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A kinetic model of the clotting of casein by rennet

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Summary. A kinetic model of casein clotting caused in milk by rennet was defined by measuring changes in viscosity and in the complex modulus of rigidity. Three separate stages of the process were distinguished: the enzymic, the flocculation and the gelification. The kinetic equations and the main activation parameters were defined for each stage.

The mechanism of the coagulation of milk casein caused by rennet has occupied the attention of scientists for many years. One of the main reasons for this continuing interest is the biological importance of the processes occurring in the proteins. Another reason is in connexion with the engineering problems that have arisen from the present-day trend towards continuous cheese-making. These continuous methods require an understanding of the quantitative effects of the chemical reactions that accompany the physical changes during milk clotting.

A great deal of research has been done both on the chemical changes and on the physical phenomena caused by the action of rennet. Rheological methods have been very useful for studying the postenzymic stages, and even the enzyme action could be followed in this way with very great accuracy (Scott Blair & Oosthuizen, 1961*a*,*b*; 1962). These methods have also been very useful for investigating the effects of various parameters such as pH, calcium content, temperature, and others (Tarodo de la Fuente & Frentz, 1966; Tuszyński, Burnett & Scott Blair, 1968).

Although so much research has been done on the problem, there is still much uncertainty about the kinetics of the clotting processes, and particularly about the stage at which visible physical changes of the substrate structure occur. There is still little known about the dependence of the clotting processes on the substrate characteristics.

The present paper reports a study on some of these aspects, and is concerned mostly with the history of the substrate and its influence on the particular stages of milk clotting. Another object of this work has been to study the kinetics of clot formation when this first takes place and when the rate of formation can be measured by the increase of the complex modulus of rigidity.

Materials and methods

Experiments were done on undiluted fat-free milk. Some of the viscometer experiments were done for comparison on a 2 % solution of sodium caseinate in 0·015 M-$CaCl_2$.

The method of preparing the samples for investigation is shown in Table 1, in which particulars of all the experiments are also given. Two instruments were used. One was a U-tube viscometer similar to that described by Scott Blair (1941). The experiments were performed as described by Tuszyński & Scott Blair (1967).

The other instrument was a torsiometer similar to that described by Burnett & Scott Blair (1963). Two types of this instrument were used. The first was as described in our earlier work (Tuszyński *et al.* 1968) and the second was built on the same pattern, but with beakers of 1·5 l capacity. The torsiometer with the smaller beakers was used for experiments in which the milk was cold-renneted and therefore had to be heated in 20–30 s, because of the very rapid coagulation.

RESULTS

The first effect to be studied was that produced on the early stages of clotting by holding the milk at 4–6 °C. It was found that the equation proposed by Tuszyński & Scott Blair (1967),

$$\frac{\tau}{\tau'} = \left(\frac{\dot{\gamma}}{\dot{\gamma}'}\right)^a, \tag{1}$$

applied to the early stages of milk coagulation by rennet and was independent of the

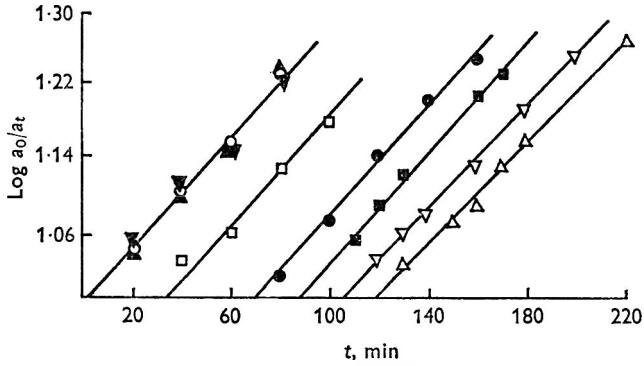

Fig. 1. Plots of the relation $t = (1/k)\log a_0/a_t$ at 16 °C. Expts: 1, ●; 2, ■; 3, ▽; 4, △; 5, □; 8, ▼; 9, ▲; 10, ○.

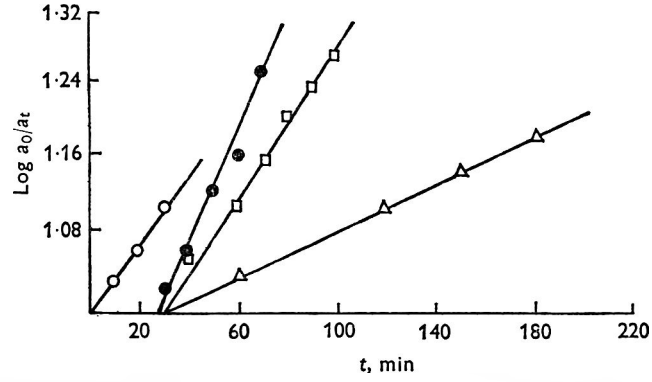

Fig. 2. Plots of the relation $t = (1/k)\log a_0/a_t$ showing the temperature dependence. Expt 6, 18 °C □; expt 7, 20 °C ◧; expt 11, 13 °C △; expt 12, 17 °C ○.

Table 1. *List of experiments*

Programme of expts	Substrate sample	No. of expt	Casein content, % (±0·1)	pH	Holding time at 4 °C before measurement, h	Temp. of rennet addition, °C	Temp. of investigation, °C
Investigation of early stages in milk and sodium caseinate clotting using a capillary viscometer	A—Skim-milk	1	2	6·6	0	4	16
		2	2	6·6	4	4	16
		3	2	6·6	24	4	16
		4	2	6·6	52	4	16
		5	2	6·6	*	4	16
		6	2	6·6	24	4	18
		7	2	6·6	24	4	20
		8	2	6·7	0	4	16
		9	2	6·7	4	4	16
	B—Sodium caseinate	10	2	6·7	24	4	16
		11	2	6·7	24	4	13
		12	2	6·7	24	4	17
Investigation of viscosity changes in milk held at 4 °C, using a capillary viscometer	Skim-milk C	13	2	6·5	†	Rennet	20
	Skim-milk D	14	2	6·3	†	was not	20
	Skim-milk E	15	2	5·9	†	add-	20
	Skim-milk F	16	2	5·8	†	ed	20
Torsiometric investigation on the complex rigidity modulus in milks differing in pH	G—Skim-milk	17	2·5	6·65	24	32	32
		18	2·5	6·62	60	32	32
		19	2·5	6·42	60	32	32
		20	2·5	6·20	60	32	32
Torsiometric investigation on the complex rigidity modulus in milk	H—Skim-milk	21	2·5	6·36	‡	32	32
		22	2·5	6·36	‡	32	32
		23	2·5	6·36	‡	32	32
Torsiometric investigation on the complex rigidity modulus in relation to the reaction temperature	J—Skim-milk	24	2	6·45	72	4	26
		25	2	6·45	72	4	27
		26	2	6·45	72	4	29
		27	2	6·45	72	4	30
		28	2	6·45	72	4	33
		29	2	6·45	72	4	37

* The experiment was performed to investigate the reversibility of changes occurring in milk as the result of holding it at 4 °C. Milk was held for 24 h at 4 °C, heated to 50 °C and held for 3 h, and cooled to 4 °C. Rennet was added and measurement begun after a further 4 h.

† Measurements were performed during 48 h at the points shown on Fig. 4.

‡ After 20 h at 4 °C, the samples were heated to 20 °C and kept at this temperature for 0, 2 and 4 h before measurements.

holding time. Furthermore, it was found that the plots $\log(a_0/a_t)$ against time were reasonably linear for all experiments in this series (a_0 is the exponent of the equation at zero time, i.e. when the sample was poured into the viscometer, and a_t is the exponent after time t, counted from the moment of filling the viscometer). These relations are shown in Fig. 1 for the experiments at 16 °C and in Fig. 2 for the experiments at 13–20 °C. Assuming that the exponent is proportional to the concentration, the relation found could be shown as a typical equation of first-order reactions,

$$t = \frac{1}{k} \log \frac{a_0}{a_t}, \tag{2}$$

where k is the velocity constant, and a_0 and a_t are the concentrations of the substrate at the beginning and after time t.

