with an individual casein.

Skean & Overcast (1961) showed by means of continuous paper electrophoresis that lipase activity is associated with the α-casein fraction. Saito & Hashimoto (1963) observed similarities in the chromatographic behaviour on DEAE cellulose of milk lipase activity, and a casein component which they consider to be β-casein. In contrast, Yaguchi, Tarassuk & Abe (1964) suggested that milk lipase is a part of the κ-casein complex or κ-casein itself since both are eluted in the same position from DEAE columns. Subsequently, Fox, Yaguchi & Tarassuk (1967) suggested that lipase in milk is normally associated with κ-casein and is a discrete minor component of the casein system. On the other hand Gaffney, Harper & Gould (1966), employing similar chromatographic conditions to the previous investigators, detected

lipase activity in all fractions eluted from DEAE cellulose and concluded that the milk lipase activity is associated with other proteins besides κ-casein.

These divergent results may be attributed to the use of different lipase preparations by the various investigators. The propensity of lipases to adsorb at interfaces (Desnuelle, 1961) could lead to adsorption of the lipase on to finely dispersed proteins produced during the fractional precipitation steps employed prior to chromatography. It has been observed (Downey, 1965) that milk lipases tend to adsorb on to protein precipitates obtained during fractionation of milk proteins. Because of these intrinsic difficulties there is need for caution in relating the status of lipases in purified milk preparations to that in the original milk.

In an attempt to resolve these anomalous results an alternative approach was explored in the present investigation. Pancreatic lipase, which appears to interact with casein micelles in a manner similar to milk lipases (Downey & Andrews, 1965*b*), was added to milk and to colloidal phosphate-free milk, and the resultant lipase-casein complexes investigated by gel-filtration. The use of a lipase preparation containing no milk proteins and in which the enzyme is known to exist as a monomer (Downey & Andrews, 1965*a*) enabled us to study the association of free lipase with casein micelles and complexes. To maintain conditions comparable to those prevailing in milk, and to minimize decomposition of the lipase-casein complexes during gel-filtration, the columns were equilibrated with a buffer (referred to as synthetic milk serum) similar in composition to milk ultrafiltrate.

MATERIALS AND METHODS

Milk preparations

Whole milk was collected from individual Friesian cows in mid lactation, cooled to 4 °C and the cream immediately separated by centrifugation at 500 *g* at 4° C for 20 min, care being taken not to sediment casein micelles.

Supernatants were prepared by centrifuging skim-milk at 34000 *g* for 2 h at 4 °C. Most of the clear solution was removed by pipette to exclude the slowly sedimented casein micelles and the residual fat.

NaCl supernatants were prepared by similar procedures from skim-milk in which NaCl had been dissolved to a final concentration of 0·75 M before centrifugation.

The method of preparation of colloidal phosphate-free milk was similar to that described by Pyne & McGann (1960). The pH of skim-milk (100 ml, 0 °C) was slowly reduced from pH 6·8 to 4·9 by the dropwise addition, with stirring, of concentrated HCl. Following stirring for a further 30 min the acidified skim-milk was dialysed at 4 °C for 48 h against 2 changes of skim-milk (5 l). The pH of the resultant preparations was within 0·05 units of that of the original skim-milk.

Synthetic milk serum (McGann, 1960) was prepared by the addition of 8·8 m-moles citric acid, 11·7 m-moles potassium dihydrogen orthophosphate, 25 m-moles sodium chloride, 10 m-moles calcium chloride and 2·5 m-moles magnesium chloride to 800 ml distilled water. The pH was adjusted to 6·7 by addition of 60 ml 0·5 M-NaOH, and 0·12 moles lactose were added. The pH was then readjusted to 6·7 and the volume made up to 1 l. The buffer was stored at 5 °C and filtered immediately before use.

Pig pancreatic lipase. The juice was expressed from chilled fresh pancreas, centrifuged at 34000*g* for 2 h at 4 °C and the supernatant carefully decanted and frozen until required. Samples were diluted before use with 0·75 M-NaCl containing 0·05 M-$MgCl_2$, or with synthetic milk serum.

Purified proteins. Details of other protein preparations used are given in Table 1.

Gel-filtration. Sephadex G-200 (lot no. To-5212, particle size 40–120 μ) and Sepharose 2B (lot no. 4203) (Pharmacia, Uppsala, Sweden) were suspended in synthetic milk serum and allowed to swell at 5 °C for about 2 weeks before use and

Table 1. *Details of the purified protein preparations*

Protein	Mol. wt.	Reference	Supplier
Phosvitin (hen egg)	21000 monomer 38000 dimer	Mecham & Olcott (1949)	Nutritional Biochemical Corp.
Thyroglobulin (bovine, purified)	670000 1330000*	Spiro & Spiro (1963)	Nutritional Biochemical Corp.
γ-globulins (human, Fraction II)	160000 205000*	Phelps & Putnam (1960)	Sigma Chemical Co.
Cytochrome *c* (horse heart, Type II)	12000*	Margoliash (1962)	Sigma Chemical Co.

* Apparent molecular weights on gel-filtration when referred to typical carbohydrate-free globular proteins (Andrews, 1965).

the smallest particles removed by decantation. Columns (70 cm × 2·5 cm diam.) were packed at 5 °C as described by Andrews (1964) and equilibrated with synthetic milk serum for at least 24 h before use. Samples were applied by layering under the buffer on the top of the column following addition of sucrose to facilitate even application. Flow rates were maintained at about 15–20 ml/h. After about 6 fractionations, however, the flow rates of the Sepharose 2B columns had so decreased as to necessitate their repacking. To minimize bacterial contamination and possible protein degradation during fractionation, the column materials were suspended overnight in a solution of 1 M-NaCl containing 0·003 M sodium azide before repacking. Blue dextran 2000 was used to determine the void volumes of the columns. Effluent was collected in 5 ml fractions with a collector (Aimer Products Ltd., London). To estimate the molecular weight of the milk proteins the marker proteins listed in Table 1 were mixed with some milk preparations before gel-filtration. Precise molecular weight determinations, similar to those obtainable with Sephadex (Andrews, 1964, 1965), were not possible with Sepharose 2B columns, due to the lack of suitable macroproteins of known molecular size.

Examination of column effluent. Proteins in the effluent fractions were estimated spectrophotometrically. Phosvitin and cytochrome c were estimated at 220 and 412 nm, respectively. The other proteins listed in Table 1 and the milk proteins were estimated at 280 nm. Effluent fractions which were opalescent due to the presence of casein micelles were also read at 360 nm and their protein content expressed as the difference between the absorbance at 280 and 360 nm. When required, the protein content was also determined by micro-Kjeldahl analysis.

Assays for milk and pancreatic lipase activity were performed at 25 °C in the presence of 3 ml 0·75 M-NaCl containing 0·025 M-$MgCl_2$, by automatic titration at

pH 8·5, using 1 ml tributyrin emulsion as substrate (Downey & Andrews, 1965*a*, 1966). One unit of lipase activity is defined as the quantity of enzyme required to liberate 1 micro-equivalent of acid from the substrate/min. Because of the buffering capacity of the column eluent, it was necessary to dialyse the effluent fractions overnight at 5 °C with stirring against 5 l 0·75 M-NaCl containing 0·025 M-$MgCl_2$ before assay. When 0·1 M-NaCl was employed, considerable protein precipitation occurred during dialysis.

The individual milk proteins in the column effluent were identified by urea-starch gel electrophoresis (Aschaffenburg & Thymann, 1965; Murphy & Downey, 1969) of pooled fractions. Consecutive fractions, corresponding to selected regions of the protein elution diagrams, were combined so that 4 to 6 pooled fractions (20–60 ml) were obtained from each fractionation. The pooled fractions were adjusted to pH 4·6 at 4 °C by careful addition of 2 M-HCl and held at 20 °C for 1 h to ensure complete precipitation of the casein, and centrifuged at 34000 *g* for 15 min at 20 °C. The supernatants were decanted and concentrated (Korn, 1955) by dialysis overnight at 4 °C against a saturated solution of polyethylene glycol ('Carbowax' 20 M, made by Union Carbide and supplied by G. T. Gurr & Co., London) and adjusted to a final volume of 2 ml with 0·1 M phosphate buffer of pH 7·5. The casein precipitates were dissolved in 1 ml of the same buffer. Approximately 1 h before electrophoresis, 0·65 g urea and 0·08 ml mercaptoethanol/ml were added to the samples. Following electrophoresis the protein bands were located by staining with Amido black and quantified by means of a Chromoscan densitometer (Joyce, Lobel & Co. Ltd., London), using the following instrument settings: aperture, 1 mm diam.; specimen/drive gear ratio, 1:3; cam no. 5–077/C; filter, 620.

Dialysis. Visking dialysis tubing (size 18/32 in.) was used throughout the investigation.

RESULTS

Inhibition of lipase

Up to 70% of the lipase activity was lost when 10–100 units of pancreatic lipase dissolved in 0·75 M-NaCl containing 0·025 M-$MgCl_2$ or in synthetic milk serum were added to any of the following: whole milk, skim-milk, skim-milk containing 0·75 M-NaCl, colloidal phosphate-free milk, or to high speed supernatants prepared in the presence or absence of NaCl. A further 30% of the residual lipase activity was sedimented together with the casein micelles when whole milk or the skim-milk preparations were centrifuged at 34000 × *g* for 2 h at 5 °C. In contrast, addition of the same quantity of pancreatic lipase to 0·75 M-NaCl containing 0·025 M-$MgCl_2$, to synthetic milk serum, to milk dialysate or to solutions of phosvitin (1–20 mg in synthetic milk serum) resulted in no reduction in activity and, furthermore, none of the lipase was sedimented on centrifugation under the above conditions.

Gel-filtration on Sephadex G-200

Similar protein elution diagrams were obtained by fractionation of 4 ml of skim-milk (Fig. 1*b*) or of colloidal phosphate-free milk (Fig. 1*c*) on Sephadex G-200 columns at 5 °C. With both preparations, a large peak containing casein micelles or complexes of molecular weight > 10^6 (Downey & Andrews, 1965*a*) was eluted at

the void volume and followed by 2 or 3 ill-defined peaks. The first of these peaks (V_e 135 ml) contained milk globulins as well as β-casein aggregates of mol. wt. *ca.* 200000 (Downey & Andrews, 1966). With colloidal phosphate-free milk this peak

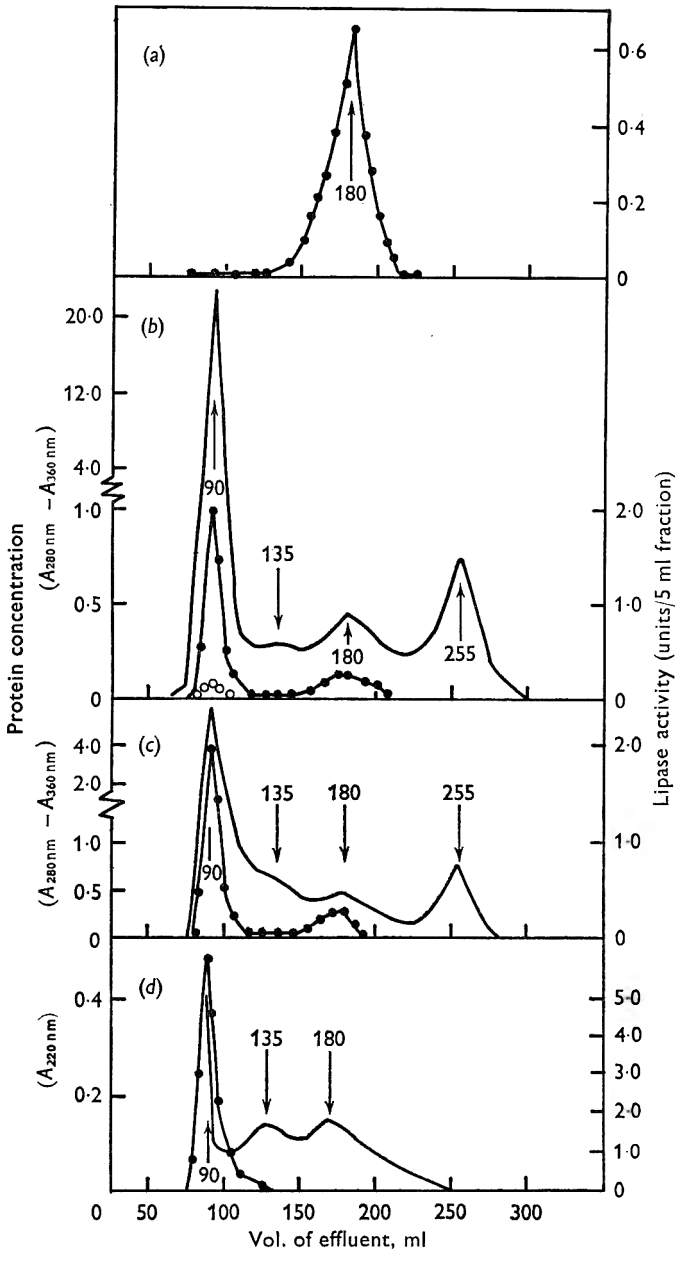


Fig. 1. Gel-filtration on Sephadex G-200 columns equilibrated with synthetic milk serum, of pancreatic lipase ●—●: (*a*) alone; (*b*) in the presence of 4 ml skim-milk; (*c*) in the presence of 4 ml colloidal phosphate-free milk; (*d*) in the presence of phosvitin (10 mg in 4 ml synthetic milk serum). ——, Protein content of fractions; ○—○, endogeneous milk lipase activity. The elution patterns of endogenous milk lipase and pancreatic lipase which had been admixed with skim-milk were determined in separate fractionations, and the results are superimposed here to facilitate comparison.

was slightly larger (Fig. 1*c*). The subsequent peaks obtained with both milk preparations contained β-lactoglobulin and α-lactalbumin (V_e 180 ml) and low molecular weight dialysable material (V_e 255 ml), respectively. Because of turbidity the optical density of the peak eluted at the void volume on fractionation of skim-milk (Fig. 1*b*) was higher than that of the corresponding peak obtained with colloidal phosphate-free milk (Fig. 1*c*). However, as was shown by nitrogen analysis, both peaks contained the same amount of protein. On gel-filtration on Sephadex G-200 (Fig. 1*a*) of the pancreatic lipase preparation which had been diluted 50-fold with synthetic milk serum (30 units of lipase activity in 4 ml), the enzyme was eluted as a single peak (V_e 180 ml) corresponding to a molecular weight of 42000 (Downey & Andrews, 1965*a*) with a recovery of 20 %. The intrinsic lipase activity of skim-milk (2·0 units of lipase in 4 ml) was eluted together with the casein micelles at the void volume (V_e 90 ml, Fig. 1*b*) of Sephadex G-200. Little or no lipase activity was detectable in colloidal phosphate-free milk. On gel-filtration of pancreatic lipase (25 units in 0·5 ml of synthetic milk serum) that had been mixed with 4 ml of skim-milk containing 2·0 units of lipase (Fig. 1*b*) or with colloidal phosphate-free milk (Fig 1*c*), over 90 % of the eluted lipase emerged at the void volume (V_e 90 ml) with the casein micelles or complexes, and was followed by a minor peak of lipase activity (V_e 180 ml) in the expected position for free pancreatic lipase. Approximately 25 % of the added pancreatic lipase was recovered in the effluent fractions. When pancreatic lipase (32 units in 0·5 ml of synthetic milk serum) was mixed for 5 min at 5 °C with a solution containing 10 mg phosvitin in 4 ml of synthetic milk serum, the enzyme was eluted from Sephadex G-200 (Fig. 1*d*) as a sharp peak at the void volume with a recovery of 70 %.

Gel-filtration on Sepharose 2B

Two well-separated protein peaks (V_e 80 and 235 ml, respectively) were obtained on fractionation of skim-milk on Sepharose 2B columns (Fig. 2*b*). The major peak (V_e 80 ml) emerged at the void volume and contained α_{s1}-, α_{s2}, α_{s3}, β- and κ-caseins (Fig. 3*a*). The whey proteins (Fig. 3*c*) together with β-casein and traces of α_s-casein (Fig. 3*a*) were eluted in the second peak (V_e 235 ml). The elution volumes of thyroglobulin, γ-globulin and cytochrome *c* (see Table 1), which were incorporated in some milk samples before fractionation, were 190 ml, 220 ml, and 235 ml, respectively (Fig. 2*b*).

Pig pancreatic lipase (30 units in 4 ml of synthetic milk serum) was eluted as a single peak (V_e 235 ml, recovery 25 %) from Sepharose 2B columns (Fig. 2*a*). The intrinsic lipase of skim-milk was eluted (Fig. 2*b*) with the casein micelles at the void volume of Sepharose 2B, with recovery of 20 %. On the other hand, pancreatic lipase (28 units in 0·5 ml synthetic milk serum) that had been added to 4 ml skim-milk at 5 °C approximately 5 min before fractionation was eluted (Fig. 2*b*) as 3 well-defined peaks at V_e 80, 170 and 235 ml, respectively, each of which contained more lipase activity than could be attributed to the intrinsic milk lipase. Recovery of the added lipase was 33 %. The first lipase peak (V_e 80 ml) emerged at the void volume and would appear to be casein micelle-bound lipase, while the third lipase peak (V_e 235 ml) was eluted with the whey proteins in the expected position for free pancreatic lipase (Fig. 2*a*). The middle lipase peak (V_e 170 ml) was eluted approximately 20 ml before thyroglobulin (V_e 190 ml) and generally contained less detectable lipase

activity than the other 2 enzyme peaks and its elution volume corresponded to the trough between the 2 protein peaks.

The proteins in 4 ml colloidal phosphate-free milk were fractionated into 3 well-defined peaks on Sepharose 2B columns (Fig. 2*c*). A relatively small peak emerged at the void volume. Its protein content was too low to permit electrophoretic identification, but it did appear to contain some residual micellar casein. The height of this peak ($A^{1\,cm}_{280\,nm} - A^{1\,cm}_{360\,nm}$) varied about 2-fold between different samples of colloidal

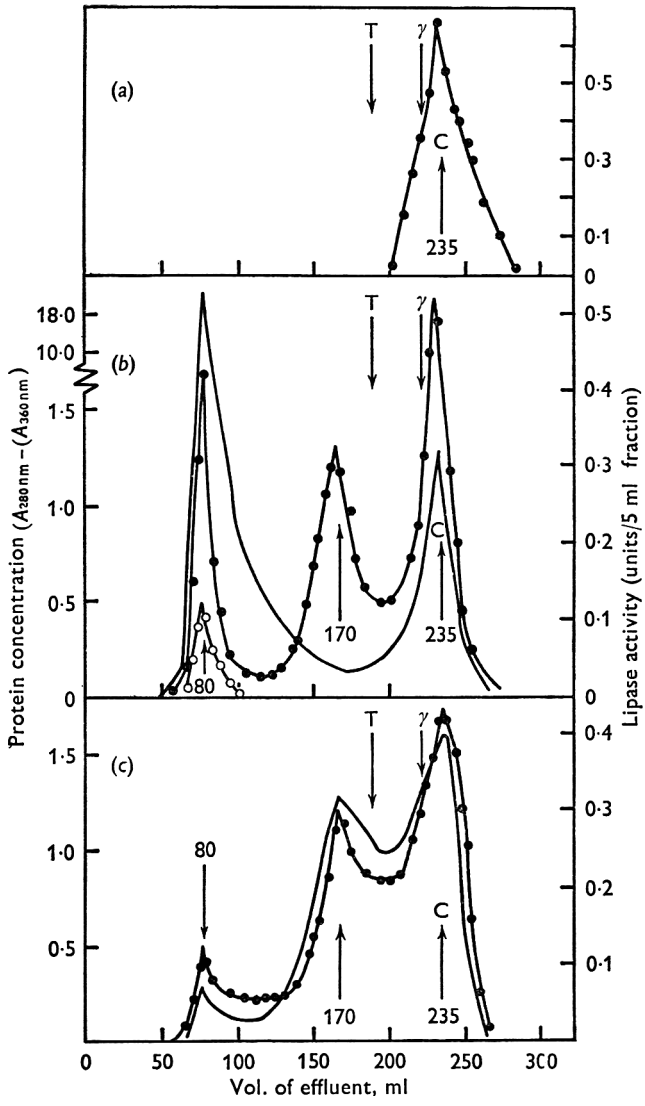


Fig. 2. Gel-filtration on Sepharose 2B columns equilibrated with synthetic milk serum, of pancreatic lipase ●—●: (*a*) alone; (*b*) in the presence of 4 ml skim-milk; (*c*) in the presence of 4 ml colloidal phosphate-free milk. ——, Protein content of the fractions; ○—○, endogenous milk lipase activity. T, γ and C indicate the elution volumes of thyroglobulin, γ-globulin and cytochrome *c*, respectively, (see Table 1) used as markers. The elution patterns of endogenous milk lipase and pancreatic lipase which had been admixed with skim-milk were determined in separate fractionations and the results are superimposed here to facilitate comparison.

phosphate-free milk prepared from milk of the same cow, but in general was less than 0·5. Most of the protein was eluted as 2 overlapping peaks of V_e 170 and 235 ml, respectively, the first of which emerged approximately 20 ml before thyroglobulin and contained all the major casein fractions (Fig. 3*b*), apparently in aggregated form. The final protein peak (V_e 235 ml) was similar in composition (Fig. 3*b*, *c*) to the second protein peak obtained with skim-milk, but contained more α-casein.

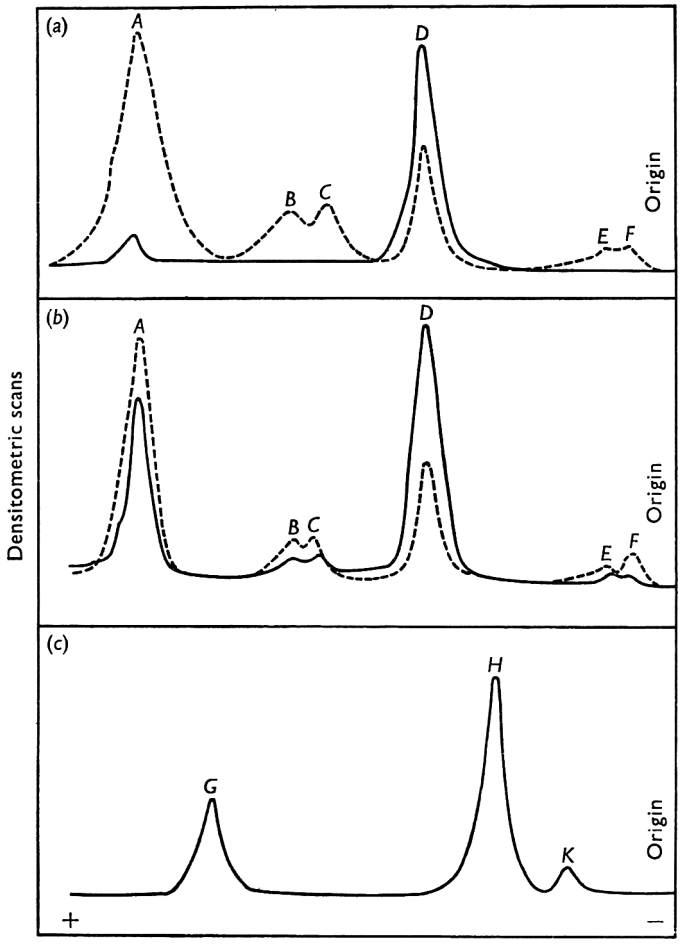


Fig. 3. Electrophoretic analysis of the proteins eluted from Sepharose 2B columns (see Fig. 2*b*, *c*) on gel-filtration of skim- and colloidal phosphate-free milks. (*a*), Casein in skim-milk; (*b*), caseins in colloidal phosphate-free milk; (*c*), whey proteins in both milk preparations. —, Proteins eluted in the final peaks (V_e 235 ml) of Fig. 2*b*, *c*; - - -, proteins eluted at the void volume (V_e 80 ml, Fig. 2*b*) and at 170 ml (Fig. 2*c*). *A*, α_{s1}-casein *B*; *B*, α_{s2}-casein; *C*, α_{s3}-casein; *D*, β-casein *B*; *E*, κ-casein *A*; *F*, κ-casein *B*; *G*, α-lactalbumin; *H*, β-lactoglobulin *A*; *K*, serum albumin.

Pancreatic lipase (25 units in 0·5 ml of synthetic milk serum) which had been mixed with 4 ml colloidal phosphate-free milk was fractionated (Fig. 2*c*) on Sepharose 2B columns into 3 peaks, of V_e 80, 170 and 235 ml, respectively, which were eluted in the same positions as the 3 protein peaks. The recovery of lipase was 20%. While the elution volumes of the lipase peaks in Fig. 2*b* and *c* were similar, the relative

sizes of the enzyme peaks obtained with the 2 milk preparations were different. In particular, the lipase peak eluted at the void volume on fractionation of colloidal phosphate-free milk containing pancreatic lipase (Fig. 2*c*) was relatively small, and most of the enzyme was detected in the expected position (V_e 235 ml) for free pancreatic lipase. The other lipase peak (V_e 170 ml) was eluted with the casein complexes. Furthermore, the relative sizes of the 3 enzyme peaks varied slightly with different samples of colloidal phosphate-free milk: the magnitude of the lipase peak at the void volume (Fig. 2*c*) was markedly influenced by the content of residual casein micelles in the milk preparation.

The recoveries of lipase activity in the experiments with both Sephadex G-200 and Sepharose 2B were lower than those previously reported (Downey & Andrews, 1965*a*). However, in the present investigation a considerable amount of activity was lost because of the delay involved in dialysing the fractions before assay.

DISCUSSION

Size of casein micelles and sub-units

The protein elution patterns obtained on fractionation of skim-milk on Sephadex G-200 is markedly different from that reported by Yaguchi & Tarassuk (1967). Considerable disintegration of the casein micelles appears to have occurred during gel-filtration under the conditions employed by these workers, as evidenced by the large quantities of both α_s- and β-casein aggregates in the fractions eluted subsequent to the casein micelle peak. In contrast, the synthetic milk serum used as column eluent in the present investigation appears to have preserved the integrity of the casein micelles during gel-filtration.

Based on the fractionation of dextrans, the molecular weight exclusion limit of Sepharose 2B is stated by the manufacturers to be approximately $2{\cdot}5 \times 10^7$. As with Sephadex G-200, the upper exclusion limit for globular proteins is likely to be about 4 times higher. Consequently, the molecular weights of the casein micelles in skim-milk appear to exceed 10^8, in agreement with the results obtained from electron microscopy by Nitschmann (1949) and Carroll, Thompson & Nutting (1968), who showed that approximately 80% of the casein micelles of milk had particle sizes in the molecular weight range 10^7–10^9. In contrast, over 95% of the casein of colloidal phosphate-free milk (Fig. 2*c*) appears to exist as complexes which are considerably smaller than the casein micelles. Since most of the casein of colloidal phosphate-free milk is eluted at the void volume of Sephadex G-200, the molecular weights of these casein complexes appear to be $< 10^6$, which is the upper exclusion limit of Sephadex-G-200 (Andrews, 1965). The relative elution volumes on Sepharose 2B (Fig. 2*c*) of thyroglobulin (V_e 190 ml) which has an apparent molecular weight as judged by gel-filtration of $1{\cdot}33 \times 10^6$ (Andrews, 1965), and of the casein complexes present in colloidal phosphate-free milk (V_e 170 ml), suggest that the molecular weight average of the latter may be about 2×10^6. Lack of suitable macromolecules of known molecular weight makes impracticable a more precise estimate of size. However, Morr (1967) suggests that on removal of colloidal phosphate the casein micelles of skim-milk are disaggregated to casein complexes of diameter less than 30 mμ. Based on the results of Nitschmann (1949), this suggests a molecular weight of less than 8×10^6. Since the

removal of colloidal phosphate from skim-milk results in disintegration of the casein micelles to smaller complexes, casein micelles (mol. wt. $> 10^8$) appear to be composed of sub-units of mol. wt. *ca.* 2×10^6, containing α_s-, β- and κ-casein which are bonded together in the micelle by the colloidal phosphate. This hypothesis accords with the models for casein micelles recently proposed by Morr (1967) and Rose (1969). These sub-units of the micelle are considerably larger than the spherical complexes of α_s-, β- and κ-casein (mol. wt. *ca.* 300000), which Shimmin & Hill (1964) consider to be the building units of casein micelles. However, the experimental conditions employed by the latter investigators were more severe than those used in the present study.

Lipase-casein interactions

The elution of pancreatic lipase with the casein micelles of skim-milk (Fig. 1*b*) or the soluble casein complexes of colloidal phosphate-free milk (Fig. 1*c*) indicates that the loss of enzyme activity of lipase added to the various milk preparations is due to binding of the enzyme to the casein micelles or complexes. Based on ultracentrifugal studies at pH 8·5 in Tris-maleate buffer, Shahani & Chandan (1965) also report that the inhibition of purified clarifier slime lipase (Chandan & Shahani, 1963) by β-casein, appears to result from the formation of a complex with the enzyme. It is suggested that the relatively large casein micelles (mol. wt. $> 10^8$), or complexes (mol. wt. *ca.* 2×10^6), to which the lipase binds inhibit enzyme activity by impairing the accessibility of substrate to enzyme so that adsorption of the lipase at the fat globule interface, which appears to be a prerequisite to lipolysis (cf. Desnuelle, 1961), is hindered. The marked affinity of pancreatic lipase for casein complexes and particularly for phosvitin, in contrast to the various whey proteins of milk, suggests that esterified phosphate groups of casein may be involved in the binding of lipase.

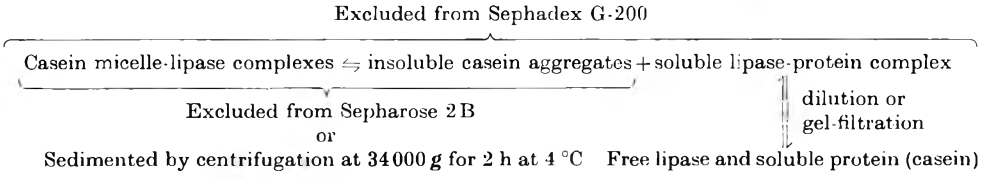
The presence of a lipase peak (Fig. 2*b*) in a position (V_e 170 ml) intermediate between that of the casein micelle-bound lipase (V_e 80 ml) and that of the free enzyme (V_e 235 ml) indicates that in addition to binding to casein micelles the pancreatic lipase added to the skim-milk also formed a complex with soluble protein. The nature of the soluble protein associated with the lipase is uncertain. However, it does appear to be casein, small amounts of which were eluted with the lipase. Furthermore, on fractionation of pancreatic lipase in the presence of a milk preparation, from which all casein had been removed, only free lipase was eluted from Sephadex G-200 (Downey & Andrews, 1966). The relative elution volumes on Sepharose 2B (Fig. 2*b*) of the soluble lipase-casein complex and thyroglobulin (V_e 170 ml) indicate that the molecular weight of the complex is about 2×10^6. The principal complex formed (V_e 80 ml) on addition of pancreatic lipase to colloidal phosphate-free milk (Fig. 2*c*) is of similar molecular size.

The comparable losses in enzyme activity obtained on addition of pancreatic lipase to both skim-milk and colloidal phosphate-free milk, together with the relatively small amounts of free pancreatic lipase obtained on gel-filtration of both milk preparations of Sephadex G-200 (Fig. 1*b*, c), indicates that colloidal calcium phosphate is not required for binding of lipase to casein. Furthermore, contrary to the suggestion of Downey & Andrews (1965*b*) the casein need not be present in micellar form for association with lipase to take place. While removal of colloidal calcium

phosphate from skim-milk resulted in a marked increase in the quantities of casein eluted in the same fractions as the soluble lipase-casein complex the heights of the latter enzyme peaks (V_e 170 ml) were similar with both milk preparations (Fig. 2*b*, *c*, respectively). However, since the casein-bound lipase appears to be considerably less active than the free enzyme (Downey & Andrews, 1966; Gaffney, Harper & Gould, 1968), more lipase is likely to be present in the fractions (V_e 170 ml) containing the α_s-, β-, κ-casein complexes of colloidal phosphate-free milk than is apparent from the elution diagram (Fig. 2*c*).

The relationship of the soluble lipase-protein complex (mol. wt. *ca.* 2×10^6) to the non-sedimentable lipase-protein complexes (mol. wt. *ca.* 350000) detected in high speed supernatants from skim-milk (Downey & Andrews, 1966) is difficult to decide since the 2 complexes were detected when different milk preparations were fractionated under different experimental conditions. However, the dissociation of both complexes to yield free lipases appears to be influenced markedly by the casein micelle content of the preparations. Downey & Andrews (1966, 1969) noted that as the casein micelle content of high speed supernatants prepared from skim-milk was decreased by prolonged centrifugation, the tendency for the non-sedimentable lipase-protein complexes to dissociate during subsequent gel-filtration was enhanced. A similar phenomenon may account for the relatively larger peak of free lipase (V_e 235 ml) obtained during the gel-filtration experiments on Sepharose 2B columns (Fig. 2*b*, *c*), compared with that obtained as a result of comparable fractionations on Sephadex G-200 (Fig. 1*b*, *c*). On Sepharose 2B columns the casein micelles are rapidly separated from the soluble lipase-protein complex. In contrast, both the casein micelles and the soluble lipase-protein complex pass through Sephadex G-200 together.

Scheme 1. *Suggested equilibrium between soluble and casein micelle-bound lipase*



The suggestion by Noble & Waugh (1965) and Waugh & Noble (1965) that some of the micellar casein is in equilibrium with casein in solution provides a possible explanation for the manner in which the presence of casein micelles affects the dissociation of lipase-protein complexes. Since pancreatic lipase apparently binds both to micellar and soluble casein, the enzyme is presumably involved in this equilibrium. If the micelle-lipase complex equilibrates with the soluble lipase-protein complex only, removal of the former either by sedimentation or gel-filtration should result in aggregation of the soluble lipase-protein complex so as to restore equilibrium. However, as shown by gel-filtration on Sepharose 2B (Fig. 2*b*, *c*) and by ultracentrifugation studies (Downey & Andrews, 1966, 1969), this did not occur. The present results are better explained if micelle-lipase complexes are in equilibrium with insoluble casein aggregates plus a much smaller amount of soluble casein to which some lipase is bound (Scheme 1). When both the micelle-lipase complexes and the insoluble casein

aggregates are removed together, the equilibrium concentration of the soluble lipase-protein complex is retained. The complex then dissociates to yield free lipase during gel-filtration. Andrews (1964) suggested that gel-filtration enhanced the dissociation of certain proteins into smaller sub-units. The sharp separation obtained between the soluble lipase-protein complex and the free lipase is difficult to explain, unless dissociation of the lipase from soluble protein occurs mainly during the initial stages of gel-filtration on Sepharose 2B. However, it appears that all the lipase is not dissociated from the soluble protein during gel-filtration.

Since the dissociation during gel-filtration (Downey & Andrews, 1966) of the intrinsic milk lipases from non-sedimentable lipase-protein complexes of mol. wt. 350000 appears to be similarly affected by the presence of micellar casein, it is suggested that milk lipases may be involved in an analogous equilibrium between micellar and soluble casein. The position of the equilibrium would not appear to favour the existence of free lipase since very little free enzyme was obtained on gel-filtration, using G-200, of skim-milk or skim-milk containing pancreatic lipase. Consequently, it is not surprising that the lipases of milk are largely associated with casein. The reduced activity of milk lipases when bound to casein (Downey & Andrews, 1966; Gaffney *et al.* 1968) may be due to impaired accessibility of enzyme and substrate, similar to that previously discussed for pancreatic lipase.

With the recent purification of a highly active milk lipase (Fox & Tarassuk, 1968) the possibility now exists of investigating the relative affinity of milk lipases for individual caseins as well as casein complexes and micelles, under conditions similar to these prevailing in milk. In this connexion it is interesting to note that the purified lipase is not inhibited at pH 8·5 by skim-milk or isoelectric casein (Patel, Fox & Tarassuk, 1968) and so may not bind to casein micelles or complexes. However, because of the reported (Fox & Tarassuk, 1968) association of the lipase activity with κ-casein during the initial stages of purification, coupled with the marked similarity in its molecular weight to that of lipase-protein complexes (Downey & Andrews, 1966), it would be desirable to ensure that the purified lipase is not an aggregate of lower molecular weight lipases (Downey & Andrews, 1969) and in particular that the enzyme is not complexed with some residual casein.

We thank Dr P. Andrews, Dr T. C. A. McGann and Dr A. C. O'Sullivan for helpful discussions and criticism and Mr S. A. Aherne and Mr R. D. Kearney for skilled technical assistance.

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A technique for studying the build-up and prevention of milk film on hard surfaces

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Summary. A laboratory technique using glass and stainless steel slides has been developed to study the build-up of milk film on hard surfaces and its removal with detergent-disinfectant mixtures. Soiling and cleansing treatments were of similar duration to those used in practice but were repeated 6 or 7 times a day for 4 days to simulate 12 or 14 days' milking and washing procedures in the farm dairy; no mechanical assistance was used with the cleaning solutions. Soiling was followed immediately by a pre-rinse, wash and final rinse, allowing a short period for drying before further re-soiling. The technique eventually developed involved treatments repeated 18 times, using solutions at 15·5 °C. The weight of milk film and its content of fat, mineral and 'protein' were determined.

Under the conditions of the experiments the following were observed.

(1) The deposit left by all treatments consisted mainly of fat with a smaller proportion of protein and still less of mineral matter.

(2) Glass accumulated slightly less deposit than did stainless steel with an aqua-blast finish.

(3) Removal of milk film was more complete when sodium hypochlorite was added to certain detergent solutions than when the materials were used separately.

(4) With the treatments studied, the use of the detergent solution from a previous treatment as a pre-rinse did not result in a reduction in the residual film.

(5) A final rinse with 0·1 % phosphoric acid virtually eliminated the mineral matter from the residual film.

(6) The substitution of a warm (46 °C) for a cold (15·5 °C) pre-rinse resulted in a reduction of 40 % in residual film and the further use of a warm final rinse effected a reduction of 64 %.

(7) The addition of 0·05 % wetting agent (Lissapol NDB, I.C.I. Ltd.) to the pre-rinse effected only marginal improvement in the treatments studied.

(8) Omission of the pre-rinse and corresponding lengthening of the detergent treatment had no consistent effect.

Of the many factors which influence chemical disinfection of hard surfaces in the food and beverage industries, the presence of film on the product-contact surface can be one of the most important. This film, which usually consists of residues of the pro-

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duct being handled together with traces of minerals from water used in cleaning, may provide a protective barrier against chemical disinfection for micro-organisms and may permit their proliferation. The chemical and physical properties of such films will obviously vary, but it is convenient to consider 2 general groups, the soft film which can readily be removed by mechanical action and the hard film or 'stone' which cannot. The soft film is mainly the result of inefficient cleaning whereas the hard film is generally caused by an incompatibility between the detergent and the water used for cleaning. Thus, the means of removal and of prevention of the 2 types of film are different, but their effects on the bacteriological quality of the product may not be dissimilar. The present paper describes studies on the soft film which is easily induced in the laboratory.

Most work on film removal has been devoted either to the removal of film from equipment in the field once it has been built up, or to the application in the laboratory of a film which is subsequently removed. Recently, a laboratory milking simulator which reproduces in 2 days the effects of milking and cleaning on the farm for 4 weeks (Whittlestone, Fell, Calder & Galvin, 1963) has been used for evaluating commercial detergents (Whittlestone & Twomey, 1966) but as far as we are aware the results have not been published. The present study involved a repeated cycle of soiling with fresh raw bulk milk and cleaning with various detergent-disinfectant mixtures to simulate repeated milking and cleaning under controlled conditions, with the aim of developing a method for observing the build-up and prevention of milk film using different detergent and disinfectant treatments.

MATERIALS AND METHODS

Soiling

Resistance glass slides (3 × 1 in.) with ground edges (Chance Bros. Ltd, Birmingham) and stainless steel strips of similar size with an aquablast finish (surface blasted with small glass beads in a jet of water) were washed, immersed in 25 % (v/v) nitric acid for 30 min, rinsed in tap and distilled water, dried for 30 min at 37 °C and weighed. Six slides, suspended from a rod by means of 'paper clips' specially made from stainless steel wire, were soiled by immersing them in fresh raw bulk milk for 30 min. The milk was contained in a round glass tank placed on a combined hot plate and magnetic stirrer (J. W. Towers & Co. Ltd, Widnes) by means of which the milk was maintained at 33 ± 1 °C and was slowly agitated to prevent the fat from rising. Four sets of 6 slides were soiled simultaneously and the milk was used for 3 soilings before being discarded.