As may be seen from Fig. 1, all the lines are parallel at constant temperature, indicating that the constant k does not depend on the holding time of the samples, but the points where the curves cut the time-axis do depend on it.

To explain the latter observation it was assumed that the enzymic stage of the process occurred only partly at 4 °C, the other part occurring at 16 °C, i.e. in the viscometer.

The amount of substrate changed by rennet at 4 °C depended on the holding time before rennet was added. If the above assumption is true the following equations would apply:

$$t_4 = \frac{1}{k_4} \log \frac{1}{a_c'}, \tag{3}$$

$$t_{16} = \frac{1}{k_{16}} \log \frac{a_c'}{a_c}, \tag{4}$$

$$k_{16} = k_4 Q_{10}^{(16-4)/10}, \tag{5}$$

where

k_4 and k_{16} are the velocity constants for the enzymic stage at 4 and 16 °C,

a_c' is the fraction of unchanged substrate after the time t_4, i.e. the time of rennet action on milk at 4 °C (t_4 = 4 h = 14400 s),

a_c is the fraction of unchanged substrate when the reaction reached equilibrium (a_c was taken as the fraction of NPN released; a_c = 0·087),

t_{16} is the time taken from the plot on Fig. 1 as the intercept on the time axis,

Q_{10} is the temperature coefficient for the enzymic reaction.

Equations (3) and (4) are first-order reaction equations, found by viscometric methods (Scott Blair & Oosthuizen, 1961*b*; 1962) to apply also to the enzymic stage. The values k_4 and k_{16} calculated from these equations (taking Q_{10} = 2·12) were found to be $k_4 \simeq 1{\cdot}0 \times 10^{-4}$ s^{-1} and $k_{16} \simeq 2{\cdot}46 \times 10^{-4}$ s^{-1} for expt 1 (holding time = 0 h).

Taking the same Q_{10} coefficient, at 32 °C the value would be $k_{32} \simeq 8{\cdot}2 \times 10^{-4}$ s^{-1}, which is in good agreement with the value found by Scott Blair & Oosthuizen (1961*b*).

Having ascertained the dependence on holding time of the velocity constant for the enzymic stage, it was interesting to find an explanation for this phenomenon. When equation (1) is written as

$$\tau = K.\dot{\gamma}^a, \tag{6}$$

K is the Newtonian viscosity η when a = 1.

Plotting $\log K$ against a for each experiment of the series, the value of η was estimated for the milk at the beginning of the reaction at 16 °C, i.e. when a part of the substrate had been changed by rennet at 4 °C.

Finding the value of $K(\eta)$ for water in the same viscometer at 16 °C, the specific viscosity η_{sp} of the milk was estimated at that stage of the reaction. For all the experiments done the following relation was found,

$$\frac{1}{\eta'_{sp}} \log \frac{1}{a'_c} = \text{constant.} \tag{7}$$

When η_{sp} for expt 1 is taken as unity, the values of η_{sp} marked as η'_{sp} for the other experiments are larger and the relation

$$\eta'_{sp} = f(t_p^{\frac{1}{2}}), \tag{8}$$

where t_p is the holding time, was found to be linear, as is shown in Fig. 3.

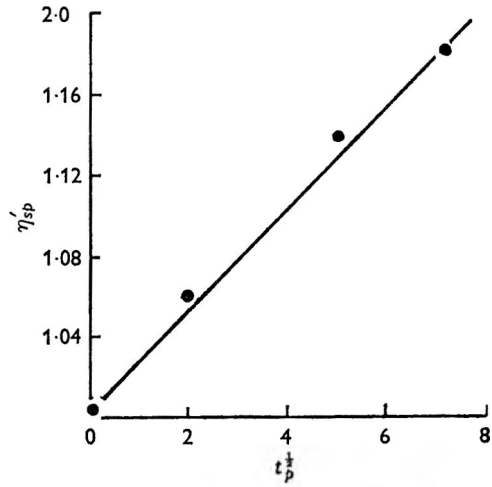

Fig. 3. Plot of the relation $\eta'_{sp} = f(t_p^{\frac{1}{2}})$, ●.

This observation led to the assumption that while holding the milk at a low temperature and at pH > 6 the viscosity of the milk increased and that this increase caused a decrease in the velocity constant for the enzymic part of the coagulation process. The plots on Fig. 4 seem to support this assumption, although for short holding times the plots are not very regular.

The second phenomenon to be studied was the relation of the complex modulus of rigidity G to time t of the reaction. Two examples of typical coagulation curves are shown in Fig. 5, where the experimental points and the points calculated from the equation described below are marked.

The shape of the curves is similar to that for simple autocatalytic reactions. This type of reaction is very possible for the coagulation process and its kinetic equation is given by the relation,

$$\frac{dc}{dt} = kc(c_0 - c), \tag{9}$$

where

c is the concentration of the product at the time t,
c_0 is the concentration of the substrate at the beginning of the reaction,
k is the velocity constant for the process.

Taking $c/c_0 = x$ and putting $x = x_0$ when $t = 0$, the integrated form of equation (9) will be

$$t = \frac{1}{kc_0} \log \frac{x}{x_0(1-x)}, \tag{10}$$

This function is symmetrical because when taking

$$\frac{d^2x}{dt^2} = 0, \tag{11}$$

the inflexion point is $x = \frac{1}{2}$.

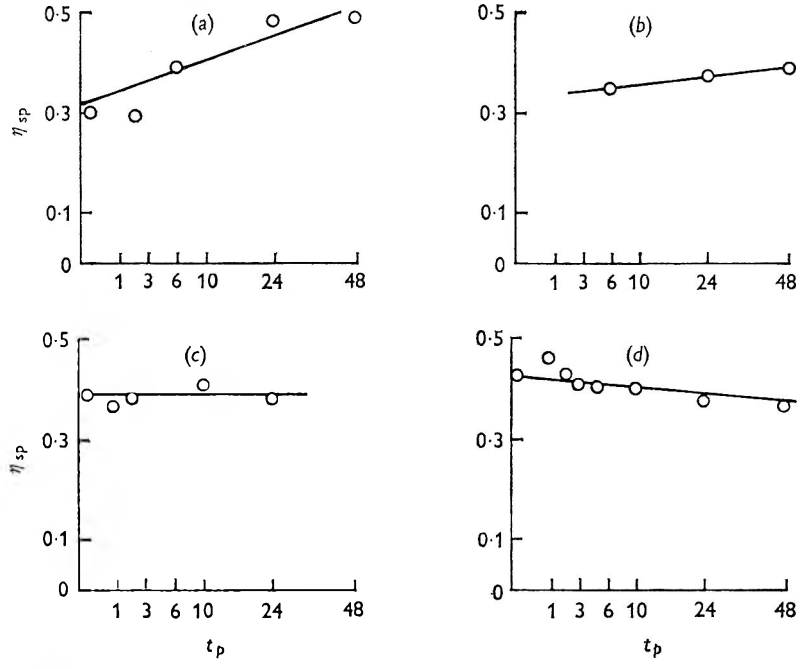

Fig. 4. Plots of the relation $\eta_{sp} = f(t_x)$ for the holding temperature 4 °C. (*a*) Expt 13, pH 6·5; (*b*) expt 14, pH 6·3; (*c*) expt 15, pH 5·9; (*d*) expt 16, pH 5·8.

Assuming that the modulus G at time t shows the value of c from equation (9), it is easy to find the inflexion point on the curve shown in Fig. 5. To calculate k from equation (10), the fraction G/G_∞ was taken as x, and the unknown x_0 was eliminated by combination of this equation for two different values of x and t of the curve. As seen from Fig. 5, equation (10) represents fairly well the process of gelling caused by rennet.

In Fig. 6 the plots of the values $\log x/(1-x)$ against time are shown for milk samples at different pH values. The time t_c at which the reaction started, estimated by the method of Scott Blair & Burnett (1963), is marked on the graph. All the t_c points

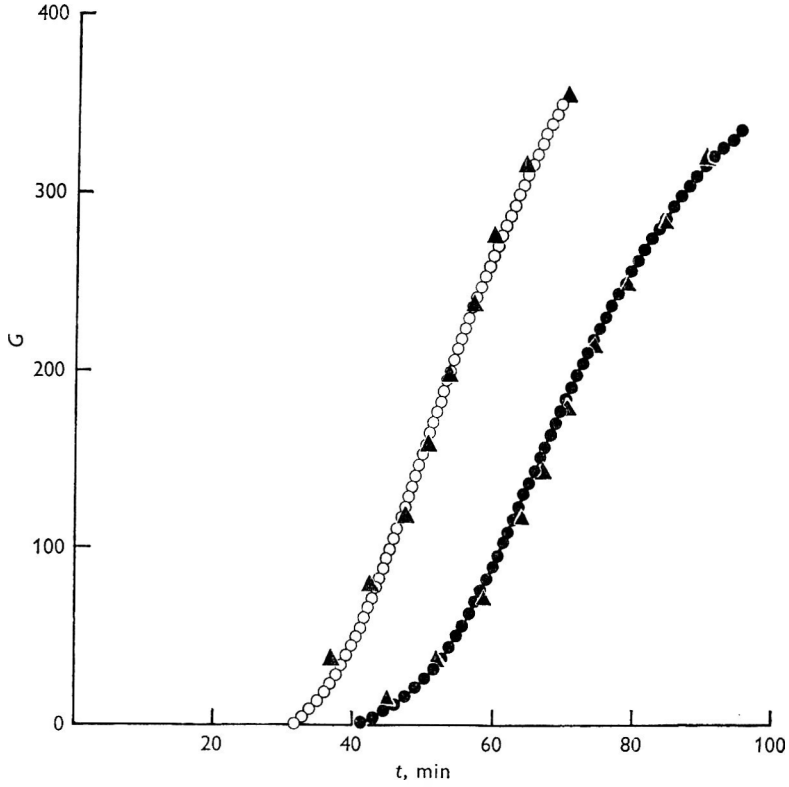

Fig. 5. Plots of the relation $G = f(t)$. ▲, Calculated points. Experimental points: expt 17, ○; expt 18, ●.

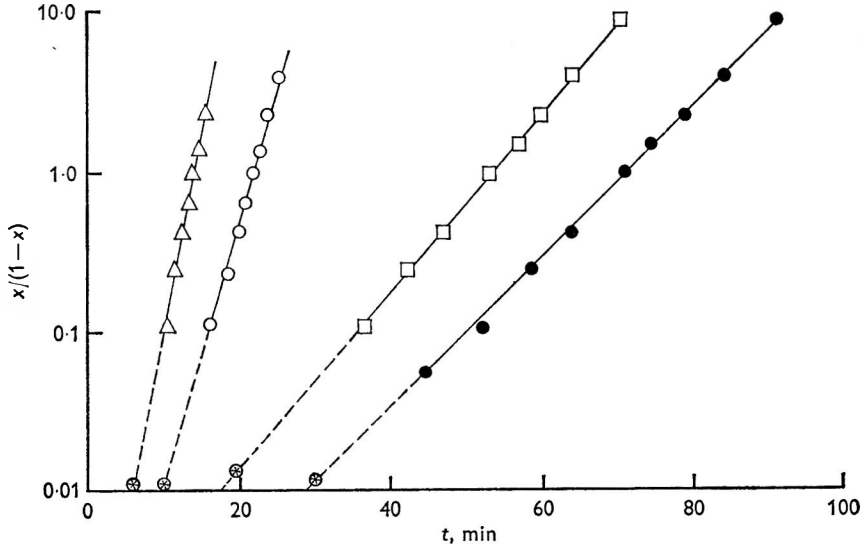

Fig. 6. Plots of the relation $t = 1/k \log x/[x_0(1-x)]$. Expt. 17, □; expt 18, ●; expt 19, ○; expt 20, △. ⊛, Start of reaction, t_c.

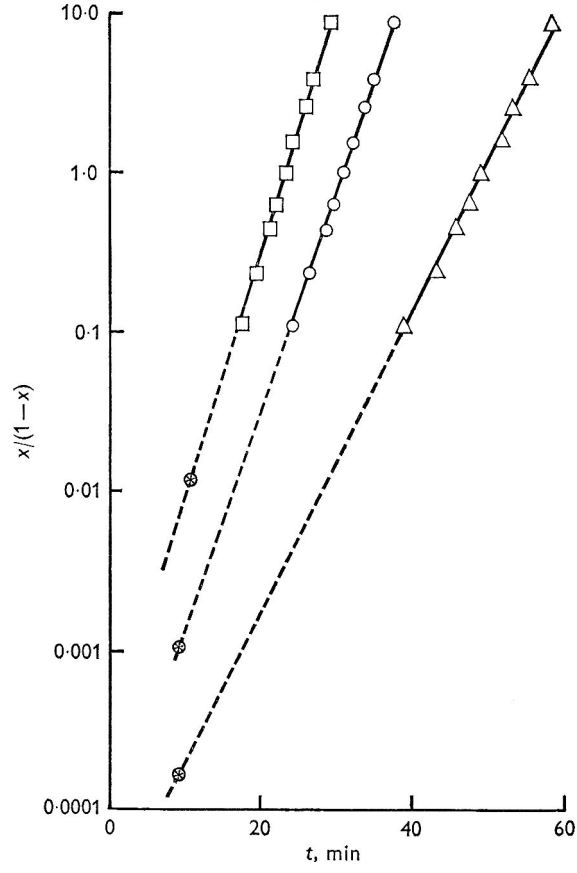

Fig. 7. Plots of the relation $t = 1/k \log x/[x_0(1-x)]$ for milk samples held for different periods at 20 °C at constant pH 6·36. Expt 21, □; expt 22, ○; expt 23, △. ⊛, Start of reaction, t_p.

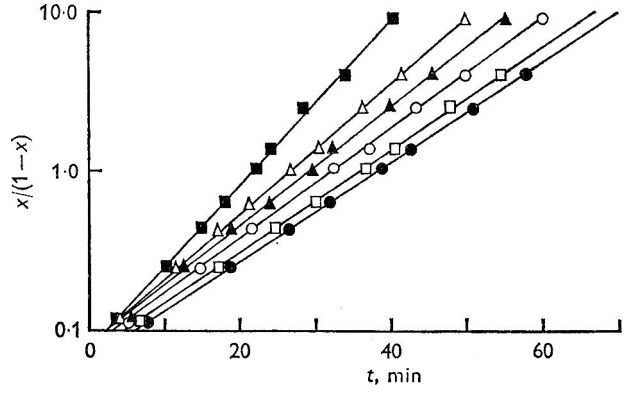

Fig. 8. Plots of the relation $t = 1/k \log x/[x_0(1-x)]$ showing the temperature dependence. ●, 26 °C, $k = 1{\cdot}2 \times 10^{-3}$; □, 27 °C, $k = 1{\cdot}23 \times 10^{-3}$; ○, 29 °C, $k = 1{\cdot}3 \times 10^{-3}$; ▲, 30 °C, $k = 1{\cdot}35 \times 10^{-3}$; △, 33 °C, $k = 1{\cdot}53 \times 10^{-3}$; ■, 37 °C, $k = 1{\cdot}94 \times 10^{-3}$.

show the value of $x_0 \sim 10^{-2}$, which means that in these experiments the value of x_0 did not depend on the pH of the sample, but the value of k depended on pH.

In Fig. 7 another series of experiments is shown, in which the effect of keeping the milk at 20 °C for 0–4 h before renneting was investigated.

As may be seen from the plots, keeping the milk at 20 °C did not change the value of t_c and the value of k decreased slightly from $6{\cdot}4 \times 10^{-3}$ for sample 21 to $3{\cdot}7 \times 10^{-3}$ for sample 23.