Washing

Washing and rinsing solutions were freshly prepared immediately before use, with tap water. The solutions were dispensed in 600-ml beakers in each of which 6 slides were suspended. The cleaning treatment was carried out without any mechanical agitation.

The detergents and disinfectants and the concentrations at which they were used were: (*a*) anhydrous sodium carbonate (AR), 0·25 % (w/v); (*b*) a mixture of (*a*) and Calgon (sodium hexametaphosphate, Albright & Wilson Mfg Ltd), 4 parts Na_2CO_3 and 1 part Calgon, 0·25 % (w/v); (*c*) Teepol (Shell Chemicals Ltd), sodium salt of a

secondary alkyl sulphate, 0·25 % (v/v); (*d*) an iodophor detergent-disinfectant (Iodogen, Pinkstone Ltd, Bristol, 1), 0·31 % (v/v); (*e*) sodium hypochlorite (Chloros Agricultural Grade, I.C.I. Ltd), 200 ppm available chlorine; (*f*) Lissapol NDB (I.C.I. Ltd), a propylene oxide-ethylene oxide condensate, 0·05 % (v/v).

The water used in all experiments was from a local borehole and had a total hardness of 200–210 ppm, expressed as $CaCO_3$, all of which was alkaline (temporary) hardness.

Immediately after the soiling period each set of slides was drained for 10 s, immersed in the rinsing solution for a determined period, drained for 10 s, immersed in the washing solution for a set period at 46 ± 1·5 °C (unless otherwise stated), drained for 10 s, immersed in a rinsing solution for a set time, drained and dried at 37 °C for 15 min.

Slides were soiled and washed several times (7 in the first and 6 in later experiments) in a day and the deposited soil determined by weighing after drying for 30 min in a forced air-circulation incubator at 37 °C. Higher temperatures were not used for drying because of the possibility of physical alteration of the soiled surface. Soiling was usually continued for 4 days, simulating 24 or 28 milkings.

Examination of deposit

At the end of the soiling and washing treatments the deposit was analysed for fat, mineral matter and 'protein'. Fat was determined by extracting the film with diethyl ether for 1 h at room temperature, and drying and re-weighing the slide. Mineral matter was estimated by loss in weight following treatment with either 2 % sulphamic acid or 2 % phosphoric acid for 1 h at room temperature. Treatment in a 1 % (approx.) solution of sodium hypochlorite for 1 h at room temperature removed the residue, which was presumably proteinaceous material. It was observed that 5 % NaOH, either cold or at 70 °C, was much less effective in removing this residue. The above 3 treatments removed all the deposit from the slides and weights of slides so treated were normally found to be within 0·5 mg of those of the original untreated slides.

Preliminary experiments are detailed here, because the results clearly showed differences between some detergent treatments and also indicated those factors which most influenced the accuracy of the technique, thus enabling the precision to be improved.

EXPERIMENTS

1. *Rate and type of milk film accumulation.* The basic treatment, repeated 28 times, consisted of a cold pre-rinse for 1 min, a wash at 46 °C for 2 min and a cold final rinse for 1 min. The solutions used for treatments 1–4 were: 1, water, Na_2CO_3, NaOCl; 2, water, Na_2CO_3 + NaOCl, water; 3, water, Teepol, NaOCl; 4, water, Teepol + NaOCl, water.

2. *Extended soiling and treatment.* Treatment 2 was used 48 times in this experiment but the times for the pre-rinse, wash and final rinse were extended to 2, 4 and 2 min, respectively.

3. *Effect of variations in treatment.* The basic treatment, repeated 24 times, was a cold pre-rinse for 2 min, a wash at 46 °C for 4 min and a final cold rinse for 2 min. The

solutions used for treatments 5–11 were: 5, water, Na_2CO_3, NaOCl; 6, water, Na_2CO_3 + NaOCl, water; 7, water, Na_2CO_3 + Calgon, NaOCl; 8, water, Na_2CO_3 + Calgon + NaOCl, water; 9, wash from 7, Na_2CO_3 + Calgon, NaOCl; 10, wash from 8, Na_2CO_3 + Calgon + NaOCl, water; 11, water, Na_2CO_3 + NaOCl, 0·1 % H_3PO_4.

4. *Reproducibility of the test and the effects of heat and of additives to the pre- and final rinses.* The basic treatment, repeated 24 times, was a pre-rinse for 2 min, a wash at 46 °C for 4 min and a final rinse for 2 min. The wash solution was the same in all the treatments, namely Na_2CO_3 + Calgon + NaOCl; the solutions used for the pre- and final rinses of treatments 12–18 were: 12, cold water, wash, cold water; 13, warm water, wash, cold water; 14, warm water, wash, warm water; 15, warm water, wash, cold 0·1 % H_3PO_4; 16, warm water, wash, warm 0·1 % H_3PO_4; 17, cold 0·05 % Lissapol NDB, wash, warm 0·1 % H_3PO_4; 18, warm 0·05 % Lissapol NDB, wash, warm 0·1 % H_3PO_4.

5. *Influence of temperature of solution on film retention.* After soiling and drying 8 times slides were immersed in water alone at controlled temperatures in the range 15·5–52 deg C. Six slides were immersed at each temperature and removed successively at intervals of 1 min.

6. *Variations in washing treatments at* 15·5 °C. The basic treatment, repeated 24 times, was a cold pre-rinse for 2 min, a cold wash for 4 min and a cold final rinse for 2 min. The solutions used for treatments 19–26 were: 19, water, Na_2CO_3, NaOCl; 20, water, Na_2CO_3 + NaOCl, water; 21, water, Teepol, NaOCl; 22, water, Teepol + NaOCl, water; 23, water, iodophor, water; 24, no pre-rinse, Na_2CO_3 + NaOCl (6 min), water; 25, no pre-rinse, Teepol + NaOCl (6 min), water; 26, no pre-rinse, iodophor (6 min), water.

RESULTS

Rate and type of milk film accumulation

In the first experiment a comparison was made of treatments with Na_2CO_3 or with Teepol, both in conjunction with sodium hypochlorite (p. 63, treatments 1–4). The 24 slides, 6 for each treatment, were soiled and washed 7 times on each of 4 successive days. At the end of each day's treatments the slides were dried, weighed and stored at room temperature overnight.

The progressive accumulation of milk film on the slides following the soiling-washing treatments is shown in Fig. 1 and the composition of the deposited film in Fig. 2. The lines in Fig. 1 were drawn by inspection.

Figs 1 and 2 show the greater efficiency of the Teepol solutions in reducing build-up of film and removing fat. However, perhaps of more importance, and shown clearly in Fig. 1, the combination of hypochlorite with both detergents was consistently more effective than the separate treatments. This phenomenon is considered more fully later.

Because the rinsing and washing treatments were without mechanical agitation so much film was built up that the detergent solutions may not have had sufficient time to act and thus demonstrate fully any differences in effectiveness. Accordingly, in the later experiments the times allowed for the pre-rinse, detergent treatment and final rinse were increased from 1, 2 and 1 min to 2, 4 and 2 min, respectively.

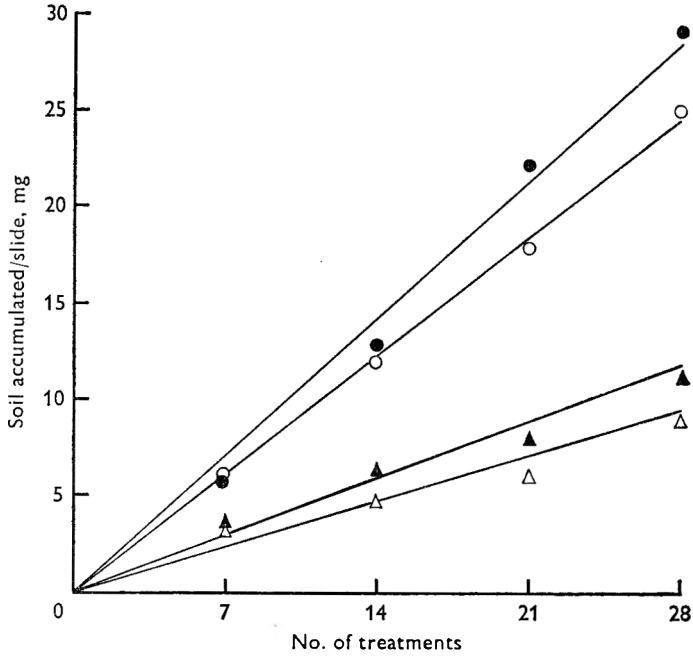

Fig. 1. Rate of accumulation of residual soil on slides during repeated soiling and various cleaning treatments (expt 1). *Basic treatment*: pre-rinse (1 min, cold); wash (2 min, 46 °C); final rinse (1 min, cold). *Treatment details*: ●, water, Na_2CO_3, NaOCl; ○ water, Na_2CO_3 + NaOCl, water; ▲, water, Teepol, NaOCl; △, water, Teepol + NaOCl, water.

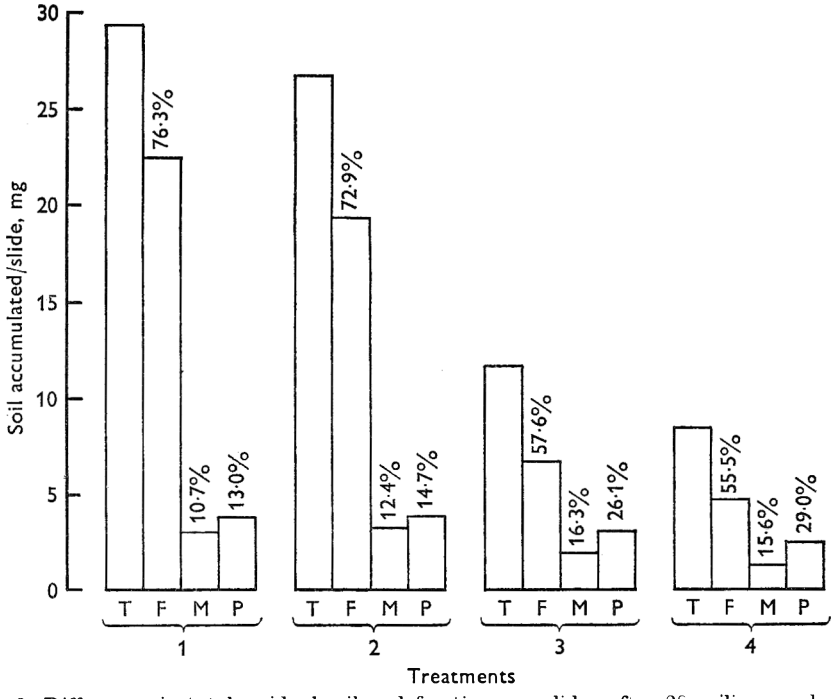

Fig. 2. Differences in total residual soil and fractions on slides after 28 soilings and various cleaning treatments (expt 1). *T*, total soil; *F*, fat; *M*, mineral; *P*, protein. *Basic treatment*: pre-rinse (1 min, cold); wash (2 min, 46 °C); final rinse (1 min, cold). *Details*: (1) water, Na_2CO_3, NaOCl; (2) water, Na_2CO_3 + NaOCl, water; (3) water, Teepol, NaOCl; (4) water, Teepol + NaOCl, water.

Extended soiling and treatment

In a second experiment the rate of accumulation of the milk film over a longer period was examined. One stainless steel slide with a softened descaled finish and one glass slide were used with a treatment similar to treatment 2, p. 63, except that the longer times of treatment mentioned above were used. Fig. 3 shows that the curves relating the accumulation of soil to numbers of treatments are not simple. The

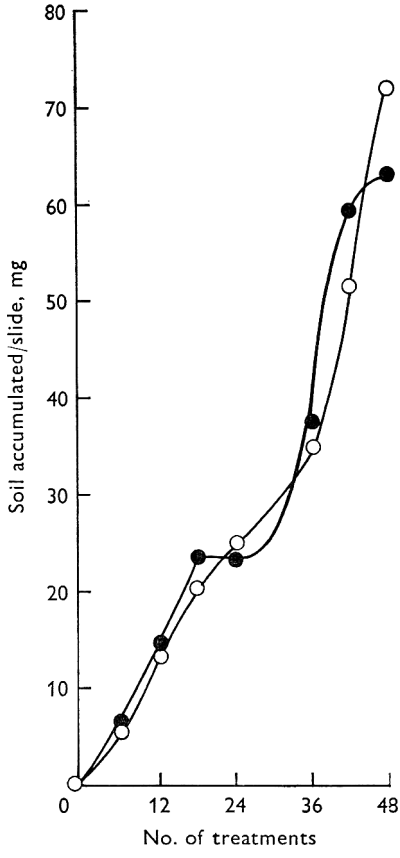

Fig. 3. Rate of accumulation of residual soil on slides during repeated soiling and treatment with $Na_2CO_3 + NaOCl$ (expt 2). ○, glass; ●, stainless steel.

'step-wise' increase of milk film was no artifact, as on the surface of the slides there could be observed firstly a 'crazing' of the film and then a gradual flaking off with simultaneous deposition on the freshly exposed surfaces. Therefore, it was considered necessary to continue to determine the weight of deposit at intermediate stages lest a crossing over of curves, as shown in Fig. 3, should obscure the true results.

Effect of variations in treatment

In a further experiment a study was made of the effect of (*a*) the addition of Calgon, a water softener, to the Na_2CO_3, (*b*) a repeat of the addition of NaOCl either

to the detergent solution or separately to the final rinse, and (*c*) the addition of phosphoric acid to the final rinse. Along with the treatments detailed on p. 64, glass slides were compared with stainless steel slides having an aquablast finish. A further modification to the technique was that the number of soilings/day was reduced from 7 to 6.

The slides were weighed after every 6 soiling-washing treatments. The between-treatment differences in the weight of residual soil, at first small, became greater with increasing numbers of treatments. Fig. 4 shows the following effects.

(*a*) The accumulation of residues on glass was in general less than that on stainless steel. However, in the comparisons of detergent-disinfectant treatments, both glass and stainless steel slides are considered together.

(*b*) One of the results of the previous experiment was confirmed, namely that NaOCl added to Na_2CO_3 in the 'detergent wash' (treatment 6) was more effective than the 2 materials acting separately (treatment 5) and the weight of residual soil was about 40 % less than that resulting from the separate treatments, although the composition of the deposits was similar.

(*c*) A similar effect was noted when NaOCl was added to the Calgon-Na_2CO_3 detergent (treatment 7 *vs.* 8) and the deposit resulting from treatment 8 was about 30 % less than that from treatment 7. As might be expected, adding Calgon to the Na_2CO_3 effected a reduction in residual soil (amounting to about 10 %) as compared with Na_2CO_3 alone (treatments 7 and 8 *vs.* 5 and 6).

(*d*) The re-use of the detergent from treatments 7 and 8 as a pre-rinse in treatments 9 and 10 proved to be a detriment rather than an advantage, showing an increase of 25 % in accumulated film.

(*e*) The simple modification of treatment 6 by the addition of 0·1 % phosphoric acid to the final rinse (treatment 11) brought about a reduction of 20 % in residual soil because of the virtual absence of mineral matter and reductions in the weight of fat and protein in the film.

Reproducibility of the test and the effects of heat and of additives to the pre- and final rinses

The previous experiments had confirmed certain known facts such as the value of a water softener with an alkaline detergent and had also established certain new facts where the differences in results were large. It was appreciated, however, that it might become necessary to establish the significance of smaller differences between treatments requiring a determination of the experimental error between replicates. An experiment was therefore set up with 6 replicate glass slides for each of the treatments listed on p. 64.

The standard errors of the means of replicates, given in Table 1, indicated that similar replications would be necessary in future experiments. The following findings are apparent from Fig. 5. (*a*) The substitution of a warm water pre-rinse (treatment 13) for a cold rinse (treatment 12) resulted in a 40 % reduction in accumulated film. (*b*) Treatment 14 with warm pre- and final rinses resulted in a reduction of 64 % in the deposit as compared with treatment 12, and a marked improvement over treatment 13. (*c*) The addition of 0·1 % phosphoric acid to the final cold rinse in treatment 15 brought about a further marked improvement, but a warm final acid rinse (treatment

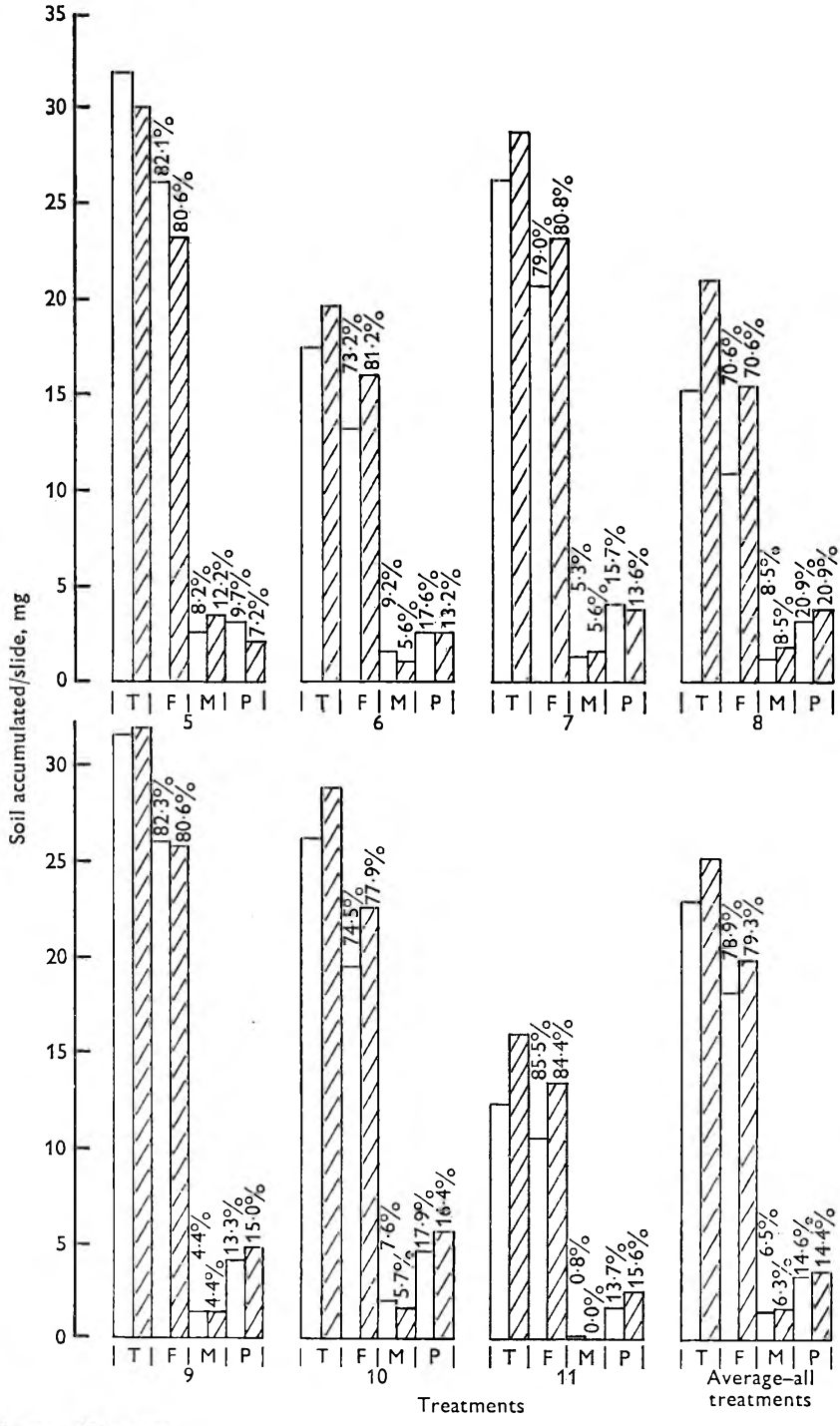

Fig. 4. Differences in total residual soil and fractions on glass and stainless steel slides after 24 soilings and various cleaning treatments (expt 3). Plain, glass; hatched, stainless steel. *T*, total soil; *F*, fat; *M*, mineral; *P*, protein. *Basic treatment*: pre-rinse (2 min, cold), wash (4 min, 46 °C), final rinse (2 min, cold). *Details*: (5) water, Na_2CO_3, NaOCl; (6) water, Na_2CO_3 + NaOCl, water; (7), water, Na_2CO_3 + Calgon, NaOCl; (8) water, Na_2CO_3 + Calgon + NaOCl, water; (9) wash from (7), Na_2CO_3 + Calgon, NaOCl; (10) wash from (8), Na_2CO_3 + Calgon + NaOCl, water; (11) water, Na_2CO_3 + NaOCl, 0·1 % H_3PO_4.

Table 1. *Means of treatments in expt* 4, *with standard errors* (*Fig.* 5)

Treatment no.	Weight of soil remaining, mg/slide	
	Mean	Standard error
12	38·6	±5·0
13	23·2	±1·4
14	14·1	±1·9
15	9·1	±0·7
16	9·3	±2·4
17	8·4	±0·8
18	7·9	±0·6

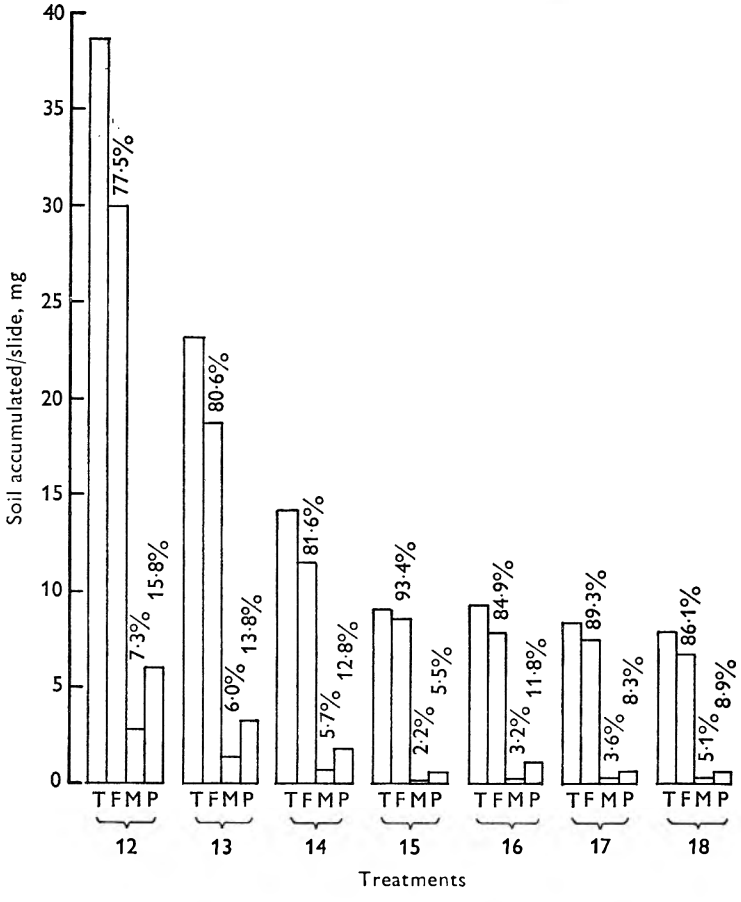

Fig. 5. Differences in total residual soil and fractions on glass slides after 24 soilings and various cleaning treatments (average of 6 replicates) (expt 4). *T*, total soil; *F*, fat; *M*, mineral; *P*, protein. *Basic treatment*: pre-rinse (2 min), wash (Na_2CO_3 + Calgon + NaOCl, 4 min, 46 °C), final rinse (2 min). *Details*: (12) cold water, wash, cold water; (13) warm water, wash, cold water; (14) warm water, wash, warm water; (15) warm water, wash, cold H_3PO_4; (16) warm water, wash, warm H_3PO_4; (17) cold Lissapol, wash, warm H_3PO_4; (18) warm Lissapol, wash, warm H_3PO_4.

16) effected no further reduction. In all the treatments with an acid final rinse (15–18) the amounts of mineral and protein matter in the residue on the slides were visibly different from those after the treatments without acid, and the reduction in mineral content was quite marked in comparison with treatments 12–14. (*d*) The addition of 0·05% Lissapol NDB to the pre-rinse (treatments 17 and 18) brought about a slight further improvement. (*e*) The step-wise increase in residual soil, noted previously, was even more apparent in this experiment (see Fig. 6). Furthermore, after

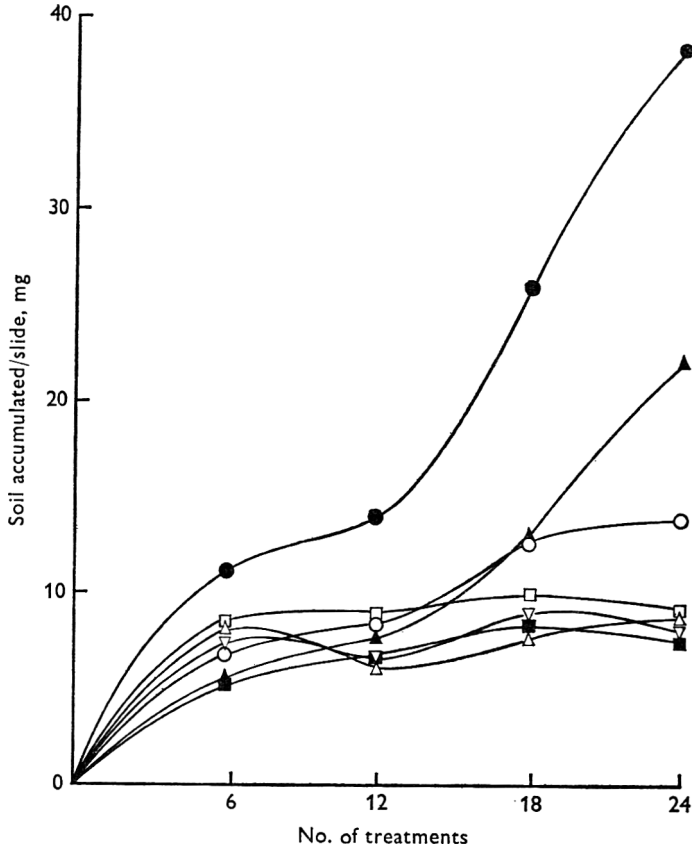

Fig. 6. Rate of accumulation of soil on glass slides during various cleaning treatments (average of 6 replicates) (expt 4). Treatments: ●, (12); ▲ (13); ○ (14); △ (15); □ (16); ▽ (17); ■ (18). (For details of treatments see Fig. 5.)

6 soilings with the most efficient treatments the amount of film deposited was little more than that lost, and the film could be seen falling off the slides into the solution in discrete droplets.

Because of the small differences in residual soil with the best treatments it seemed that experiments must be lengthened or the comparisons made in the cold to slow down reaction between detergent and soil if better discrimination was to be achieved. It therefore seemed desirable to study the effects of temperature and time on the removal of milk film.

Table 2. *Percentage removal of milk film from glass slides by immersion in still water at different temperatures for different times (expt 5)*

(*a*)

Temp. °C	Percentage removal of soil in, min 1	2	3	4	5	6	Means for all times
32	43·0	49·6	50·2	55·7	57·3	62·5	53·0
35	63·9	57·5	58·8	53·9	63·4	56·2	59·0
38	62·0	72·4	66·5	63·1	74·1	77·7	69·3
40·5	65·6	69·1	61·2	72·4	73·4	74·2	69·3
43·5	71·6	71·9	74·6	71·2	74·8	83·8	74·7
46	73·1	70·0	67·8	81·4	75·8	84·3	75·4
49	76·2	73·4	72·1	76·7	85·3	82·2	77·7
52	76·0	80·1	75·5	75·4	80·3	88·4	79·3
Means for all temps	66·4	68·0	65·8	68·7	73·1	76·2	—
(*b*)							
15·5	22·2	17·3	16·2	15·8	17·2	16·4	17·5
18	16·9	15·2	13·4	15·2	19·6	16·5	16·1
21	22·4	25·1	17·4	20·6	16·2	22·8	20·8
24	16·4	30·0	27·8	30·9	26·4	23·0	25·8
26·5	25·9	33·9	36·3	27·2	32·9	34·4	31·8
29·5	28·3	27·2	26·9	34·3	32·6	34·4	30·6
32	35·6	35·5	34·3	40·0	43·9	37·5	37·8
Means for all temps	24·0	26·3	24·6	26·3	27·0	26·4	—

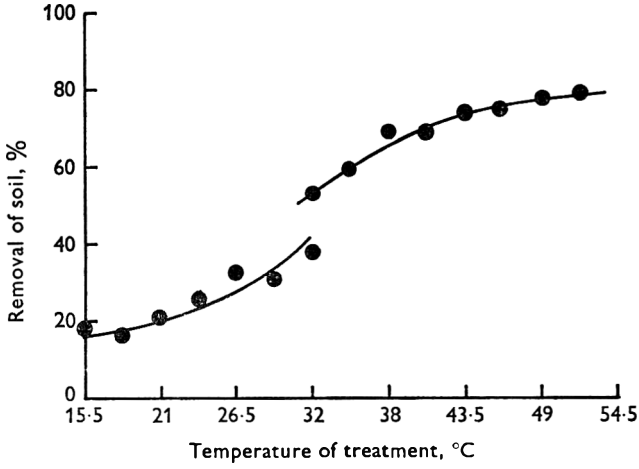

Fig. 7. Effect of temperature on removal of milk film from glass slides with water. (Expt 5, Table 2.)

The influence of temperature of solution on film retention

Forty-eight glass slides were soiled 8 times with fresh raw milk at 34 °C for 30 min with 30 min intervals of drying at 37 °C between each soiling. After determining the weight of soil the slides were immersed for 1–6 min in water at different temperatures in the range 32–52 °C. Six slides were immersed at each controlled temperature, which varied by $< \pm 0{\cdot}3$ °C. The results are given in Table 2*a*.

The discrepancies between individual results may have arisen through of irregular deposition of film on individual slides, or of movement in the water used for immersion.

Nevertheless, the average results show that an increment of temperature had more effect in removing film than had an increment of time. In the range 43–52 °C water alone was very effective and in the range 32–40 °C rate of film removal increased rapidly with increase in temperature. These ranges should therefore be avoided, if possible, in comparisons of detergents. Accordingly, the effect of treating slides in the range 15·5–32 °C was explored. The results are shown in Table 2*b*.

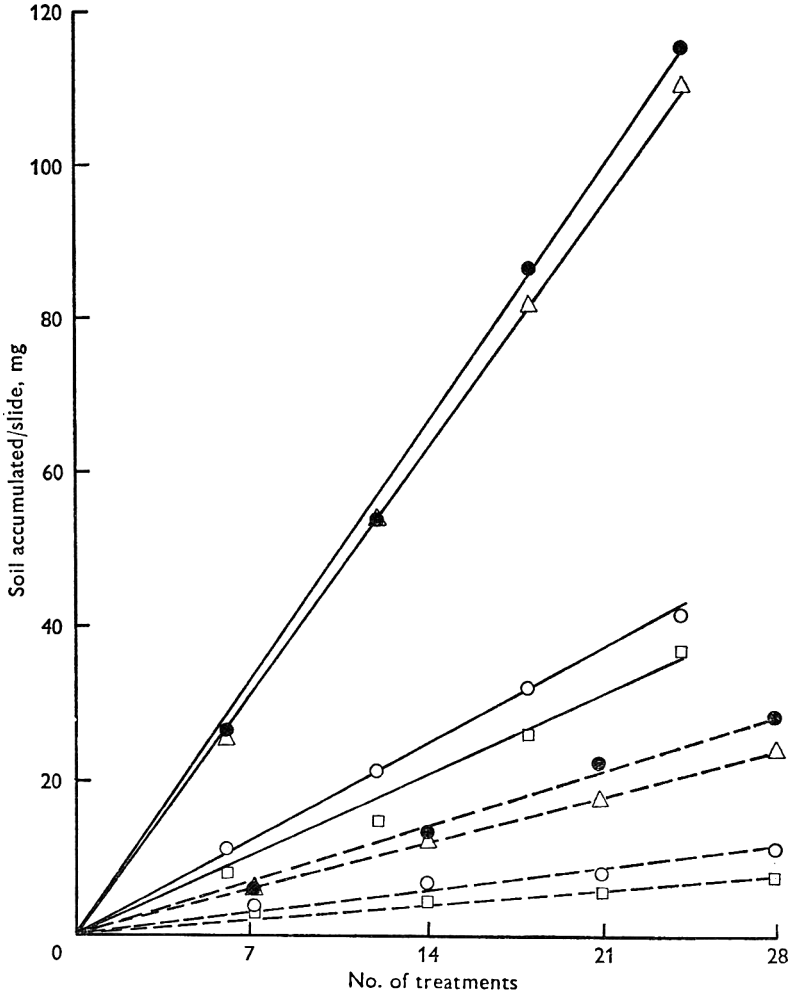

Fig. 8. Effect of temperature of treatment on rate of accumulation of soil on glass slides during various cleaning treatments (expt 6). Treatment at 15·5 °C, ——; at 46 °C, – – –. Treatments 1 and 19, ●; 2 and 20, △; 3 and 21, ○; 4 and 22, □ (See Figs 2 and 9 for treatment details).

Although great care was taken to see that the water was motionless there were still discrepancies in individual results. They were, however, sufficiently precise to indicate that the effect of increase of temperature on soil removal was least in the range 15·5–18 °C as demonstrated by the flattening of the curve in Fig. 7, which incorporates the results of Table 2*a* and *b*. In Fig. 7 the 2 series of results seem to be discontinuous.

The total amount of milk film deposited on the 48 slides treated at 15·5–32 °C was 2·6170 g as compared with only 1·5194 g for those treated at 32–52 °C. The latter were soiled with milk from one cow only instead of the bulk herd milk used for the slides treated at 15·5–32 °C and in all other experiments, and differences in composition of the milk may have accounted for the discontinuity. In addition, removal of film was perhaps less efficient with the heavier deposition.

In this experiment, more so than with the detergent treatments of earlier experiments, some of the milk film floated to the surface of the water during treatment and part of this was picked up by the slides when they were removed from the water. By including one clean slide with each temperature treatment this secondary deposit was measured and found to be, on average, 7·7 % of the original soiling. This could have accounted for some of the variation noted in Table 2 but would not have invalidated the findings.

Table 3. *Means of treatments from* 18 *and* 24 *treatments, with standard errors* (*see Fig.* 8, *expt* 6)

	Weight of soil remaining, mg/slide, after			
	18 treatments		24 treatments	
Treatment no.	Mean	Standard error	Mean	Standard error
19	86·8	±6·2	115·2	±4·5
20	81·7	±2·6	111·0	±1·2
21	32·0	±1·4	41·7	±2·7
22	26·3	±3·1	37·3	±4·8
23	36·7	±2·5	48·0	±0·8
24	78·5	±8·2	112·7	±3·4
25	46·5	±3·5	63·3	±4·9
26	20·9	±1·4	29·1	±1·9

Various washing treatments at 15·5 °C.

A separate group of 6 replicate glass slides was subjected to each of the 8 treatments (detailed on p. 64), each consisting of a pre-rinse, wash and final rinse lasting for 2, 4 and 2 min, respectively, all at 15·5 ± 0·5 °C. The deposits on the slides were considerably heavier than those occurring after treatments at 46 °C in earlier experiments.

The rates of increase in deposition for comparable detergents and disinfectants are shown in Fig. 8, in which treatments 1–4 at 46 °C are compared with treatments 19–22 at 15·5 °C. The time in the solutions at 46 °C was half that in solutions at 15·5 °C, emphasizing the greater efficiency of the detergents at the higher temperature.

The weights of residual soil after 18 and 24 soiling and cleaning treatments, shown together with standard deviations in Table 3, indicate that at 15·5 °C 18 treatments would have been adequate to differentiate between the various detergent-disinfectant treatments.

The facts brought out by the histograms in Fig. 9 and by the standard errors of the means in Table 3 are as follows. (*a*) The improved detergent effect resulting from the simultaneous use of Na_2CO_3 and NaOCl (treatment 20) in comparison with their separate use (treatment 19) was less evident at 15·5 °C than at 46 °C. The same was true when Teepol was used in conjunction with NaOCl (treatments 21 and 22).

Nevertheless, the mean intermediate values of all 4 of the above treatments (Fig. 8) gave small but consistent differences in favour of the combined use of detergent and NaOCl. (*b*) The marked superiority of Teepol over Na_2CO_3 was again evident (treatments 21 and 22 *vs.* 19 and 20) and was mainly because of a reduction in the fat content of the residues. (*c*) The iodophor treatment (23) was clearly better than both Na_2CO_3 treatments (19 and 20). However, the total deposit with the iodophor was greater than with either of the 2 Teepol treatments (21 and 22), though the mineral and 'protein' fractions were much lower than with any of the above 4 treatments. (*d*) The results obtained by omitting the pre-rinse differed according to the detergent-disinfectant treatment. With Na_2CO_3 + NaOCl (treatment 20 *vs.* 24) there was virtually

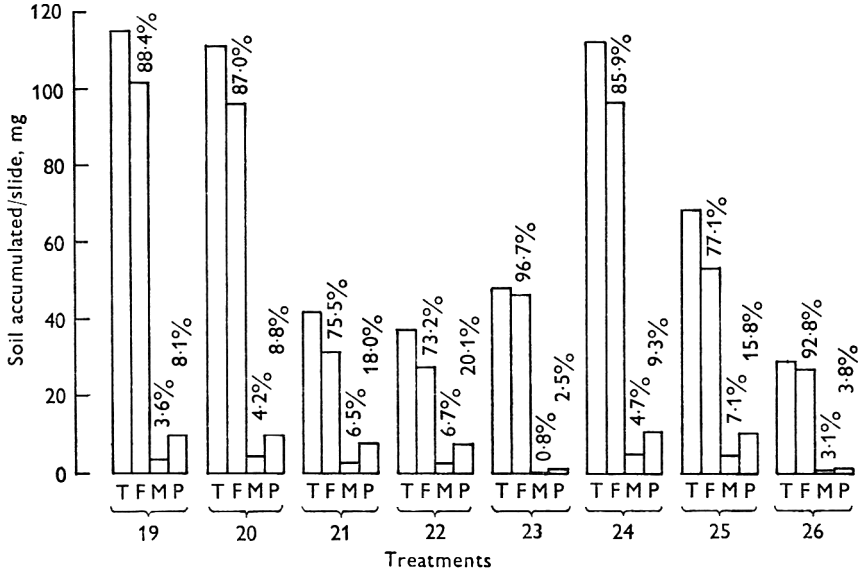

Fig. 9. Differences in total residual soil and fractions on glass slides after 24 soilings and various cleaning treatments at 15·5 °C (average of 6 replicates) (expt 6). *T*, total soil; *F*, fat; *M*, mineral; *P*, protein. *Basic treatment*: pre-rinse (2 min, cold), wash (4 min, cold), final rinse (2 min cold). *Details*: (19) water, Na_2CO_3, NaOCl; (20) water, Na_2CO_3 + NaOCl, water; (21) water, Teepol, NaOCl; (22) water, Teepol + NaOCl, water; (23) water, iodophor, water; (24) no pre-rinse, Na_2CO_3 + NaOCl (6 min), water; (25) no pre-rinse, Teepol + NaOCl (6 min), water; (26) no pre-rinse, iodophor (6 min), water.

no difference in residual soil. With Teepol + NaOCl (treatment 22 *vs.* 25) the lack of a pre-rinse was clearly detrimental. With the iodophor the absence of a pre-rinse (treatment 23 *vs.* 26) reduced the total deposit, because of a reduction in the fat fraction. It must be borne in mind that the omission of the pre-rinse did not result in a shortened treatment because the period in the 'wash' solution was increased in compensation.

DISCUSSION

Complete removal of soil from a surface by normal cleaning is not possible in practice. With inferior cleaning methods or materials, the soil may accumulate on the surface and in consequence chemical disinfection may become inefficient so that there is a bacteriological hazard to any food or beverage contacting such a surface.

It is therefore desirable to know in what circumstances a film of residual soil can accumulate or can be prevented. In the present work the amount of film built up was deliberately exaggerated in order to obtain measurable deposits after 3 or 4 days. Also, the type and composition of the film was different from that normally found under field conditions. While it is important to realize this, it was possible to distinguish between differences in cleaning methods and in detergent-disinfectant formulations. Such findings alone are not adequate for choosing cleaning materials that will be satisfactory in the field but the technique could aid the development of materials and methods for final proving tests.

The use of detergent-disinfectant mixtures as against the separate use of the components has been in question for a number of years. The present work supports the findings of, for example, Neave & Hoy (1947) and Clegg & Bačić (1968) favouring the combined use of hypochlorite and alkaline detergents but does so from a consideration of detergency rather than disinfection, thus confirming the work of Merrill, Jensen & Bass (1962). It is of added interest that an apparent synergistic action was also observed with the combined use of NaOCl and Teepol. Teepol left a residue containing much less fat than that left by the Na_2CO_3-based detergents, and thus, in combination with NaOCl, it was the most effective of all the treatments involving hypochlorite. It is probable that the germicidal efficiency of the NaOCl could be reduced less by Teepol (the mixture had a pH of 9·1) than by the more alkaline detergents.

In practice, detergent and disinfectant solutions are seldom completely expended after use and it seems unfortunate that such solutions, with unspent detergent and disinfecting ability, should be wasted. Therefore, the re-use of the 'wash' solution as a pre-rinse seemed worth investigating. The treatments, however, resulted in more deposit than did the use of plain water (see Fig. 4, treatments 9 and 10). This finding may not be generally applicable, as the wash solutions were among the least effective of those tested and they might have deposited as much soil as they removed.

The effectiveness of 0·1 % phosphoric acid as a final rinse, particularly in the reduction of the mineral content of the residues, supports recommendations (e.g. Abele, 1966) to use an acid rinse in practice.

The marked reductions in deposit resulting from the use of warm instead of cold water for the pre- and final rinses were borne out by the results of the experiment showing the effectiveness of warm water alone in removing a milk film built up by repeated soiling, but information is lacking concerning the advantages, if any, of warm rinses in practice.

No significant improvement was noted when a wetting agent was added to the pre-rinse water (treatments 17 and 18 *vs.* 16) although Calder & Twomey (1966) reported that the similar use of such an additive reduced milkstone build-up to only a third of that found when no additive was used.

The omission of the pre-rinse, if not deleterious, would simplify cleaning in practice. The results of expt 6 suggest that this simplification might be possible, but not, for example, with Teepol solution (treatment 25), which presumably lacked the good suspending properties required for the increased soil load in the detergent solution. With the iodophor (treatment 26), omitting the pre-rinse was, however, advantageous and reduced the fat content of the residual soil; this was presumably associated with

the wetting agent in the formulation. These results suggest that it would be worth while to investigate further variations and combinations of rinses and washes. With combined detergent-disinfectants such as iodophors, however, the depletion of the disinfectant by milk residues resulting from the omission of the pre-rinse might adversely affect the bactericidal properties of the solution. This aspect would therefore need to be considered.

The findings are sufficient to illustrate some of the ways in which the multi-soiling washing technique can be used. It should also be possible to gain useful information on differences, if any, between the cleanability of stainless steel, rubber and plastics, the effects of different surface finishes and, further, on the suitability of detergents for use with refrigerated surfaces.

The basic multi-soiling washing technique can be modified as required for different purposes. In the present work where detergent-disinfectant treatments were compared, the test conditions considered favourable for satisfactory differentiation are summarized below. (*a*) A standard reference treatment should be included in each experiment because the weight of film deposited may vary from one experiment to the next. (*b*) For good differentiation each treatment should be replicated. The standard errors suggest that 6 slides/treatment are necessary. (*c*) Determination of the weight of residual soil after every 6 soiling-washing treatments provides useful information on the rate of deposition and may permit a reduction in the total number of treatments, e.g. from 24 to 18. (*d*) Treatment times of 2 min for the pre-rinse, 4 min for the wash and 2 min for the final rinse are used to compensate for the absence of agitation. (*e*) The use of detergent solutions in the cold (15·5 °C) increases the rate of deposition of soil. Where this accentuates differences between treatments a reduction in the number of soiling-washing treatments is possible.

It will be necessary to study bacteriological aspects of the technique before more comprehensive information can be obtained on detergent and disinfectant treatments.

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With 2 plates

Studies on the mechanism of the Whiteside mastitis test reaction*

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Summary. The contribution of certain components of leucocytes and milk to the viscosity and precipitate which develop in the Whiteside mastitis test was studied to obtain information on the mechanism of the reaction. The viscosity obtained in the Whiteside test reaction was considerably less than that in the California mastitis test reaction and the Whiteside test was found to be more dependent on precipitation than increased viscosity.

Leucocyte nuclei were mainly responsible for the formation of the precipitate in the Whiteside reaction and $CaCl_2$ dispersed the precipitate formed by the leucocyte nuclei into small clumps. Leucocyte protein and fibrinogen increased the amount of precipitate formed by the leucocyte nuclei. Fat globules also increased the amount of precipitate, probably by being trapped in the precipitate.

The addition of non-gelable or gelable nuclei resulted in the formation of similar amounts of precipitate in the modified Whiteside reaction. Furthermore, storage of milk for 5 days did not decrease the amount of precipitate formed, indicating that the DNA-protein complex of the leucocyte nuclei need not be present in the native form to contribute to the precipitate in this reaction.

Whiteside (1939) described a test for the detection of mastitis milk. The test was conducted by adding 2 ml 1·0 N-NaOH to 10 ml milk, followed by beating the mixture with a glass rod. A viscid mass was formed by milk from cows suffering from mastitis and the test was invariably negative with normal milk. This reaction took place immediately and at room temperature. The reaction did not occur if the test mixture was boiled. Murphy & Hanson (1941) modified the test by mixing 1 drop of 1·0 N-NaOH and 5 drops of milk on a glass plate and stirring the mixture vigorously for 20 s. These investigators suggested scoring the reaction as − to 4+. Reactions of 2+ or more were found to occur with milk containing more than 5×10^5 leucocytes/ml. In a subsequent study, Murphy (1942) stated that the intensity of the reaction

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was increased by refrigerating the milk before the test. He proposed the use of 2 drops of 1·0 N-NaOH and 5 drops of milk when the test was conducted on non-refrigerated milk. This test is known as the modified Whiteside test (MWT). Several workers have studied the mechanism of this reaction, but their results are somewhat conflicting. Dunn, Murphy & Garret (1943) concluded that the reaction was due to leucocytes in the milk and the intensity of the reaction varied directly with the number of leucocytes present. Peterson, Grimmell & Schipper (1950) believed that the reaction was not due solely to leucocytes but was caused by the absorption of fibrin on the white cells. Schalm & Noorlander (1957) indicated that fat precipitation occurred in the Whiteside reaction.

The purpose of the work reported in the present paper was to determine more precisely the contribution of various components of leucocytes and milk to the viscosity and precipitate formed in the Whiteside mastitis test reaction.

MATERIALS AND METHODS

Modified Whiteside test (MWT). Approximately 0·15 ml of the sample was placed on a glass plate having $(3·3 \text{ cm})^2$ divisions and 0·06 ml of 1·0 N-NaOH was added. The 2 liquids were mixed vigorously with a flat toothpick for 20 s over a circular area of 2·5 cm diam. The glass plate was illuminated by a light source underneath to facilitate reading the reaction, which was scored according to Schalm & Gray (1954).

Determination of viscosity. The samples were warmed to room temperature. In the California mastitis test (CMT) reaction 70 ml of the sample were mixed with 70 ml of CMT reagent (Mastest solution, Norden Laboratories, Lincoln, Neb.) in a round 14·6 × 5 cm (250 ml capacity) glass jar. In the Whiteside reaction, 100 ml of the sample were mixed with 40 ml of 1·0 N-NaOH. The viscosity was determined with a Brookfield viscometer (Brookfield Engineering Laboratories, Stoughton, Mass.) with spindle 2 at 60 rev/min. Triplicate determinations were made for each sample and the viscosity was noted at the highest reading.

The score in the CMT reaction was determined according to the procedure of Schalm & Noorlander (1957).

Isolation of milk leucocytes. Milk leucocytes and their nuclei were isolated according to the procedure described elsewhere (Nageswararao & Derbyshire, 1969*a*). The leucocyte protein used in this study was prepared by lyophilization of the supernatant obtained after centrifugation of homogenized leucocytes at 550 × *g* for 20 min.

RESULTS

Comparison of viscosity in the Whiteside and California mastitis test reactions

Milk from a cow with mastitis was divided into 6 samples, one of which was used as a control. To 3 samples, bovine fibrinogen was added in concentrations of 0·25, 0·5 and 0·75%. To another sample 2% EDTA was added, and 0·3% $CaCl_2$ was added to the sixth sample of milk. The viscosity of each sample was then estimated in the Brookfield viscometer with NaOH and CMT reagent and, in addition, the MWT and CMT scores were determined. The results of this experiment are given in Table 1, from which it will be seen that the viscosity obtained after the addition of CMT

reagent to the control mastitis milk was more than twice that obtained after the addition of NaOH. The addition of increasing concentrations of fibrinogen to the milk increased the viscosity obtained in the MWT reaction, although the MWT score remained the same, while in the CMT reaction viscosity decreased with increasing fibrinogen concentrations. The addition of EDTA to the milk slightly increased the viscosity and slightly decreased the precipitate obtained in the MWT reaction. EDTA also increased the viscosity obtained in the CMT reaction. $CaCl_2$ markedly decreased the viscosity in both the MWT and CMT reactions, but while the CMT score was reduced, the MWT score was unaffected in the presence of $CaCl_2$.

Table 1. *Comparison of viscosity obtained in the Whiteside reaction and in the California mastitis test (CMT)*

Preparation	Viscosity* in Whiteside reaction	Modified Whiteside test score	Viscosity* in CMT reaction	CMT score
Mastitis milk (control)	47	1+	121	3
Control + 0·25 % fibrinogen	58	1+	106	3
Control + 0·5 % fibrinogen	69	1+	107	3
Control + 0·75 % fibrinogen	72	1+	99	3
Control + 2 % EDTA	64	1+	138	3
Control + 0·3 % $CaCl_2$	9	1+	61	2

* Determined by Brookfield viscometer, spindle 2, 60 rev/min.

Effect of fibrinogen, leucocyte protein, leucocyte nuclei, milk, calcium and EDTA on the modified Whiteside test reaction

The following were prepared, and each preparation was subjected to the MWT: *A*, normal whole milk; *B*, whole milk + 10 mg/ml fibrinogen; *C*, whole milk + 5 mg/ml leucocyte protein; *D*, whole milk + 10 mg/ml leucocyte protein; *E*, whole milk + 0·5 mg/ml leucocyte nuclei; *F*, whole milk + 1·0 mg/ml leucocyte nuclei; *G*, whole milk + 10 mg/ml fibrinogen + 5 mg/ml leucocyte protein; *H*, whole milk + 10 mg/ml fibrinogen + 5 mg/ml leucocyte protein + 0·5 mg/ml leucocyte nuclei. The reactions obtained are illustrated in Plate 1*a*. No precipitate was obtained with normal milk; the addition of fibrinogen resulted in the formation of a few dispersed particles in the MWT, and when leucocyte protein was added a well marked precipitate occurred. The degree of precipitation obtained with leucocyte nuclei in milk was greater than that obtained with leucocyte protein.

Skim-milk was used in place of whole milk in a similar experiment to that described above, and the following preparations were subjected to the MWT: *A*, skim-milk; *B*, skim-milk + 10 mg/ml fibrinogen; *C*, skim-milk + 5 mg/ml leucocyte protein; *D*, skim-milk + 10 mg/ml leucocyte protein; *E*, skim-milk + 0·5 mg/ml leucocyte nuclei; *F*, skim-milk + 1·0 mg/ml leucocyte nuclei; *G*, 0·15 M-NaCl + 20 mg/ml fibrinogen; *H*, 0·15 M-NaCl + 20 mg/ml leucocyte protein. The reactions obtained with these samples are shown in Plate 1*b*. No precipitation occurred with skim-milk or fibrinogen in skim-milk. Skim-milk containing leucocyte protein or leucocyte nuclei produced precipitation in the MWT, but this was less marked than with the corresponding whole milk preparations. Fibrinogen in 0·15 M-NaCl produced no precipitate in the MWT, and leucocyte protein in 0·15 M-NaCl formed only finely dispersed particles.

In the next experiment, leucocytes isolated from milk were suspended in 0·15 M-

NaCl at 2 concentrations, and $CaCl_2$, fibrinogen, leucocyte protein or whole milk was added and each preparation was subjected to the MWT. The samples tested were as follows: *A*, 5×10^6 leucocytes/ml; *B*, 5×10^6 leucocytes/ml + 0·3 % $CaCl_2$; *C*, 10×10^6 leucocytes/ml; *D*, 10×10^6 leucocytes/ml + 0·3 % $CaCl_2$; *E*, 5×10^6 leucocytes/ml + 10 mg/ml fibrinogen; *F*, 5×10^6 leucocytes/ml + 10 mg/ml leucocyte protein; *G*, 5×10^6 leucocytes/ml + 0·15 ml whole milk/ml + 0·3 % $CaCl_2$; *H*, 5×10^6 leucocytes/ml + 0·3 ml whole milk/ml + 0·3 % $CaCl_2$. The results are shown in Plate 2*a*. Leucocyte suspension alone formed a small clump of white precipitate, but when $CaCl_2$ was present well defined opaque particles of precipitate were formed. The addition of fibrinogen increased the amount of clumped precipitate formed by the leucocytes, but when leucocyte protein was present a finely dispersed precipitate was formed. The addition of whole milk to the leucocyte suspension resulted in the formation of finely dispersed particles against an opaque background in the MWT.

The addition of graded concentrations of leucocytes, from 5×10^6 to 25×10^6 cells/ml, to normal whole milk resulted in a gradual increase in MWT score from 1+ to 4+ (Plate 2*b*, *B–F*). When 2 % EDTA was added to leucocyte suspensions which gave 3+ or 4+ scores in the MWT, the samples no longer precipitated in the MWT, but formed white, viscous material (Plate 2*b*, *G–H*).

Effect of pH of nuclei preparations on the precipitate formed in the modified Whiteside test reaction

Preparations of leucocyte nuclei isolated at pH 4·0, 5·9 or 8·0 were added to samples of normal whole milk at a concentration of 1·0 mg/ml. There was no appreciable difference in the precipitate formed by the 3 samples in the MWT, but in the CMT the samples containing nuclei isolated at pH 4·0 or 8·0 gave no reaction, although the pH 5·9 nuclei gave a CMT score of 2.

Effect of storage of milk at 5 °C on the modified Whiteside test

Milk samples were obtained from 4 cows; the leucocyte counts ranged from 7×10^5 to 9×10^6 cells/ml. The samples were stored at 5 °C for 5 days, and the MWT and CMT scores were determined daily. On the day of collection, the samples gave MWT scores of ±, 1+, 2+ and 3+, respectively, and there was no appreciable change in the reaction on storage for 3 days. After 4 days storage the amount of precipitate formed in the MWT increased slightly. The CMT score on each sample decreased on storage, as has been reported previously (Nageswararao & Derbyshire, 1969 *b*).

DISCUSSION

The viscosity obtained in the Whiteside reaction was considerably less than that in the CMT reaction (Table 1), even though the volume of milk used was greater in the Whiteside reaction. The viscosity in the Whiteside reaction was eliminated by the addition of Ca^{2+}, but the precipitate formed in the modified Whiteside reaction remained the same except when EDTA was added. This indicates that the Whiteside reaction depends more upon precipitation than increased viscosity.

The addition of fibrinogen to Whiteside negative milk resulted in the formation of a slight precipitate. The addition of leucocyte protein caused the formation of con-

siderably more precipitate in the Whiteside reaction, but the addition of only one tenth as much of the nuclei preparation formed considerably more precipitate than leucocyte protein (Plate 1 *a*, *B–F*). The precipitates formed by the same materials in whole milk and in skim-milk were markedly different and the amount of precipitate formed was considerably more in whole milk as shown in Plate 1. This indicates that fat globules contribute to the amount of precipitate formed in the MWT. Our results suggest that the leucocyte nuclei are primarily responsible for the formation of precipitate and that Ca^{2+}, normally present in milk, disperses the clump of precipitate formed by the leucocytes as shown in Plate 2 *a*, *A–D*. Leucocyte protein and fibrinogen contribute to the amount of precipitate formed by leucocyte nuclei. The fat globules present in whole milk increased the amount of precipitate formed, probably by being trapped in the precipitate formed by leucocyte nuclei. Thus, in order of importance, the following constituents of mastitis milk contribute to the formation of precipitate in the MWT reaction: leucocyte nuclei, Ca^{2+}, leucocyte protein, fat globules and fibrinogen.

Although our results indicate that leucocyte nuclei make a major contribution to the precipitate formed in the MWT, it was of interest that leucocyte nuclei which fail to give a gel in the CMT were nevertheless capable of reacting in the MWT in the same way as gelable nuclei. Moreover, storage of milk for 5 days at 5 °C did not decrease its MWT score. These findings suggest that the DNA-protein complex of the leucocyte nuclei need not be present in the native form to cause precipitation in the MWT reaction, in contrast to the CMT reaction (Nageswararao & Derbyshire, 1969*b*).

The authors wish to thank Dr David T. Berman for his suggestions during this investigation.

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EXPLANATION OF PLATES

Plate 1

(*a*) Effect of proteins and leucocyte nuclei added to normal whole milk on the modified Whiteside reaction. *A*, Whole milk; *B*, whole milk + 10 mg fibrinogen/ml; *C*, whole milk + 5 mg leucocyte protein/ml; *D*, whole milk + 10 mg leucocyte protein/ml; *E*, whole milk + 0·5 mg leucocyte nuclei/ml; *F*, whole milk + 1·0 mg leucocyte nuclei/ml; *G*, whole milk + 10 mg fibrinogen and 5 mg leucocyte protein/ml; *H*, whole milk + 10 mg fibrinogen, 5 mg leucocyte protein and 0·5 mg leucocyte nuclei/ml.

(*b*) Effect of proteins and leucocyte nuclei in skim-milk on modified Whiteside reaction. *A*, Skim-milk; *B*, skim-milk + 10 mg fibrinogen/ml; *C*, skim-milk + 5 mg leucocyte protein/ml; *D*, skim-milk + 10 mg leucocyte protein/ml; *E*, skim-milk + 0·5 mg leucocyte nuclei/ml; *F*, skim-milk + 1·0 mg leucocyte nuclei/ml; *G*, fibrinogen 20 mg/ml in 0·15 M-NaCl; *H*, leucocyte protein 20 mg/ml in 0·15 M NaCl,

PLATE 2

(*a*) Effect of leucocytes, $CaCl_2$, proteins and whole milk in 0·15 M-NaCl solution on modified Whiteside reaction. *A*, Leucocytes 5×10^6/ml; *B*, leucocytes 5×10^6/ml + 0·3 % $CaCl_2$; *E*, leucocytes 5×10^6 + 10 mg fibrinogen/ml; *F*, leucocytes 5×10^6 + 10 mg leucocyte protein/ml; *G*, leucocytes 5×10^6 and 0·15 ml whole milk/ml + 0·3 % $CaCl_2$; *H*, leucocytes 5×10^6 and 0·3 ml whole milk/ml + 0·3 % $CaCl_2$.

(*b*) Effect of leucocytes and EDTA in whole milk on modified Whiteside reaction. *A*, Whole milk; *B*, whole milk + 5×10^6 leucocytes/ml; *C*, whole milk + 10×10^6 leucocytes/ml; *D*, whole milk + 15×10^6 leucocytes/ml; *E*, whole milk + 20×10^6 leucocytes/ml; *F*, whole milk + 25×10^6 leucocytes/ml; *G*, whole milk + 20×10^6 leucocytes/ml and 2 % EDTA; *H*, whole milk + 15×10^6 leucocytes/ml and 2 % EDTA.

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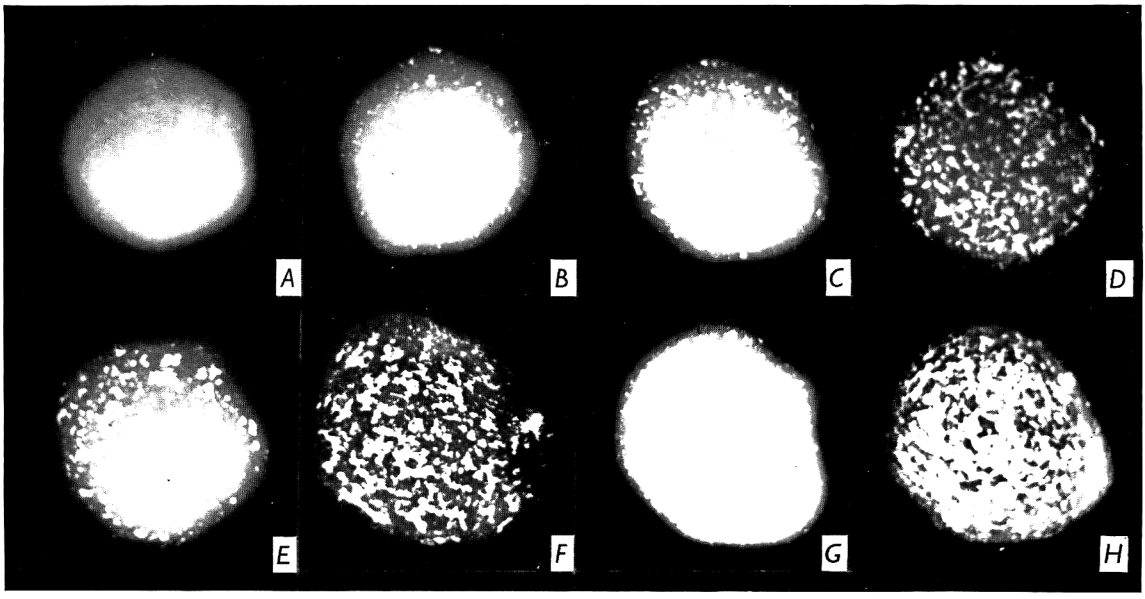

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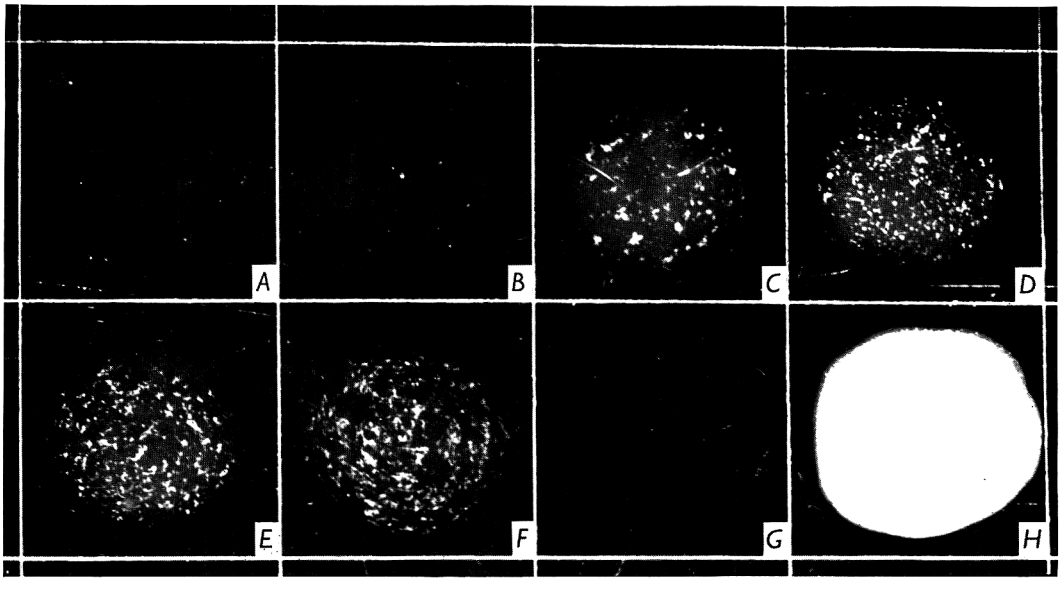

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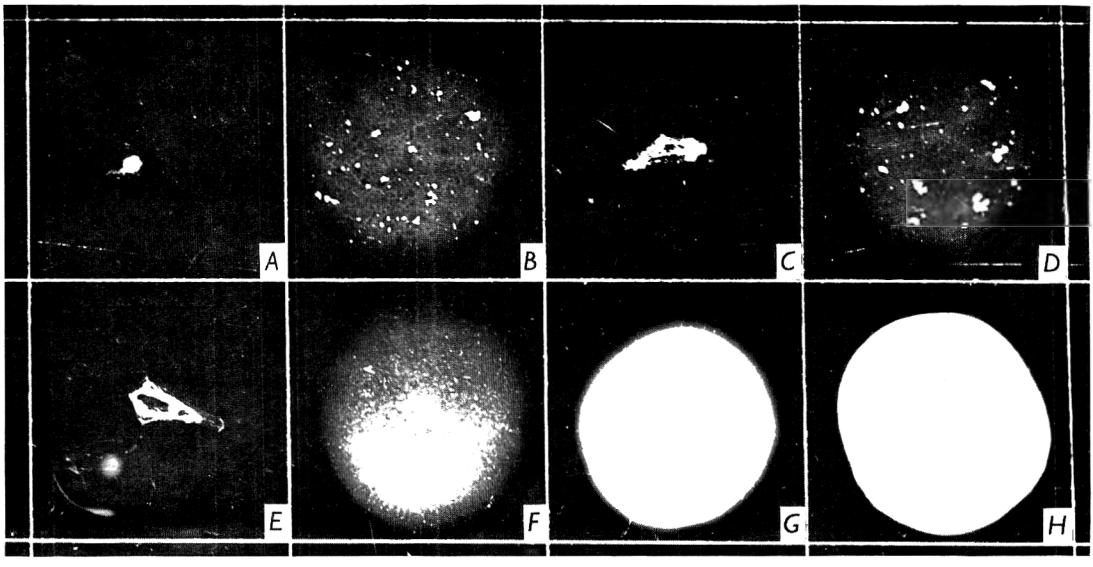

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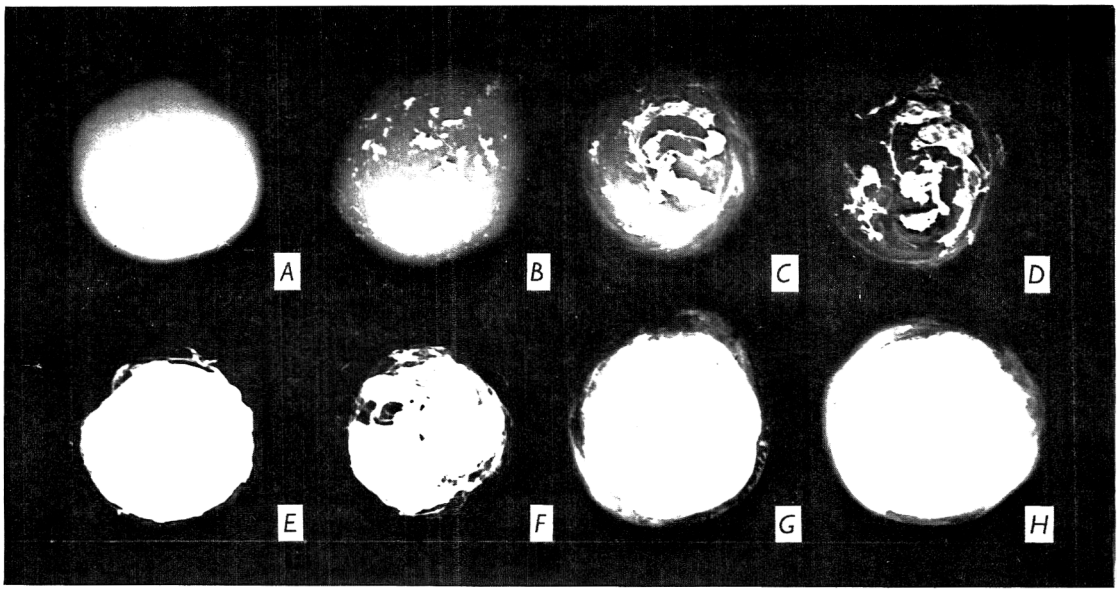

(*b*)

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The pattern of release of free fatty acids from milk fat under the action of intrinsic and added lipases

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Summary. (1) The rate of release of fatty acids from milk by porcine pancreatic lipase was studied as a model system for the development of lipase taint. The rate was low initially but then increased logarithmically and later declined. Addition of milk which had been subjected to the action of lipase and then boiled to inactivate the enzyme enhanced fatty acid release. An extract containing monoglycerides from lipolysed milk had a similar action whereas an extract of free fatty acids had an effect mainly in the early stages, increasing the initial rate. Addition of Ca^{2+}, which accelerated lipolysis of fresh milk, enhanced the action of fatty acids and depressed that of monoglycerides.

(2) In the early stages of lipolysis there was a preferential release of short-chain fatty acids and a high proportion of these passed into the aqueous phase. These effects were more pronounced at low temperature and at acid to neutral pH. At alkaline pH a greater proportion of the short-chain acids was present in the aqueous phase but the total release of these acids was less.

(3) Fresh milks differed in their response to 'aeration' and storage at 4 °C. In those in which taint was induced there was a considerable release of fatty acids in response to aeration, and a further release and also a redistribution between the aqueous and fat phases during storage. No relationship was apparent between the amount of fatty acids released and the initial fatty acid content.

(4) The findings are discussed in relation to the development of lipase taint in commercial milks.

The off-flavour of milk known as 'lipase taint' is assumed to result from the release of free fatty acids (FFA) from milk fat which may occur through the action of lipases naturally occurring in milk. An association between taint and the presence in milk of FFA has been demonstrated, and there is some evidence that the concentration of butyric acid in the aqueous phase of milk may have an especially marked influence on flavour (Kosikowski, 1959; Harper, Gould & Hankinson, 1961; Kintner & Day, 1965). The present paper reports an investigation of factors which, during lipolysis, affect the rate of release of the fatty acids and their distribution between aqueous and fat fractions. The rate of lipolysis of milk fat under the action of natural lipases is slow, and in certain of the experiments the rate of lipolysis was increased artificially by the addition of porcine pancreatic lipase.

EXPERIMENTAL

Experimental details

Expt 1. *The course of lipolysis.* Samples of fresh milk (10 ml each) were introduced into the titration vessel of a pH stat (Radiometer, Copenhagen) maintained at 37 °C, and the volume adjusted to 13 ml with distilled water, with or without the previous addition of one of the following materials, the preparation of which is described below: 1, 2 ml boiled 'lipolysed milk'; 2, 2 ml of a solution of fatty acids as sodium soaps; 3, 2 ml of a solution of monoglycerides; 4, calcium ions (as calcium chloride) to a final concentration of 1·0 M.

The pH was adjusted to 8·0, 1 ml of porcine pancreatic lipase (Koch Light Laboratories Ltd., Colnbrook, Bucks) in aqueous suspension (4 %, w/v) was added and the pH maintained at 8·0 by the controlled addition of 1·0 N-NaOH.

Lipolysed milk was prepared by subjecting milk to the action of pancreatic lipase at pH 8·0 until the rate of lipolysis was markedly decreased. Fatty acids and 'monoglycerides' were obtained by adjusting the pH of lipolysed milk to 2·0 and extracting with diethyl ether. Fatty acids were extracted from the ether layer with alkali and monoglycerides obtained by evaporation of the residual ether. Fatty acids and monoglycerides were diluted to a volume equivalent to that of the milk from which they were extracted.

Expt 2. *The pattern of release of FFA and their distribution between aqueous and fat fractions during lipolysis.* A sample of milk was obtained by hand-milking from a single quarter of a Friesian cow. The milk was collected directly into a 1-l sterile bottle. Ten ml portions were lipolysed until 7·3, 14·5, 36·4, 50·9 and 727·0 μmol acid had been released (equivalent to 1, 2, 5, 7 and 100 % hydrolysis of triglyceride to monoglyceride and FFA) in a pH stat by the addition of 1 ml of a 4 % aqueous suspension of porcine pancreatic lipase. The acids liberated were titrated automatically to pH 8·0 with 1·25 N-sodium hydroxide solution. When the desired degree of lipolysis had been achieved, 5 ml were pipetted immediately into 35 ml of methanol (for lipid extraction, see later). The remainder was transferred to a tube chilled to 0 °C and immediately separated into aqueous and fat fractions as outlined below. Samples were analysed for triglyceride, diglyceride, monoglyceride and FFA, as appropriate.

Expt 3. *Effect of pH and temperature on the pattern of release of FFA and their distribution between aqueous and fat fractions.* A sample of milk was obtained as described for expt 2. Ten ml portions were lipolysed by pancreatic lipase until 40 μmol of acid had been released (equivalent to about 5% of hydrolysis of triglyceride to monoglyceride and FFA) at temperatures of 0, 10, 20, 30 and 40 °C and at each temperature at pH values of 6·0, 6·5, 7·0, 7·5 and 8·0. Other details were as described for expt 2.

Expt 4. *Effect of aeration and storage at 4 °C on the release of FFA, lipase activity and lipolysability.* Samples of milk from 10 cows (A–J) were taken by hand into 1-l sterile bottles.

Sixty ml subsamples were aerated in the laboratory in a 100 ml measuring cylinder at 37 °C for 5, 15 or 30 min, using compressed air at 30 p.s.i. forced through a thick-walled glass capillary tubing of 1 mm I.D. Samples (11 ml) were removed for analysis immediately. Additional samples were removed for analysis after storage at 4 °C for

24 and 48 h. All samples were analysed for total lipid and FFA in the aqueous fraction and samples A–F for lipase activity and G–J for lipolysability (Hemingway, Smith & Rook, 1970).

Methods of analysis

Lipase activity. The method of Parry, Chandan & Shahani (1966) was used. Ten ml of butter-oil emulsion stabilized by the addition of gum arabic (10 %, w/v) and containing NaCl (0·057 M) were held at pH 8·8 and 37 °C in the titration vessel of an automatic titrator. The initial rate of release of FFA following the addition of 1 ml of milk was measured by the automatic addition of 0·1 NaOH to maintain the pH at 8·8.

Lipolysability. Ten ml of milk were incubated with 1·0 ml of 4 % porcine pancreatic lipase at pH 8·0 and 37 °C. Release of FFA from the milk was measured by automatic titration with 1·25 N-NaOH. The reaction is autocatalytic and the determination of initial rate of lipolysis was impracticable. Results were therefore expressed as the reciprocal of the time taken to reach maximum rate.

Separation of aqueous and fat fractions. Twenty-five ml portions of milk were centrifuged at 11 000 × *g* for 15 min at 2 °C. The solid pad of fat was removed and the aqueous layer decanted from the overlying remnants of fat and the underlying pellet of protein and debris. Fat and aqueous fractions were stored at −20 °C.

Extraction of lipids. The lipids of whole milk were extracted by a modification of the method of Folch, Lees & Sloane Stanley (1957). The fatty acids of the aqueous fractions were extracted and concentrated as follows. Protein was precipitated with metaphosphoric acid (final concentration 2·5 %, w/v) and centrifuged off. The supernatant liquid was extracted twice with 2 volumes of a mixture of diethyl ether and *n*-pentane (1:1, v/v) which contained heptadecanoic acid as an internal standard. The aqueous phase was saturated with sodium sulphate to assist extraction. The solvent layers were filtered through anhydrous sodium sulphate and evaporated to a small volume at room temperature under a stream of nitrogen.

Separation of lipid fractions. Separation of milk lipids into FFA, mono-, di- and triglycerides was accomplished by thin-layer chromatography. Glass plates (20 × 20 cm) coated with silica gel and sodium carbonate (Duthie, 1965) were used. A solution of extracted lipid in chloroform (5–10 µl according to the fat content) was applied to the prepared thin-layer plates which were then developed for 30 min in a mixture containing diethyl ether, benzene, ethanol and glacial acetic acid in the relative proportions (40:50:2:0·2, v/v) (Freeman & West, 1966).

Analysis of fatty acids. FFA fractions were methylated with methanol containing 14 % (w/v) boron trifluoride (Metcalfe & Schmitz, 1961). After addition of saturated, ice-cold brine, the esters were extracted into *n*-pentane. Triglycerides, diglycerides and monoglycerides were transesterified by the use of sodium methoxide (Storry, Rook & Hall, 1967).

Methyl esters were analysed by gas-liquid chromatography using a dual column gas chromatograph with flame-ionization detectors (series 104, Pye-Unicam Ltd., Cambridge). The column packing material was diethylene glycol succinate adsorbed on silanized Chromosorb W, 80–100 mesh (20:80, w/v), and the temperature was programmed as follows: 3 min at 85 °C followed by a rise of 32 deg C/min to 180 °C and maintained for about 35 min, until methyl linolenate appeared in the effluent.

RESULTS

The action of pancreatic lipase on milk

Time course of the reaction

The results of a typical series of experiments, in which the course of lipolysis of whole milk by pancreatic lipase and the effects of various additions were studied, are given in Figs 1 and 2. The rate of release of fatty acids during lipolysis was at first low, then accelerated logarithmically, and later declined (Fig. 1, curve 1). In the presence of Ca^{2+} the acceleration was more rapid and the decline in rate was delayed (Fig. 1,

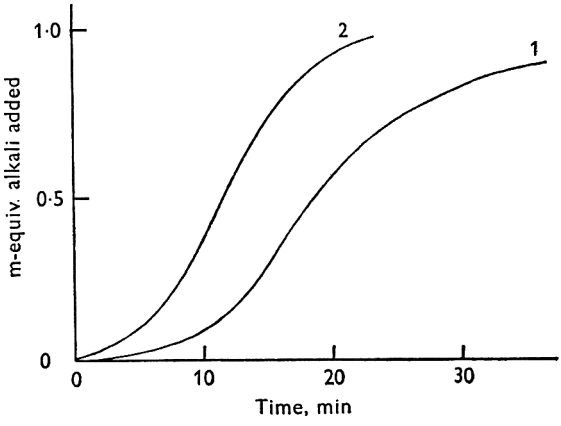

Fig. 1

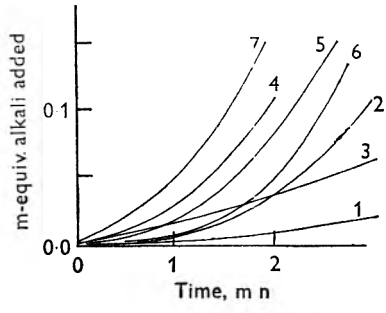

Fig. 2

Fig. 1. The course of lipolysis of whole milk by pancreatic lipase alone (1) and with the addition of Ca^{2+} (2).

Fig. 2. The effects of various additions on the initial phase of lipolysis of milk by pancreatic lipase: 1, no addition; 2, Ca^{2+}; 3, soaps; 4, soaps + Ca^{2+}; 5, monoglycerides; 6, monoglycerides + Ca^{2+}; 7, lipolysed milk.

curve 2). Addition of lipolysed milk (Fig. 2, curve 7) reduced the time required to reach maximum rate from 15 to 2 min. Of the components present in lipolysed milk, the addition of monoglycerides gave an effect most closely resembling that of the addition of boiled lipolysed milk itself (curve 5). The effects of the fatty acids as soaps (curve 3) were less dramatic but they seemed to have a more marked effect in the early stages, increasing the initial rate. In the presence of Ca^{2+}, the effects of fatty acids and monoglycerides were modified, that of fatty acids being enhanced (curve 4) and that of monoglycerides depressed (curve 6).

Products of the reaction

The release of FFA during pancreatic lipolysis at pH 8·0 and 37 °C is shown in Table 1. Though the results are not entirely self consistent, owing to the difficulties attached to the analysis of the trace amounts of acids present in the early stages of lipolysis, they nevertheless show a clear pattern. There was a preferential release of short-chain fatty acids (C_4–C_{12}) in the early stages of lipolysis, and the shorter the chain length the earlier the release and the greater the proportion of FFA in the aqueous fraction.