The greatest change was observed for x_0, which had decreased to one hundredth of its original value at the end of 4 h. This was probably due to the native enzymes of milk, which can be very active at 20 °C.

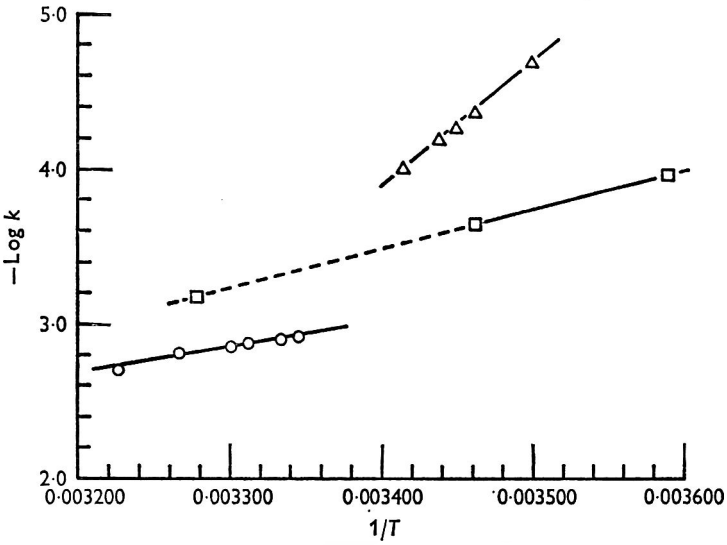

Fig. 9. Plots for the Arrhenius equation. □, Enzymic stage; △, flocculation stage; ○, gelling stage.

The temperature dependence of k was also investigated and the results are shown in Fig. 8. Rennet was added at 4 °C. The sample was kept at this temperature for 4 h and then heated very quickly to the temperature of investigation before placing the milk in the torsiometer.

In Fig. 9 the plots of $\log k$ against the reciprocal of the absolute temperature are shown, according to the Arrhenius equation,

$$k = B\exp(-E/RT), \tag{12}$$

where
- k is the velocity constant (s^{-1}),
- B the constant characteristic of the process (s^{-1}),
- E the activation energy (cal mol^{-1}),
- R the gas constant (1·986 cal mol^{-1} deg^{-1}),
- T the absolute temperature.

Analysing all the 3 processes (the enzymic stage, the flocculation and the gelling) according to the Eyring theory of the active complex, the increases in activation

enthalpy $\Delta H^{\ddagger}$, activation entropy $\Delta S^{\ddagger}$, and of the free energy of activation $\Delta F^{\ddagger}$, were estimated from the equations

$$\Delta H^{\ddagger} = RT^2 \frac{d(\log k)}{dT} - RT \simeq E - RT, \tag{13}$$

$$\Delta S^{\ddagger} = -R\left(\log \frac{k'T}{hk} - T\frac{d(\log k)}{dT} + 1\right), \tag{14}$$

$$\Delta F^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \simeq RT \log \frac{kT}{h'k}, \tag{15}$$

where k is the velocity constant s^{-1},

k' the Boltzmann constant $(1{\cdot}38 \times 10^{-16}$ erg deg$^{-1})$,

h the Planck constant $(6{\cdot}62 \times 10^{-27}$ erg s).

The results were as follows:

	Q_{10}	E (kcal mol^{-1})	H (kcal mol^{-1})	S (kcal mol^{-1}) deg^{-1}	F (kcal mol^{-1})
Enzymic stage	2–4*	11·9	11·3	−0·0366	22·0
Flocculation	11·8	38·7	38·2	+0·0548	22·1
Gelling	1·6	8·1	7·4	−0·0479	21·4

* Estimated by Berridge (1942).

Discussion

The relation $\eta_{sp} = f(t_p^{\frac{1}{2}})$ found for the milk samples kept at 4 °C is rather difficult to interpret at this stage of the investigation. It seems right to assume only that the phenomenon found is due to the changes in the interaction of the milk components.

The phenomena occurring with casein after addition of rennet show a remarkable similarity to the conversion of fibrin to fibrinogen caused by thrombin, although the third part of this conversion (clotting) has not until now been explained in kinetic terms. Scheraga (1961) suggested that all 3 stages of fibrin conversion are reversible. As shown in our experiments, at the gelling stage of milk clotting there is an initial concentration of casein converted into the gel, as shown by the value $x_0 > 0$ in equation (10). This supposition is thermodynamically correct and leads to the assumption that the process of casein gelling is reversible. The assumption that the first 2 processes (the enzymic and the flocculation) are also reversible is even more probable.

The experiments performed enabled the activation parameters to be estimated; the value for the increase of free energy of activation $\Delta F^{\ddagger}$ was found to be almost identical for all 3 stages ($\Delta F^{\ddagger} \simeq 22$ kcal mol^{-1}). Joly (1965) reported that all the denaturation terms showed an almost constant value $\Delta F^{\ddagger} \simeq 22 \pm 5$ kcal mol^{-1}. This conformity of the values reported by Joly for other proteins with those found in our experiments could support our interpretation of casein clotting phenomena and on the other hand it leads to the interesting suggestion of an equilibrium between the active complexes in milk at body temperature (37 °C). At this temperature the velocity constants for all 3 stages are of the same magnitude, $k \sim 10^{-3}$ s^{-1}.

The equilibrium constants $K^{\ddagger}$ for the active complexes of all the stages must be of the same magnitude, as shown by the relations,

$$-\Delta F^{\ddagger} = RT \log K^{\ddagger}, \tag{16}$$

$$K^{\ddagger} = \frac{kh}{k'T}. \tag{17}$$

If the above assumption is correct the value of $K^{\ddagger}$ for all stages at 37 °C would be

$$K^{\ddagger} \simeq 1{\cdot}6 \times 10^{-16}.$$

When decreasing the temperature to 4 °C, these values would change to

$$K^{\ddagger}_{\text{enz}} \sim 10^{-17}, \quad K^{\ddagger}_{\text{floc}} \sim 5 \times 10^{-19}, \quad K^{\ddagger}_{\text{gel}} \sim 8 \times 10^{-17}.$$

It seems to be correct to assume that changes in the values of $K^{\ddagger}$ as the temperature diverges from 37 °C are due to the appearance of gradients of chemical potentials which cause the changes of viscosity during storing, and to other consequences of the process as described above.

The author is much indebted to Dr G. W. Scott Blair, who initiated him some years ago into the world of rheology, for very helpful discussions and suggestions and for his kind help in preparing this paper for publication. Some of the experiments described in the paper were performed under his direction.

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Growth of *Streptococcus lactis* in milk in a continuous fermenter

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Summary. Milk was added to a continuous fermenter to maintain a selected operating pH in the range 5·4–6·0, a fermentation system controlled in this way being called a pH-stat. Below pH 5·4 casein coagulated in the fermenter and on the pH electrodes. At pH 6·0 stirring inhibited the growth of the test culture, *Streptococcus lactis* C 10, but the inhibitory effect was reduced and the culture grew satisfactorily when the milk reservoir was sparged with 5 % CO_2 in N_2 or with N_2 alone. The dilution rate was highest near pH 6·0, which was close to the optimum pH for the growth of the organism. The rate of production of lactic acid and of bacterial cells in the fermenter was highest at pH 5·4.

The lactic acid fermentation of milk is traditionally a batch process, but the development of continuous methods in other fermentation industries has drawn attention to the possibilities of using continuous fermentation to produce fermented dairy products. The advantages of continuous culture have been discussed by Herbert, Elsworth & Telling (1956), but it does not appear to have been applied commercially in the dairy industry. The present authors have developed an effective laboratory scale pH-stat continuous fermenter, details of which have been given elsewhere (Linklater & Griffin, 1971*a*). The present report is concerned with the application of continuous culture techniques to dairy fermentation processes.

MATERIALS AND METHODS

Organism. Streptococcus lactis C 10 was used in all the experiments.