Table 1. *The concentrations (mg/100 ml of whole milk) of free fatty acids (FFA) in aqueous and fat fractions and of the fatty acids of mono-, di- and triglycerides during the course of pancreatic lipolysis*

	Extent of lipolysis*	Fatty acids†												Total fatty acids
		4:0	6:0	8:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	
Triglycerides	0	83·2	51·9	47·6	93·6	116·3	415·6	989·4	147·0	394·0	1097·1	28·6	24·9	3489·1
FFA (aqueous)	0	1·0	0·4	0·1	0·0	0·0	0·2	0·3	0·0	0·2	0·7	0·1	0·0	3·0
	1	0·9	0·3	0·1	0·0	0·0	0·1	0·3	0·0	0·1	0·4	0·0	0·0	2·2
	2	5·2	1·9	0·7	0·2	0·1	1·3	1·8	0·2	1·0	3·5	0·4	0·2	16·5
	5	8·3	2·5	2·6	1·3	1·6	2·3	5·0	0·3	1·7	5·9	0·1	0·1	25·7
	7	8·6	2·8	1·5	1·8	4·7	7·4	13·1	0·7	4·3	10·5	0·5	0·1	56·0
	100	69·6	14·8	17·8	68·1	63·6	137·6	552·0	57·7	260·5	741·5	17·5	14·6	2015·3
FFA (fat)	0	1·0	0·4	1·5	1·0	0·9	0·6	1·3	0·3	1·2	1·2	0·3	0·2	9·9
	1	1·5	1·7	2·2	2·3	2·0	4·5	8·7	2·6	6·1	8·4	0·7	0·2	40·9
	2	4·2	1·8	7·3	4·8	4·3	2·9	9·0	2·5	6·0	5·8	1·6	1·2	51·4
	5	4·1	0·9	2·8	4·3	3·5	8·8	32·5	2·2	18·4	41·2	1·3	1·4	120·4
	7	8·9	1·1	3·3	3·4	6·2	5·5	40·3	5·7	12·3	61·1	0·3	0·0	148·1
Monoglycerides	0	0·3	0·1	0·2	0·6	0·5	1·3	4·5	0·5	2·3	7·8	0·1	0·1	18·3
	1	0·3	0·1	0·2	0·6	0·7	1·3	4·9	0·6	2·4	8·0	0·2	0·2	19·5
	2	0·4	0·5	0·5	1·4	2·6	4·2	9·3	1·9	7·3	15·4	0·6	1·5	45·6
	5	1·4	1·2	1·9	1·6	2·4	9·3	20·3	1·9	4·6	17·3	1·9	2·0	65·8
	7	4·0	3·2	3·0	2·0	3·7	9·5	17·4	1·8	8·7	10·3	1·4	1·4	66·4
	100	5·4	7·4	7·4	23·7	51·4	188·6	350·2	43·3	66·9	251·5	15·5	37·2	1048·5
Diglycerides	0	1·8	1·0	1·9	1·5	2·5	8·9	26·2	3·9	11·7	23·7	1·3	1·4	85·8
	1	3·5	1·5	1·9	2·6	3·5	8·3	23·3	3·2	10·0	23·4	0·7	0·3	82·2
	2	10·5	4·5	5·4	3·6	7·7	27·2	57·6	2·5	18·1	48·5	5·5	1·2	192·3
	5	7·3	5·1	8·1	7·3	10·8	27·7	64·8	11·8	25·3	73·8	9·1	1·6	252·7
	7	9·7	9·0	5·8	6·0	13·1	32·1	59·1	9·8	22·1	53·8	6·8	2·8	220·1
	100	9·9	5·6	6·0	10·9	11·3	40·0	109·8	12·9	42·3	135·5	11·2	6·0	401·4

* Corresponding to the complete breakdown of the triglyceride molecule to one molecule of monoglyceride and 2 molecules of FFA.

† Number of carbon atoms and number of double bonds (Farquhar, Insull, Rosen, Stoffel & Ahrens, 1959).

Table 2. *Effects of temperature and of pH during lipolysis on (A) the content (molar %) of individual free fatty acids, and (B) the proportion (%) of each acid in the aqueous fraction, after 3% lipolysis by pancreatic lipase*

Fatty acid*	pH	At 0 °C		At 10 °C		At 20 °C		At 30 °C		At 40 °C	
		A	B	A	B	A	B	A	B	A	B
4:0	6·0	18·3	34·4	25·2	40·9	10·1	38·9	13·7	34·6	17·7	23·9
6:0 to 14:0		37·3	20·9	31·9	20·0	20·6	62·0	11·6	61·9	22·8	39·9
16:0		9·2	34·8	10·5	24·2	16·9	44·9	30·8	34·2	24·5	45·1
18:0		14·4	40·9	7·0	38·6	15·3	46·3	16·4	32·4	13·1	90·0
18:1		19·1	2·4	21·4	11·5	33·1	24·9	24·5	35·4	20·1	17·0
Others		1·7	16·9	4·0	20·0	4·0	29·5	3·0	26·7	1·8	42·5
4:0	6·5	17·5	41·2	26·5	66·7	20·2	13·3	13·2	36·6	32·3	21·1
6:0 to 14:0		48·8	33·2	41·6	42·0	15·4	45·5	22·1	24·7	28·9	18·0
16:0		9·6	32·9	8·8	27·0	22·7	17·1	17·3	6·6	7·6	11·4
18:0		8·3	38·5	7·1	34·8	15·2	46·5	15·3	9·2	11·2	15·3
18:1		13·9	31·4	13·4	21·9	25·3	19·6	24·6	7·1	17·0	5·1
Others		1·9	69·7	2·6	57·0	1·2	51·9	7·5	5·8	3·0	16·1
4:0	7·0	35·2	31·9	17·0	18·3	10·7	73·8	15·4	12·8	14·0	29·4
6:0 to 14:0		40·2	32·7	27·5	53·4	23·9	20·9	23·9	13·6	21·0	11·6
16:0		8·1	49·0	25·0	38·5	26·8	8·0	19·8	8·6	18·7	2·1
18:0		5·8	66·0	11·4	35·4	11·9	12·6	15·1	8·1	12·2	2·2
18:1		8·9	68·4	17·0	82·2	24·0	12·4	20·9	10·5	28·1	1·8
Others		1·8	33·3	2·1	19·0	2·6	15·2	4·9	4·4	6·0	1·6
4:0	7·5	13·8	89·5	23·2	71·5	15·6	79·6	17·2	30·0	7·7	27·7
6:0 to 14:0		13·1	79·3	23·4	73·1	39·6	35·4	22·2	34·6	22·7	19·3
16:0		27·6	22·1	17·3	24·6	12·8	70·2	17·7	24·3	26·6	18·3
18:0		17·1	23·7	13·3	44·9	12·1	50·0	13·8	21·4	17·3	30·6
18:1		26·5	42·9	19·2	23·8	85·9	67·2	26·8	20·6	23·6	8·9
Others		1·8	28·0	3·6	18·4	4·0	36·5	2·3	18·3	2·1	17·8
4:0	8·0	8·8	91·9	23·1	46·0	14·4	79·6	10·5	36·6	8·2	53·5
6:0 to 14:0		20·8	37·1	23·8	48·6	24·2	43·0	17·5	37·7	6·7	85·5
16:0		23·5	14·2	20·6	38·9	15·8	28·5	23·8	9·6	25·3	7·1
18:0		15·9	36·4	10·0	70·4	18·5	19·2	19·0	6·3	16·2	34·0
18:1		25·4	8·2	19·6	79·4	22·6	19·6	26·4	13·0	41·1	4·7

The trace amounts of monoglycerides present when lipolysis began contained a high proportion of oleic acid. As lipolysis progressed, the proportion of saturated fatty acids, and especially of palmitic, myristic and lauric acids, increased. Diglycerides, present initially in greater amounts than either FFA or monoglycerides, showed a 5-fold increase throughout the period of lipolysis but the fatty acid composition was fairly constant.

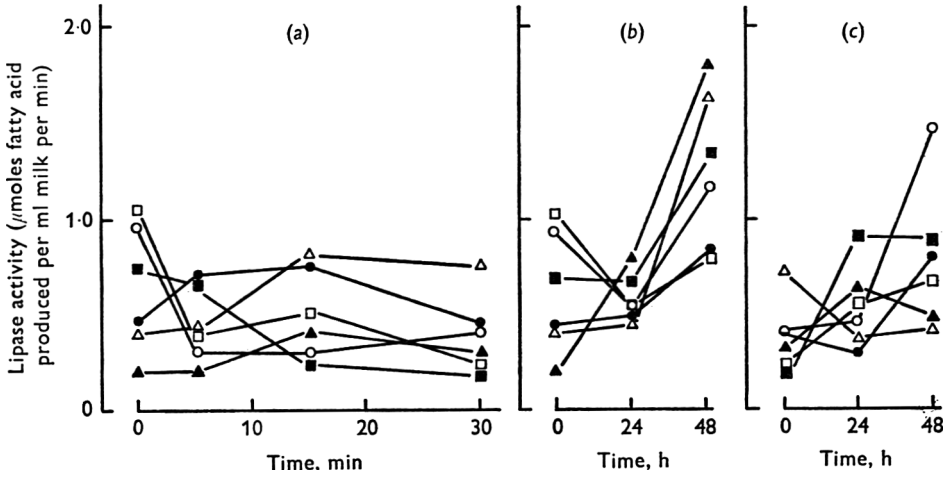

Fig. 3. The effects of aeration and storage on lipase activity: (*a*) effect of aeration; (*b*) effect of storage without aeration: and (c) effect of storage after 30 min aeration. ○, Milk A; ●, milk B; △, milk C; ▲, milk D; □, milk E; ■, milk F.

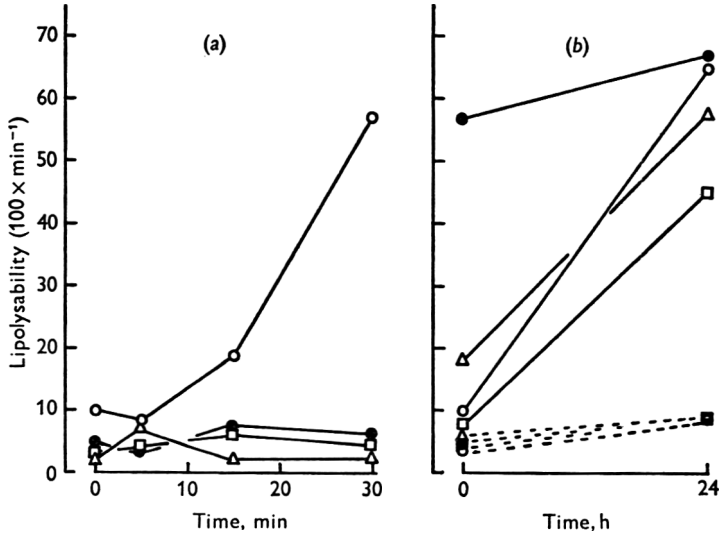

Fig. 4. The effects of aeration and storage on lipolysability: (*a*) effect of aeration. ○, Milk G; □, milk H; △, milk I; ●, milk J. (*b*) Effect of storage time after varying periods of aeration. ——, Milk G after 0 (○), 5 (□), 15 (△) and 30 (●) min aeration; – – –, milk H after 0 (○), 5 (□), 15 (△) and 30 (●) min aeration.

Effects of temperature and pH

The effects of temperature and pH during pancreatic lipolysis on the composition of the FFA released and their distribution between aqueous and fat fractions is shown in Table 2. The composition of FFA varied considerably with the conditions of lipolysis. Palmitic acid accounted for a higher proportion of the total at high temperatures and pH values. The proportion of oleic acid was highest at high temperatures and at a minimum at low pH values. The proportion of short-chain fatty acids (C_4–C_{14}) was at a maximum at low temperatures and at an acid to neutral pH, although at 40 °C high proportions were recorded at pH values up to 7·5.

At 0 °C and 10 °C the proportion of the total FFA in the aqueous fraction tended to increase with change of pH from 6·0 to 8·0, whereas at higher temperatures the proportion was at a minimum at pH 7·0. The trend with temperature at pH values between 6·5 and 8·0 was for the proportion in the aqueous fraction to decrease with increase in temperature, but at pH 6·0 the effect of temperature was reversed. The proportion of palmitic and oleic acids in the aqueous fraction was highest at pH 7·0 and at low temperatures, whereas a high proportion of the short-chain fatty acids, butyric acid in particular, in the aqueous fraction was favoured by alkaline conditions and low temperatures.

The action of intrinsic lipases on milk

As judged by a taste panel, sample G was already highly tainted before aeration and low temperature treatment, whereas other samples were untainted. After treatment, samples A, B and I were untainted, C and D moderately tainted, E, F, H and J highly tainted.

Effect of aeration and storage at 4 °C on the products of lipolysis

Aeration of milks gave a consistent increase in total FFA, which was most marked in samples C–F: the composition of the acids was not changed in samples A–C but in D–F there was a preferential release of short-chain acids (Table 3). There was, however, no consistent corresponding increase in the aqueous fraction of FFA nor of short-chain fatty acids. Sample E, with the highest initial concentration of FFA in the aqueous fraction, showed in fact a decrease with aeration.

Storage without aeration was associated with limited lipolysis and in samples D–F the increase in FFA was proportionately greater in the aqueous fraction than in the cream fraction: with samples D and F there was an especially marked increase in the concentration of butyric acid in the aqueous fraction. The effects of storage after aeration varied considerably between milks and the differences were not accounted for by differences in the concentrations of FFA at the beginning of the storage period. Total FFA increased notably in milks D and F, the effect increasing with the length of aeration. Samples D–F all showed considerable increases in the concentrations of FFA, and of butyric acid in particular, in the aqueous fraction.

Effect of aeration and storage at 4 °C on lipase activity

The effects of aeration on lipase activity (Fig. 2) were variable. Even a short period of aeration produced a fall in activity in milks with high initial values (A, E, F) but

Sample		A								B							
Aeration time, min		0		5		15		30		0		5		15		30	
Storage time, h	Fatty acid†	(1)*	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
0	4:0	1·3	0·2	2·1	0·0	2·8	0·1	2·7	0·2	0·4	0·1	0·4	0·2	2·0	0·1	2·4	0·3
	6:0	0·3	0·1	1·2	0·0	1·3	0·0	1·7	0·1	0·1	0·0	0·1	0·1	0·6	0·0	1·1	0·1
	8:0	0·4	0·0	0·5	0·0	1·0	0·0	1·2	0·1	0·1	0·0	0·1	0·0	0·2	0·0	1·1	0·0
	10:0	0·4	0·0	0·4	0·0	1·0	0·0	1·6	0·1	0·1	0·0	0·1	0·0	0·3	0·0	0·9	0·0
	12:0	0·5	0·1	0·6	0·0	1·3	0·0	1·2	0·1	0·1	0·0	0·1	0·0	0·3	0·0	0·7	0·0
	14:0	1·7	0·1	1·9	0·1	3·6	0·1	3·9	0·1	0·2	0·1	0·5	0·1	1·3	0·1	1·4	0·1
	16:0	3·2	0·3	4·1	0·3	6·7	0·3	7·3	0·2	0·3	0·2	1·0	0·3	2·9	0·2	3·3	0·3
	16:1	0·2	0·0	0·2	0·0	0·5	0·0	0·5	0·0	0·0	0·0	0·2	0·0	0·3	0·0	0·6	0·0
	18:0	2·0	0·2	2·2	0·2	2·5	0·2	3·0	0·1	0·3	0·1	0·4	0·2	1·2	0·2	1·6	0·2
	18:1	2·8	0·2	3·7	0·3	5·3	0·3	6·9	0·1	0·3	0·2	0·9	0·2	2·1	0·2	4·3	0·2
	18:2	0·1	0·0	0·1	0·0	0·3	0·0	0·4	0·0	0·0	0·0	0·1	0·0	0·1	0·0	0·3	0·0
	18:3	0·1	0·0	0·1	0·0	0·3	0·0	0·3	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·2	0·0
	Total	13·0	1·2	17·1	0·9	26·6	1·0	30·7	1·1	1·9	0·7	3·9	1·1	11·3	0·8	17·9	1·2
	Combined total	14·2		18·0		27·6		31·8		2·6		5·0		12·1		19·1	
24	4:0	4·0	0·1	3·4	0·1	4·4	0·2	7·1	0·1	0·6	0·2	2·0	1·1	3·5	0·9	4·4	0·5
	6:0	1·0	0·0	1·2	0·0	2·0	0·2	1·7	0·0	0·4	0·1	0·6	0·4	1·8	0·1	9·1	0·1
	8:0	0·5	0·0	1·8	0·0	1·3	0·0	1·3	0·1	0·2	0·0	0·4	0·1	2·1	0·0	6·0	0·1
	10:0	0·4	0·0	1·3	0·0	2·0	0·0	1·9	0·1	0·1	0·0	0·2	0·1	2·0	0·1	5·3	0·0
	12:0	0·4	0·0	1·1	0·0	2·2	0·0	1·5	0·1	0·1	0·0	0·1	0·1	2·3	0·1	3·3	0·0
	14:0	1·2	0·0	2·1	0·0	3·7	0·1	3·9	0·3	0·3	0·0	0·6	0·3	2·5	0·3	5·2	0·1
	16:0	3·6	0·1	6·4	0·1	6·5	0·5	6·4	1·1	0·7	0·1	2·0	0·9	4·4	1·2	13·4	0·6
	16:1	0·4	0·0	0·9	0·0	0·4	0·1	1·0	0·1	0·1	0·0	0·4	0·1	0·8	0·0	0·7	0·0
	18:0	2·1	0·1	1·8	0·1	3·1	0·3	4·5	0·6	0·2	0·1	1·1	0·6	2·0	0·7	5·1	0·3
	18:1	2·3	0·1	5·7	0·1	5·5	0·3	6·4	0·7	0·4	0·1	1·3	0·6	3·0	1·0	9·2	0·4
	18:2	0·2	0·0	0·3	0·0	0·4	0·0	0·5	0·0	0·0	0·0	0·3	0·0	0·2	0·1	0·4	0·0
	18:3	0·1	0·0	0·1	0·0	0·2	0·0	0·2	0·0	0·0	0·0	0·2	0·0	0·2	0·0	0·4	0·0
	Total	16·2	0·4	26·1	0·4	31·7	1·7	36·4	3·2	3·1	0·6	9·2	4·3	24·8	4·5	62·5	2·1
	Combined total	16·6		26·7		33·4		39·6		3·7		13·5		29·3		64·6	

Table 3 (*cont.*)

Sample		C								D							
Aeration time, min		0		5		15		30		0		5		15		30	
Storage time, h	Fatty acid†	(1)*	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
0	4:0	3·9	0·4	5·8	0·3	7·7	0·4	19·3	0·6	1·6	0·1	7·5	0·2	9·6	0·4	10·7	0·2
	6:0	1·6	0·1	1·4	0·1	1·6	0·1	8·3	0·3	0·7	0·0	2·3	0·1	3·3	0·2	3·4	0·1
	8:0	0·6	0·1	0·9	0·0	1·0	0·0	3·7	0·2	0·7	0·0	1·4	0·0	2·1	0·0	2·4	0·0
	10:0	0·2	0·1	0·6	0·0	0·7	0·0	2·6	0·1	0·5	0·1	0·8	0·2	0·7	0·4	0·9	0·3
	12:0	0·2	0·1	0·2	0·2	0·5	0·1	1·9	0·1	0·9	0·1	1·1	0·2	1·6	0·4	2·7	0·4
	14:0	0·6	0·2	0·4	0·6	2·3	0·4	4·0	0·2	2·7	0·2	2·9	0·6	2·7	1·3	4·3	0·8
	16:0	1·5	0·7	0·6	1·8	2·4	1·3	8·0	0·7	6·2	0·5	3·9	1·8	3·4	3·3	5·0	1·7
	16:1	0·0	0·2	0·2	0·3	0·4	0·1	1·4	0·1	0·2	0·0	0·5	0·1	0·5	0·2	0·7	0·1
	18:0	0·6	0·4	0·2	1·5	2·3	1·0	3·0	0·4	1·1	0·2	0·6	1·2	0·9	1·8	2·8	1·2
	18:1	1·7	0·7	2·0	1·5	4·7	1·1	6·2	0·6	2·4	0·3	1·9	1·5	2·0	1·7	2·0	1·8
	18:2	0·2	0·0	0·2	0·1	0·3	0·0	0·2	0·1	0·2	0·0	0·2	0·0	0·2	0·0	0·1	0·1
	18:3	0·1	0·0	0·1	0·0	0·2	0·0	0·2	0·0	0·2	0·0	0·1	0·0	0·1	0·0	0·1	0·1
	Total	11·2	3·0	12·6	6·4	24·1	4·5	58·8	3·4	17·4	1·5	23·2	5·9	27·1	9·7	35·1	6·8
	Combined total	14·2		19·0		28·6		62·2		18·9		29·1		36·8		41·9	
24	4:0	7·1	0·3	10·2	0·2	7·4	1·0	20·1	0·6	3·0	0·6	6·2	2·5	9·0	10·8	38·2	2·5
	6:0	3·6	0·2	3·5	0·1	3·7	0·2	9·1	0·2	1·1	0·2	3·0	0·3	6·4	2·4	16·1	1·0
	8:0	1·0	0·1	2·0	0·1	1·1	0·1	3·8	0·1	0·8	0·1	1·6	0·2	5·0	1·5	9·2	0·4
	10:0	0·6	0·2	2·9	0·0	0·8	0·0	3·1	0·1	0·9	0·1	1·0	0·2	3·1	0·8	9·2	0·4
	12:0	0·6	0·2	2·4	0·3	0·8	0·1	4·5	0·0	0·9	0·1	1·0	0·2	3·0	0·7	13·4	0·7
	14:0	1·8	0·2	3·2	0·6	2·5	0·3	5·4	0·2	3·3	0·3	3·7	0·5	15·6	2·0	36·0	2·3
	16:0	4·4	0·9	2·6	2·7	5·3	1·1	15·8	0·6	6·6	1·0	5·4	1·0	32·7	4·6	93·2	7·2
	16:1	0·9	0·1	0·8	0·1	1·0	0·1	1·6	0·1	0·5	0·1	0·5	0·1	3·5	0·2	5·6	0·5
	18:0	2·0	0·9	0·5	1·4	2·9	0·6	4·6	0·5	3·6	0·5	2·4	0·5	21·0	1·7	34·3	3·4
	18:1	2·5	1·2	1·4	2·1	6·5	0·7	7·7	0·5	5·7	0·7	3·2	0·8	24·2	4·0	59·8	4·7
	18:2	0·2	0·1	0·1	0·1	0·3	0·1	0·9	0·1	0·1	0·0	0·1	0·1	3·4	0·1	2·6	0·4
	18:3	0·2	0·1	0·1	0·0	0·3	0·0	1·1	0·0	0·1	0·0	0·1	0·1	2·5	0·2	2·1	0·4
	Total	24·9	4·5	29·7	7·7	32·6	4·3	77·7	3·0	26·6	3·7	28·2	6·5	129·4	29·0	319·7	23·9
	Combined total	29·4		37·4		36·9		80·7		30·3		34·7		158·4		343·6	

Aeration time, min		0		5		15		30		0		5		15		30	
Storage time, h	Fatty acid†	(1)*	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
0	4:0	0·4	2·1	5·6	0·5	14·8	2·8	17·7	0·6	0·9	0·4	3·0	0·5	6·0	0·8	10·3	2·5
	6:0	0·4	0·6	1·8	0·2	11·2	1·0	17·9	0·3	0·4	0·1	0·8	0·3	1·0	0·3	5·6	0·8
	8:0	0·8	0·2	0·8	0·2	4·5	0·6	7·6	0·1	0·2	0·0	0·9	0·1	0·9	0·2	5·7	0·3
	10:0	0·4	0·2	0·2	0·2	1·6	0·8	2·5	0·1	0·2	0·0	0·5	0·1	0·8	0·2	3·8	0·3
	12:0	0·4	0·2	0·4	0·3	1·2	0·7	3·0	0·2	0·2	0·0	0·4	0·2	0·5	0·2	3·9	0·3
	14:0	1·4	0·8	1·4	0·8	1·5	1·9	8·4	0·4	0·6	0·1	0·4	0·5	0·3	0·7	3·5	1·2
	16:0	4·5	2·9	5·0	2·1	3·4	3·9	28·8	1·4	2·7	0·2	3·0	1·5	2·3	2·6	6·0	3·2
	16:1	0·6	0·1	0·9	0·3	0·9	0·7	1·4	0·3	0·2	0·0	0·0	0·2	0·1	0·2	0·7	0·2
	18:0	4·1	1·5	5·6	1·2	5·1	1·7	13·2	1·0	1·9	0·1	1·3	1·7	1·7	1·7	4·7	1·8
	18:1	4·6	2·2	6·4	2·0	5·3	3·2	16·6	1·4	7·2	0·1	6·0	1·4	6·1	2·3	8·1	2·7
	18:2	0·2	0·1	0·1	0·1	0·0	0·2	0·2	0·0	0·1	0·0	0·0	0·1	0·1	0·1	0·2	0·1
	18:3	0·1	0·0	0·1	0·1	0·0	0·1	0·1	0·0	0·1	0·0	0·1	0·1	0·1	0·0	0·1	0·1
	Total	17·9	10·9	28·3	8·0	49·5	17·6	117·4	5·8	14·7	1·0	16·4	6·7	19·9	9·3	52·6	13·5
	Combined total	28·8		36·3		67·1		123·2		15·7		23·1		29·1		66·1	
24	4:0	1·7	1·1	5·4	1·4	17·1	6·5	18·3	6·3	5·5	2·3	6·2	1·2	8·5	3·2	13·9	5·4
	6:0	1·1	0·5	1·2	0·9	13·5	1·9	13·9	4·9	0·4	0·5	3·7	0·3	4·4	3·5	5·7	1·2
	8:0	0·4	0·3	1·1	0·2	2·8	1·5	6·6	1·2	0·9	0·3	1·6	0·1	5·9	1·4	6·5	0·5
	10:0	0·4	0·2	0·5	0·2	2·3	0·4	3·2	0·7	0·4	0·2	1·5	0·1	4·1	0·7	5·8	0·4
	12:0	0·4	0·3	0·6	0·2	2·7	0·4	3·8	0·9	0·4	0·2	1·4	0·1	2·4	0·9	6·3	0·4
	14:0	0·9	1·3	1·6	0·6	3·0	1·8	6·6	2·6	0·6	0·4	2·8	0·3	5·2	2·2	8·6	1·8
	16:0	3·9	3·9	4·3	2·9	4·0	3·2	27·1	7·5	1·9	0·8	5·7	0·7	12·5	7·3	36·3	7·5
	16:1	0·4	0·2	0·5	0·1	0·6	0·1	2·0	0·4	0·1	0·2	0·4	0·1	1·1	0·4	1·9	0·4
	18:0	3·5	2·3	5·7	1·4	2·6	4·4	12·1	4·2	6·6	0·5	3·3	0·5	3·2	5·0	23·2	4·8
	18:1	3·6	2·8	8·5	1·1	4·4	4·3	15·6	7·0	6·6	0·8	7·6	0·7	10·1	6·3	27·9	5·7
	18:2	0·1	0·1	0·1	0·1	0·1	0·1	0·4	0·1	0·1	0·0	0·1	0·0	0·2	0·1	0·2	0·0
	18:3	0·1	0·1	0·3	0·1	0·2	0·2	0·3	0·0	0·1	0·0	0·1	0·0	0·4	0·2	0·4	0·1
	Total	16·5	13·1	29·8	9·2	53·3	24·8	109·9	35·8	23·6	6·2	34·4	4·1	58·0	31·2	136·7	28·2
	Combined total	29·6		39·0		78·0		145·7		29·8		38·5		89·2		164·9	

* (1), fat; (2), aqueous.

the other milks showed slight increases. There was a similar pattern of change for the effects of storage without aeration for a storage period of 24 h, but in all samples after 48 h of storage the activity had increased beyond the initial value. Storage after 30 min aeration gave variable effects after 24 h, but with one exception (milk C) activity was higher at the end of 48 h of storage than at the end of aeration.

Effect of aeration and storage at 4 °C on lipolysability

Aeration and storage had a negligible effect on lipolysability in sample I, but H and J showed small increases. Milk G, however, had an initially high value which increased sharply after aeration for 15 and 30 min, and at the end of 24 h of storage it showed high values, which were to some extent unrelated to the period of aeration. This milk had an exceptionally high initial concentration of FFA in total and in the fat fraction (Table 4). Aeration and low-temperature treatment consistently increased the content of FFA but a high proportion was retained within the cream fraction. The composition of the FFA was similar to that in other milks.

Table 4. *The effects of aeration and storage on the total concentration of free fatty acids (mg/100 ml milk) in milk H*

Storage time, h	Aeration time, min...	0	5	15	30
0	Fat fraction	50·8	70·2	82·5	126·6
	Aqueous fraction	6·8	2·3	2·3	4·9
	Total	56·6	72·5	84·8	131·5
24	Fat fraction	87·8	94·3	102·4	171·6
	Aqueous fraction	9·0	6·7	5·6	15·0
	Total	96·8	101·0	108·0	186·6

DISCUSSION

Attempts to use the pH-stat technique to follow the lipolysis of milk by intrinsic lipases were unsuccessful because the rate of release of fatty acids was below the sensitivity of the instrument. Pancreatic lipolysis at pH 8·0 and 37 °C was therefore studied as a model system to assist in the understanding of the action of natural lipases on milk fat.

The course of lipolysis of milk by pancreatic lipase was complex (Fig. 1, curve 1). The rate was low initially but increased at first logarithmically and then later declined. The early logarithmic phase, which is of special interest in relation to the limited lipolysis which occurs under the action of intrinsic lipases, appears to reflect an autocatalytic action of the products of lipolysis. Of these, the monoglycerides had an especially marked effect on the rate of lipolysis while the FFA had a smaller one, but these effects were modified by Ca^{2+} which considerably enhanced the action of the fatty acids and depressed that of monoglycerides.

The decline in the rate of hydrolysis is probably due both to a reduction in the availability of substrate and an inhibition caused by the accumulation of the products of the reaction. Pancreatic lipase is specific for the ester links at the 1- and 3-position of glycerol and the plateau in the curve of lipolysis does in fact occur at a point corresponding approximately to the complete hydrolysis of triglyceride to monoglyceride and FFA. An inhibitory action of fatty acids as soaps when present

in high concentrations is suggested by the effect of the addition of Ca^{2+} which, probably due to the formation of insoluble calcium salts, delays the decline.

Lipase acts exclusively at a fat/aqueous interface, suggesting that the active centre of the enzyme is uncovered only after the twisting of the enzyme, which results from the movement of the non-polar side chains of certain amino acids towards the fat phase. For this intimate contact to be achieved, it is necessary to strip off the globule membrane and expose the milk fat, and the accelerating effects of boiling and homogenization are probably the result of such disruption. Surface-active materials may also have a similar action in displacing the globule membrane. However, such displacement would be ineffective in accelerating lipolysis if these materials then occupied the interface to the exclusion of the lipase molecules. This mechanism would lead to complexity in the observed effects of adding surface active agents, and the ambivalent effects of adding the soaps of fatty acids in the experiments described here may be a case in point. The transition from an activating effect to an inhibitory effect may result from changes in concentration, while the addition of Ca^{2+} ions may be effective in the selective precipitation of inhibitory soaps. A more analytical series of experiments would be necessary to explain these observations in detail.

Individual fatty acids were released by pancreatic lipase at different rates. In the early stages there was preferential release of short-chain fatty acids and a high proportion of these passed into the aqueous fraction. The effect was most marked at low temperatures and at acid or neutral pH. At alkaline pH, the proportion of fatty acids in the aqueous phase was extremely high, but the total amount of short-chain acids released was less.

Analogies between the course of lipolysis in the system studied and that occurring under natural conditions must be accepted with caution, but there is evidence that similar phenomena occur. The addition to 10 ml of fresh milk at pH 8·0 of 1 ml of milk that had been lipolysed with pancreatic lipase and then boiled to inactivate the enzyme, induced a rate of lipolysis measurable on the pH stat, and over a 2-h period there was an acceleration, indicating that the reaction was autocatalytic. Under natural conditions, the FFA content of milk as secreted and also the distribution, modified by pH, between aqueous and fat phases, may influence the further release of FFA.

Fresh milks were found to differ in their response to aeration and storage. Those in which taint could be induced by aeration and low-temperature treatment showed a considerable increase in FFA content during aeration whereas other milks showed little change. Storage at 4 °C for 24 h resulted in a further release of FFA but there was also a redistribution of fatty acids between aqueous and fat fractions. The increase in FFA content in response to aeration and low-temperature treatment was not, however, obviously related to the initial concentration of total or individual FFA either in the aqueous or in the fat fractions. Other factors such as, for example, the content and composition of fat, the globule size and membrane characteristics, or the milk pH, must be of greater importance.

Aeration and low-temperature treatment (up to 24 h) had variable effects on lipase activity and no relationship with the release of FFA was established. Effects on lipolysability, as assessed by the action of pancreatic lipase at pH 8·0 and 37 °C, were small, with the exception of milk H. This milk had an exceptionally high initial FFA

concentration in the cream and exhibited strong taint prior to aeration and low-temperature treatment, but showed only a limited further lipolysis as a result of this treatment.

The conditions chosen for the study of pancreatic lipolysis were optimum, since the rate of lipolysis falls off with reduction in pH or temperature so as to make it impracticable to measure lipolysability as a routine procedure at a pH and temperature corresponding to those under which taint develops naturally. In a survey of the bulk milks of herds milked by pipeline-bulk tank storage systems (Hemingway *et al.* 1970) lipolysability was correlated with taint score as judged organoleptically, but under the present conditions it did not correlate well with FFA release induced by aeration and low-temperature treatment.

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The digestion of fatty acids in the stomach and intestines of sheep given widely different rations

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Summary. The amounts of total lipid and fatty acids consumed, leaving the stomach and excreted in the faeces were examined in 4 sheep fitted with rumen and re-entrant duodenal cannulas. Diets of high (HM 1) and low (CM 1) roughage content were given at 0·9 times maintenance and the low-roughage diet was also given at 1·7 and 2·3 times maintenance. With all the rations more fatty acid left the abomasum than was consumed in the food, the difference being greater on ration CM 1 than on ration HM 1 and increasing irregularly with the amount of the low-roughage ration fed. Of the fatty acid entering the duodenum in the chyme, 72–89 % was digested in the intestine. About 80 % of the increase in fatty acids in the stomach was stearic acid and most of the remainder was palmitic acid. Almost all the polyunsaturated C_{18} acids ingested in the food were hydrogenated in the stomach, and the amounts of oleic acid were also greatly reduced, although more oleic acid entered the duodenum in the chyme with ration CM 1 than with ration HM 1. The possible origins of the increase in fatty acids in the stomach are discussed.

Substitution of starchy concentrates for most of the roughage of rations causes marked changes in the lipid metabolism of dairy cows, and in particular it results in substantial decreases in the output of milk fat. These changes appear to be due primarily to alterations in the proportions of short-chain fatty acids produced in the rumen by microbial fermentation. There is little evidence as yet about changes in the long-chain fatty acids in the rumen with different rations. Dietary lipids are known to be readily hydrolysed in the rumen and unsaturated fatty acids hydrogenated. There is evidence which suggests that the degree of hydrogenation of unsaturated acids is less on high-concentrate than on high-roughage rations (Tove & Matrone, 1962; Viviani, Borgatti, Monetti & Mordenti, 1967).

It has been reported that the amounts of lipid entering the duodenum of cattle (McGilliard, 1961; Sineshchekov, 1965) and sheep (Sheremet & Mikhailova, 1953) are greater than the amounts ingested in the diet but little appears to be known about the factors affecting the size of the increase. Furthermore, the composition of the lipid requires more careful examination. Reports of increases of lipid during passage of digesta through the stomach have mostly been based on the determination of 'ether extract', an ill-defined chemical fraction, but where, in sheep, the flow of long-

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chain fatty acids has been specifically measured (Scott, Ulyatt, Kay & Czerkawski, 1969; Leat & Harrison, 1969), only small differences between the amounts ingested and the amounts entering the duodenum have been found.

As part of a programme of study on the effect of dietary changes on milk fat secretion, Nicholson & Sutton (1969) measured the amounts of various nutrients digested in the stomach (rumen, reticulum, omasum and abomasum) and in the intestines of sheep given rations containing widely different proportions of hay and starchy concentrates. Changes in the amount and composition of lipid during passage of digesta through the gastrointestinal tract are now reported.

EXPERIMENTAL

Full details of the procedures were described earlier (Nicholson & Sutton, 1969).

The experimental animals were 4 sheep, each fitted with a rumen cannula and a re-entrant duodenal cannula inserted about 4–5 cm posterior to the pylorus and anterior to the point of entry of the common bile duct. Four rations were given in successive periods (amounts in g air-dry feed/day): (HM 1) 450 chopped hay, 150 dairy cubes; (CM 1) 90 hay, 150 dairy cubes, 200 flaked maize; (CM 2) 160 hay, 150 dairy cubes, 500 flaked maize; and (CM 3) 230 hay, 150 dairy cubes, 850 flaked maize. The ratio of hay to concentrates was 75:25 for HM 1 and 20:80 for the other 3 rations. Small amounts of ration HM 1 were refused occasionally. Rations HM 1 and CM 1 provided about 0·9 times maintenance, CM 2 1·7 times maintenance and CM 3 2·3 times maintenance (Agricultural Research Council, 1965). Two sheep (numbers 13 and 33) received all the rations; sheep 48 received ration HM 1 and sheep 83 rations CM 1 and CM 2.

The overall digestibility of the rations was determined by total collection of faeces for 7–10 days. Digestibility in the stomach was determined by two 24-h total collections of duodenal contents from each sheep on each diet. A proportion, 20–30 %, of the collected digesta was kept for analysis and the remainder was made up to volume with material from a donor sheep and returned to the duodenum via the distal cannula.

Two inert markers were administered to sheep 13 and 33. Chromic oxide, incorporated in paper, and polyethylene glycol (PEG, average mol. wt. 4000) in aqueous solution (25 mg/ml) were introduced directly into the rumen via the cannula at the rate of 1·75 and 18·0 g/day, respectively. Sheep 48 and 83 were given chromic oxide only.

Calculations. Overall digestibility was calculated from the difference between food ingested and faeces excreted during each collection period. Flow of digesta at the duodenum was corrected to give 100 % recovery of chromic oxide. Digestibility in the stomach was calculated from the difference between food ingested and the corrected amounts of digesta entering the duodenum.

Analyses. Samples of digesta and faeces were put into cold storage at −17 °C immediately after collection. Subsequently, a portion of each sample was freeze-dried and stored in closed jars.

Total lipids from samples of ground, freeze-dried food and digesta were extracted for 6 h with chloroform-methanol (2:1, v/v) in a Soxhlet apparatus. The extracted

lipids were then made up to a known volume with more chloroform-methanol and washed with 0·88 % (w/v) KCl solution, as described by Folch, Lees & Sloane Stanley (1957). The lipids were recovered from the lower phase by rotary evaporation and drying to constant weight under a stream of nitrogen. Samples of ground, freeze-dried faeces were mixed with 10 ml dilute H_2SO_4 and the mixture (pH 2) extracted by shaking with warm (55 °C) chloroform-methanol (2:1, v/v) for 30 min. After filtering the mixture, the chloroform-methanol extract was made up to a known volume, washed with 0·88 % (w/v) KCl and the lipids recovered as described above.

Total fatty acids were extracted from the total lipid samples, after saponification, as described by Lough, Navia & Harris (1966), except that hexane was used instead of light petroleum. Methyl esters of the total fatty acids were prepared with boron trifluoride in methanol (Metcalfe & Schmitz, 1961) and their composition determined by gas-liquid chromatography using a Perkin Elmer 880 chromatograph and a column (6 ft × $\frac{1}{8}$ in.) packed with 20 % ethylene glycol succinate on 80/100 mesh acid- and alkali-washed celite.

Details of other analyses conducted on these samples have been given by Nicholson & Sutton (1969).

RESULTS

The mean total lipid and fatty acid content and composition of the fatty acids of the foods is shown in Table 1. The 'other' fatty acids in hay consisted of 1·1 % 15:0*, 4·7 % 16:1, 4·0 % 22:0 and 2·5 % 24:0.

The amounts of total fatty acids ingested, entering the duodenum and excreted in the faeces are shown in Table 2 and the amounts of total lipid in Table 3. With all the rations the amounts of fatty acids leaving the abomasum were greater than the amounts ingested. The difference was over twice as great with the high-concentrate

Table 1. *Lipid composition of the foods*

Food	Total lipid, g/100 g D.M.	Total fatty acids, g/100 g D.M.	Composition of the fatty acids (g/100 g total fatty acids)								
			12:0	14:0	16:0	18:0	18:1	18:2	18:3	20:0	Other
Hay	3·71	1·12	0·4	1·9	48·0	14·7	9·4	4·7	5·2	3·5	12·3
Dairy cubes	4·50	2·78	9·8	6·3	21·3	4·3	25·4	30·7	0·1	2·1	0
Flaked maize	4·12	1·97	0	0·1	17·6	3·4	30·0	47·3	0·2	1·3	0

ration as with the high-roughage ration given at the same level, and it increased, though not regularly, with the amount of the high-concentrate ration eaten. Thus, the amount of fatty acid apparently absorbed from the tract, as calculated by the conventional procedure (amount in food minus amount in faeces), was only about half the amount absorbed when calculated from use of the present technique. The total fatty acids reaching the duodenum were highly digestible (Table 4) and so were readily available to the animal. Digestibility in the intestines and in the complete tract was higher with the high-concentrate ration than with the high-roughage ration given at the same level, but declined as the intake of the high-concentrate ration increased.

* Designation of fatty acids according to Farquhar, Insull, Rosen, Stoffel & Ahrens (1959).

With sheep 13 and 33 extraction of total lipids from duodenal digesta and faeces with 2:1 chloroform-methanol resulted in quantitative extraction of PEG also. Amounts of the samples were too small to permit removal of the PEG by further extraction. However, when the amounts of PEG in these samples, determined previously, were deducted from the amounts of total lipid, the values agreed well with those for sheep 48 and 83, which did not receive the PEG infusion. The presence of PEG in the total lipid extracts was found not to interfere with the subsequent determination of total fatty acids.

Table 2. *The amounts (g/24 h ± standard error of the means) of total fatty acids ingested, entering the duodenum and excreted in the faeces and the change in the amounts during passage of digesta through various parts of the gut*

	Flow, g/24 h			Change, g/24 h		
Ration	Food	Duodenum	Faeces	Stomach	Intestines	Total
HM1	8·2	11·5 ± 0·6	3·2 ± 1·2	+3·3	−8·3	−5·0
CM1	8·0	16·3 ± 2·1	1·8 ± 0·4	+8·3	−14·5	−6·2
CM2	14·4	23·9 ± 1·5	4·7 ± 0·8	+9·5	−19·2	−9·7
CM3	21·2	37·8 ± 1·5	8·4 ± 0·2	+16·6	−29·4	−12·8

Table 3. *The amounts (g/24 h ± standard error of the means) of total lipids ingested, entering the duodenum and excreted in the faeces and the change in the amounts during passage of digesta through various parts of the gut*

	Flow, g/24 h			Change, g/24 h		
	Food	Duodenum	Faeces	Stomach	Intestines	Total
HM1	20·6	28·5 ± 2·1	11·0 ± 1·2	+7·9	−17·5	−9·6
CM1	16·5	25·1 ± 3·8	5·2 ± 1·0	+8·6	−19·9	−11·3
CM2	31·3	48·5 ± 2·5	9·9 ± 1·4	+17·2	−38·6	−21·4
CM3	42·6	64·5 ± 3·2	18·0 ± 0·2	+21·9	−46·5	−24·6

Table 4. *Percentage change in the amounts of total lipid and total fatty acid during passage through sections of the digestive tract*

	Total lipid			Total fatty acids		
Ration	Stomach	Intestines	Total	Stomach	Intestines	Total
HM1	+38·3	−61·3	−46·6	+38·9	−72·0	−60·8
CM1	+51·5	−79·1	−68·4	+104·6	−88·8	−77·2
CM2	+55·3	−79·5	−68·2	+65·6	−80·3	−67·3
CM3	+51·4	−72·1	−57·8	+78·6	−77·7	−60·2

Total fatty acids did not form a constant proportion of the total lipids, the proportions tending to be greater at the duodenum than in the food and greater with high-concentrate than with high-roughage rations. As a consequence, although the broad pattern of the changes in the gut was similar for the 2 fractions, the percentage increase at the duodenum was greater for fatty acids than for total lipids with all the high-concentrate rations, and total apparent digestibility throughout the tract was greater for fatty acids than for total lipids with all the rations except CM2 (Table 4). However, the absolute increases in the stomach and decreases in the intestines were greater for total lipids than for fatty acids.

The amounts of the major fatty acids ingested and entering the duodenum are shown in Table 5, but since faecal fatty acids are mainly endogenous in origin, their composition was not determined in the present work.

Changes in the flow of individual fatty acids were broadly similar for all the rations. Of the saturated acids, the amounts of 12:0 at the duodenum decreased, 14:0 remained largely unchanged, 16:0 increased a little, especially at the higher levels of intake, and 18:0 increased very considerably; 20:0 tended to decrease. The amounts of the C_{18} unsaturated fatty acids were greatly reduced, 18:2 and 18:3 disappearing almost completely. The amount of 18:1 leaving the abomasum was greater with the high-concentrate ration than with the high-roughage ration and increased with the amount of the high-concentrate ration eaten. With all the rations a fraction identified as 14:1 was found in appreciable amounts at the duodenum but not in the food. Traces, less than 1 %, of 15:0 and up to 2·1 % of 17:0, were found at the duodenum with most rations. Trace amounts of 15:0 branch-chain were identified on 2 occasions only.

Table 5. *Means amounts (g/24 h ± standard error of the means) of the major fatty acids ingested and reaching the duodenum with each ration*

		Fatty acid									
Ration		12:0	14:0	14:1	16:0	18:0	18:1	18:2	18:3	20:0	Other
HM1	Ingested	0·3	0·3	0	2·8	0·8	1·5	1·3	0·2	0·2	0·8
	Duodenum										
	Mean	0·1	0·4	0·4	2·9	6·3	0·4	0	0	0·2	0·8
	S.E. mean (±)	0·03	0·08	0·10	0·30	0·54	0·12	—	—	0·08	—
CM1	Ingested	0·4	0·2	0	2·1	0·5	2·2	2·3	0·1	0·1	0·1
	Duodenum										
	Mean	0·1	0·3	0·3	2·9	11·6	0·9	0	0	0·1	0·1
	S.E. mean (±)	0·02	0·05	0·09	0·43	1·60	0·17	—	—	0·05	—
CM2	Ingested	0·4	0·3	0	3·0	0·6	3·7	6·0	0·1	0·2	0·1
	Duodenum										
	Mean	0·1	0·4	0·7	4·7	15·8	1·7	0	0	0·1	0·4
	S.E. mean (±)	0·07	0·10	0·24	0·86	0·62	0·47	—	—	0·09	—
CM3	Ingested	0·4	0·4	0	3·8	1·0	5·0	9·8	0·2	0·4	0·2
	Duodenum										
	Mean	0·1	0·4	0·7	6·3	26·0	3·5	0	0·1	0·1	0·6
	S.E. mean (±)	0·10	0·06	0·20	0·31	1·17	0·91	—	—	0·08	—

DISCUSSION

Composition of the fatty acids

The high degree of hydrogenation of dietary fatty acids in the stomach is in agreement with previous results from similar experiments (Bath & Hill, 1967; Leat & Harrison, 1969). There have been suggestions that hydrogenation is less effective with high-concentrate than with high-roughage rations (Tove & Matrone, 1962; Viviani *et al.* 1967; Storry & Sutton, 1969). In the present experiment, considerably greater amounts of unsaturated C_{18} acids entered the duodenum in the chyme with the high-concentrate rations than with the high-roughage rations. This was due primarily to the much greater intake of these acids in the food with the high-concentrate ration. In addition, a greater proportion of the unsaturated bonds escaped

hydrogenation with rations CM1 (13%), CM2 (11%) and CM3 (14%) than with ration HM1 (8%).

In contrast to the virtual disappearance of ingested polyunsaturated C_{18} fatty acids at the duodenum, there was an apparent increase in a mono-unsaturated C_{14} acid. This acid occurs in milk fat (Storry & Rook, 1965) and in adipose tissue (Tove & Matrone, 1962) of ruminants but was not reported to occur in duodenal digesta of sheep given hay and oats (Bath & Hill, 1967) or in microbial lipids (Keeney, Katz & Allison, 1962).

Only part of the increase in stearic acid at the duodenum could be accounted for by hydrogenation of unsaturated C_{18} fatty acids. The remainder of the increase and the increase in palmitic acid may have been due to elongation of shorter chain acids by the addition of two-carbon units and in this respect it is interesting that the amounts of lauric acid reaching the duodenum were less than the amounts ingested. However, the possibility that the reduced amounts of lauric acid at the duodenum may have been due to absorption from the rumen cannot be entirely eliminated (McCarthy, 1962; Wood, Bell, Grainger & Teekel, 1963).

Increase in fatty acids in the stomach

The results of the present experiment have clearly shown that a considerable increase in long-chain fatty acids can occur during passage of ingesta through the stomach of ruminants. Most previous results showing an increase in lipid have relied on extraction of lipids by ether which does not remove lipids from all materials with equal efficiency (Lough *et al.* 1966). Nevertheless, our values for the flow of total lipids, obtained by extraction into chloroform-methanol, agree reasonably well with results of Sheremet & Mikhailova (1953) with sheep, and McGilliard (1961) and Sineshchekov (1965) with cattle, all of whom relied on ether extraction.

The factors contributing to increased flow of fatty acids are not yet clearly understood. In our experiment the increase was greater with high-concentrate than with high-roughage rations and increased with the amount of the high-concentrate ration eaten. Measurements of lipid flow by McGilliard (1961) were too few to establish any clear pattern. Increases in total lipid of over 400% were found by Sheremet & Mikhailova (1953) in sheep given a ration containing very little fat, and Sineshchekov (1965) reported that equally large increases occur occasionally in cattle but few details were given. In contrast, when sheep were given hay and oats (Leat & Harrison, 1969) or dried grass, with or without supplementary linseed oil fatty acids (Scott *et al.* 1969) only small differences were found between the amounts of fatty acids ingested and the amounts reaching the duodenum.

The digestibility of the fatty acids entering the duodenum in the chyme was high in our experiment and greater than the digestibility of the total lipid. Work with cattle (Sineshchekov, 1965) and sheep (Lennox & Garton, 1968) also showed that about 80% of the fatty acids entering the duodenum in chyme and bile were absorbed during passage through the remainder of the tract.

Origins of the increase

It is most important to establish the origin of the increase in fatty acids in the stomach. If it is endogenous in origin, its significance to the overall energy meta-

bolism of the ruminant is probably small. Possible endogenous sources contributing to the increased flow of fatty acids are: desquamation of rumen epithelium, which is reported to contain appreciable amounts of lipid (Habel, 1959); the secretion of fatty acids in saliva or gastric juice; and diffusion of fatty acids from the blood across the gastric epithelium. The quantitative significance of these processes cannot be adequately assessed from available evidence. It is well established that considerable amounts of endogenous lipids are added to the chyme in the intestines. Adams & Heath (1963) estimated that, in adult sheep, 10–15 g phospholipid enter the duodenum daily with the bile, but in our experiment the position of the cannulas, immediately posterior to the pylorus, and the collection of samples from the proximal cannula excluded any possible contribution of fatty acids from this source.

On the other hand, the increase could reflect net synthesis by the rumen bacteria and protozoa of fatty acids from non-lipid dietary components, in which case the results would indicate that the ruminant may absorb 50–100% more long-chain fatty acid than is apparent merely from the conventional examination of food and faeces. Thus, part of the lipogenesis in ruminants could occur in the gut rather than in the tissues.

Some of the fatty acids at the duodenum are undoubtedly microbial in origin. Keeney *et al.* (1962) calculated that 142 g microbial lipid would leave the reticulorumen of a dairy cow every 24 h. Part of this microbial lipid probably represents direct incorporation of dietary lipids, but the work of Allison, Bryant, Katz & Keeney (1962) and Patton, McCarthy & Griel (1968) showed that synthesis of lipid from non-lipid substrates can occur, although its quantitative significance is not clear.

In the absence of contrary evidence, it is tempting to conclude that the increased flow of fatty acids is due largely to microbial synthesis, in which case the fatty acids reaching the duodenum should partly reflect the amounts and types of fatty acids synthesized by rumen microbes. However, the increase, in our experiment, of stearic acid in the stomach over and above that which could be attributed to hydrogenation of unsaturated C_{18} acids was always much greater than the increase in palmitic acid, whereas the results of Keeney *et al.* (1962) indicate that palmitic and stearic acids occur in approximately equal amounts in microbial lipids.

It is also difficult to account for the quantities of fatty acids apparently synthesized. Hungate (1965) estimated that bacteria convert the energy of substrates to microbial cells with an efficiency of only 10–20%. By using the higher value and the estimates for the production in the present experiments of short-chain fatty acids, methane and heat (Nicholson & Sutton, 1969), which are the main waste products of microbial fermentation in the rumen, we have calculated that 56, 58, 102 and 148 g respectively, of microbial organic matter would be synthesized from the 4 rations HM1, CM1, CM2 and CM3. Fatty acids constitute about 6–10% of the dry matter of rumen protozoa and bacteria (Gutierrez, Williams, Davis & Warwick, 1962; Katz & Keeney, 1964). From the mean value of 8%, it can be calculated that about 4·5, 4·6, 8·2 and 11·8 g of microbial fatty acids could have contributed to the increased flow at the duodenum for rations HM1, CM1, CM2 and CM3, respectively. The corresponding observed increases in fatty acid flow (3·2, 8·3, 9·5 and 16·8 g, respectively) considerably exceeded the calculated amounts for all the high-concentrate

rations. The discrepancy would be even greater if the lower efficiency of energy conversion reported by Hungate (1965) were correct.

There is clearly insufficient evidence to indicate the origin of the increased flow of fatty acids that occurs during passage of digesta through the stomach of ruminants. It is important that the origin be firmly established in order that the significance of the increase to the overall energy metabolism of the ruminant may be determined.

Conclusions

Direct comparison of the present results, obtained with sheep, with those for cows given production rations is not possible. Ration HM 1 contained a greater proportion of hay and was fed in smaller amounts, relative to metabolic body size, than a normal production ration for dairy cows. The high-concentrate rations contained a smaller proportion of concentrates than is necessary to induce severe falls in milk fat content (Storry & Sutton, 1969) although the amount of metabolizable energy provided by ration CM 3 was approximately equivalent to that in a production ration for cows.

Within these limitations the results suggest that more fatty acids reach the duodenum with high-concentrate than with high-roughage rations, and yet it is with very high-concentrate rations that severe falls in the amount of fat secreted in the milk of cows may occur. In view of this apparent increase in the amount of long-chain fatty acid absorbed from the digestive tract on high-concentrate rations, it is unlikely that an absolute shortage of long-chain fatty acid precursors would contribute to the reduced milk-fat secretion in cows given these rations. The results also indicate that more oleic acid is absorbed from the gut when high-concentrate rations are given, supporting the finding that higher concentrations of this acid occur in the plasma triglycerides (Storry & Sutton, 1969).

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Note added in proof

Heath & Hill (1969) have recently estimated that, in sheep fed lucerne or oaten chaff, about 6 g fatty acid enter the duodenum in the bile and that a further 3 g are added to the digesta from other endogenous sources which they were unable to identify. Heath, T. J. & Hill, L. N. (1969). *Aust. J. biol. Sci.* **22**, 1015.

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Determination of moisture in dairy products by near infra-red absorption of methanol extracts

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Summary. Absorbance measurements at the 1·93 μm water band on methanol extracts from butter, cheese and dried milk enabled moisture contents to be determined with coefficients of variation of 1·0, 1·1 and 2·0 %, respectively. Butter, cheese and cheese curd analyses could be completed within a few minutes, but a 2-h standing period was required for dried milk to overcome difficulties associated with lactose precipitation.

The use of conventional oven-drying methods for the analysis of complex materials such as food products gives rise to a number of fundamental difficulties, since the loss of volatile components under prescribed conditions of heating cannot always be taken as loss of water. This difficulty is particularly apparent both for products in which water molecules can be bound in different ways and also for products in which thermal decomposition reactions can lead to the production and consequent loss of water. For such materials, alternative methods for moisture determination are required, not only to provide more rapid analysis, but also to provide results which are specific for water.

Hart, Norris & Golumbic (1962) determined the moisture content of seeds by a method based upon the extraction of water with methanol or other solvent, followed by the spectrophotometric determination of water in the extract. The same method has been used successfully for vegetables (Gold, 1964), dried vegetables and spices (Rader, 1966) and for dried coffee powder (Brandenberger & Bader, 1961). Although the spectrophotometric methods for water determination have been known for several years, their use has been restricted to laboratories where expensive recording spectrophotometers are available. In principle, the method can be used with suitable low-cost filter photometers which are now being developed.

A number of solvents other than methanol have been proposed by Mamiya (1965) but several reasons have prompted the choice of this solvent in the present investigation. Methanol has a high affinity for water and is one of the few solvents which will remove the water of hydration from lactose occurring as the monohydrate. In the presence of water, methanol is a poor solvent for organic compounds such as fats which would otherwise cause interference in the spectrophotometric determination. Methanol is relatively transparent in the near infra-red region and the transmittance of a 10-mm path-length is about 10 % at the 1·93 μm water band. Methanol is cheap and is less unpleasant than the other solvents suggested by Mamiya.

Goulden & Manning (1969) made a detailed study of the spectrophotometric method for the determination of water in methanol and showed that a coefficient of variation of 0·4 % could be obtained by direct spectrophotometry. This could be reduced to 0·04 % by use of the transmittance-ratio technique combined with scale-expansion. The over-all accuracy for the dairy products so far examined was limited, by the effects of sample inhomogeneity, to that obtained by the direct spectrophotometric method.

The preparation of clear methanol extracts from dairy products presented a number of difficulties which were circumvented by the appropriate pre-treatment. Initially the product had to be disintegrated sufficiently to allow penetration of the solvent and to ensure complete removal of the water. For products with a high fat content (e.g. butter) or with a high protein content (e.g. cheese), it was necessary to immerse the vessel containing the methanol solution in an acetone-solid carbon dioxide bath for a short while. This procedure accelerated the precipitation of components which otherwise were precipitated from the solution during the spectrophotometric part of the analysis. Dried milk, and products with a high lactose content, presented a special difficulty since the lactose in its original state was partially soluble in methanol but was slowly converted into an insoluble form which deposited on the cell windows of the spectrophotometer. As described later, this difficulty was overcome by allowing the reaction to go to completion and then filtering the clear solution.

MATERIALS AND METHODS

The spectrophotometer used was a single beam non-recording instrument based on the monochromater of a Unicam SP500 spectrophotometer that had been modified as described by Goulden & Manning (1969).

Butter

In order to obtain a reproducible sample, butter was melted at the lowest possible temperature and the flask shaken vigorously until the butter became almost solid. Approximately 3 g were removed with the aid of a wide-tipped pipette and transferred to a weighed stoppered 100-ml conical flask. Methanol (75 ml) was added and the flask reweighed before warming it sufficiently to melt the butter. After shaking the flask for about 10 s, it was immersed in an acetone-solid carbon dioxide bath for about 1 min. This procedure caused most of the fat to separate as a solid, leaving a fairly clear solution which was filtered through the dried cotton wool plug of the filtration assembly shown in Fig. 1. A syringe was then used to transfer a portion of the clear filtrate to the spectrophotometer cell. Cells with a 10-mm path-length were used in both sample and reference beams and the absorbance difference at 1·93 μm was measured, using methanol in the reference beam.

Samples of butter covering a wide range of moisture content were obtained by the addition of known amounts of water to a butter containing 10·7 % moisture. The butter samples were then mixed thoroughly in a small mechanical blender.

Cheese and cheese curd

Absorbances in the region of 0·5 were ensured by taking the appropriate quantities of sample and solvent. For hard cheeses, a representative sample was taken from a carefully mixed portion of grated cheese and about 1 g placed in a weighed stoppered test-tube. The tube and contents were then reweighed and 35 ml methanol added. Absorption of water from the atmosphere was minimized by disintegrating the cheese in a test-tube by means of a glass rod sliding in a glass sleeve inserted in the rubber

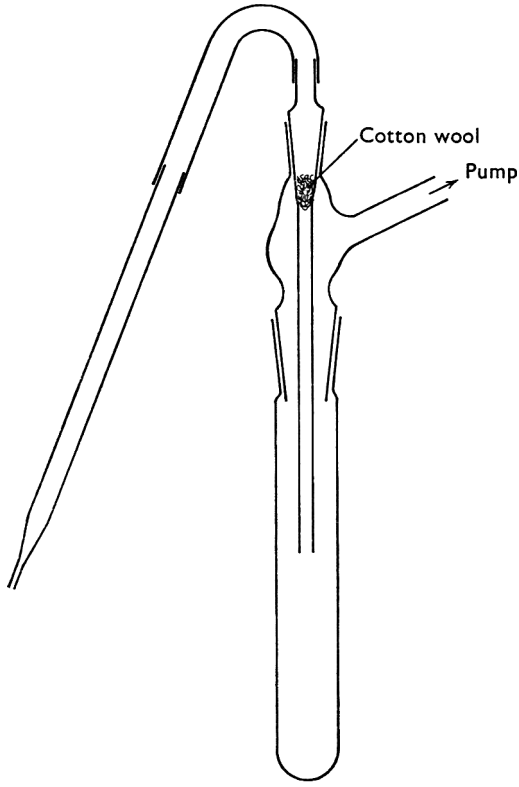

Fig. 1. Filtration assembly.

bung. The rod and bung were then removed, a stopper inserted, and the contents of the tube mixed by shaking. The tube was immersed in an acetone-solid carbon dioxide bath for about 1 min after which a portion of the clear filtrate was removed and analysed in a 10-mm path-length cell as for the butter extract. For processed cheese and soft cheeses, a sample quantity of about 0·5 g was taken up in 50 ml solvent.

Samples of cheese (3 g in 50 ml methanol) were mixed in an M.S.E. homogenizer (Measuring and Scientific Equipment Ltd, 25–28 Buckingham Gate, London, S.W. 1). A path-length of 2 mm was required to give absorbance readings of about 0·5.

Dried milk

Approximately 4 g milk powder were weighed into a stoppered 100-ml conical flask. Methanol (30 ml) was added and the mixture agitated for 20 min on a mechanical

shaker. After removal from the shaker, the flask was allowed to stand for 2 h to ensure that lactose precipitation was complete. A clear solution was obtained with the aid of the filtration assembly and was examined using a 10-mm path-length cell.

Chemical methods of analysis

The moisture contents of cheese and cheese curd were determined by standard oven-drying procedures, as outlined by Davis & MacDonald (1953) in Richmond's Dairy Chemistry. Method 5 of Richmond's Dairy Chemistry was used to determine the moisture content of butter. The moisture content of dried milk was determined by the oven-drying method (British Standards Institution, 1968) and by the toluene-distillation method (Dean & Stark process, Richmond's Dairy Chemistry) using a receiver of capacity 2 ml.

Table 1. *Comparison of results and reproducibilities*

Expressed as coefficients of variation (%) for mid-range water contents

	Reproducibilities			Comparison of spectrophotometric and conventional method
Sample	Spectrophotometric	Toluene-distillation	Oven-drying	
Butter	0·5	—	0·3	1·0
Cheese and cheese curd	0·7	—	0·6	1·1
Dried milk powder	0·35	—	0·7	5·0
	0·35	1·5	—	2·0

RESULTS AND DISCUSSION

Reproducibilities obtained for the moisture contents of butter, cheese and milk powders by both the spectrophotometric and chemical methods are given in Table 1. In all the methods, sample inhomogeneity appeared to be the main factor limiting reproducibility. The appropriate coefficients of variation enabled a comparison to be made between the results of the spectrophotometric and conventional methods of moisture determination.

The spectrophotometric method gave results (Fig. 2*a*) which were in good agreement with those of the oven-drying method, both for commercial butters (~ 16% moisture content) and for the experimental butters with very high and very low moisture contents. Two margarine samples examined by the spectrophotometric method gave results which also were in good agreement with those obtained by the oven-drying method.

The spectrophotometric method was also applicable to cheese and cheese curd samples covering a wide range of moisture content. Points on Fig. 2*b* are for cheese curd taken directly from the vat at various stages of the process, soft cheeses (Cottage, Colwick), hard cheeses (Cheddar, Cheshire, Stilton, Edam, Lancashire, Emmenthal) and 4 processed cheeses and cheese spreads.

Water contents obtained by the spectrophotometric method were compared with the values obtained by the toluene-distillation and oven-drying methods (Figs 3*a*, *b*) for a wide range of milk products which included dried full cream milk, dried skim-milk and whey powder. No systematic correlations were observed between the deviations shown in Figs 3*a* and *b* and the particular type of milk powder or the manufacturing

process (e.g. spray- or roller-drying). Several samples which gave low water contents by the oven-drying method gave values by the toluene-distillation method which were in closer agreement with those obtained by the spectrophotometric method. This accounts for the better correlation between the results of the spectrophotometric and toluene-distillation methods.

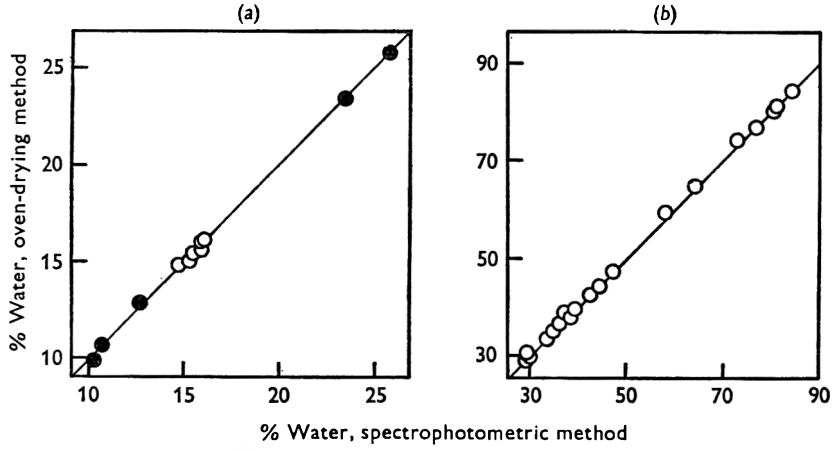

Fig. 2. Correlation of spectrophotometric and oven-drying methods for (*a*) butter samples: experimental, ●; commercial, ○, and (*b*) cheese and cheese curd samples.

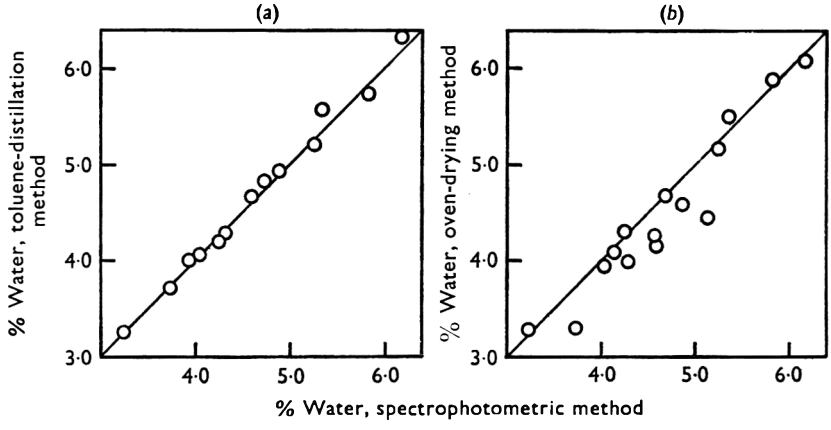

Fig. 3. Correlation of spectrophotometric with (*a*) toluene-distillation and (*b*) oven-drying methods for dried milk.

Since the spectrophotometric method is specific for water, the close agreement obtained by this method and by the oven-drying method for butter, cheese and cheese curd suggests that the water is completely removed from these products during the oven-drying process. With dried milk products, binding of water by lactose makes difficult the removal of all the water by oven-drying. For this reason, the more laborious toluene-distillation method is generally used when high accuracy is required. Since both the spectrophotometric and the toluene-distillation methods remove water from hydrated lactose, better agreement was found between the results

obtained by these 2 methods than between the results obtained by spectrophotometric and oven-drying methods.

The spectrophotometric method would seem to be particularly applicable to process control in cheese-making where it would enable the moisture content of cheese curd from the vat to be determined in less than 5 min. It may also find application in the analysis of milk powders, particularly if some way can be found of reducing the 2-h period at present needed to ensure complete precipitation of lactose.

We thank Miss M. Alder for technical assistance.

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Carbohydrate analysis of the glycopeptides released by the action of rennin on whole milk

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Summary. A gas-liquid chromatographic method is described for the simultaneous estimation of the carbohydrates attached to the glycopeptides released by the action of rennin on whole milk. The method can also be used for κ-casein. Certain of the findings may be applicable to other glycoproteins or glycopeptides. In particular: (*a*) the conditions for methanolysis of the carbohydrates from the glycopeptides must be chosen carefully to avoid decomposition of *N*-acetyl neuraminic acid; (*b*) it is not possible to obtain a satisfactory calibration for *N*-acetyl neuraminic acid using the free acid as a standard.

It is now recognized that bovine κ-casein is heterogeneous and that this heterogeneity is partly due to variation in the amounts of sugars that are attached to the protein (Mackinlay & Wake, 1965). When rennin acts on whole milk the κ-casein is hydrolysed (Waugh & Hippel, 1956; Wake, 1959) with the release of peptides and glycopeptides which contain almost all of the sugars attached to the κ-casein (Jollès, Alais & Jollès, 1961). The glycopeptides are soluble in trichloroacetic acid (TCA). Consequently it should be possible to obtain information on the sugars attached to κ-casein by studying the sugars of the glycopeptides which are soluble in the TCA filtrate of whole milk after the action of rennin. The use of gas-liquid chromatography (GLC) makes it possible to estimate individual sugars simultaneously. The present paper describes a GLC method which we have developed for the study of these sugars and which can be used for identification and quantitative analysis. The method can also be used for κ-casein.

Experimental

The satisfactory estimation of sugars by GLC has been achieved using the volatile trimethyl silyl (TMS) derivatives (Sweeley, Bentley, Makita & Wells, 1963). This procedure has been adapted for estimation of the sugars in glycopeptides and glycoproteins (Bolton, Clamp, Dawson & Hough, 1965; Clamp, Dawson & Hough, 1967).

For the estimation of the sugars in the glycopeptides released by rennin action on whole milk, there are special problems to be overcome. (*a*) Lactose is present in the TCA filtrate of whole milk in considerable excess of the sugars present in the glycopeptides and must be removed, otherwise it will mask the glycopeptide sugars. We have found that by dialysing against running tap-water for 14 days, the lactose, glucose, amino acids and other compounds of small molecular weight originally

present in the filtrate are satisfactorily removed. The dialysed filtrates can easily be checked to ensure that all free sugars have been removed. (*b*) Glycopeptides may be present in the TCA filtrate of milk before the action of rennin. We have allowed for this possibility by including a control sample, which had not been subjected to rennin action. The sugars attached to the glycopeptides could then be determined by difference. We have usually found that sugars attached to peptides before rennin action were present in relatively small amounts. (*c*) The concentration of glycopeptides in the dialysed filtrate is so low that the sugars released could not be detected with the Pye Panchromatograph which we used initially. By starting with a large amount of dialysed filtrate and then reducing the volume it was possible to obtain a sufficiently high concentration of sugars for detection by the instrument. In later work, a more sensitive instrument was available and therefore much less starting material was required. (*d*) The optimum conditions for methanolysis of the sugars may differ between glycopeptides.

Method

To 200 ml whole milk at 37 °C were added 2 ml of a 1 % (w/v) solution of rennin (Koch-Light Laboratories Ltd, Colnbrook, Bucks.). Clotting was first observed after about 5 min, and after 10 min TCA was added to a final concentration of 12 % (w/v) to stop the rennin action and precipitate the milk proteins. A control sample was also prepared in which the TCA was added before the rennin. The mixture was filtered and 100 ml of filtrate dialysed for 14 days against running tap-water at 4 °C. The volume was then made up to 250 ml with distilled water. A 50 ml sample of this solution was evaporated to dryness and kept in a desiccator over P_2O_5 for at least 12 h. The methyl glycosides of the sugars attached to the peptides were prepared by methanolysis (0·16, 0·32 or 0·64 N-HCl in methanol for periods of 2, 6, 12, 24 or 36 h at 80 °C on an oil bath). A few alumina chips were added to facilitate suspension of any glycopeptides adhering to the sides of the flask. The reaction mixture was neutralized with Ag_2CO_3 and filtered. Two ml of a 0·001 % (w/v) solution of D-mannitol in methanol-water (3:1, by volume) was added to the filtrate to act as an internal standard. The filtrate and methanol washings were evaporated under reduced pressure. TMS derivatives were prepared from methyl glycosides by the addition of 1·0 ml of a mixture of pyridine, trimethyl silyl chloride and hexamethyl disilazane (5:1:1, by wt.). The reaction mixture was allowed to stand, with occasional shaking, for 30 min and then centrifuged at $500 \times g$ for 5 min. A portion of the supernatant, (2 μl) was injected onto the column. The gas chromatograph was programmed from 140 to 220 °C at 1 deg C/min. All samples were checked to ensure that any free sugars had been completely removed. This was done by taking an additional sample of diluted dialysis residue and treating it in exactly the same way as described above but omitting the methanolysis. By this means any sugars in the filtrate except those released from the glycopeptides by methanolysis would be detected. No free sugars were detected in any of the samples after dialysis.

The analyses were carried out on a Pye Panchromatograph (Pye-Unicam Ltd, Cambridge) equipped with a flame ionization detector and temperature programming unit. The stationary phase was 3 % methyl silicone gum (SE 30) supported on Celite (mesh size 100–120). The column was 150 cm long. A Pye 104 Series Gas-Liquid

Chromatograph (Pye-Unicam Ltd, Cambridge) was used in the later stage of the investigation. For this instrument, the amount of milk used as a starting material was reduced from 200 to 40 ml.

Calibration. Accurate calibration is dependent on the quantitative preparation of the methyl glycoside from the free sugar. This can be achieved without difficulty for D-galactose and 2-acetamido-2-deoxy-D-galactose but we found that the methyl glycoside cannot be prepared quantitatively from the free *N*-acetyl neuraminic acid. In our experiments, the peak areas for *N*-acetyl neuraminic acid on the chromatograms were calibrated against values obtained independently on selected samples using the method of Warren (1959).

N-acetylation. In some experiments *N*-acetylation was done after neutralization with Ag_2CO_3 (1 ml acetic anhydride at room temperature for 6 h).

Reagents. It is essential that the methanolysis is performed under anhydrous conditions to ensure quantitative formation of the methyl glycosides. Pyridine was redistilled once. Small amounts of iodine and of magnesium turnings were added to the methanol before redistillation. H_2SO_4 was dropped on to NaCl crystals and the HCl gas evolved was bubbled through the anhydrous methanol to produce methanolic-HCl.

Table 1. *Effect of varying the time and the concentration of HCl used in methanolysis on the amount of carbohydrate produced from the glycopeptides*

Values are expressed as peak areas, arbitrary units.

	HCl conc., N	Time of methanolysis at 80 °C, h: 2	3	6	12	24	36
Expt no. 1							
D-galactose	0·16	5·6	—	8·1	8·6	—	—
	0·32	8·5	—	8·3	—	—	—
	0·64	8·2	—	8·3	8·0	—	—
N-acetyl neuraminic acid	0·16	1·5	—	1·9	1·8	—	—
	0·32	2·1	—	1·9	—	—	—
	0·64	1·9	—	1·9	1·1	—	—
2-Acetamido-2-deoxy D-galactose	0·16	0·0	—	0·26	—	—	—
	0·32	0·20	—	0·55	—	—	—
	0·64	0·55	—	0·61	0·58	—	—
Expt no. 2.							
D-galactose	0·64	—	12·1	—	—	12·4	—
N-acetyl neuraminic acid	0·64	—	9·6	—	—	6·7	—
Expt no. 3.							
D-galactose	0·64	—	12·8	—	—	—	11·6
N-acetyl neuraminic acid	0·64	—	12·3	—	—	—	7·0

Note—when methanolysis continued for 24 or 36 h, the chromatogram base line was very uneven, presumably due to the presence of break-down products and so it was not possible to obtain accurate values for 2-acetamido-2-deoxy-D-galactose, which produces a comparatively poor response.

RESULTS

Conditions of methanolysis. The effects of varying the conditions of methanolysis for glycopeptides in the trichloroacetic acid filtrate of milk after rennin action are shown in Table 1. These show that the maximum peak response was obtained for

all the sugars with 0·64 N-HCl for periods of methanolysis between 2 and 6 h. When the period of methanolysis with 0·64 N-HCl was increased to 12 h or more, there was a definite decrease in the response for *N*-acetyl neuraminic acid. For periods up to 24 h the response for D-galactose was not affected although there was perhaps a slight decrease after 36 h. On the basis of the results we selected methanolysis for 4 h with 0·64 N-HCl.

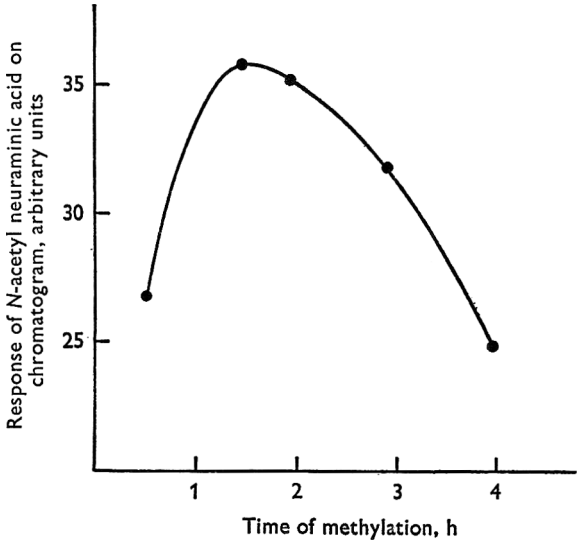

Fig. 1. Changes in the response of free *N*-acetyl neuraminic acid on gas-liquid chromatogram with time of methylation. Conditions of methanolysis and preparation of trimethyl silyl derivative are described in the text.

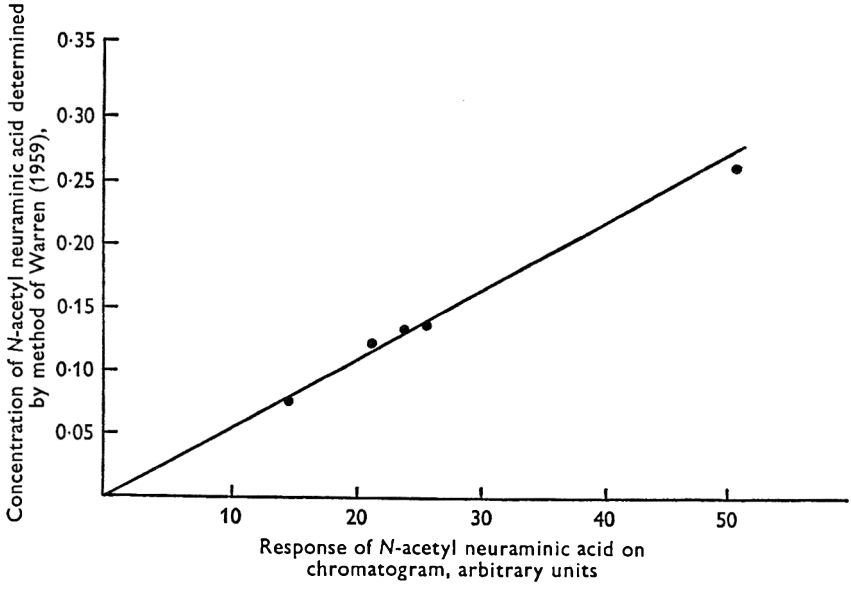

Fig. 2. Calibration of response of *N*-acetyl neuraminic acid on gas-liquid chromatogram by the method of Warren (1959).

Calibration. Fig. 1 shows how the response of free N-acetyl neuraminic acid on GLC varied with time of methylation. As the time of methylation increased there was an increase in the response until a maximum was reached and then the response decreased again. It appears from this result that decomposition of the *N*-acetyl neuraminic acid began before the formation of the methyl glycoside was completed. Fig. 2 shows that a satisfactory calibration could be obtained using the method of Warren (1959).

Reproducibility. There was good agreement between duplicates. Some examples are shown in Table 2.

Table 2. *Reproducibility of carbohydrate analyses of the glycopeptides*

Values are expressed as peak areas, arbitrary units.

Sample no.	D-galactose	*N*-acetyl neuraminic acid	2-Acetamido-2-deoxy-D-galactose
1A	5·5	3·4	0·95
1B	5·3	3·5	1·00
2A	6·2	3·2	2·1
2B	6·0	3·1	2·1
3A	4·9	2·5	0·98
3B	5·0	2·8	0·96
4A	9·1	5·4	2·2
4B	8·9	5·4	2·2
5A	8·0	4·5	1·9
5B	8·0	4·7	1·8
6A	6·0	3·7	1·6
6B	6·0	3·6	1·6

In samples 1, 2 and 3, A and B are independent duplicate analyses. In samples 4, 5 and 6, A and B are duplicate analyses on the dialysed filtrate.

N-*acetylation.* Because of the possibility of cleavage of the *N*-acetyl group in 2-acetamido-2-deoxy-D-galactose during methanolysis, Clamp *et al.* (1967) included a *N*-acetylation step. However, we have observed that when this step is omitted 2-amino-2-deoxy-D-galactose is not detected, which would be expected if any of the acetyl groups were cleaved. When the *N*-acetylation step was included there was no change in the response for 2-acetamido-2-deoxy-D-galactose. It appears therefore that under our conditions of methanolysis the 2-acetamido-2-deoxy-D-galactose was released from the sugar moiety without any loss of *N*-acetyl groups. This could be a useful method for determining whether or not a hexosamine is fully *N*-acetylated.