Bacterial counts. These were made by the standard plate count method of the American Public Health Association (1960), using Oxoid Milk Agar, code number CM 21.

Pasteurized skim-milk. This was collected from a commercial plate-type pasteurizer into a sterile 20-l glass storage vessel for transport to the laboratory fermenter.

Reconstituted milk medium. This was prepared by dissolving skim-milk powder in water at the rate of 10 g/100 ml. The reconstituted milk, in 3-l quantities, was heated in an autoclave in an atmosphere of saturated steam for 20 min at 121 °C. Measurement of the temperature of the milk with a thermocouple showed that this combination of time and temperature gave an effective heat treatment of about 101 °C for 12 min.

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Table 1. *Fermentation of reconstituted skim-milk* (10 % *solids*) *at various pH values*

Parameter	Units	Non-sparged, control				Sparged, N_2				Sparged, CO_2 in N_2			
		pH 5·4	pH 5·6	pH 5·8	pH 6·0	pH 5·4	pH 5·6	pH 5·8	pH 6·0	pH 5·4	pH 5·6	pH 5·8	pH 6·0
Average flow rate	ml/h	200	217	218	—	200	214	232	230	221	238	268	266
Dilution rate and specific growth rate	h^{-1}	0·66	0·72	0·73	—	0·66	0·71	0·77	0·76	0·74	0·80	0·90	0·89
Plate count	10^6 organisms/ml	1200	730	480	412	850	675	570	330	950	710	470	223
Bacteria production rate	10^9 organisms per h per l	800	525	350	—	560	480	440	250	665	578	420	200
Acid concentration as lactic acid	m-equiv/l	22·3	17·2	12·7	8·6	22·4	17·0	12·7	9·0	22·3	16·9	12·2	8·2
Acid production rate	m-equiv per h per l	14·7	12·4	9·25	—	14·8	12·1	9·8	6·8	16·5	13·5	11·0	7·3
Specific acid production rate	m-equiv per h per l, 10^6 organisms/ml	0·0122	0·017	0·0192	—	0·0174	0·0179	0·0172	0·0206	0·0174	0·019	0·0234	0·033

Flow rate measurement. The milk pumped out of the fermenter was directed into 500-ml measuring cylinders through a glass distributor spout fitted onto the rotator of an LKB fraction collector, which changed hourly.

Table 2. *Relationship between the pH of pasteurized skim-milk and the concentration of HCl added*

pH	Acid concentration m-equiv/l
6·0	7·2
5·8	10·1
5·6	13·2
5·4	16·3
5·2	19·4

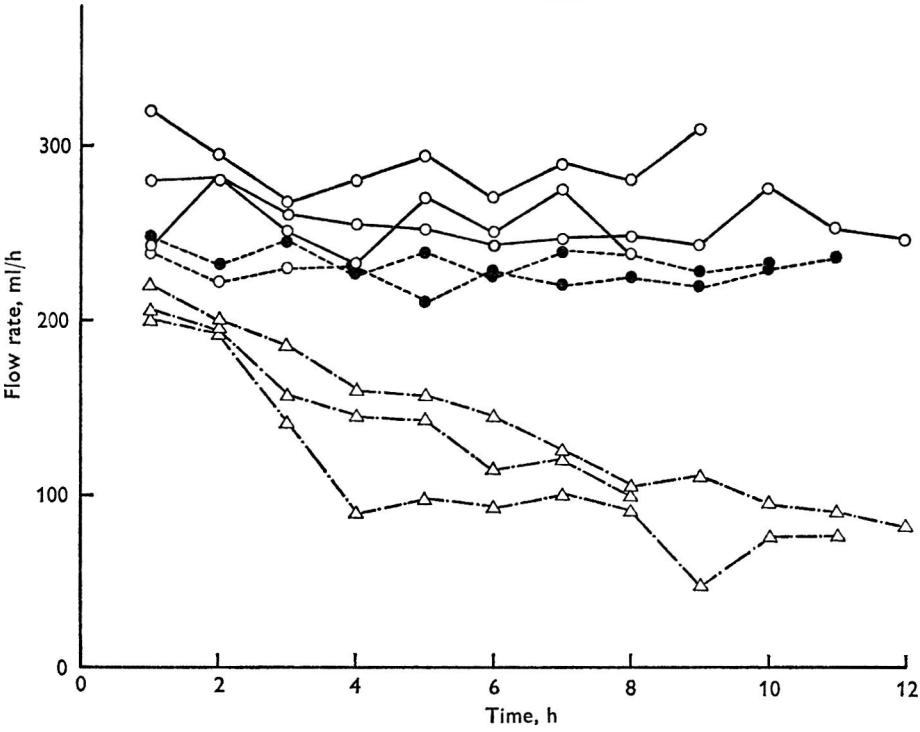

Fig. 1. Growth of *Streptococcus lactis* in reconstituted skim-milk (10% solids) in a continuous fermenter at pH 6·0. ○, Milk reservoir sparged with 5% CO_2 in N_2; ●, milk reservoir sparged with N_2; △, control (not sparged).

Fermenter operation. The fermenter used in these experiments has been described by Linklater & Griffin (1971*a*). It was not possible to measure the amount of acid produced in the fermentation by titration of the outflowing milk from the fermenter because of the continuing rapid production of acid by the large numbers of bacteria still present in the milk. Standard titration curves both for the reconstituted and for the pasteurized skim-milk were obtained by adding 0·1 N-HCl to samples of milk and allowing 30 min at 30 °C for the pH to equilibrate before making the measurement. This procedure was repeated over a range of acid additions to provide a titration

curve, and the results in Tables 1 and 2 show the corresponding lactic acid concentrations at the various operating pH values used in these experiments. The rate of acid production was computed by multiplying the acid concentration of the milk medium by the dilution rate of the fermentation. In Table 1 these rates have been expressed as m-equiv acid per litre of culture. The rate of production of bacteria was also calculated per litre of culture. Where sparging of the fermentation with N_2 or with CO_2 in N_2 was required, the gas was bubbled through the cold milk in the main reservoir (Linklater & Griffin, 1971*a*).

The fermenter usually reached steady state, as indicated by a steady flow rate, approximately 5 h after inoculation. The measurement of the hourly output was continued for not less than 8 h and samples were then taken to determine the bacterial count. After measurements had been completed at pH 5·4, the operating pH was changed to 5·6 and subsequently to 5·0 and 6·0; in each case at least 5 h was allowed for the fermenter to reach steady state, after which the flow rate was measured and samples were taken for estimation of the bacterial count.

The experiments were arranged so that they could be completed in the shortest possible time to minimize the build-up of coagulated casein in the fermenter. In all cases the experiment was completed within 60 h of inoculation. The reconstituted skim-milk experiments were replicated twice for the N_2-sparged treatment and 3 times for the other treatments. The results for N_2 sparging in Fig. 1 show hourly flow rates typical of steady state conditions.

RESULTS

Flow rate, plate count, and acid concentration were measured. All the remaining data in Table 1 were derived from these observations. The dilution rate was obtained by dividing the average hourly flow rate by the working volume of the fermenter, which was 300 ml. Specific growth rate is the ratio of the increase in bacterial numbers per unit time to the number initially present. In a continuous fermenter at steady state, the increase in numbers equals the number removed in the flow of culture from the fermenter, so that specific growth rate is numerically equal to the dilution rate.

In the first series of experiments the reconstituted milk medium was not sparged. The results are given in Table 1. The dilution rate tended to increase as the pH was increased. The lactic acid concentration in the fermenter was reduced as the pH was increased because of the decrease in the buffering capacity of the milk medium, as is shown in Table 1. The bacterial count also decreased as the lactic acid concentration decreased. The implications of these relationships are explained in the Discussion. A steady-state equilibrium was not obtained at pH 6·0 and there was a gradual decline in the dilution rate in all replicates, as is shown in Fig. 1. No evidence of contamination of the culture was found and an inoculum from the fermenter produced normal growth when subcultured in sterile milk. Normal growth was observed again when the operating pH in the fermenter was lowered to 5·8. These results show that the upper limit for the operation of the pH-stat continuous fermentation with this medium, with no gas sparging or other treatment, was between pH 5·8 and 6·0.