Identification of sugars. In many cases the TMS derivatives are formed from more than one isomer, and the relative proportions of the isomers are usually constant. Consequently, there is a characteristic response for each sugar and so a tentative identification can be made by comparing the response of an unknown sugar with those of standards. This procedure is feasible with glycoproteins because of the limited range of sugars present. Confirmation may be obtained by adding some of the standard to the unknown. Where any doubt remains, a different derivative may be prepared, which will give rise to different peaks in the chromatogram. This can be done by substituting hydrolysis (Neuberger & Marshall, 1966) for methanolysis. In this way

the free sugar is released instead of the methyl glycoside (see Fig. 3). For hexosamines, the *N*-acetyl derivative may also be prepared. Typical chromatograms obtained are shown in Fig. 4.

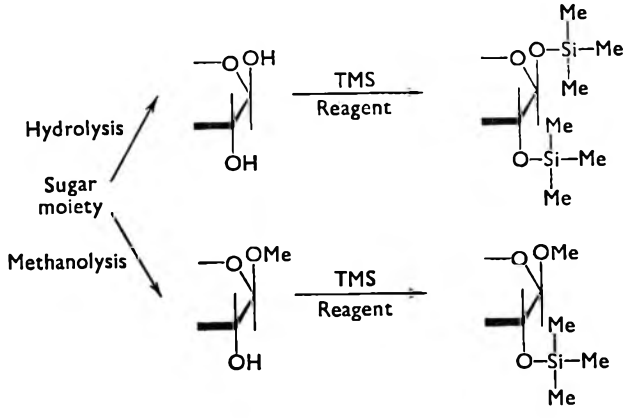

Fig. 3. Diagram to show trimethyl silyl derivatives formed as a result of methanolysis and hydrolysis.

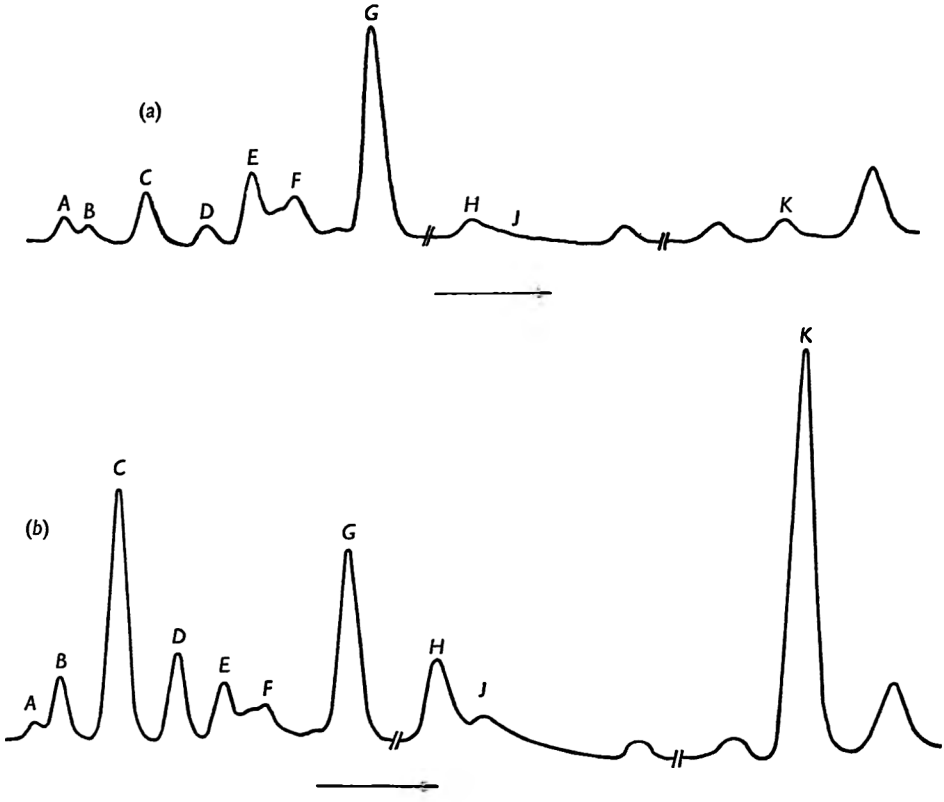

Fig. 4. (*a*) Chromatogram of sugars attached to glycopeptides soluble in the trichloroacetic acid filtrate of whole milk before rennin action. (*b*) Chromatogram of sugars attached to glycopeptides soluble in the trichloroacetic acid filtrate of whole milk after 10 min of rennin action. Identification of peaks. *A*, D-mannose; *B*, *C*, *D*, D-galactose; *E*, *F*, unidentified; *G*, D-mannitol (internal standard); *H*, *J*, 2-acetamido-2-deoxy-D-galactose; *K*, *N*-acetyl neuraminic acid.

DISCUSSION

Our results show that a satisfactory method can be obtained for the simultaneous estimation of the carbohydrates attached to the κ-casein glycopeptides released by rennin action. To achieve this, it has been necessary to make a number of modifications to the method originally developed by Clamp *et al.* (1967).

We found that the optimum conditions for methanolysis of the κ-casein glycopeptides were 2–6 h at 80 °C with 0·64 N-HCl. These conditions are somewhat milder than those used by Clamp *et al.* (1967), who concluded that there was no significant loss when the concentration of HCl was increased from 0·2 to 1·0 N and when the duration of methanolysis was increased from 6 to 24 h. However, the mildest conditions they used which would give maximum release of carbohydrates were 0·5 N-HCl for 24 h and 1·0 N-HCl for 12 h. Examination of our results suggests that if these conditions were used with κ-casein glycopeptides, there might be some decomposition of *N*-acetyl neuraminic acid. Although Clamp *et al.* (1967) used a selection of glycopeptides and glycoproteins they did not report any difference between them in the conditions of methanolysis needed for optimum release of carbohydrates. Our results suggest that this may not always be the case.

For most sugars relatively severe conditions can be used to ensure that all the carbohydrates are released. However, with *N*-acetyl neuraminic acid it is apparent that the methyl ester of the methyl glycoside decomposes if the duration of the methanolysis is extended for a considerable time after the carbohydrate is released from the glycopeptide. It follows from these observations that the optimum conditions of methanolysis must be established for each glycoprotein or glycopeptide being investigated.

Under the conditions of methanolysis employed some of the *N*-acetyl neuraminic acid is decomposed before it can all be converted into the methyl glycoside for use as a standard. However, when the methyl glycoside is formed from the bound *N*-acetyl neuraminic acid it is stable for a considerable period. It is possible that the methyl ester is formed before methanolysis and that the methyl glycoside of the ester is more stable than that of the free acid under these conditions. It has been shown that *N*-acetyl neuraminic acid decomposes in aqueous 0·1 N-HCl at 100 °C (Karkas & Chargaff, 1964). Although we have used the method of Warren (1959) to calibrate the GLC estimation of *N*-acetyl neuraminic acid, an alternative method would be to use the methyl glycoside of methyl *N*-acetyl neuraminate instead of the free acid. This compound can be prepared from *N*-acetyl neuraminic acid (Blix, Lindberg, Odin & Werner, 1956).

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With 2 plates

Fractionation of bovine serum lipoproteins and their characterization by gradient gel electrophoresis

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Summary. Bovine serum lipoproteins were fractionated by precipitation with dextran sulphate followed by ultracentrifugation and were examined by paper electrophoresis and by disk electophoresis in acrylamide gels. Gels containing concentration gradients of both sucrose and acrylamide gave better resolution of lipoproteins than did gels of uniform composition or gels with a concentration gradient of sucrose and a uniform concentration of acrylamide.

The densities of the lipoprotein classes isolated (listed in order of increasing mobility on electrophoresis in acrylamide gels) were as follows:

I, $d < 1{\cdot}019$; II, $1{\cdot}039 < d < 1{\cdot}050$;

III, $1{\cdot}019 < d < 1{\cdot}039$; IVa and IVb, $d > 1{\cdot}050$.

Lipoproteins of classes I, II and III were precipitated by dextran sulphate. On paper electrophoresis, lipoproteins of class II had β mobility and lipoproteins of classes IVa and IVb had α mobility. Lipoproteins of class I remained at the origin.

The lipoprotein classes of bovine sera resembled those of sheep and goat sera in that class IV predominated. In contrast, the sera of several non-ruminant species showed a predominance of lipoprotein classes of low electrophoretic mobility.

Extensive investigations have been made of procedures for fractionating and characterizing human serum lipoproteins employing analytical and preparative ultracentrifugations, electrophoresis and precipitation techniques (Lindgren & Nichols, 1960; Fredrickson, Levy & Lees, 1967). In contrast, less attention has been paid to the development of separation and characterization procedures for bovine serum lipoproteins (Griel & McCarthy, 1969). Preparative ultracentrifugation at a density of 1·063 (Evans, Patton & McCarthy, 1961) and precipitation with dextran sulphate (Glascock *et al.* 1966) have been used to separate bovine serum lipoproteins into 2 fractions. A technique combining both these methods has been investigated with a view to the separation of bovine serum lipoproteins into more fractions on a scale sufficiently large to allow their further examination.

In order to test the purity of the fractions of lipoproteins separated, a suitable analytical technique was required. The method of electrophoresis in polyacrylamide gel, known as disk electrophoresis (Davis, 1964), was selected, as it had already been

shown to give good resolution when applied to the separation of serum lipoproteins of other species (Narayan, Narayan & Kummerow, 1965; Narayan, Creinin & Kummerow, 1966).

MATERIALS AND METHODS

Chemicals

All the solvents used for lipid extraction and thin layer chromatography were redistilled. Clinical grade dextran sulphate was obtained from Glaxo Laboratories, Greenford, Middlesex. Amido black (Naphthol blue black) manufactured by Eastman Organic Chemicals Ltd., was supplied by Kodak Ltd., Kirkby, Liverpool. Glycine was purchased from Cambrian Chemicals Ltd., 59 Macks Road, London, S.E.16. Merck silica gel 254 + 366 was supplied by Camlab (Glass) Ltd., Cambridge. Analar grades of sodium citrate, calcium chloride, Tris and sucrose, reagent grades of acrylamide, *N*:*N*:*N*′:*N*′—tetramethyl-ethylene-diamine, *N*:*N*′—methylene-bis-acrylamide, Sudan black B, and Oil red 0, and riboflavine (biochemical grade), were purchased from British Drug Houses, Poole, Dorset. Whatman 3 MM paper was used for paper electrophoresis.

Concentrations of sodium citrate have been expressed as percentages (w/v) of the dihydrated salt.

Lipoprotein fractionation

Whole blood was allowed to clot at 22 °C for 2–3 h and the serum separated from the clot by centrifugation for 30 min at 1400 *g*. Where plasma was examined, sodium citrate was used as the anticoagulant in preference to heparin, which is known to react with lipoproteins (Cornwell & Kruger, 1961; Dangerfield & Faulkner, 1964; Pratt & Dangerfield, 1969).

Dextran sulphate precipitation

The method based on that of Burstein & Samaille (1957, 1958, 1960) as previously described (Glascock *et al.* 1966) was used with further modifications. Lipoproteins were precipitated by incubating serum with dextran sulphate reagent at 22 °C for 2 h. The precipitated lipoproteins were centrifuged at 35000 × *g* (max.) for 15 min and the precipitate redissolved in 3·4 % sodium citrate, density 1·019 (10 ml/100 ml serum). Reprecipitation was carried out by adding a volume of 0·1 M-$CaCl_2$ equal to that of the serum used. Lipoproteins were redissolved in 3·4 % sodium citrate (7 ml/100 ml serum).

Preparative ultracentrifugation

The density of the serum was adjusted by the addition of solid potassium bromide, and in the early stages of this work potassium bromide solutions were used to dissolve dextran sulphate precipitates. It was found, however, that the precipitated lipoproteins were more soluble and when redissolved were more stable in solutions of sodium citrate. An improvement in stability has also been found by including a chelating agent, EDTA, in the sodium chloride solutions used for the ultracentrifugation of lipoproteins (Lossow, Lindgren, Murchio, Stevens & Jensen, 1969). For most of the studies, therefore, dextran sulphate precipitates were dissolved in 3·4 % sodium citrate and adjusted to an appropriate density with sodium citrate solution of density 1·060 (10·2 %).

Samples were centrifuged in the 10 × 10 ml angle rotor of an MSE Superspeed 50 at 157000 × *g* (av.) for 22 h at 12 °C. Fractions were removed serially from the tubes into 10-ml plastic syringes (Becton, Dickinson & Co. Ltd., Drogheda, Ireland) having the same bore size as the tube. A syringe, fitted with an 18 G needle, curved at the tip, was clamped by the plunger above the tube so that the tip of the needle just touched the meniscus. The top fraction was drawn into the syringe by gently rotating and lowering the barrel, the needle remaining in the meniscus during this operation. Subsequent fractions were taken in a similar way.

Electrophoresis techniques

Reagents

The reagents used were mainly those described by Davis (1964).

(A) *Gel buffer.* This was made by mixing 36·6 g Tris in distilled water with 0·23 ml *N*:*N*:*N*′:*N*′—tetramethyl-ethylene-diamine adjusted to approximately pH 8·9 by the addition of 48 ml of N-HCl, and making up the volume to 100 ml with distilled water.

(B) *Sample buffer.* Tris (1·196 g) and 12 g sucrose in distilled water adjusted to pH 6·7 with N-HCl and the volume made up to 80 ml with distilled water.

(C) *Concentrated acrylamide.* Acrylamide (28 g) and 0·735 g *N*:*N*′—methylene-bis-acrylamide made up to 100 ml with distilled water.

(D) *Dilute acrylamide.* Acrylamide (11 g) and 1·5 g *N*:*N*′—methylene-bis-acrylamide made up to 100 ml with distilled water.

(E) Riboflavine in distilled water (0·004 % w/v).

(F) Sucrose in distilled water (40 %, w/v).

(G) *Electrophoresis buffer.* Tris (6 g) and 28·8 g glycine dissolved in distilled water and the volume made up to 1 l.

(H) *Sudan black.* A saturated solution of Sudan black B in propylene glycol prepared according to McDonald & Ribeiro (1959).

(I) *Amido black.* Amido black (1 %) in acetic acid:methanol: water, 10:30:60 (v/v) (Margolis & Kenrick, 1967).

Table 1. *Preparation of uniform gels*

The reagents were mixed in the indicated proportions by volume

Gel concentration, ...	11·0	7·0	5·0	4·0	3·5	3·0
Reagent A	1·0	1·0	1·0	1·0	1·0	1·0
C	3·0	2·0	1·0	0·5	0·2	0
D	0	0	1·0	1·5	1·8	2·0
E	1·0	1·0	1·0	1·0	1·0	1·0
Distilled water	3·0	4·0	4·0	4·0	4·0	4·0

Procedure

Acrylamide gels of several types were used in the electrophoresis of lipoproteins. Gels of uniform acrylamide concentration without sucrose were prepared using the reagents given in Table 1. Gels containing 15 % sucrose were prepared by replacing 3 ml of the distilled water with 3 ml of reagent F. Measured volumes (1·2 ml) of the appropriate mixture were placed in standard disk electrophoresis tubes overlayered with water and photopolymerized (Davis, 1964). The method of Margolis & Kenrick

(1967) was used to prepare the gradient gels. Those containing gradients in sucrose concentration of 5–15% and acrylamide of uniform concentration were prepared from solutions containing 5 and 15% sucrose. These solutions were prepared according to the proportions given in Table 1 with the exceptions that 1-ml and 3-ml volumes of distilled water were replaced by corresponding volumes of reagent F. Gels with gradients of both sucrose and acrylamide were prepared using the reagents given in Table 2.

Table 2. *Preparation of sucrose and acrylamide gradient gels*

The reagents were mixed in the indicated proportions by volume.

	Low concentration acrylamide	High concentration acrylamide
Reagent A	1·0	1·0
C	0	3·0
D	2·0	0
E	1·0	1·0
F	1·0	3·0
Distilled water	3·0	0

Preparation of the mixtures and fitting of the gel tubes was carried out in dim light. The acrylamide was then photopolymerized and individual tubes separated from the gel block for insertion into the disk electrophoresis chamber (Shandon Scientific Company Ltd). The surface of the gel was rinsed and the buffer compartments filled with the electrophoresis buffer, reagent G, diluted 1 to 5.

Lipoprotein fractions dissolved in potassium bromide solutions of densities greater than 1·063 were first dialysed against 0·9% (w/v) sodium chloride because the electrophoretic mobility was found to be reduced by these high salt concentrations. Lower salt concentrations had no appreciable effect. Lipoprotein samples were pre-stained by adding 0·1 to 0·2 parts of solution H carefully and with constant mixing to 1 part of each sample. The quantity of solution H used was varied according to the lipoprotein content of the sample, since the use of too large an excess of Sudan black caused losses of lipoprotein, presumably due to adsorption by the precipitated stain. After 1 h at 20 °C excess stain was removed by centrifugation and an equal volume of reagent B was added to the supernatant liquid. Samples for staining after electrophoresis (post-staining) were diluted with an equal volume of reagent B. In these samples the stains used were either Amido black for protein, or Oil red O for lipoprotein (Beaton, Selby & Wright, 1961).

Prepared samples of 4–40 μl were layered directly on the surface of the gels beneath the electrophoresis buffer. Electrophoresis was carried out at room temperature by passing a current of 2·5 mA/tube for 3 min, followed by 4 mA/tube for 12 min. Samples post-stained with Oil red O showed the same pattern as the pre-stained samples except for a reduced amount of dye uptake at the origin and just below, indicating the presence in the pre-stained samples of colloidal particles of Sudan black in this area. An assessment of the significance of any bands at or near the origin could therefore only be made after post-staining. Amido black staining demonstrated the absense of non-lipoprotein components in dextran sulphate precipitates.

Lipid Separations

Lipids were extracted from the serum and from the lipoprotein fractions by methanol and chloroform according to the method of Folch, Lees & Sloane Stanley (1957) essentially as described by Storry & Rook (1965).

The extracted lipids were separated by thin layer chromatography on a 0·5 mm layer of silica gel (Merck HF 254 + 366) with hexane : ether : formic acid 80 : 20 : 1 in a vapour saturated tank. The positions and intensities of the lipid bands were observed after exposure to iodine vapour by illuminating them with light of 366 nm wavelength.

RESULTS AND DISCUSSION

Electrophoretic patterns obtained by disk electrophoresis of human and bovine sera pre-stained with Sudan black B are shown in Plate 1 *a*. The patterns obtained on uniform gels at various acrylamide concentrations were the same in the presence or absence of sucrose. In the 7 and 5 % gels, only the faster lipoprotein classes had entered and separated; in the 3 % gel the slower classes had migrated well into the gel, but resolution of faster classes was not achieved. Similar results were obtained by Narayan *et al.* (1965, 1966) and Narayan (1967). It has been demonstrated by Margolis & Kenrick (1967, 1968) and Epstein, Houvras & Zak (1968), that improved resolution was achieved by the use of gels having a gradient in the concentration of polyacrylamide. When such a gradient gel system was applied to the separation of serum lipoproteins (Plate 1 *a*, *d* and *h*), the slower moving lipoprotein classes were able to enter and separate as in the 3 % gel, whilst resolution of the faster moving classes occurred as in more concentrated gels. During the course of this work similar results showing the advantages of using gradient gels for lipoprotein separations have been reported by Pratt & Dangerfield (1969).

Gels with a gradient in sucrose concentration, but uniform acrylamide concentration, gave good resolutions of lipoprotein classes in human and bovine sera on 5 % gels (Plate 1 *b*). Slightly improved resolution, especially of the slowest moving classes, was attained by using gels with concentration gradients both in sucrose and acrylamide. Sucrose gradient gels of either 7 or 3 % gave poorer resolution, again the slow-moving classes migrated too slowly in 7 % gel, and the faster moving classes failed to separate on 3 % gel. The extent to which the sucrose gradient affected the electrophoresis of proteins in the systems of Margolis & Kenrick (1967, 1968) and Pratt & Dangerfield (1969) was not determined. Sucrose gradients in those systems were 0–10 % (Margolis & Kenrick, 1967); 1–4 % (Margolis & Kenrick, 1968) and 0–4 % (Pratt & Dangerfield, 1969) compared with 5–15 % used in this study. The reason why better resolution was achieved on 5 % gels with a sucrose gradient of 5–15 % than on 5 % gels without the sucrose gradient may be due to the effect of the gradient in suppressing convective disturbances during gelation.

Comparisons of electrophoretic patterns of serum lipoproteins of several species of animal on gradient gels

The electrophoretic patterns of lipoproteins in sera or plasma of human, pig, rabbit, rat, goat, sheep and cow are shown in Plate 2 *a*. Marked differences between

species can be clearly seen, although it must be remembered that variations from sample to sample from a single species can occur. For example, Fredrickson *et al.* (1967) have drawn attention to the different lipoprotein patterns that can occur in sera of humans who are suffering from various abnormalities of lipid metabolism. In the present study, an examination of the faster moving plasma lipoprotein classes of cow 13 compared with those of cow 381 showed that the latter had an additional class (III) which migrated slightly more slowly than did the 2 classes of greatest mobility (IVa & IVb). Lipoproteins of class III were present in the serum of several cows and will be further considered later. Class IVa was sometimes resolved into 2 subclasses.

Other differences may be related to the physiological state of the animal, as was shown by a greatly enhanced intensity of the band just below the origin on electrophoresis of serum of a starved rabbit. In addition to this, some preliminary evidence was obtained which showed that the electrophoretic pattern of the serum of an animal may depend upon its state in the reproductive cycle, e.g. whether pregnant, lactating or dry (R. W. Smith & V. A. Welch, unpublished results). Major changes must have been responsible for such observations, since very little difference was observed between the electrophoretic patterns of bovine sera from jugular and mammary veins, although there were large differences in triglyceride content between the dextran sulphate-precipitable lipoproteins (precipitable lipoproteins) from these sites (Bishop, Davies, Glascock & Welch, 1969). Despite the occurrence of such variations, analyses made on the sera of several representatives showed that each species had a characteristic lipoprotein pattern. Inspection of the patterns obtained with sera of ruminant and non-ruminant animals showed that the former had a relatively low content of slow moving lipoprotein classes but a relatively high content of classes of high mobility. Significant differences were also apparent between the electrophoretic patterns obtained with sera of different ruminant species showing, in particular the presence of 2 or more class IV lipoproteins in bovine serum compared with the single class IV lipoproteins in the sera of sheep and goats.

Electrophoresis of bovine serum lipoproteins isolated by dextran sulphate precipitation and by ultracentrifugal flotation

The pattern obtained from the precipitable lipoproteins of bovine serum is shown in Plate 2*b*. The effect of reprecipitation in removing traces of faster running lipoprotein classes can be clearly seen; that which remained coincided with the position of class III of whole serum. All the precipitable lipoproteins floated when centrifuged in a medium of density 1·050. Only the slowest migrating class (I) floated when the precipitable lipoproteins were centrifuged at a density of 1·019. After removal of the lipoprotein class of density $< 1{\cdot}019$, the 2 remaining precipitable lipoprotein classes of density $> 1{\cdot}019$ were separated by adjustment of the density to 1·039 followed by ultracentrifugation; the class with the greater electrophoretic mobility (III) floated under these conditions. Thus, in order of increasing electrophoretic mobility, the densities (d) of the 3 precipitable lipoprotein classes were as follows: (I) $d < 1{\cdot}019$; (II) $1{\cdot}039 < d < 1{\cdot}050$; (III) $1{\cdot}019 < d < 1{\cdot}039$. The presence of these 3 classes was also demonstrated in the upper layer of whole serum twice centrifuged at density 1·050.

Electrophoresis on paper showed that the non-precipitable lipoproteins (classes IVa and IVb) migrated in the α position; lipoproteins of class II migrated in the β position and those of class I did not migrate. The mobility on paper of the lipoprotein of class III was not examined. A summary of the characteristics of bovine serum lipoproteins is given in Table 3.

Table 3. *Characterization of bovine serum lipoproteins*

Electrophoresis on gradient polyacrylamide. Lipoprotein class:	Electrophoresis on paper	Density, g/ml	Dextran sulphate fractionation
I	Origin	$< 1{\cdot}019$	Precipitated
II	β	$1{\cdot}039 < d < 1{\cdot}050$	Precipitated
III	Not examined	$1{\cdot}019 < d < 1{\cdot}039$	Precipitated
IV*a*	α	$> 1{\cdot}050$	Not precipitated
IV*b*	α	$> 1{\cdot}050$	Not precipitated

Thin layer chromatography of lipids extracted from the precipitable lipoproteins of bovine jugular serum showed that their triglyceride contents were inversely related to their density. Details of the lipid composition of each fraction are not yet available.

We are grateful to colleagues at the Institute for providing blood samples and to Dr J. D. Cumming of Reading University Health Centre for drawing a sample of human blood. We also acknowledge the interest and encouragement of Dr G. C. Cheeseman and Dr R. F. Glascock.

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EXPLANATION OF PLATES

Disk electrophoretic separations of samples pre-stained with Sudan black B. The direction of migration was from top to bottom. This was also the direction of increasing concentration in gradient gels.

PLATE 1

(*a*) Electrophoretic separation of human serum, a–d, and bovine serum (cow 381), *e–h*: *a* and *e*, 7 % acrylamide, 15 % sucrose; *b* and *f*, 5 % acrylamide, 15 % sucrose; *c* and *g*, 3 % acrylamide, 15 % sucrose; *d* and *h*, 3–11 % acrylamide, 5–15 % sucrose.

(*b*) Electrophoretic separation of human serum, *a–d*, and bovine serum (cow 381), *e–h*: *a* and *e*, 7 % acrylamide, 5–15 % sucrose: *b* and *f*, 5 % acrylamide, 5–15 % sucrose; *c* and *g*, 3 % acrylamide, 5–15 % sucrose; *d* and *h*, 3–11 % acrylamide, 5–15 % sucrose.

PLATE 2

(*a*) Electrophoretic separation of serum (S) or plasma (P) from different species on gels containing 3–11 % acrylamide and 5–15 % sucrose.

(*b*) Electrophoretic separation on gels containing 3–11 % acrylamide and 5–15 % sucrose of whole bovine serum (cow 24), and of the lipoproteins precipitated by dextran sulphate: *a*, serum; *b-d*, precipitated lipoproteins in sodium citrate solution (4·1 %); *b*, after initial precipitation; c, after reprecipitation; *d*, after a further reprecipitation.

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h
g
f
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d
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b
a
(a)
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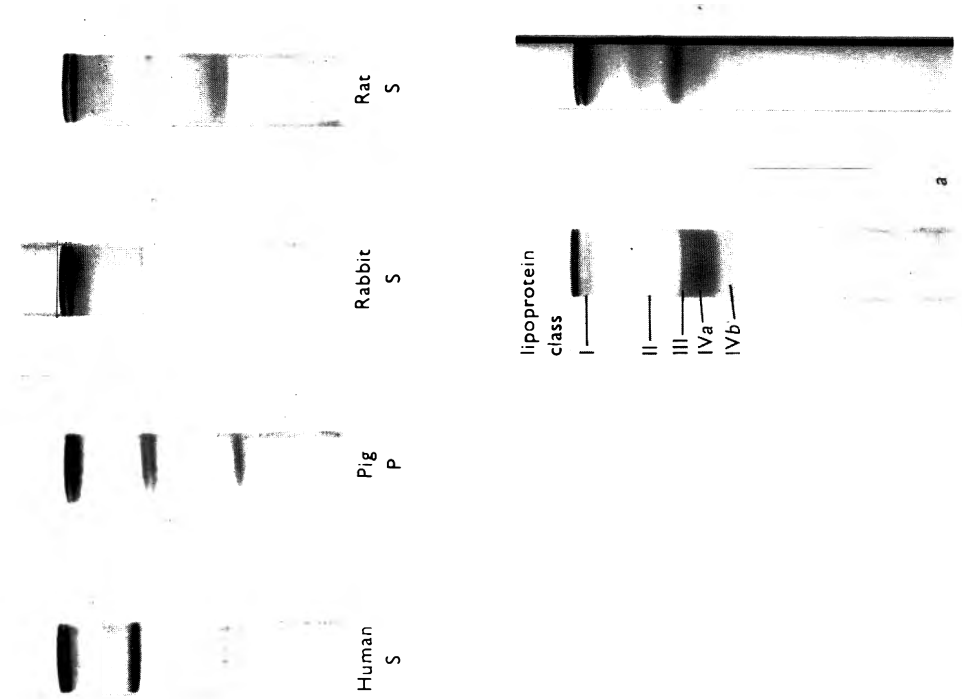
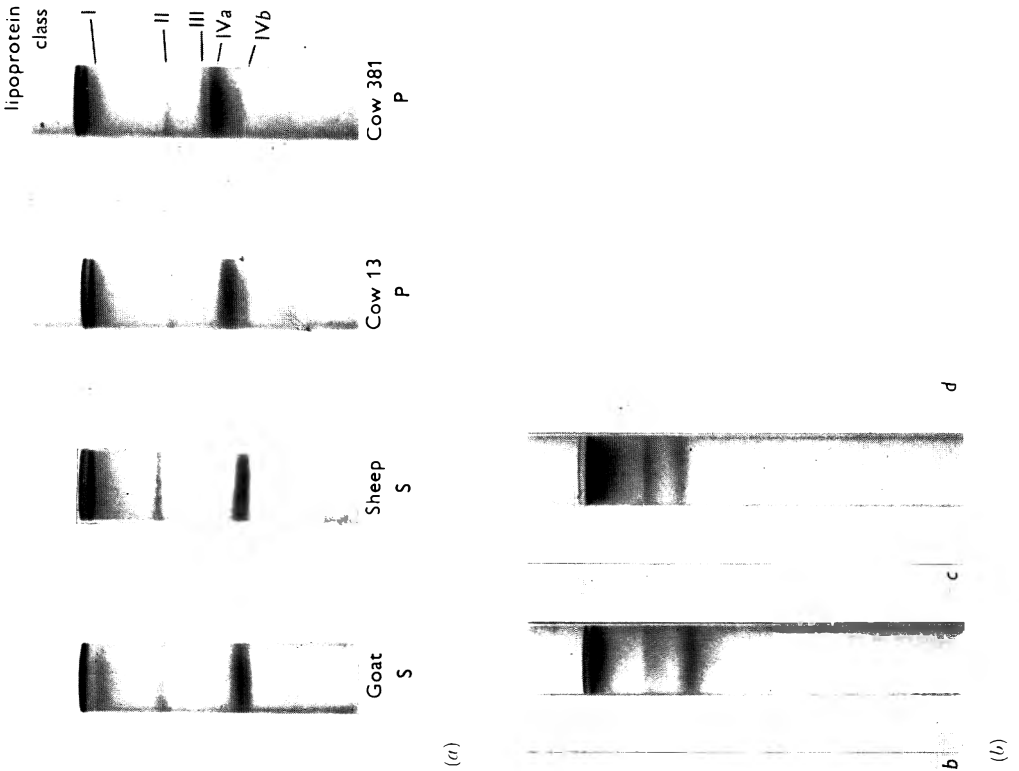


P. E. BRUMBY AND V. A. WELCH


lipoprotein class
I
II
III
IVa
IVb
Cow 381 P
Cow 13 P
Sheep S
Goat S
(a)
d
c
b
(b)

The estimation of diacetyl in the presence of other carbonyl compounds

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Summary. An examination of the spectra of some volatile carbonyl semicarbazones indicated that only compounds containing vicinal carbonyl groups formed semicarbazones which absorbed at 270 nm. This observation was used for the quantitative estimation of diacetyl. The presence of both acetaldehyde and acetoin did not interfere with the estimation of diacetyl by this method.

During a study of the metabolism of acetaldehyde in Group N streptococci, the formation of acetaldehyde was estimated by the Conway microdiffusion technique in which the acetaldehyde was trapped by semicarbazide in the centre well of the Conway unit. The semicarbazone formed was estimated by the method of Burbridge, Hine & Schick (1950). While this method of estimating acetaldehyde was relatively specific under the conditions used, the concurrent trapping in the semicarbazide of other volatile carbonyls, formed in the reaction mixtures, presented a potential source of error.

As Group N streptococci do produce, in addition to acetaldehyde, such volatile carbonyls as diacetyl and acetoin during the metabolism of glucose or pyruvate (Wilssens & Defloor, 1963; Keenan & Bills, 1968; Speckman & Collins, 1968; G. J. Lees & G. R. Jago, unpublished), a study was made of the spectral properties of a number of semicarbazones including those of the above carbonyl compounds. The results indicated that it is possible to estimate diacetyl free of interference by acetaldehyde and acetoin. A correction factor can be applied to adjust for any interference by diacetyl in the estimation of acetaldehyde by the same method.

MATERIALS AND METHODS

Carbonyl semicarbazones were formed by the addition of 1 ml of a solution of the carbonyl compound (approximately 1 mM) to 1 ml of 6·7 mM semicarbazide. The tubes were sealed with parafilm and allowed to stand for 90 min before the addition of distilled water to make a total volume of 10 ml. Absorption spectra were determined against a semicarbazide blank, prepared as above without the addition of carbonyl compounds, and recorded on a Zeiss spectrophotometer, model PMQII or on a Beckman recording spectrophotometer, model DK2.

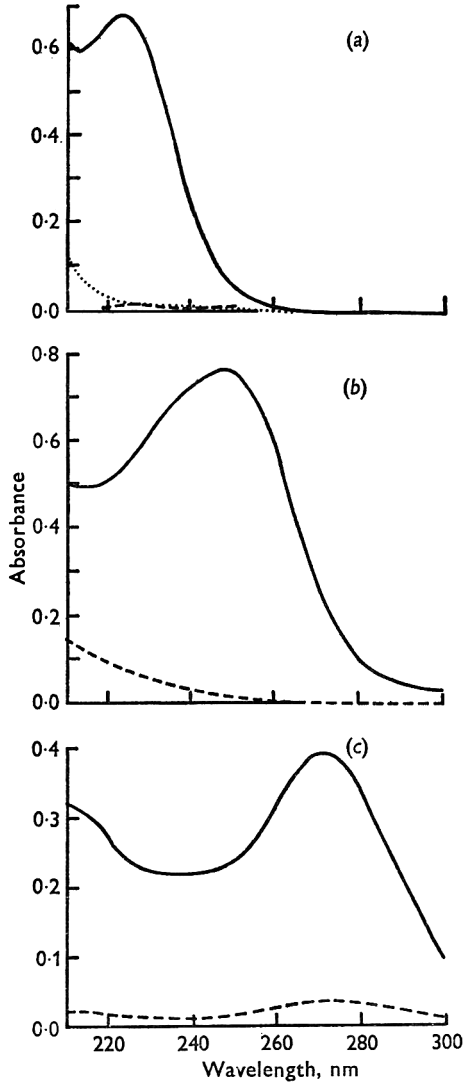

Fig. 1

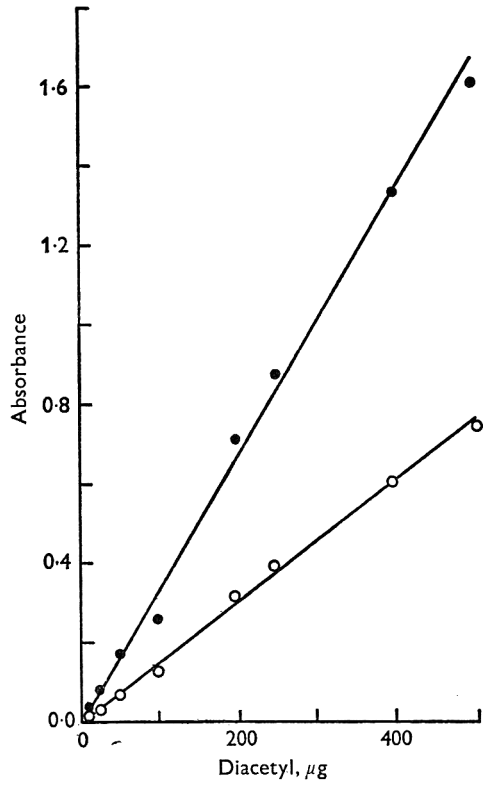

Fig. 2

Fig. 1. Absorption spectra of carbonyl semicarbazones. (*a*), Acetaldehyde; (*b*), pyruvate; (*c*), diacetyl. ——, Semicarbazone; ----, carbonyl compound; ....., semicarbazide (determined against distilled water).

Fig. 2. Standard curve for the estimation of diacetyl. ●, Absorbance at 270 nm; ○, absorbance at 224 nm.

Results and Discussion

The results of the present investigation showed that carbonyl compounds could be classified into 3 groups according to the spectral properties of their semicarbazones. The spectra representative of each group are shown in Fig. 1 *a*, *b*, *c*. The carbonyl compounds whose semicarbazones had similar spectral properties are grouped below.

Group 1. Acetaldehyde, n-butyraldehyde, phenylacetaldehyde, glutaraldehyde, acetylacetone, acetone, 2-butanone, acetoin.

Group 2. Pyruvate, α-ketobutyrate, oxaloacetate, glyoxylate.

Group 3. Diacetyl glyoxal, methyl glyoxal.

A list of the wavelengths of the absorption maxima and the difference absorption maxima (where the carbonyl compounds themselves absorbed at these wavelengths) is given in Table 1. It can be seen from this Table that the absorption maximum of a carbonyl semicarbazone depends on the class to which the carbonyl compound belongs. For example, acetaldehyde semicarbazone with one carbonyl group does not absorb at 272 nm, the absorption maximum of diacetyl semicarbazone which has 2 carbonyl groups in the vicinal position. A separation of 2 carbonyl groups by one carbon atom, as in acetylacetone, is sufficient to cause a loss in the absorbance at 270 nm of the semicarbazone.

Table 1. *Spectral properties of carbonyl semicarbazones*

Carbonyl semicarbazone	Absorption peaks, λ max., nm
Monocarbonyl compounds	
Acetaldehyde	224
n-Butyraldehyde	225
Phenylacetaldehyde	230
Acetone	223
Butanone	224
Hydroxycarbonyl compounds	
Acetoin	224
Ketoacids and derivatives	
Pyruvate (sodium salt)	248
α-Ketobutyrate (sodium salt)	236 (245)*
Oxaloacetate (sodium salt)	247
Glyoxylate (sodium salt)	255
Dicarbonyl compounds	
Diacetyl	272
Methyl glyoxal	272
Glyoxal	281
Acetylacetone	228
Glutaraldehyde	227

* λ max of difference spectrum obtained from spectrum of carbonyl semicarbazone minus spectrum of carbonyl compound, at the same concentrations.

As shown in Fig. 2, a linear relationship between the absorbance at either 224 nm or 270 nm and the amount of diacetyl semicarbazone was obtained for diacetyl in concentrations up to 500 μg/ml in the test sample. The ratio of the absorbance at 270 nm to the absorbance at 224 nm did not fall below a value of 2 for any concentration of diacetyl tested. This observation is useful as a means of indicating the absence of acetaldehyde or acetoin in a test sample. Any interference by diacetyl, when estimating acetaldehyde, can be corrected by subtracting half the absorbance at 270 nm from the absorbance at 224 nm.

The use of the Conway microdiffusion technique as described by Burbridge *et al.* (1950) with semicarbazide in the centre well of the Conway units was found to be a convenient method for separating volatile carbonyl compounds from other substances present in the reaction mixtures.

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With 4 plates

The detection of clinical mastitis with in-line filters

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(*Received* 13 *October* 1969)

Summary. A simple in-line filter is described which can be fitted into the milk tube of a milking machine in order to detect clinical mastitis. It consists of a stainless steel mesh, on which the clots are retained, and it is fitted obliquely into a short length of clear plastics tube. The filter has been developed as an alternative to the fore-milk cup. Tests made over 9 months in a 60-cow herd show good agreement between the fore-milk cup and the in-line filter recordings. For general service production the filters are made by moulding.

Stockmen normally detect clinical mastitis by udder palpation during machine stripping and examination of the fore-milk for abnormality on a black disk or other surface. Skilled stockmen can often detect the inflammation by udder palpation before the first milk abnormalities are found (Neave, Phillips & Mattick, 1952) but in most cases the first detection of the disease is from clots in the fore-milk (A. Meek, F. K. Neave & F. H. Dodd, unpublished). Both methods have the disadvantage that they add to the milker's routine work and therefore reduce the number of cows milked/hour. Also, because they will lead to the spread of pathogens from cow to cow on the milker's hands, they prevent the development of fully effective hygiene routines. A further important disadvantage of these methods is that, in herds with a low incidence of disease, abnormal milk will be detected in less than one in a thousand examinations, and under these conditions stockmen often stop using the fore-milk cup, and clinical cases that occur may remain undetected and untreated. For these reasons other methods of detecting clinical mastitis have been examined and the present paper describes the use of an in-line filter.

Development and operation of an in-line filter

In the course of experiments carried out in 1954–60 records were made of the detection of mastitis using a fore-milk cup, by filtering the milk through a 1·5 mm (0·06 in.) stainless steel gauze and by udder palpation. These results indicated that filtering the milk from individual cows provides a sensitive test for the milk abnormalities associated with udder disease. More recently, clamshell transparent plastics holders containing a filter pad have been tested when fitted into the long milk tube of the milking machine (Plate 1). For this work the white filters used in the U.S.A. were replaced by black cellulose fabric filters which enabled the milk clots to be seen more easily. These in-line filters were readily accepted by the stockmen who pre-

ferred them to the use of the fore-milk cup, but the persistent milk films and foam on the plastics surface prevented clear observation of the filter pad, and the films were not readily removed by the air which was let into the teatcup clusters at the end of the milking because the air velocity through the filter was low. This was due to the clamshells giving a 6 in. enlargement in what was basically a $\frac{1}{2}$ in. diam. rubber tube system. Further disadvantages are that these filters could not be cleaned in-place, and they required careful fixing in the milking parlour with good lighting for observation.

In attempting to develop improved in-line filters a number of different designs were made. They were fixed into the long milk tube leading from the milking machine cluster to the milk collecting jar or pipeline. The initial models all had the disadvantage that milk film and foam prevented quick and accurate reading at the end of milking. The idea of the simpler and more effective in-line filter came from our observation of a comparatively coarse filter that had been fitted into a main milk transfer line to prevent foreign material reaching the farm bulk tank. This filter was made from $1\frac{1}{2}$ mm expanded stainless steel mini-mesh and was equivalent in area to the cross-section of a $1\frac{3}{4}$ in. pipe, being set at an angle in a $1\frac{1}{2}$ in. pipe. The filter had to take the full pump flow-rate into the tank until such time as it was blocked. Obviously a much smaller filter area would be sufficient for the flow from one cow.

The first filters for use with each milking machine cluster were made on the same principle except that perspex tube was used to give good visibility, the stainless steel mesh was fitted at a more acute angle to increase the area of the filter to 0·5 in.^{2} and the diameter of the tube was $\frac{1}{2}$in., which was similar to the internal bore of the milking machine rubber tube (Plate 2). With a filter of this type the air velocity through the filter at the end of milking was sufficient to remove most of the milk film and foam, so that abnormalities could readily be seen.

It was not expected that acrylic tube would withstand the high temperatures and detergent concentrations which are used on some farms; nor would a joint held with perspex cement be mechanically satisfactory. However, these hand-made filters, and their successors made with the use of resorcinol adhesive instead of perspex cement and with flame-darkened mesh, quickly demonstrated their simplicity and effectiveness, and they were readily accepted by the stockmen.

These hand-made filters have been used continuously since December 1968, in 2 herds, one of 60 cows milked in a 6-unit double tandem parlour and the other of 90 cows milked in a 6-unit abreast parlour. The stockmen find that if clots are retained in the mesh they are readily seen when the cow is milked and the cluster removed (Plate 3). In the more acute cases the filter may become blocked and have to be removed during milking to be replaced by a reserve filter or plain tube. When all the cows have been milked the filters are removed, back flushed on a jet of water to remove debris and replaced before the plant is cleaned using the acidified boiling water method (Clough, Akam & Cant, 1965). Clearly the filter does not indicate the quarter affected but this can usually be found by udder palpation or by examining the strippings from each quarter with a fore-milk cup at the end of milking.

Comparison of in-line filter and fore-milk cup

In the herd with 60 cows the stockmen continued to examine fore-milk, drawing 2 or 3 streams of milk onto a fore-milk cup and recording abnormalities found during

palpation when machine stripping. From the records collected a first assessment of the value of the in-line filter was made. Grading of clots for the fore-milk cups and the filters followed arbitrary scales.

In-line filter: C_1, one small clot; C_2, several clots but not completely covering mesh; C_3, mesh covered with thin layer of clots; C_4, mesh retaining thick layer of clots or milking interrupted.

Fore-milk cup: C_1, 1–3 small clots; C_2, 1 large clot, or several small clots; C_3, several large clots or many small clots; C_4, many large clots.

To help the stockmen photographs of a fore-milk cup plate showing the various classes were supplied.

RESULTS

During the 36-week period 156 clinical cases of mastitis were detected. Some of these followed experimental infusions of bacteria or endotoxin and therefore the pattern of clinical signs may not have been completely normal.

The method used to compare the in-line filter and fore-milk cup had limitations. If the milker detected clots on the fore-milk cup he might look more carefully at the filter; if a cow showed clots on the filter the milker would normally examine the fore-milk cup more carefully at the next milking; and it is possible that all the clots might be removed in the first fore-milk so that none would be seen in the filter.

At the 27000 separate cow milkings an abnormality was found on either or both the fore-milk cup or the in-line filter on 727 occasions. This is approximately 2·7 % of milkings which is higher than the average because of the experimentally induced infections and infusions. For all the cow milkings for which clinical mastitis was detected, clots were found on the fore-milk cup but not on the in-line filter in 19% of them, and in another 26% of them filter positives followed normal fore-milk cup readings. This means that at 45% of the milkings for which abnormalities were found the readings by the 2 methods differed. If, however, the occasions were omitted when one method indicated C_1 and the other normal, then agreement rose to 62%, and when the occasions were omitted for which one method showed C_2 and the other normal it rose to 80%.

The degree of agreement of the readings obtained using the 2 methods can be seen from Fig. 1. The 4 lines show the frequency distribution of in-line filter readings when the fore-milk cup readings were normal, C_1, C_2, and C_3+C_4. It can be seen that if the fore-milk cup reading was C_3 or C_4 then 15% of filter readings were normal, 4% were C_1, 13% were C_2 and 68% were C_3 or C_4. The results of C_3 and C_4 have been combined because it is clear from Table 1 that using either method the C_3 class tended to be ignored. This may mean that more instruction should have been given or different classes recognized. When questioned, the stockmen were not clear about the use of the C_3 class, at least with the in-line filter.

In using either of these techniques we were interested in the results both for individual cow milkings and also for each clinical case when clots might be found in the milk of a quarter for several days. As many as 156 clinical cases were found which lasted for 1–39 milkings with a mean of 4·8 milkings. Of these 156 cases 22 were found at 1 milking on the in-line filter only, and 23 cases were found once on the fore-milk cup only. If these 45 are excluded, 25 were first found on the in-line filter, 14 on the

fore-milk cup and 72 simultaneously by both methods. On 33 occasions the last recorded abnormality was with the in-line filter, in 38 cases with the fore-milk cup and on 40 occasions it was recorded by both methods simultaneously. In-line filter readings of C_3 or C_4 were found at least once in 83 of the 102 clinical cases which lasted for more than one milking, and in 50 of the 56 which lasted for 4 or more milkings.

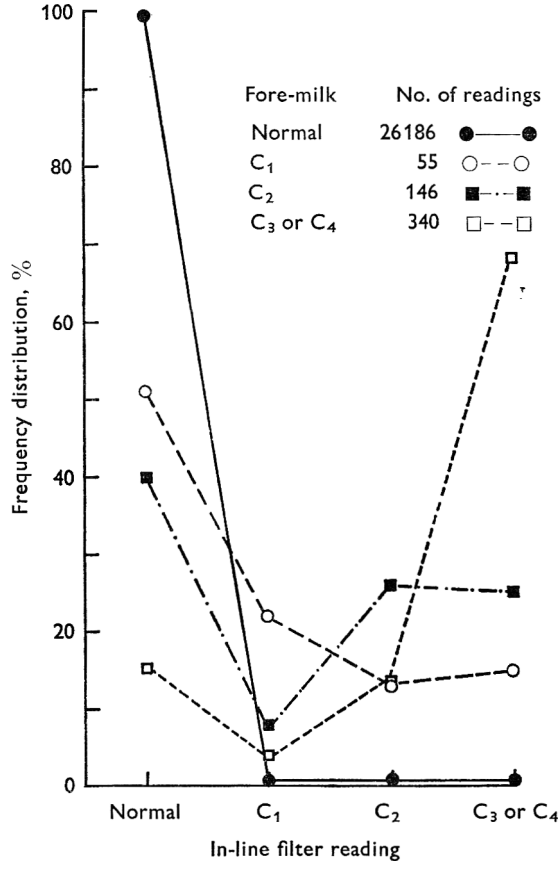


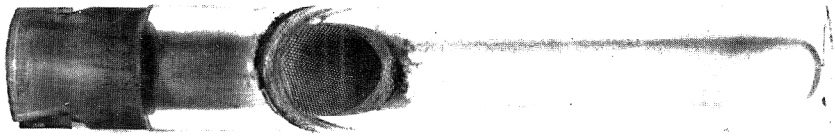
Fig. 1. The frequency distributions of in-line filter readings shown separately for each grade of fore-milk cup reading.

Table 1. *The proportion of fore-milk cup and in-line filter readings* (%) *falling into each recording class, excluding recordings when no clots were found*

	C_1	C_2	C_3	C_4
Fore-milk cup	10	27	12	51
In-line filter	15	29	9	47

In severe cases the filters were blocked and the clusters fell from the cows. The filters were then replaced by spares or for the remainder of that milking by clear tubes of the same length.

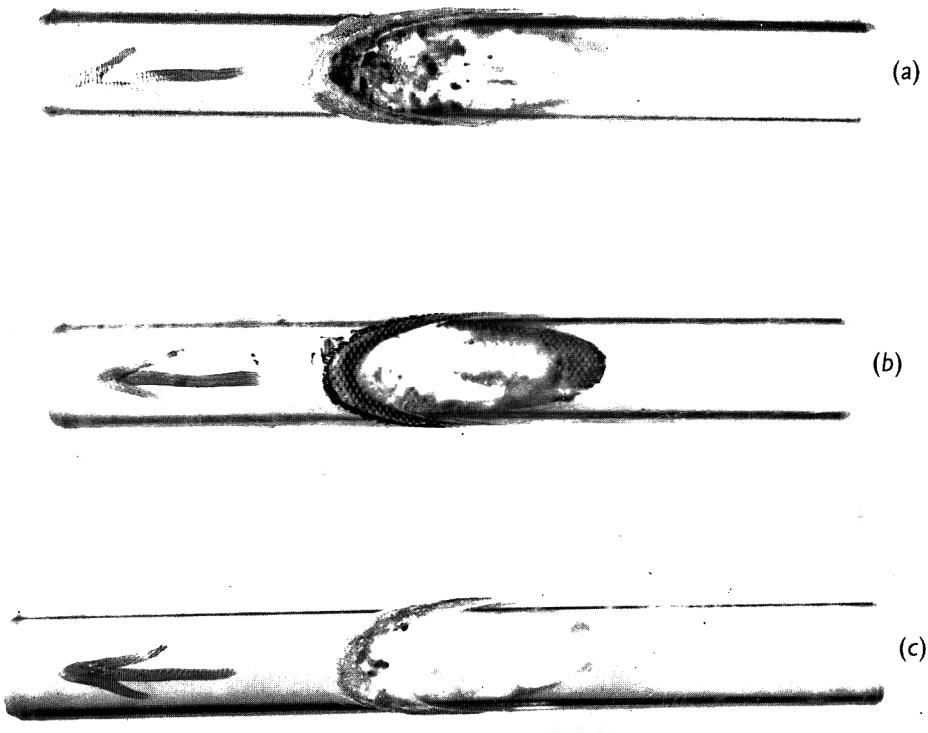
For general service, injection moulding offers a cheap method of quantity production and the stainless steel mesh may be of $\frac{3}{4}$ or $1\frac{1}{2}$ mm mesh within a clear plastics tube. The first moulded filters have been made with an int. diam. of 9/16 in.

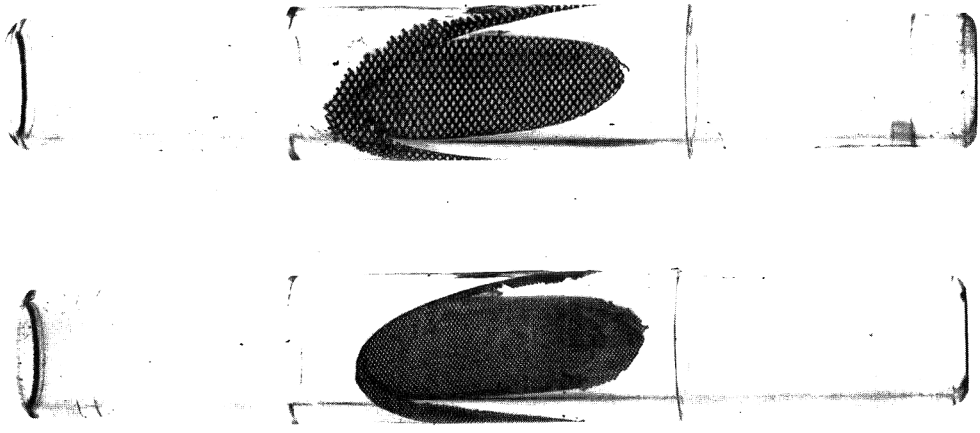


(a)

(*b*)

(*c*)


(a)
(b)
(c)



at the filter so that the cross-sectional area of the tube minus the 'area of the mesh' is similar to the cross-sectional area of the milk tube (Plate 4). The pressure drop across the filters measured at a vacuum of 15 inHg and a constant milk flow rate of 10 lb/min is 0·5 inHg for the moulded in-line filter fitted with 1·5 mm (0·06 in.) expanded mesh and 1 inHg for the clamshell type or for the tubes fitted with $\frac{3}{4}$ mm (0·03 in.) mesh.

CONCLUSION

At present, antibiotic therapy is normally given during lactation only to quarters detected by the stockmen as being inflamed or giving abnormal milk. In the past various alternative methods for detecting infected quarters have been suggested; these measure changes in the chemical composition or electrical properties of the milk of affected quarters. They can be much more sensitive than the methods stockmen use but so far have not proved to be practical alternatives. Their main advantage is that it may be possible to develop them for fully automatic recording which would enable antibiotic therapy to be given sooner and thereby increase its effectiveness. However, these systems have yet to be developed and initially they will be used only in the largest herds. The in-line filter described in this paper is not an alternative to a fully automated system. It is a simple inexpensive device, suitable for all herds, which can be used with advantage to replace the current methods, providing the milker observes the filter at each milking.

We recognize that filters of this type will not detect clinical mastitis resulting in discolouration of milk or when the milk forms a sediment but in practice these can rarely be detected using a fore-milk cup.

Smaller versions of the same design have been produced for experimental work with an individual quarter milking machine.

We are grateful to Mr N. Jackson for supervising the recordings on the farm and tabulating the data and to Mr J. Dawkins for making and maintaining the prototype filters. We also thank Johnson & Johnson, Slough, Bucks., who provided the 'Rapid-Flo' filter holder and various cellulose fabrics for filters used in the development work.

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EXPLANATION OF PLATES

Plate 1

Johnson & Johnson 'Rapid-Flo' filter holder with black cellulose fabric filter.

Plate 2

Three handmade prototype filters using $1\frac{1}{2}$ mm mesh in (*a*) $1\frac{1}{4}$ in. int. diam. tube for milk to the farm tank; (*b*) $\frac{1}{2}$ in. int. diam. tube for use in the long milk tube; (*c*) $\frac{5}{16}$ in. int. diam. tube for individual quarter milking.

Plate 3

Prototype filters after use showing (*a*), general debris; (*b*), C_2 clots; (*c*), C_4 clots.

Plate 4

Injection moulded filters; $\frac{9}{16}$ in. int. diam. tube with $1\frac{1}{2}$ mm and $\frac{3}{4}$ mm mesh.

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Reviews of the progress of Dairy Science

Section A. Physiology. Ruminant metabolism in relation to the synthesis and secretion of milk fat

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(*Received* 22 *October* 1969)

CONTENTS

INTRODUCTION

The subject of milk fat was last reviewed in this journal 10 years ago when emphasis was given to methods of analysis and factors affecting the composition and utilization of butterfat (Pont, 1960). Since then much progress has been made in the physiology and biochemistry of milk fat secretion and the present review is an attempt to integrate this knowledge with a view to providing a basis for understanding the role of nutrition in milk fat secretion of the dairy cow. Certain aspects of lipid metabolism in ruminants have not been studied extensively, however, and if interest is stimulated in these directions the present review will have achieved its objective.

STRUCTURE AND COMPOSITION OF MILK FAT

Milk fat globule

In freshly secreted milk the fat occurs dispersed throughout the aqueous phase as minute globules, the composition and properties of which have been reviewed by King (1955), Smith (1962), Brunner (1965) and Prentice (1969). The gross chemical composition of the globule membrane is now reasonably well established and small

differences between the findings of many workers can be attributed to the use of differently treated samples of milk as starting material or to the use of different techniques for isolating the membrane. However, much less is known about the way in which the various components are organized to form the membrane around the triglyceride core of the globule and 3 arrangements have been described: a series of highly orientated adsorbed layers of high melting triglycerides, phospholipids and proteins (King, 1955); a layer of cytoplasmic protein on which is adsorbed a dense layer of microsomes (Morton, 1954) and an adsorbed layer of cytoplasmic protein covered with cell membrane (Bargmann & Knoop, 1959). In the light of more recent findings these theories now appear inadequate explanations of the structure of the fat globule membrane.

Hayashi & Smith (1965) desorbed about half of the surface membrane from the globule with sodium deoxycholate and found that the desorbed material contained 45 % of the protein and 67 % of the lipid phosphorus originally present in the washed cream. The lipoproteins which remained as a structural matrix round the triglyceride core contained 32 % of the protein and 33 % of the lipid phosphorus in the original cream. The deoxycholate-soluble lipoproteins, when fractionated on the ultracentrifuge, were heterogeneous and too small to be microsomes. Chien & Richardson (1967*a*) also concluded that the globule membrane consisted of several separate lipoproteins. Their desorbed membrane material, obtained by washing cream with water until phase inversion occurred, accounted for 54 % of the total membrane and was further fractionated by centrifugation into a reddish-brown pellet (60 % protein, 40 % lipid) and a fluffy-white supernatant aggregate (13 % protein, 87 % lipid). The non-desorbed inner membrane was fractionated into 3 lipoproteins; a green-white pellet (57 % protein, 43 % lipid), a supernatant aggregate (28 % protein, 72 % lipid) and interfacial material (60 % protein, 40 % lipid). In all the isolated lipoproteins, particularly the 'lipid-rich' lipoproteins, the neutral lipids, which contained monoglycerides, diglycerides, triglycerides and free cholesterol, were the major lipids. In all the lipoproteins the relative concentrations of the neutral lipid fractions were similar except that cholesterol was higher in the pellet material from both the inner and outer membranes. The phospholipids contained phosphatidyl choline, phosphatidyl ethanolamine and sphingomyelin, with no obvious differences in their relative proportions between different fractions of the membrane. The phospholipids were rich in palmitic, stearic, oleic, linoleic and longer chain saturated acids whereas the neutral lipids were high in myristic, palmitic, stearic and oleic acids.

The proportions and fatty acid compositions of different lipid classes in the total membrane prepared by freeze-thawing and centrifugation of washed cream have also been extensively investigated by Huang & Kuksis (1967). The membrane contained 0·5–1·0 % of the total lipid in milk and was composed of 21–44 % phospholipid, and 56–79 % neutral lipid and free fatty acid. The phospholipids contained equal proportions of phosphatidyl ethanolamine, phosphatidyl choline and sphingomyelin, whereas the neutral lipids contained 83–88 % triglyceride, 5–11 % diglyceride, 1–5 % free fatty acid and 0·4–2·0 % cholesterol. The fatty acid compositions of the different phospholipids were similar and the triglycerides and diglycerides of the membrane contained more palmitic and stearic acids but no short chain acids when compared with the glycerides of the globule core.

In addition to lipids, the distribution and character of proteins, enzymes and metals in the various lipoprotein fractions of the fat globule membrane have been studied. Thus, hexosamines have been found in all fractions indicating the presence of glycoproteins (Chien & Richardson, 1967*b*; Swope & Brunner, 1969). Also the amino acid composition of the proteins did not differ from one lipoprotein to another. Ribonucleic acid occurs in all fractions of the membrane, the highest concentrations being in the supernatant aggregate from the inner insoluble layer of the membrane (Chien & Richardson, 1967*b*; Swope & Brunner, 1968). Alkaline phosphatase, acid phosphatase, reduced NAD dehydrogenase, Mg^{2+} activated ATPase, acetylcholinesterase, xanthine oxidase, aldolase and glucose-6-phosphatase are all present in the globule membrane (Dowben, Brunner & Philpott, 1967). Alkaline phosphatase and xanthine oxidase are located almost entirely in the outer soluble lipoprotein layer of the membrane (Hayashi, Erickson & Smith, 1965). Copper and iron are distributed throughout all fractions of the membrane with highest concentrations of iron in the outer layers (Richardson & Guss, 1965; Chien & Richardson, 1967*b*).

Electron microscope studies indicate that the membrane of the secreted fat globule is about 9 nm thick and composed of 3 layers (Dowben *et al.* 1967) which may represent true lipoprotein micelles adsorbed on to a basal protein layer (Brunner, Swope & Carrol, 1969). The presence in the membrane of several enzymes which are also characteristic of cell microsomes and the ability of fat globule membrane antisera to agglutinate and haemolyse bovine erythrocytes (Dowben *et al.* 1967) suggests that the globule membrane has its origin in a true cell membrane. The identification, by electron microscopy, of remnants of the endoplasmic reticulum at the periphery of fat droplets in the lumen of mouse alveolar cells also suggests that cell structures form part of milk lipoproteins (Stein & Stein, 1966). A similarity in chemical composition but difference in morphological appearance of the membranes of milk fat globules and cells of bovine mammary tissue has resulted in the conclusion that the milk fat globule membrane is derived from the plasma membrane by structural rearrangement of the cell membrane components (Keenan, Morré, Olson & Yunghans, 1969; Keenan, Morré, Olson & Patton, 1969).

Phospholipids

Phospholipids, which may represent up to 1 % of the total lipids in bovine milk, are found in both the fat globule membrane and the serum. In addition to their function in membrane structure, as already indicated, phospholipids are also an important source of oxidative off-flavours which may develop during the storage or processing of milk (Kinsella, 1969).

Although the major phospholipid components in milk have been known for some time (see Garton, 1963) recent more sophisticated methods, in particular 2-dimensional thin-layer chromatography, have led to a more detailed identification. It is now recognized that a significant proportion of the phospholipids are present in milk as a variety of glycolipid and possibly proteolipids (Galanos & Kapoulas, 1965; Morrison, Jack & Smith, 1965; Mohammadzadeh-K. & Smith, 1968). Phospholipids in cow's milk are composed of 32–42 % phosphatidyl ethanolamine, 34–35 % phosphatidyl choline, 2–3 % phosphatidyl serine, 4–5 % phosphatidyl inositol, 18–25 % sphingomyelin (Parsons & Patton, 1967; Morrison, 1968).

The phospholipids lack short chain acids (< 10 carbon atoms) but the individual phospholipids tend to have definite fatty acid compositions. Sphingomyelin and glycolipids ceramide monohexoside and ceramide dihexoside contain mainly saturated acids with chain lengths ranging from 14 to 24 carbon atoms (Badings, 1962; Morrison *et al.* 1965). Phosphatidyl ethanolamine, phosphatidyl serine and phosphatidyl choline on the other hand contain C_{10} to C_{22} normal and branched chain saturated acids and C_{15} to C_{20} unsaturated acids containing mainly one double bond (Badings, 1962; Morrison *et al.* 1965; Moore, Rattray & Irvine, 1968). In these diacyl phospholipids the unsaturated acids tend to be esterified more in the β-position (Morrison *et al.* 1965; Moore, Rattray & Irvine, 1968).

Although proportions of the different phospholipids are similar in the milk of a wide range of species (Morrison, 1968) there appears to be a difference between ruminants and non-ruminants in the composition and specific distribution of fatty acids in the corresponding phospholipids.

Triglycerides

Ruminant milk triglycerides are the most complex in fatty acid composition of all naturally occurring fats. During the last decade the use of more sensitive techniques of gas chromatography in conjunction with ultraviolet and infrared spectrophotometry, nuclear magnetic resonance, X-ray diffraction and mass spectrometry have led to the identification of over 140 fatty acids in milk. These acids, of which many are present in only trace amounts, may contain as few as 2 or as many as 28 carbon atoms and may be broadly classified into saturated, unsaturated (cis- or trans-), branched (mono- or multi-), keto, hydroxy or cyclic acids. For information on the detailed composition of these fatty acids in milk, the reader is referred to comprehensive reviews by Garton (1963) and Jensen, Quinn, Carpenter & Sampugna (1967).

In addition to the composition of fatty acids in milk, however, their arrangement in the triglyceride molecule itself has also been the subject of intensive investigation. The arrangements of fatty acids is known to influence the geometry of the triglyceride molecule and hence to be of vital importance in the chemical and physical properties of milk fat. Furthermore, elucidation of milk triglyceride structure has given rise to interesting theories regarding the way in which milk triglycerides are synthesized within the mammary gland.

Studies on the structure of milk triglycerides are complicated by the wide range of fatty acids present. Fractional crystallization, distillation, countercurrent distribution, gas-liquid, liquid-liquid and thin-layer chromatography have therefore been used at various times to separate milk triglycerides into groups of lesser complexity according to their molecular weight, polarity or degree of unsaturation. By subsequent digestion of the fractionated triglycerides with pancreatic lipase, which specifically hydrolyses acids esterified in the terminal positions of the molecule, and by analysis of the resulting free fatty acids, diglycerides and monoglycerides, the distribution of fatty acids in the original triglycerides has been determined. An integrated system for such fractionation and enzymatic analysis of milk fat has been proposed by McCarthy & Kuksis (1964).

This use of pancreatic lipase is subject to certain limitations. Valid results are only obtained if no triglyceride species is preferentially hydrolysed, if complete hydrolysis

of the triglycerides occurs to an insignificant extent and if no appreciable acyl migration from the 2-position occurs during digestion. More rapid digestion by pancreatic lipase of both synthetic and milk triglycerides containing butyric acid has been demonstrated (Jensen, Sampugna & Pereira, 1964; Sampugna *et al.* 1966). Acyl migration of short and intermediate chain acids from the 2- to 1- and 3-positions in synthetic and milk triglycerides also occurs (Jensen, Sampugna & Parry, 1963; Sampugna *et al.* 1966). However, intermolecular specificity and acyl migration are likely to be less in milk triglycerides where short chain acids are complexed with long chain acids in the same molecule (Jensen & Sampugna, 1966) and when short incubation times restricting the extent of hydrolysis to about one third are used (Jack, Freeman, Smith & Mickle, 1963; Freeman, Jack & Smith, 1965). Preparation of the phosphatidyl phenol derivatives of milk diglycerides followed by their digestion with phospholipase A has been used in conjunction with pancreatic lipase studies, to distinguish between fatty acids in the 1- and 3-positions (Pitas, Sampugna & Jensen, 1967; Breckenridge & Kuksis, 1968*b*, 1969).

Thus, milk triglycerides can be broadly fractionated into groups of different chain lengths by fractional crystallization (Patton & Keeney, 1958; Thompson, Brunner & Stein, 1959; Wolf & Dugan, 1964; Smith, Freeman & Jack, 1965; Woodrow & deMan, 1968), by countercurrent distribution (Smith *et al.* 1965) and by thin-layer or column chromatography (Blank & Privett, 1964; Dimick & Patton, 1965; Dimick, McCarthy & Patton, 1965; Breckenridge & Kuksis, 1967, 1968*a*, *b*, 1969; Nutter & Privett, 1967). Proportions of short, medium and long chain triglycerides are about 45, 17 and 38% respectively. More critical separation of milk triglycerides, using gas-liquid chromatography, has shown that they contain from 24 to 54 acyl carbon atoms including small amounts of odd numbered triglycerides (Kuksis, McCarthy & Beveridge, 1963; Dimick & Patton, 1965; Jensen & Sampugna, 1966; Breckenridge & Kuksis, 1967, 1968*a*, 1969). Separation of milk triglycerides according to their degree of saturation has also been effected by column and thin-layer chromatography (Kerkhoven & deMan, 1966; Nutter & Privett, 1967; Breckenridge & Kuksis, 1968*a*, *b*) and they are composed of about 39, 40, 18 and 3% tri-, di-, mono- and non-saturated glycerides, respectively.

In milk, there is clearly a non-random distribution of the different triglyceride types, each of which differs widely in its fatty acid composition. The diverse fatty acid composition of fractionated milk triglycerides has been confirmed by gas-liquid chromatography (Patton & Keeney, 1958; Thompson *et al.* 1959; McCarthy & Kuksis, 1964; Blank & Privett, 1964; Smith *et al.* 1965; Dimick & Patton, 1965; Kerkhoven & deMan, 1966; Nutter & Privett, 1967; Breckenridge & Kuksis, 1967, 1968*a*).

These various experimental approaches have led to the following conclusions on triglyceride structure. The highest molecular weight triglycerides contain no short chain fatty acids (Dimick & Patton, 1965). Of those triglycerides in which short chain acids do occur, most contain only one molecule of short chain acid (Kuksis *et al.* 1963; Blank & Privett, 1964; Dimick & Patton, 1965; Smith *et al.* 1965) while the others contain 2 molecules of short chain acids (Blank & Privett, 1964; Nutter & Privett, 1967; Breckenridge & Kuksis, 1968*a*). Apart from traces of short chain acids in the 2-position (Clement, Clement, Bézard, Di Costanzo & Paris, 1963; Smith *et al.*

1965; Freeman *et al.* 1965; Sampugna *et al.* 1966) these acids are located in the terminal positions, and mainly in the 3-position (Kumar, Pynadath & Lolka, 1960; Clement *et al.* 1963; Jensen *et al.* 1964; Smith *et al.* 1965; Pitas *et al.* 1967; Breckenridge & Kuksis, 1968*b*). Saturated acids of intermediate chain length (C_{10-14}) tend to be preferentially esterified in the 2-position of the triglyceride molecule (McCarthy, Patton & Evans, 1960; Patton, Evans & McCarthy, 1960; Wolf & Dugan, 1964; Smith *et al.* 1965; Dimick *et al.* 1965; Freeman, *et al.* 1965; Breckenridge & Kuksis, 1969). Palmitic acid is unique in that its distribution in the molecule depends on the molecular weight of the triglyceride; it is predominantly in the 2-position for the high and predominantly in the 1- and 3-positions for the low molecular weight triglycerides (Dimick, McCarthy & Patton, 1966). This dependence of the distribution of palmitic acid on triglyceride type is probably related to the 2 sources from which palmitate is derived (see later), and probably accounts for the varied or random distribution found by other workers analysing unfractionated milk triglycerides. The C_{18} acids are located mainly in the 1- and 3-positions of the molecule (Patton, Evans & McCarthy, 1960; Dimick *et al.* 1965; Woodrow & deMan, 1968; Breckenridge & Kuksis, 1969).

SYNTHESIS AND SECRETION OF MILK FAT

In view of their complex composition, it is perhaps not surprising that the fatty acids in milk result from the integration of several biochemical pathways. The origin of milk fat has been extensively reviewed (Folley & McNaught, 1961; Garton, 1963; Barry, 1964, 1966; Rook & Storry, 1964; Linzell, 1968; Jones, 1969) and it is now generally accepted that in ruminants the short chain acids (4–10 carbon atoms) are derived entirely by synthesis within the mammary gland from acetic and β-hydroxybutyric acids, the long chain acids (18 carbon atoms) from the triglycerides of the low density β-lipoproteins of blood plasma and the intermediate chain acids (12–16 carbon atoms) from both of these sources. Less is known, however, about the relative contributions of the various pathways to the formation of total milk fatty acids especially under different nutritional and physiological conditions.

Intramammary synthesis of fatty acids

The utilization by the ruminant mammary gland of acetic and β-hydroxybutyric acids for the synthesis of short and intermediate chain acids in milk has been demonstrated in arterio-venous difference studies with cows (Hartmann & Lascelles, 1964) and goats (Barry, Bartley, Linzell & Robinson, 1963; Annison & Linzell, 1964; Annison, Linzell, Fazakerley & Nichols, 1967) and in perfusions of isolated bovine (Kumar, Lakshmanan & Shaw, 1959) and caprine (Hardwick, Linzell & Price, 1961; Hardwick, Linzell & Mepham, 1963; Linzell, Annison, Fazakerley & Leng, 1967) mammary glands. Also intraruminal (Storry & Rook, 1965*a*) or intravenous (Ahrens & Luick, 1964; Storry & Rook, 1965*b*; Luick & Kameoka, 1966; Gerson, Shorland, Wilson & Reid, 1968) infusions of these metabolites into lactating cows have resulted in either extensive labelling or net increases in the yields of the C_{4-16} fatty acids in milk.

The biochemical pathways by which fatty acids are synthesized have been elucidated by studying the metabolism of labelled acetic and β-hydroxybutyric acids in either tissue slices, dispersed cultured cells or isolated subcellular fractions of actively

secreting mammary glands. Both ruminant and non-ruminant tissues have been studied, and although generally similar pathways appear to operate in both some important differences do exist. For example, ruminants differ from non-ruminants in not being able to use glucose for fatty acid synthesis. This metabolic distinction is now known to be due to a lack in ruminants of citrate lyase (Hardwick, 1966; Hanson & Ballard, 1967; Bauman, Brown & Davis, 1969) which provides acetyl CoA from citrate derived within the mitochondria from glucose (see Jones, 1969; Ballard, Hanson & Kronfeld, 1969).

Malonyl pathway

Synthesis of fatty acids from acetate by the malonyl pathway has been demonstrated in mammary glands of rats, (Abraham, Matthes & Chaikoff, 1961; Coniglio & Popják, 1962; Dils & Popják, 1962; Spencer & Lowenstein, 1962; Matthes, Abraham & Chaikoff, 1963; Hülsman & Dow, 1964; Spencer, Couman & Lowenstein, 1964; Coniglio & Bridges, 1966; Bartley, Abraham & Chaikoff, 1967; Bogin & Katz, 1967); rabbits, (Singh & Kumar, 1963; Bu'Lock & Smith, 1965; Lachance & Morais, 1965; Kumar, Singh & Keren-Paz, 1965; Becker, 1966; Smith, Easter & Dils, 1966; Smith & Dils, 1966*a*, *b*; Easter & Dils, 1968); goats, (Becker & Kumar, 1965; Kumar *et al.* 1965; Becker, 1966) and cows (Ganguly, 1960; Hibbitt, 1966; Kinsella, 1968*a*; Kinsella & McCarthy, 1968*a*; McCarthy & Smith, 1969). The malonyl pathway operates in the cytosol of the cell although subcellular particles have been shown in certain instances to influence the rate of synthesis. The overall reaction may be conveniently divided, as follows:

(1) formation of malonyl CoA by carboxylation of acetyl CoA:

$$ATP + HCO_3^- + \text{biotin enzyme} \xrightarrow{Mg^{2+}} CO_2 \sim \text{biotin enzyme} + ADP + P,$$

$$CO_2 \sim \text{biotin enzyme} + CH_3COSCoA \rightarrow \text{biotin enzyme} + COOH\,CH_2\,COSCoA;$$

(2) the condensation of acetyl CoA and malonyl CoA. The following equation describes the formation of palmitic acid:

$$CH_3COSCoA + 7COOH\,CH_2\,COSCoA + 14NADPH + 14H^+ \rightarrow$$
$$CH_3(CH_2)_{14}COOH + 7CO_2 + 8CoASH + 14NADP + 6H_2O.$$

The formation of malonyl CoA is the rate limiting step of the pathway and is catalysed by acetyl CoA carboxylase which is located in the cytosol and microsomes of the cell (Smith *et al.* 1966; Easter & Dils, 1968). The activity of this enzyme is reduced by avidin (Singh & Kumar, 1963; Hibbitt, 1966; Bogin & Katz, 1967), by free fatty acids or their acyl derivatives (Hibbitt, 1966; Smith & Dils, 1966*a*; Bartley *et al.* 1967) or by fasting (Coniglio & Culp, 1965). Its activity is increased by the presence of citrate (Spencer & Lowenstein, 1962).

The condensation of acetyl CoA and malonyl CoA is catalysed by the multienzyme complex fatty acid synthetase which is located exclusively in the cytosol of the cell (Becker & Kumar, 1965; Becker, 1966; Smith *et al.* 1966). The structure of the synthetase has been studied mainly in yeast where the complex has a molecular weight of about $2{\cdot}3 \times 10^6$ and consists of 6 or 7 enzymes, each catalysing one specific reaction step of fatty acid synthesis (Lynen, 1967*a*, *b*; Lynen, Oesterhelt, Schweizer & Willecke, 1968). The enzyme also exists as a complex in goat mammary cells (Nandedkar, Schirmer, Pynadath & Kumar, 1969). The existence of such an

enzyme complex has the advantage that diffusion of intermediates is not required nor are they available for utilization in competitive biochemical pathways.

In the condensation sequence, an acetyl residue from acetyl CoA is transferred to a 'peripheral' SH group and a malonyl residue, from malonyl CoA, to the 'central' SH group of the enzyme complex. The acetyl-malonyl-enzyme thus produced is then transformed to the acetoacetyl-enzyme, with the concomitant release of CO_2, followed by stepwise conversion, via β-oxo- and β-hydroxy acid intermediates, to the saturated butyryl enzyme. The carboxylic acid intermediates are covalently bound to the central SH group until the saturated acyl derivative is formed and the latter is then transferred to the peripheral SH group thus liberating the central SH group for introduction of the next malonyl residue and subsequent condensation cycle. Thus, in the first condensation cycle malonyl CoA condenses with the starting acyl CoA ester whereas in all subsequent cycles malonyl CoA condenses with the newly formed acyl ester of the acyl carrier protein. Finally, the acyl residue of palmitoyl- or stearyl-enzyme is transferred from the central SH group to coenzyme A which results in the liberation of free enzyme for further condensation sequences.

The reduction of fatty acid intermediates in the condensation reactions shows a specific requirement for NADPH rather than NADH (Ganguly, 1960; Becker & Kumar, 1965; Smith & Dils, 1966*a*; Bogin & Katz, 1967). The different reductive steps in the pathway may also require specific isomers of NADPH (Matthes *et al.* 1963) and citrate may also be involved in the stereospecific transfer of hydrogen from NADPH to fatty acids (Kumar *et al.* 1965).

The factors governing the chain length of fatty acids synthesized are not yet completely known and may be related to the effect of chain length on the binding of fatty acid to the enzyme (Lynen, 1967*a*, *b*; Lynen *et al.* 1968) rather than to the specificity of the enzyme responsible for the transfer of synthesized fatty acid from the SH groups of the enzyme complex to coenzyme A as suggested earlier (Lynen, 1961). Also it is noteworthy that the concentration of malonyl CoA *in vitro* directly affects the rate (Smith & Abraham, 1969) and chain length (Becker & Kumar, 1965; Becker, 1966; Smith & Dils, 1966*a*; Bartley *et al.* 1967) of fatty acids synthesized. The presence of short chain acids in ruminant milk fat is thus an interesting phenomenon in that they must reflect either release of free acyl CoA from the multi-enzyme complex at an early stage in the cycle of fatty acid synthesis or, more probably, the products of other biochemical pathways (see later).

Since *in vitro* the formation of malonyl CoA is the rate limiting step of the malonyl pathway and the concentration of malonyl CoA influences the chain length of fatty acids synthesized it is important to consider the secretory cell as a whole because processes in other parts of the cell indirectly affect the formation of malonyl CoA. Thus, microsomes increase the rate and chain length of fatty acids synthesized by binding or esterifying the long chain fatty acids released from the synthetase complex and which would otherwise inhibit further fatty acid synthesis through a feedback mechanism (Spencer *et al.* 1964; Hibbitt, 1966; Smith & Dils, 1966*a*, *b*; Bartley *et al.* 1967). The presence of acetyl CoA carboxylase in microsomes (Smith *et al.* 1966; Easter & Dils, 1968) may be an additional reason for the stimulating effect of these subcellular particles on fatty acid synthesis. The various possible ways in which fatty acid synthesis may be controlled *in vivo* have been recently reviewed by Zakim

& Herman (1969) but their relative physiological significance is not known. The following factors are of importance: the amount of enzymes involved in the biosynthetic system; the availability of cofactors or enzymatic stimulators; the amount of substances acting as inhibitors of fatty acid biosynthetic enzymes; the availability of substrate for the fatty acid biosynthetic enzymes.

Although citrate does not act as a source of acetyl CoA in ruminants there is the interesting possibility that the relatively higher concentration of citrate in the milk of these animals may reflect a higher intracellular concentration of citrate which in turn could aid fatty acid synthesis, through its known role in activating acetyl CoA carboxylase and in promoting hydrogen transfer from the nucleotides (see above).