In a second series of experiments, the milk reservoir was sparged with N_2 to reduce the concentration of dissolved O_2. The results are shown in Table 1. The same trends

were observed as in the previous series of experiments, but steady-state conditions were observed at pH 6·0, as is shown in Fig. 1.

In a third series of experiments, the milk in the reservoir was sparged with 5% CO_2 in N_2. The means of values obtained in 3 replicate experiments are given in Table 1. The steady-state dilution rates increased with increase in the operating pH. The results at pH 6·0 showed that the system approached a steady state; this finding contrasts with the decline in growth rate observed when the milk was not sparged (see Fig. 1). The increasing trend in dilution rate over the range pH 5·4–6·0 was the same as with the unsparged and N_2-sparged milks (Table 1) but the rates at all pH values were increased by sparging with CO_2 in N_2.

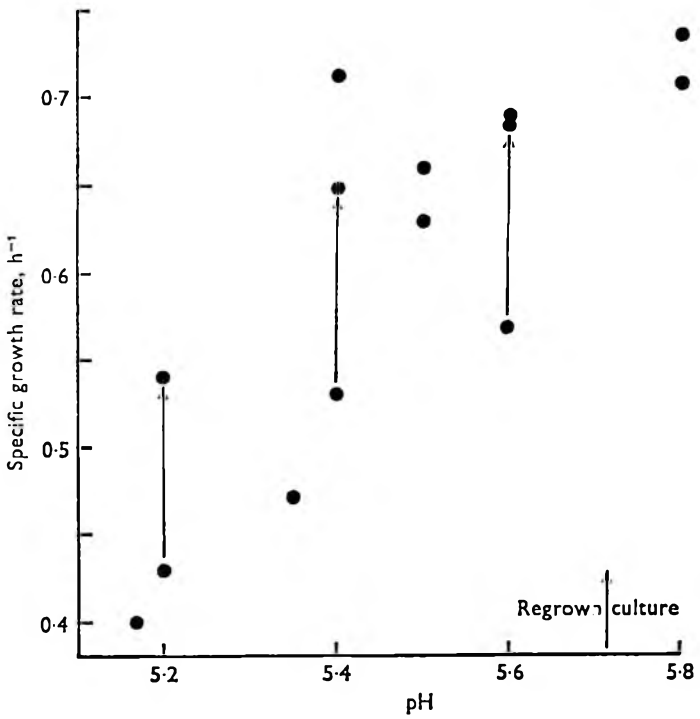

Fig. 2. Growth of *Streptococcus lactis* in pasteurized skim-milk at various operating pH values. Milk reservoir sparged with N_2. Arrows indicate increased growth rates obtained on re-growing the culture (see below).

The use of pasteurized skim-milk in a pH-stat continuous fermentation was examined in a fourth series of experiments. The milk reservoir was sparged with N_2 because the earlier experiments, discussed below, had shown the advantage of this treatment. The steady-state dilution rates showed wide variation and so the results of the 4 runs have been presented in Fig. 2, with the specific growth rate plotted against the operating pH. The bacterial numbers are shown in Fig. 3, plotted against the acid concentration. These results show that steady-state dilution rates were obtained, but were not always reproducible in successive runs, or even during the same run. If, after steady state had been obtained, the culture was substantially washed out and regrown, the new growth rate always exceeded the original. This is

shown in Fig. 2, by the arrows from one specific growth rate to another. In one experiment at pH 5·8 the culture eventually ceased to grow, behaving in the same way as in the control experiments at pH 6·0 in reconstituted milk.

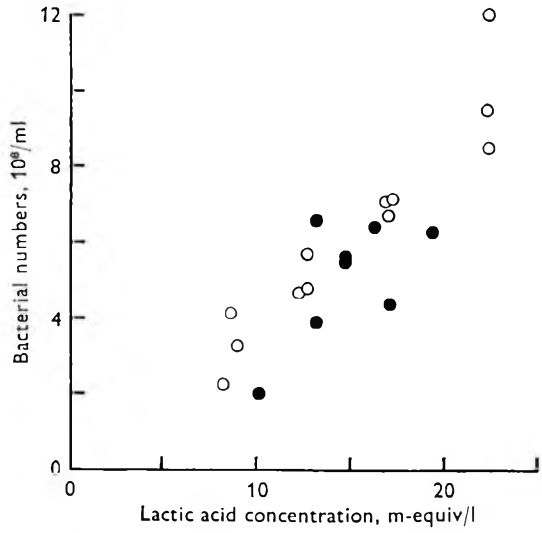

Fig. 3. The relationship between bacterial numbers and lactic acid concentration. ○, Heat-treated reconstituted skim-milk; ●, pasteurized skim-milk.

DISCUSSION

Autoclaved reconstituted skim-milk with 10% solids was used in the first 3 series of experiments because it was expected to give repeatable results and stable fermentation, and to allow the elucidation of the factors governing the operation of the pH-stat.

Stirring was an essential feature of the continuous fermenter used. In unheated milk this may lead to inhibition of the growth of lactic streptococci by lactoperoxidase, thiocyanate and hydrogen peroxide (Oram & Reiter, 1966). This growth inhibition was eliminated in the present experiments by heat treatment of the milk to inactivate lactoperoxidase.

Jago (1957) demonstrated that stirring of batch cultures of *Str. lactis* inhibited growth in heated milk and that the inhibition could be removed by bubbling 5% CO_2 in N_2 through the culture. This mixture, as well as N_2 alone, was therefore used in these experiments.

Fig. 4 shows a comparison of the specific growth rates given in Table 1. Growth was inhibited and the cultures almost died out in the presence of air at pH 6·0, but sparging the milk reservoir with N_2 and excluding O_2 from the fermenter allowed the culture to grow at pH 6·0. G. R. Jago (personal communication) pointed out that stirring in the presence of O_2 leads to the formation of H_2O_2, which inhibits the growth of *Str. lactis* C10. The N_2 treatment was designed to prevent any such formation of H_2O_2.

Stable growth in the control was obtained at pH 5·4, 5·6 and 5·8 but not at pH 6·0. Treatment with N_2 increased the specific growth rate at pH 5·8 in comparison with

the control, but it had no effect at pH 5·4 or 5·6. However, this N_2 treatment did not entirely remove the inhibition observed in the control at pH 6·0, as is shown by the failure of the specific growth rate for the N_2 treatment to continue to rise from pH 5·4 and 5·6 towards an optimum (Harvey, 1965) at pH 6·3.

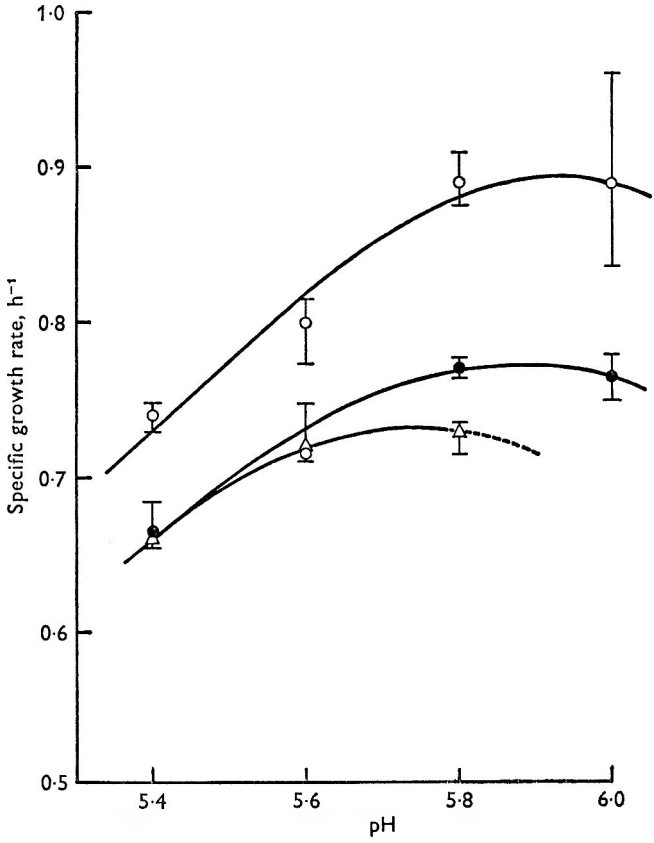

Fig. 4. Growth of *Streptococcus lactis* in reconstituted skim-milk (10 % solids) in a continuous fermenter at various operating pH values. ○, Milk reservoir sparged with 5 % CO_2 in N_2; ●, milk reservoir sparged with N_2; △, control (not sparged). Range of values is also shown at each plotting point.