Non-malonyl pathways

There is much circumstantial evidence which indicates that the short chain acids in milk are also synthesized by pathways not involving malonyl CoA. For example, the synthesis of butyrate and short chain acids is little affected by avidin, a specific inhibitor of biotin enzymes (Singh & Kumar, 1963; Kumar *et al.* 1965; Smith & Dils, 1966*a*; Smith & McCarthy, 1969), and by the concentration of malonyl CoA and does not specifically require NADPH (Becker & Kumar, 1965). One of these pathways probably involves the synthesis in the cytosol of short chain acids by direct incorporation of β-hydroxybutyrate (Popják, French & Folley, 1951; Kumar *et al.* 1959; Ahrens & Luick, 1964; Luick & Kameoka, 1966; Linzell *et al.* 1967; McCarthy & Smith, 1969; Palmquist, Davis, Brown & Sachan, 1969; Smith & McCarthy, 1969). Cleavage of β-hydroxybutyrate into C_2 units and incorporation of these into longer chain acids by the malonyl pathway also operates to a varying degree (Ahrens & Luick, 1964; Luick & Kameoka, 1966; Linzell *et al.* 1967; McCarthy & Smith, 1969; Smith & McCarthy, 1969; Palmquist *et al.* 1969). Butyric acid may also be synthesized in goat and rabbit mammary tissue by the reversal of β-oxidation (Nandedkar & Kumar, 1969).

Another non-malonyl pathway, which is insensitive to avidin and which specifically incorporates acetate into short chain acids, has been located in the mitochondria of bovine, but not rat, mammary glands (McCarthy & Smith, 1969; Smith & McCarthy 1969) and supports findings of experiments where labelled acetate was continuously infused into the jugular vein of lactating cows (Gerson *et al.* 1968). Such synthesis of fatty acids from acetate by mitochondria of bovine mammary tissue may be of particular significance in view of the lack of citrate lyase in ruminants but is difficult to reconcile with the non-utilization of glucose for fatty acid synthesis (see above).

Studies with perfused bovine mammary glands (James, Peeters & Lauryssens, 1956) and intact lactating cows (Storry, Tuckley & Hall, 1969) have shown that short and intermediate chain acids are elongated by the successive addition of 2-carbon units. A mechanism for elongating fatty acids, which involves acetyl CoA, reduced pyridine nucleotides and the enzymes acetoacetyl-CoA thiolase, 3-hydroxyacyl CoA dehydrogenase, enoyl-CoA hydrolase and enoyl-CoA reductase, has been demonstrated in the mitochondria of bovine liver (Seubert, Lamberts, Kramer & Ohley, 1968). The incorporation of small amounts of labelled acetate into milk stearate following the perfusion of bovine mammary glands (Cowie *et al.* 1951), the intravenous infusion of lactating cows (Gerson *et al.* 1968) and goats (Popják, French, Hunter & Martin, 1951) and the incubation of slices of bovine mammary tissue (Smith

& McCarthy, 1969) suggest that this pathway may also operate in the mammary gland but its unequivocal existence in this tissue awaits proof.

Although the C_{18} acids in milk are derived almost entirely from the C_{18} acids of the triglycerides of the plasma β-lipoproteins, a considerable proportion of the oleic acid is derived from stearic acid by dehydrogenation within the mammary gland (Lauryssens, Verbeke & Peeters, 1961; McCarthy, Ghiardi & Patton, 1965; West, Annison & Linzell, 1967*b*; Annison *et al.* 1967; Kinsella, 1968*a*). The enzymes responsible for this desaturation are located in the microsomes of the cell and they are also active to a lesser extent for palmitic acid but inactive for lauric and myristic acids. Desaturation does not proceed beyond the monoenoic acids (Bickerstaffe & Annison, 1968).

Contribution of plasma lipids to milk fat

A positive correlation between the concentration of plasma phospholipids and milk fat secretion has led to the suggestion that plasma phospholipids are involved in milk fat secretion (Riis, 1964) but more critical experiments have shown that neither plasma phospholipids nor sterol esters are utilized by the mammary gland (Hartmann & Lascelles, 1964; Annison *et al.* 1967; Varman & Schultz, 1968*a*; Varman, Schultz & Nichols, 1968; Bishop, Davies, Glascock & Welch, 1969). Arterio-venous difference studies in conscious lactating goats (Barry *et al.* 1963), perfusion of isolated goat mammary glands (Lascelles, Hardwick, Linzell & Mepham, 1964; Linzell *et al.* 1967) and feeding radioactive triglycerides to cows (Glascock *et al.* 1966; Bishop *et al.* 1969) have clearly demonstrated the importance of triglycerides of chylomicra and low-density lipoproteins and of non-esterified fatty acids of plasma as precursors of milk fat.

Detailed knowledge on the mode of uptake of these large molecules by the mammary gland is, however, lacking and much evidence suggests that the triglycerides of plasma lipoproteins are hydrolysed during their uptake from blood plasma. For example, the appearance of tritium labelled non-esterified fatty acids in mammary venous blood of goats (West *et al.* 1967*b*) and cows (Bishop *et al.* 1969) and the disproportionate uptake of glycerol and fatty acid by rabbit (McBride & Korn, 1964*b*) and goat (West, Annison & Linzell, 1967*a*) mammary glands following the infusion of doubly labelled chylomicra have been demonstrated. Lipoprotein lipase is presumed to be involved in the hydrolysis of these lipoproteins since there is a close correlation between the physiological activity of the mammary gland and its content of this enzyme (McBride & Korn, 1963, 1964*c*; Robinson, 1963). Histochemical investigations in rats suggest that lipoprotein lipase is located in the mammary gland at the lumenal surface of the capillary endothelium (Schoefl & French, 1968) which is in line with the findings of a 3- to 4-fold increase in lipoprotein lipase in the venous blood leaving the mammary glands of goats (Barry *et al.* 1963). The possibility that lipoprotein lipase activity of the mammary gland may be under hormonal control and more particularly of prolactin has recently been demonstrated in rabbits (Falconer & Fiddler, 1968; Fiddler & Falconer, 1968). The effect of different substrates on lipoprotein lipase activity is currently being investigated in the author's laboratories and Brumby (1969) has shown that the activity in bovine and guinea-pig mammary glands is inhibited by long chain polyunsaturated fatty acids. The mechanism by which fatty acids, released from lipoprotein triglycerides, are transported across the capillary endothelium into the alveoli remains unknown.

Synthesis of milk triglycerides, phospholipids and cholesterol esters

Most of the fatty acids derived from the various biochemical pathways described above are esterified with glycerol to form triglycerides before their subsequent incorporation into milk fat globules. The glycerol for this esterification is derived mainly from the plasma triglycerides with the remainder being either synthesized from glucose in the mammary gland or derived directly from plasma glycerol (see Rook & Storry, 1964; Barry, 1966; Linzell, 1968). Triglyceride synthesis in guinea-pig (McBride & Korn, 1964*a*; Kuhn, 1967*a*, *b*) rat (Dils & Clark, 1962) goat (Patton & McCarthy, 1963*a*; McCarthy & Patton, 1964; Pynadath & Kumar, 1964; Wood, 1966) and bovine (Patton & McCarthy, 1963*a*; Kinsella, 1968*b*; Kinsella & McCarthy, 1968*a*, *b*) mammary glands occurs in the particulate fraction of the cell by the pathway involving progressive acylation of α-glycero-phosphate, phosphatidic acid and diglycerides. In the utilization of free glycerol prior conversion to α-glycerophosphate is necessary and the enzyme which catalyses this reaction has been found in guinea-pig (McBride & Korn, 1964*a*) and bovine (Kinsella, 1968*a*, *b*), but surprisingly not in goat (Pynadath & Kumar, 1964) mammary tissue. In the synthesis of glycerol from glucose the normal product would be α-glycerophosphate derived by the Embden–Meyerhof pathway. From the configurational analysis of milk triglycerides Dimick *et al.* (1965) have suggested that a 2-mono- or 1-2-diglyceride, derived by partial hydrolysis of blood triglycerides, contributes to milk fat. However, the negligible synthesis of triglycerides in goat mammary glands (Pynadath & Kumar, 1964; Patton, McCarthy, Evans, Jensen & Gander, 1962) when monoglyceride is used as the acyl acceptor is difficult to reconcile with these hypotheses.

Findings by various workers suggest that the short chain acids are incorporated at the terminal stages of milk triglyceride formation and possibly by a specific pathway. For example, the monoglycerides and diglycerides of bovine and goat mammary tissue, in contrast to the triglycerides of these tissues and their secretions, are characterized by a lack of short chain fatty acids (Patton & McCarthy, 1963*a*; Kinsella & McCarthy, 1968*b*). Furthermore, *in vitro*, the rate of incorporation of butyrate is similar to that for palmitate when a diglyceride is the acyl acceptor but much lower when α-glycerophosphate is the acceptor and in the latter instance incorporation of butyrate does not proceed beyond the stage of diglyceride (Pynadath & Kumar, 1964). Wood (1966) has also found that short, in contrast to long, chain fatty acids are only incorporated to a small extent into synthetic 1-2-diglycerides by particulate fractions of goat mammary glands. The possible existence of a special pathway may also be reflected in the almost exclusive location of butyric acid in the 3 position of the triglyceride molecule (see earlier), but in any event it is clear that the pathway for incorporation of short chain fatty acids into milk triglycerides needs establishing.

The cholesterol esters and phospholipids, which are only minor constituents of milk fat, are thought to be synthesized within the mammary gland which is in line with earlier discussion on the non-utilization of plasma cholesterol esters and phospholipids for milk fat synthesis. Intramammary infusion of cows with labelled fatty acids and *in vitro* cell culture studies with labelled acetate and glycerol have all shown that there is a net incorporation of these metabolites into the cholesterol ester and phospholipid fractions of milk lipids (Patton *et al.* 1962; Patton & McCarthy, 1963*b*;

McCarthy & Patton, 1964; Kinsella, 1968*b*; Kinsella & McCarthy, 1968*a*; Al-Shabibi, Tobias & Brown, 1969).

Formation and secretion of milk fat globules

In an elegant series of experiments Kinsella & McCarthy (1968*a*) observed, by phase microscopy, the simultaneous formation and secretion of lipid droplets in cultured bovine mammary cells. The whole sequence from the beginning of formation of the droplet to its secretion into the culture medium took about 5 h. Fat droplets accumulated in all of the cells studied but active extrusion of these droplets through the cell membrane only occurred in young cells where this latter process took about 1 h to complete. These observations thus support the original theories of Bargmann & Knoop (1959) on the mode of milk fat secretion in rat mammary cells. It is not yet known, however, at which stages in the sequence of events the various membrane lipoproteins are incorporated into the fat globule. The appearance in the apical region of bovine mammary secretory cells of lipid droplets surrounded by intense osmiophilic staining (Feldman, 1961) or electron dense (Stewart & Irvine, 1969) material suggests that part of the membrane is already surrounding the droplet before the time of its expulsion from the cell. Also, in electron microscopy studies of ruminant mammary glands, cell ribosomes have been frequently observed at the interface of cell cytoplasm and lipid droplets (Hood & Patton, 1967) and it therefore seems reasonable to suppose that such an association may be related to globule membrane formation. On the other hand, the absence of phosphatidyl ethanolamine and carotenoids in unsecreted fat droplets (cell cream) in contrast to their presence in secreted milk fat globules has lead Patton & Fowkes (1967) to conclude that these membrane components were actually incorporated during the process of expulsion from the cell. This latter finding is however inconsistent with an equal distribution of phosphatidyl ethanolamine throughout all fractions of the globule membrane (see earlier). Cholesterol esters may also be incorporated into milk fat globules at or near the time of their expulsion from the cell (Keenan & Patton, 1969). Electron microscopic autoradiography of lactating mouse mammary glands has shown that fat droplets are secreted directly into the alveolar lumen and not via the golgi apparatus as in the case of the liver (Stein & Stein, 1966). The virtual absence in milk of cardiolipin, in spite of relatively high concentrations in the mitochondria of mammary tissue, has also been used to support the concept of little disintegration of the secretory cell or disruption of the plasma membrane during milk fat secretion (Patton, Hood & Patton, 1969). Furthermore, histological studies on bovine mammary tissue have shown that individual cells lining the alveolus appear to be synchronized at the same stage of droplet secretion (Saacke & Heald, 1969).

NUTRITION IN RELATION TO THE YIELD AND COMPOSITION OF MILK FAT

In view of trends to use increased proportions of concentrate foods, particularly oil cakes or oil supplements, in diets for intensive milk production there is a growing interest in the effects of dietary fat on the composition of milk fat, especially in relation to the suitability of milk for use in manufactured products. Also, since the fat content of milk forms part of the basis of the milk payments scheme in this country, correct manipulation of the dairy cow's nutrition is also of economic interest

to the milk producer. Over the past decade detailed biochemical studies with ruminants have done much to further understanding on the role of nutrition in the synthesis and secretion of milk fat.

Effect of dietary fat on milk fat secretion

It has been known for a long time that the composition of cow's milk fat is influenced by the type of fat in her diet; the changes in composition have been demonstrated indirectly by changes in Reichert–Meissl, Polenske and Iodine values. The relation between the level of fat in the diet and the content or yield of fat in milk is, however, less obvious for several reasons. First, in some instances only changes in the content of total fat in milk have been measured and frequently any increased fat production has been masked by simultaneous increases in milk yield. Variations in response to dietary fat may be due to differing experimental conditions, as for example the quantity, frequency and length of period of feeding with fat. The quantity and quality of the fat in the basal diet may also be an important factor and in most investigations basal diets already supplying a 'normal' quantity of fat have been used, rather than solvent-extracted or low-fat basal diets; it is likely that under these conditions animals would respond less markedly to further increases in dietary fat. In more recent studies where individual fatty acids have been determined, a lack of response to dietary fat in terms of total milk fat yield has been shown to be due to increased yields of certain acids being offset by decreased yields of other acids. For the purpose of the present review, therefore, only the effects of dietary fat on the secretion of individual fatty acids in milk will be discussed. The role of dietary fat in milk fat secretion has recently been reviewed by Moore & Steele (1968) and Storry (1968).

Digestion and absorption of dietary lipids

The digestion and absorption of lipids in ruminants have been extensively reviewed elsewhere (Garton, 1965, 1967; Lough & Garton, 1968) so that only those aspects necessary for understanding the effects of dietary fat on milk fat secretion are outlined here.

The lipids in the diet of the cow are mainly in the form of glycerides with smaller amounts of phospholipids and sterol esters. Fatty acids are released from these lipids as a result of the hydrolytic activity of micro-organisms in the rumen. The liberated unsaturated acids of 18 carbon atoms, which form a considerable proportion of the fatty acids of typical dairy rations, are then modified by the hydrogenating activity of the rumen micro-organisms to give either transisomers of less unsaturated C_{18} acids or stearic acid. The extent to which hydrogenation occurs in the rumen varies with the amount (Czerkawski, 1967) and type (Noble, Steele & Moore, 1969*a*; Moore, Noble, Steele & Czerkawski, 1969) of fat in the diet and the frequency with which it is fed. Also, other dietary factors such as the type and amount of roughage foods will influence the microflora and thus the extent of hydrogenation of unsaturated acids (see later).

In addition to fatty acids arising directly from the food, fatty acids synthesized *de novo* by rumen organisms and incorporated into their own structural lipids also contribute to the pool of fatty acids entering the small intestine. These synthesized

fatty acids become liberated as a result of further digestion in the abomasum and duodenum and it is thought that the trace amounts of branched chain acids secreted in milk originate from this microbial source. As a result of these processes in the rumen the composition of fatty acids entering the duodenum differs from that in the diet. Though the exact biochemical pathway by which the long chain fatty acids are absorbed from the intestine of ruminants has yet to be elucidated, they are known to be absorbed into the lymph as a lipoprotein complex which then enters the jugular vein via the thoracic lymph duct.

Dietary fat and composition of lymph and blood lipids and rumen VFAs

The net result of the processes of lipid digestion is reflected in the composition of the triglycerides of intestinal and thoracic lymph and, to a lesser extent, in the composition of the blood plasma triglycerides. In cows (Leat & Hall, 1968; Perry & Macleod, 1968; Wadsworth, 1968*a*) and sheep (Felínski, Garton, Lough & Phillipson, 1964; Heath, Adams & Morris, 1964; Bath & Hill, 1967) for example the fatty acids in digesta entering the small intestine or in the triglycerides of lymph are characterized by the virtual absence of polyunsaturated C_{18} acids in spite of large intakes of these acids in the diet. Such observations must reflect the overall effectiveness of hydrogenation in the rumen and, as might be expected, only when large single doses of unsaturated oils are given do significant amounts of the polyunsaturated acids escape hydrogenation to give increased outputs of them in lymph (Wadsworth, 1968*b*). The polyunsaturated acids normally present in lymph phospholipids and cholesterol esters probably originate endogenously from the resorption of the polyunsaturated acids secreted in bile and pancreatic juice (Lennox, Lough & Garton, 1968; Lennox & Garton, 1968; Leat & Harrison, 1969).

The addition of fat to the diet of ruminants increases the concentration of total lipids in blood plasma, largely due to increases in phospholipids and cholesterol esters (Bohman, Wade & Torell, 1962; Bohman & Lesperance, 1962; Storry, Rook & Hall, 1967; O'Kelly, 1968; O'Kelly & Robinson, 1968; Moore, Steele & Noble, 1969). The increased concentrations of phospholipids and cholesterol esters resulting from the feeding of cod-liver oil to lactating cows occur regardless of the degree of saturation of the cod-liver oil and they are confined to the plasma lipoproteins which are not precipitated by dextran sulphate (Brumby, Storry & Sutton, 1969). The concentrations in plasma of total or dextran sulphate precipitable triglycerides (Leat & Gillman, 1964; Tove, 1965; Hartmann, Harris & Lascelles, 1966; Storry *et al.* 1967; Brumby *et al.* 1969) seem to be little influenced by the amount of fat in the diet, presumably because of rapid clearing of the absorbed triglycerides from plasma.

The effects of dietary lipids on the fatty acid composition of the various lipid fractions of plasma have been studied less extensively in ruminants and the significance of the available findings is difficult to assess for various reasons. Dietary supplements of oils containing C 12:0, 14:0, 16:0, 18:0, 18:1 and 18:2 acids, when fed to 2 lactating cows, gave increased concentrations of all these acids except 18:2 in the blood plasma triglycerides of one cow whereas in the other cow the concentrations of only the shorter chain acids were increased (Storry *et al.* 1967). Increasing the dietary intakes of linoleic and linolenic acids by feeding sheep dried grass instead of hay, or by infusing the free acids into their rumens, did not significantly increase the pro-

portions of these polyunsaturated acids in their blood plasma triglycerides (Moore, Noble & Steele, 1968). On the other hand, the proportions of 18:0 and 18:1 acids in their plasma triglycerides were increased on the diet of dried grass in spite of lower dietary intakes of these acids, which probably could be attributed to increased absorption of 18:0 and 18:1 acids arising from hydrogenation of the polyunsaturated acids in the diet. However, it should be pointed out that since the concentrations of the various plasma lipid fractions were not quantitatively determined it cannot be unequivocally concluded that the absolute concentrations of these acids in the blood plasma triglycerides followed their compositional changes. The changes in the dietary intakes of polyunsaturated acids were also associated with parallel changes in the proportions of the corresponding acids in the plasma cholesterol esters and phospholipids but, again, in the absence of data on the levels of these lipid fractions in blood plasma, it is difficult to know whether the absolute concentrations of these acids in the blood plasma followed their changes in composition. Moore, Noble & Steele (1968) suggested that the higher proportions of polyunsaturated acids in the phospholipid and cholesterol ester fractions of blood plasma associated with an increased dietary intake of these acids may reflect incomplete hydrogenation of polyunsaturated acids in the rumen, but these findings are at variance with those of Brown & Stull (1967) and with the opinion that events in the intestine do not influence the formation of cholesterol linolenate in bovine blood plasma (Leat & Hall, 1968). In a further series of experiments, Moore, Noble & Steele (1969) bypassed the rumen by infusing linseed oil, maize oil and linolenic acid directly into the abomasum of sheep on hay diets. In contrast to their earlier feeding experiments, these infusions led to immediate increases in the proportions of 18:2 and 18:3 acids in the plasma triglycerides whereas the proportions of these acids in the plasma phospholipids and cholesterol esters did not increase until lapses of 8 and 24 h, respectively. While bearing in mind the dangers of extrapolating from mere changes in fatty acid proportions these experimental findings emphasize the role of the rumen in influencing the levels of unsaturated fatty acids in plasma triglycerides and confirm the suggested importance of the liver in maintaining the composition of plasma cholesterol esters and phospholipids (Leat & Hall, 1968).

The effects of supplementing a low-fat diet with stearic or palmitic acids on the composition of blood plasma lipids and on the yield of fatty acids in milk of cows has also been investigated by Moore, Steele & Noble (1969) but in these experiments samples of blood were taken from the subcutaneous abdominal vein. In view of the high proportion in the subcutaneous vein of blood draining from the mammary gland (Linzell, 1961) and of the avid mammary metabolism of triglycerides and non-esterified fatty acids their data would appear to reflect mainly the fatty acids not taken up by the gland rather than those presented to it for possible utilization in milk fat formation. Perhaps their experimental approach helps to explain their findings of a positive curvilinear relationship between the yield of total fatty acids in milk and the concentration of total non-esterified fatty acids, but surprisingly not of triglycerides, in blood plasma.

The effects of dietary supplements of variously hydrogenated cod-liver oils on the composition of blood lipids of lactating cows is being investigated in our laboratory (Brumby *et al.* 1969). The feeding of non-hydrogenated cod-liver oil resulted in the

appearance of polyunsaturated acids of 20 and 22 carbon atoms in all fractions of blood plasma lipids, suggesting that these acids, unlike those with 18 carbon atoms, are not easily hydrogenated in the rumen.

Addition of lipids to the diet also influences the pattern of rumen fermentation which in turn may have important effects on milk fat secretion. The observed responses in proportions of rumen volatile fatty acids to dietary fat differ from one worker to another and much of this variation can be attributed to differences in the nature of the basal diet, in the fatty acid composition of the fat supplement and in the level and way in which the fat is fed (Nicholson & Sutton, 1967; Devendra *et al.* 1968). In general, however, it may be said that additions to the diet of tallow, of cotton seed, safflower and cod-liver oils and of lauric, myristic, palmitic, stearic and oleic acids tend to change fermentation in the rumen to a pattern characterized by increased proportions of propionic and valeric acids and decreased proportions of acetic and sometimes of butyric acids (Shaw & Ensor, 1959; Brown, Stull & Stott, 1962; Nottle & Rook, 1963; Nicholson, Cunningham & Friend, 1963; Steel & Moore, 1968*b–d*; Varman *et al.* 1968; Noble, Steele & Moore, 1969*b*). The proportion of polyunsaturated fatty acids in the oil supplement appears to be an important factor governing the pattern of rumen volatile fatty acids obtained with additions of cod-liver oil to the diet (Brumby *et al.* 1969).

Dietary fat and the yields of fatty acids in milk

As already discussed, a proper understanding of the role of dietary fat on milk secretion can only be obtained by considering the effects of dietary fat on the yields of individual fatty acids in milk. For the present discussion it is convenient to consider milk fatty acids in 2 groups.

Long chain acids (> 16 *carbon atoms*). Increased dietary intakes of linolenic, linoleic, oleic and stearic acids, through feeding to cows the free acids or oils containing these acids, are associated with increased yields of oleic and stearic, but not of linoleic and linolenic, acids in milk (Storry *et al.* 1967; Storry, Hall & Johnson, 1968; Steele & Moore, 1968*a–d*; Perry & Macleod, 1968; Noble *et al.* 1969*b*). The lack of increased yields of these polyunsaturated acids in milk is consistent with the hydrogenation of these acids in the rumen prior to their absorption from the digestive tract, since, when the rumen is bypassed by intravenously infusing these polyunsaturated acids, their yields in milk increase (Tove & Mochrie, 1963; Storry & Rook, 1964, 1965*b*; Storry, Hall, Tuckley & Millard, 1969). The increased yields of stearic and oleic acids observed in the above feeding experiments could arise by a number of pathways. First, stearic acid could be derived directly from the increased dietary intake of stearic acid and secondly indirectly through the ruminal hydrogenation of unsaturated C_{18} acids in the diet. Similarly, the increased yields of oleic acid could have arisen directly from increased dietary intake of oleic acid and indirectly through hydrogenation in the rumen of more unsaturated dietary C_{18} acids, or through increased desaturation of stearic acid within the mammary gland (see above).

In contrast to acids containing 18 carbon atoms those of 20 or 22 carbon atoms do not appear to be secreted in more than trace amounts in the milk of cows given intravenous infusions (Storry, Hall, Tuckley & Millard, 1969) or dietary supplements

(Hilditch & Thompson, 1936; Beitz & Davis, 1964; Brumby *et al.* 1969) of oils containing these acids.

Short and intermediate chain acids (4–16 *carbon atoms*). The effects of dietary fat on the yields in milk of intermediate chain acids (12–16 carbon atoms) are more variable because these acids are derived by intramammary synthesis from acetate and β-hydroxybutyrate and also directly from the intermediate chain acids of the plasma triglycerides. Thus, supplementing the diet of cows with coconut oil, which is rich in lauric and myristic acids, increases the concentration of these acids circulating in the plasma triglycerides and the yields of them in milk (Storry *et al.* 1967). Increasing the concentration of lauric and myristic acids in blood plasma triglycerides by intravenous infusion also results in increased yields of these acids in milk (Storry, Tuckley & Hall, 1969) as does adding them to a diet as free acids (Steele & Moore, 1968*d*).

The correlation between dietary intake and yield of palmitic acid in milk is much more variable, however, and sometimes there is even a decreased yield of palmitic acid in milk in spite of increased dietary intakes of this acid (Storry *et al.* 1967, 1968; Steele & Moore, 1968*a–d*; Noble *et al.* 1969*b*). This variable yield of palmitic acid is probably related to the fact that intramammary synthesis of palmitic acid is depressed as a result of dietary additions of fat, and the net yield of palmitic acid will depend upon the balance between the amounts derived from *de novo* synthesis and from plasma triglycerides. Depressed intramammary synthesis of other short and intermediate chain acids have also been frequently observed with additions of oils and long chain acids to the diet (Steele & Moore, 1968*a–d*; Storry *et al.* 1968; Noble *et al.* 1969*b*). Two mechanisms have been suggested for this depressed intramammary synthesis of fatty acids (Moore & Steele, 1968). First, an altered production of volatile fatty acids in the rumen in response to dietary fat supplements may decrease the supplies of acetate and β-hydroxybutyrate to the mammary gland; as discussed earlier, there is evidence to support this hypothesis. Secondly, an increased uptake of long chain acids from plasma triglycerides as a result of dietary fat supplement could depress synthesis of the shorter chain acids through an inhibition of acetyl CoA carboxylase; again, there is considerable support at the tissue level for this second hypothesis. However, in contrast to the results from feeding experiments, the intravenous infusion of specific triglycerides containing a wide range of fatty acids up to 18 carbon atoms (Storry, Tuckley & Hall, 1969) did not depress the yields of short and intermediate chain acids in milk which may indicate that the effect on rumen fermentation is the main mechanism involved *in vivo*. (See end of paper.)

Milk fat depressing effect of cod-liver oil

A characteristic result of feeding cod-liver oil to dairy cows is a reduced content and yield of fat in milk. This effect appears to be related to the oil's content of C_{20} and C_{22} polyunsaturated acids since feeding cod-liver oil in which these acids have been removed by hydrogenation does not reduce milk fat secretion (Brumby *et al.* 1969). Once again, ruminal and non-ruminal mechanisms are probably involved in the depressed secretion of milk fat. First a change in the pattern of rumen fermentation (see above) may reduce the supplies of acetate and β-hydroxybutyrate for the *de novo* synthesis of fatty acids. Secondly, the long chain polyunsaturated acids of cod-liver oil may be incorporated as such into plasma lipids (Brumby *et al.* 1968), resulting in a

reduced uptake of fatty acids from the plasma triglycerides (Brumby *et al.* 1969; Varman *et al.* 1968; Storry, Hall, Tuckley & Millard, 1969).

Effect of dietary roughage on milk fat secretion

Diets in which the roughage is ground or diets with low proportions of roughage and high proportions of concentrates can produce a dramatic fall in the content and yield of fat in milk and significant changes in its fatty acid composition. Since the original demonstrations by Powell, some 30 years ago, of the importance of dietary roughage in maintaining milk fat secretion, the properties of a much wider range of milk fat depressing diets have been studied and an understanding of their mechanism of action sought. Reviews on the role of dietary roughage in milk fat secretion have shown that many areas of metabolism are implicated in the decreased output of milk fat (see Van Soest, 1963; Rook & Storry, 1964; Storry, 1968). Although findings differ in detail from one worker to another, owing to the wide variety of diets and breeds of cow used, it is now becoming evident that several basic mechanisms are involved in the low milk fat syndrome.

Metabolism in the rumen

Milk fat depressing diets produce a fall in rumen pH and changes in the proportions of rumen volatile fatty acids, generally characterized by decreased proportions of acetate and sometimes of butyrate and by increased proportions of propionate and valerate (King & Hemken, 1962; Hawkins, Paar & Little, 1963; Beitz & Davis, 1964; Jorgensen & Schultz, 1965; Jorgensen, Schultz & Barr, 1965; Storry & Rook, 1966; Davis & Sachan, 1966; Colenbrander, Bartley, Morrill, Deyoe & Pfost, 1967; Davis, 1967; Hawkins & Little, 1967; Thomas, Bartley, Pfost & Meyer, 1968; Varman & Schultz, 1968*a*; Storry & Sutton, 1969). The initial fall in pH is thought to be due to a diminished secretion of saliva and hence buffering capacity of rumen digesta, which then allows the survival of a microflora producing the change in pattern of volatile fatty acids. Although the effects of milk fat depressing diets on the bacterial flora of the rumen have not been measured directly there is a marked fall in the rate of cellulose digestion (Storry & Sutton, 1969) and an absence of protozoa (Chalupa, Odell, Kutches & Lauker, 1967) in the rumens of cows on low roughage diets and with depressed milk fat secretion. Also, the feeding to non-lactating cows of ground and pelleted, as opposed to long, dried grass has been shown to influence the types and numbers of bacteria in the rumen (Thorley, Sharpe & Bryant, 1968).

In addition to affecting the pattern of rumen fermentation these changes in rumen microflora may also have important repercussions on the synthesis and metabolism of lipids in the rumen, especially in view of the known role of protozoa in the hydrogenation of unsaturated acids (Wright, 1959; Klopfenstein, Purser & Tyznik, 1966; Lough, 1968; Chalupa & Kutches, 1968). Thus, hydrogenation of dietary unsaturated fatty acids in the rumen is less in calves (Viviani, Borgatti, Monetti & Mordenti, 1967) and sheep (Tove & Matrone, 1962) when they are fed all-concentrate as compared with hay and concentrate diets. Our own unpublished observations also indicate that *in vitro* the hydrogenation of soybean oil is less with rumen digesta from cows fed a low roughage diet and with depressed milk fat than with digesta from the same cows fed normal roughage diets and with normal milk fat contents. In addition to hydro-

genation, the actual synthesis by microflora of fatty acids from dietary carbohydrate may also be affected by the type of diet. For example, in sheep there is a net increase in the total flow of long chain fatty acids leaving the abomasum over and above the amounts eaten in the food and this increase is greater on low than high roughage diets and greater with high than low levels of feeding (Sutton, Storry & Nicholson, 1969).

Composition of blood plasma and activity of enzymes in adipose and mammary tissues

The changed metabolism in the rumen which results from the feeding of low roughage diets also influences the composition of blood plasma in such a way as to reduce the supply to the mammary gland of milk fat precursors. Thus, animals on low roughage rations with depressed milk fat have reduced concentrations of acetate and β-hydroxybutyrate in blood plasma (Jorgensen *et al.* 1965; Storry & Rook, 1965*c*; Varman & Schultz, 1968*a*; Storry & Sutton, 1969). Milk-fat-depressing diets also reduce the concentrations in blood plasma of phospholipid, cholesterol ester, non-esterified fatty acid and sometimes of triglyceride (Jorgensen *et al.* 1965; Storry & Rook, 1965*c*; Varman & Schultz, 1968*a*). Of more importance to milk fat secretion, however, are the increased proportions and concentrations of unsaturated, and decreased proportions and concentrations of saturated, fatty acids in the plasma triglycerides of cows on milk fat depressing diets (Davis & Sachan, 1966; McCarthy, Dimick & Patton, 1966; Storry & Sutton, 1969).

Recently, studies on enzymes involved in fatty acid and glyceride synthesis in bovine adipose and mammary tissues have shown higher activities of these enzymes in adipose tissue, and possibly lower activities of some of them in mammary tissue, under dietary regimes which reduce milk fat secretion (Opstvedt, Baldwin & Ronning, 1967; Baldwin & Cheng, 1968; Baldwin, Lin, Cheng, Cabrera & Ronning, 1969; Benson, Askew, Emery & Thomas, 1969; Young, Thorp & de-Lumen, 1969). These findings suggest that on low roughage diets fatty acid utilization is directed towards deposition in adipose tissue rather than secretion in milk fat and is in line with the findings of higher body weight gains on low roughage than on high roughage diets (Jorgensen *et al.* 1965; Opstvedt & Ronning, 1967).

Composition and yields of fatty acids in milk

The fat from cows established on low roughage diets has increased proportions of unsaturated and decreased proportions of saturated acids (King & Hemken, 1962; Palmquist, Smith & Ronning, 1964; Beitz & Davis, 1964; Jorgensen *et al.* 1965; Storry & Rook, 1965*c*; Dronovat, Stanley, Cobb & Morita, 1966; Davis & Sachan, 1966; Storry & Rook, 1966; Opstvedt & Ronning, 1967; Steele & Moore, 1968*b*; Storry & Sutton, 1969). In terms of yields, the saturated acids are markedly decreased and the unsaturated acids decreased to a lesser extent or even increased (Storry & Rook, 1965*c*, 1966; Opstvedt & Ronning, 1967; Steele & Moore, 1968*b*; Storry & Sutton, 1969). The changes in yield of fatty acids in milk on low roughage diets are thus generally associated with corresponding changes in the concentration of their respective precursors in blood plasma as a result of changes in metabolism in the rumen and adipose tissue.

Various attempts have been made to restore the depressed fat secretion by the

addition of certain compounds to low roughage diets. Additions to the rumen of acetic and butyric acids as the free acids or their sodium salts have resulted in very varied recoveries in milk fat (Stoddard, Allen & Peterson, 1949; Tyznik & Allen, 1951; Balch & Rowland, 1959; Jorgensen, 1964; Stanley, Morita & Ueyama, 1964; Jorgensen *et al.* 1965; Storry & Rook, 1966). This is perhaps not surprising in view of the complexity of factors involved in the depressed secretion of milk fat. The incorporation of labelled acetate into milk fat following its intraruminal (Hawkins, 1969) or intramammary (Palmquist *et al.* 1969) infusion has recently been shown to be less in cows on low roughage than on high roughage diets.

Increasing the buffering capacity of the rumen by including a variety of supplements has been more successful and offers a more practical approach to the possible prevention of decreased milk fat production on low roughage diets. Thus, supplements of sodium bicarbonate, calcium carbonate, calcium hydroxide, magnesium oxide, bentonite, vermiculite and milk whey have all been associated with recovery or prevention of depressed milk fat secretion and of the associated changes in milk fatty acid composition, rumen fermentation and blood composition (Davis, Brown & Beitz, 1964; Emery, Brown & Thomas, 1964; Emery, Brown & Bell, 1965; Jorgensen & Schultz, 1965; Huber, Polan & Rosser, 1967; Benson *et al.* 1969; Bringe & Schultz, 1969*a*, *b*; Huber, Emery, Thomas & Yousef, 1969; Thomas & Emery, 1969). Some of the additives, such as magnesium oxide and whey may also exert an extraruminal effect by increasing the mammary uptake of milk fat precursors (Emery *et al.* 1965; Huber *et al.* 1969).

Effect of season and inanition on milk fat secretion

Detailed analyses of samples of milk fat collected from dairies or individual herds of several countries have shown characteristic changes in fatty acid composition throughout the year. Milk fat from animals fed typical winter diets of roughage and concentrates contains higher proportions of C_{4-16} acids and lower proportions of C_{18} acids compared with milk from summer periods when the animals are fed grass diets (Patton, McCarthy, Evans & Lynn, 1960; Jensen, Gander & Sampugna, 1962; Hawke, 1963; Boatman, Hotchkiss & Hammond, 1965; Antila, 1965; Svensen, 1967; Hutton, Seeley & Armstrong, 1969; Mattson, Swartling & Nilsson, 1969; Hall, 1970). The increased proportions of C_{18} acids probably originate indirectly through hydrogenation in the rumen of increased dietary intakes of linoleic and linolenic acids on the grass diet.

The starving of cows (Luick & Smith, 1963; Hartmann & Lascelles, 1965) and goats (Annison, Linzell & West, 1968) for periods varying from 1 to 5 days reduces the yield of milk fat and changes its composition by decreasing the proportions of C_{4-16} acids and increasing the proportions of C_{18} acids. Under conditions of starvation the flow of blood to the mammary gland is greatly reduced (Linzell, 1967; Annison *et al.* 1968; Kronfeld, Raggi & Ramberg, 1968) and contains increased concentrations of non-esterified fatty acids and β-hydroxybutyrate and decreased concentrations of acetate (Hartmann & Lascelles, 1965; Radloff, Schultz & Hoekstra, 1966; Linzell, 1967; Annison *et al.* 1968; Jackson, Black & Moller, 1968). Such changes in blood flow and composition result in decreased mammary uptake and incorporation into milk of acetate and increased uptake of non-esterified fatty acids. Changes in the mammary

uptake of β-hydroxybutyrate and triglycerides, on the other hand, are more variable probably because of differences between experiments in the severity of starvation and in the extents to which endogenous supplies of substrate from the mammary tissue contributed to fat formation during starvation. Presumably an absolute shortage of acetate precursor and a decreased rate of its carboxylation through the inhibition of acetyl CoA carboxylase by the increased mammary uptake of non-esterified fatty acids (see earlier), could both contribute to the decreased synthesis and secretion of the short chain acids during periods of starvation.

Changes in milk fat secretion with stage of lactation

With animals fed uniform diets, the composition of milk fat has been found to vary with stage of lactation, the most marked changes occurring during the first 6 weeks after calving. These are decreases in the proportions of C_{18} acids, increases in the proportions of C_{6-14} acids and decreases or little change in the proportion of C_{16} acid (Boatman *et al.* 1965; Stull & Brown, 1965; Decaen & Adda, 1966). In more detailed experiments, where milk was sampled twice weekly throughout lactation, linear, quadratic and cubic equations have been used to describe the lactational trends in proportions of individual fatty acids (Stull, Brown, Valdez & Tucker, 1966).

In terms of yields, the C_{6-14} acids increase for the first 6 weeks of lactation after which time there is a steady decline. The C_{16-18} acids, on the other hand, decline sharply during the first 6 weeks of lactation and continue to decline at a slower rate to the end of lactation (Decaen & Adda, 1966; Decaen & Journet, 1967). Our own unpublished results have also fully confirmed these changes in yield of individual fatty acids during the early weeks of lactation, and when cows were fed low, in contrast to normal planes of nutrition the yields of all acids declined markedly from the beginning of lactation.

The difference in pattern of secretion between the C_{6-14} and C_{16-18} acids at the beginning of lactation must reflect changes in metabolism of the body and mammary gland. During early lactation the animal draws heavily on body reserves for milk secretion as reflected by the decrease in body weight (Decaen & Adda, 1966; Decaen & Journet, 1967) and increase in plasma concentration and mammary uptake of non-esterified fatty acids (Decaen & Journet, 1967; Varman & Schultz, 1968*b*). The gradual increase at the beginning of lactation in the yields of those acids which are synthesized within the mammary gland are less easily explained, however. The mammary glands of cows, in contrast to the glands of rats and guinea-pigs, show no marked increase at parturition in the concentrations of enzymes and metabolites involved in fatty acid synthesis, which suggests that metabolic regulatory factors are involved in the initiation of fatty acid synthesis within the mammary gland of the cow (Baldwin, 1966, 1969; Baldwin & Cheng, 1969).

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Note added in proof

Dietary fat has recently been shown to depress fatty acid synthesis in liver but not in mammary gland of mice, suggesting that the control of lipogenesis may vary between tissues (Smith, S., Gagnet, H. T., Pitelka, D. R. & Abrahams, S. (1969). *Biochem. J.* **115**, 807).

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