Fig. 4 also allows comparison of the 5 % CO_2 in N_2 treatment with the control. Stable growth was obtained at pH 6·0 with the 5 % CO_2 in N_2 treatment used by Jago (1957). Fig. 4 also shows that 5 % CO_2 in N_2 increased the specific growth rates above those of the N_2 and control treatments at all pH values. It is clear that CO_2 stimulated the growth of this organism in continuous fermentation. The 5 % CO_2 in N_2 treatment also failed to remove completely the partial inhibition of growth at pH 5·8 and 6·0, since the specific growth rate, as for the N_2 treatment, also failed to rise towards a maximum at pH 6·3.

It is inherent in a pH-stat fermenter that operation at a lower pH increases the bacterial count, and this is illustrated in Table 1. The sparging with 5 % CO_2 in N_2 and with N_2 alone probably did not remove all O_2 and it seems likely that the reduction in specific growth rates at pH 5·8 and 6·0, for both 5 % CO_2 in N_2 and N_2 treat-

ments, was caused by H_2O_2, since Griffin (1970) was able to remove this inhibition of specific growth rate by adding catalase to the culture. Table 1 shows that at pH 5·4 and 5·6, where no inhibition was observed, bacterial numbers exceeded about 7×10^8/ml. It is postulated that the inhibitory effect of H_2O_2 was removed when a sufficient concentration of organisms was present. The existence of this cell concentration threshold effect would explain the problems that were encountered in stirring a freshly inoculated culture in the fermenter (Linklater & Griffin, 1971*a*), and would also explain why stirring in the presence of air had to be delayed until the pH reached 5·6.

This discussion of Fig. 4 involves a number of comparisons of the specific growth rates for the different sparging treatments and operating pH values. These comparisons show that the specific growth rate was influenced by pH, CO_2, and the postulated interaction between H_2O_2 and cell concentration. For convenience, this last effect has been termed a cell concentration threshold effect.

The pH range over which a pH-stat continuous fermenter can be operated is limited at the lower end by coagulation of casein (Linklater & Griffin, 1971*a*), and at the upper end by the inhibitory effect of stirring. The 10% solids reconstituted skim-milk used in these experiments as a control confine operation of the pH-stat to the pH range 5·4–5·8. This is narrow, but sufficient for the development of a continuous milk fermentation process. Sparging of the milk reservoir with N_2 increased the operating range to pH 6·0, and it might be possible to extend it to pH 6·3, which is the optimum for the growth of *Str. lactis* C 10 (Pont & Holloway, 1968).

In all these results, the specific growth rate increased as the operating pH was raised from 5·4 to 5·8. This increase parallels the increase in growth rate observed by Harvey (1965) in batch experiments using *Str. lactis* grown in a synthetic medium. The increase in the growth rate from pH 5·4 to 6·0 (Fig. 4) includes the effect of pH and all other factors influencing growth, and demonstrates the effectiveness of pH-stat control in maintaining the specific growth rate at the maximum for the conditions of growth. It follows that variations in successive batches of milk will produce variations in specific growth rate, but the rate will be maintained at the maximum for any batch of milk.

The decreased acid concentration of the substrate at higher pH (Table 1) reflects the buffering capacity of the milk. In the pH-stat, the numbers of bacteria in the milk reach an equilibrium with the acid concentration at any operating pH, and Table 1 shows how the acid concentration limited the bacterial count, and that both decreased from pH 5·4 to 6·0. This relationship between acid concentration and bacterial numbers is shown in Fig. 3. The relationships between pH, growth rate, acid concentration and cell numbers are shown in Fig. 5.

Control over the fermentation was exercised through the operating pH, which determined the acid concentration in the culture, and pH and acid concentration together controlled the specific growth rate. Fixing the acid concentration determined the bacterial count, even though the acid was a product of the bacteria; for in the pH-stat it is the acid concentration which is fixed and the count which has to equilibrate with it at steady state. A further effect of the lactic acid concentration was on growth rate. Rogers & Whittier (1928) showed that increasing lactate concentration reduced growth rate. The causal relationships between bacterial count, acid concentration

and pH would be reversed if control could be exercised by a turbidostat. In this case the bacterial count would determine the acid concentration and pH, which would then control the growth rate and hence the dilution rate.

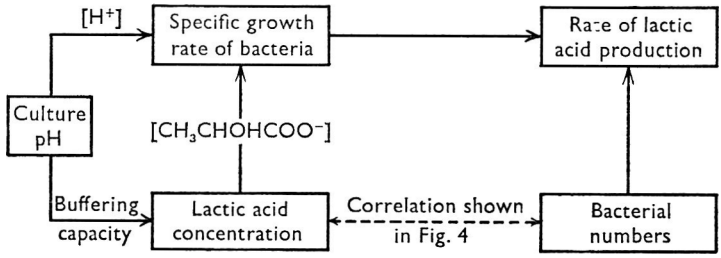

Fig. 5. pH-stat growth control relationships.

An important criterion in selecting an operating pH is the maximum productivity of the fermenter. The rates of production of bacteria and acid are both indices of productivity. The rate of acid production is the product of the dilution rate and the acid concentration in the culture. In the same way the rate of production of bacteria is the product of the dilution rate and the bacterial count in the culture. Table 1 shows that the dilution rates at pH 5·4 are somewhat lower than at pH 5·8, but the bacterial count and acid concentrations at pH 5·4 are approximately double those at pH 5·8; hence the rates of production of acid and bacteria are greatest at pH 5·4. It is concluded that the productivity of a pH-stat fermenter run on a milk medium, as measured by these criteria, is highest when operating just above the coagulation point of casein. The rates of production of bacteria and acid could be further increased by altering the medium to allow a higher lactic acid concentration at pH 5·4, for example, by the addition of alkali to the medium when it is reconstituted. Productivity, as defined here, is the rate of production of acid in milk and at any pH is increased more easily by raising the acid concentration in the culture than by altering the medium to give a higher growth rate. Fortunately, at the low pH required for maximum productivity it is unlikely that stirring will inhibit growth because bacterial numbers will exceed the threshold value mentioned above.

The final experiments were designed to investigate the application of these conclusions to the continuous fermentation of pasteurized skim-milk. The outstanding feature of the results was the variations in steady-state dilution rates as is shown in Fig. 2. Different batches of milk would have produced some of the variation in dilution rate. It is also possible that the lower buffering capacity and lower acid concentration in the milk at any pH (compare Table 2 with Table 1), with the resultant lower bacterial numbers (cell number threshold hypothesis), allowed stirring to influence growth. Growth in the reconstituted milk was unaffected by stirring at pH 5·6 when the titratable acidity was 17·0 m-equiv acid/l and the count was about 7×10^8 organisms/ml. A comparable count at the same titratable acidity in the pasteurized skim-milk would be obtained at pH 5·35. Below this pH the variation in the specific growth rate may have been caused by variations in the milk substrate, and above this pH by variation in the substrate and by the cell number threshold effect. A number of contaminants were present in the culture grown in the commerci-

ally pasteurized milk, reaching 10% in most cases. It seems likely that these would have contributed to variation in the growth rate because cell numbers in the pasteurized skim-milk tended to fall below the numbers in the reconstituted milk (Fig. 3). The combined effect of inhibition of growth by stirring different batches of milk, and a varying level of contaminants in the culture, probably produced the wide variation in growth rate shown in Fig. 2. Elimination of all these factors would perhaps give the same relationship between specific growth rate and operating pH as is shown in Fig. 4. The growth rates in Fig. 2 can be compared with those reported by Shmeleva & Jakovlev (1966) in which a constant flow fermenter was operated at a dilution rate of 0·125 and the pH equilibrated to 5·2. This dilution rate was less than one-third of that recorded in the experiments reported here.

The variation in the growth rate of the streptococci in pasteurized milk was automatically compensated for, in the pH-stat, by variation in the dilution rate. This is the important advantage of the pH-stat control. In a constant-flow continuous fermenter lacking feedback control this would not occur, and therefore it would be less suitable because of the likelihood of coagulation if the growth rate increased, and wash-out if it decreased. The stable growth obtained with reconstituted milk suggests that the sophisticated equipment and techniques for pH-stat control might not always be necessary because this substrate is a less variable growth medium. Also an increased milk solids concentration can be used to increase the concentration of bacteria in the fermenter, and make the culture less susceptible to the stirring effect. This conclusion has been utilized in later experiments (Linklater & Griffin, 1971*b*).

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A laboratory study of continuous fermentation of skim-milk for the production of sour curd cheese

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Summary. The feasibility of 2-stage continuous fermentation of skim-milk to produce sour curd cheese (quarg) has been established. The first stage was a stirred pH-stat fermenter, operated at pH 5·4, 5·6, 5·8 and 6·0, in which the operating pH was maintained by inflowing fresh milk. The remaining fermentation down to pH 4·7 was carried out by a procedure which simulated a second-stage plug flow fermenter (i.e. one in which forward velocity was as constant as possible at all points of the cross-section).

Skim-milk powder was dissolved to give 8, 10, 14 and 20% solids in the reconstituted milk. Maximum productivity of fermented milk solids was obtained with reconstituted milk containing 20% solids and operation of the first-stage fermenter at pH 5·4. This 2-stage continuous fermentation gave a total residence time of about 5 h, which represents a rate of production 4·8 times that obtainable in 24 h in a batch fermenter of the same fluid capacity filled once daily with the same reconstituted milk.

The major advantages of continuous fermentation are the introduction of automatic process control and a reduction in the size of the fermenter relative to a batch process with the same output. These advantages would be economically significant in plants processing large volumes of milk into sour curd cheese (quarg). Linklater & Griffin (1971*a*, *b*) have reported the design of a laboratory scale continuous milk fermenter and its use in a study of the growth and acid production of *Streptococcus lactis*. It was found that reconstituted skim-milk was more suitable than pasteurized skim-milk for continuous fermentation; in addition, a high level of solids in the reconstituted milk offered a method of achieving a high bacterial density in the fermenter and overcoming the inhibition caused by stirring. The findings from these experiments were applied in a study of the feasibility of continuous fermentation in some dairy manufacturing processes.

Quarg was selected in preference to other fermented dairy products because it is manufactured in plants processing up to 250000 gal/day and because it requires only centrifugation after fermentation is completed. The stirred fermenter (Linklater & Griffin, 1971*a*) cannot operate below pH 5·4, and so completion of the fermentation to pH 4·7 must be carried out in an unstirred plug-flow fermenter to give a smooth coagulum suitable for centrifugation to produce quarg. Moreover, Bischoff (1966) has shown that this combination of stirred and plug-flow fermenters would give optimum

lactic acid production and allow very considerable reduction of the size of the fermenters. An outline of a continuous fermentation process for quarg production is shown in Fig. 1. Milk is pumped into a stirred fermenter and then flows into the plug-flow fermenter where the pH is lowered to 4·7. The coagulum extruded from this fermenter is pumped to a quarg centrifuge for separation of the curd from whey.

The object of this work was to find the optimum operating pH of the first-stage fermenter, and the optimum solids level of the reconstituted milk, for maximum output of fermented milk solids.

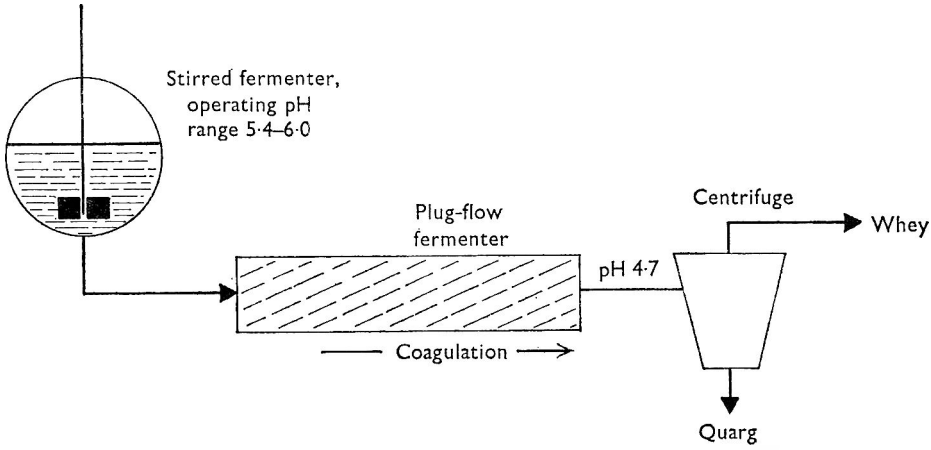

Fig. 1. Schematic outline of a process to produce sour curd cheese by continuous fermentation.

MATERIALS AND METHODS

Skim-milk substrate. Medium heat spray-dried milk powder was reconstituted to give 8, 10, 14 and 25 % total solids. The 20 % solids milk showed excessive browning when autoclaved at 121 °C for 20 min and this heat treatment caused coagulation of 25 %-solids milk. Therefore, 2 % was selected as the maximum solids content and the reconstituted milk in 3-l portions was heated for 15 min in saturated steam at 121 °C, in an autoclave. The milk temperature rose to 101 °C and was maintained at this temperature for about 7 min.

Organism. Str. lactis C10 was used in all experiments.

Fermentation apparatus and operation. Linklater & Griffin (1971*a*) have described the stirred fermenter used in this work and explained how it was operated. The bulk milk reservoir was sparged with N_2. The residence time in a second-stage fermenter was the time required for the milk flowing from the first-stage fermenter to reach pH 4·7. It was not feasible to build a plug-flow fermenter to couple with the first-stage fermenter because continuous sampling from such a small plug-flow fermenter disrupted laminar flow and caused whey separation. A plug-flow fermenter can be considered to be made up of an infinite number of batch fermentations, one of which can be selected for measurement of pH change and it was decided to use batch fermentation of samples of milk from the first-stage fermenter to simulate a second-stage plug-flow fermenter. Residence times were obtained by measuring the pH of the sample at intervals and recording the time required for the milk to reach pH 4·7.

Table 1. *Summary of results obtained for reconstituted skim-milk at different milk solids concentrations, and at different operating pH values*

S: milk solids concentration in medium, g/l	80				100				140				200			
Operating pH of first-stage fermenter	5·4	5·6	5·8	6·0	5·4	5·6	5·8	6·0	5·4	5·6	5·8	6·0	5·4	5·6	5·8	6·0
Dilution rate of first-stage fermenter, h^{-1}	0·56	0·66	0·73	0·76	0·67	0·72	0·77	0·78	0·67	0·73	0·78	0·82	0·63	0·71	0·77	0·81
$\bar{t}_1$: Mean residence time in first-stage fermenter, h	1·79	1·52	1·37	1·32	1·49	1·39	1·30	1·28	1·49	1·37	1·28	1·22	1·59	1·41	1·30	1·23
$\bar{t}_2$: Mean residence time in second-stage fermenter, h	1·55	2·10	2·60	3·20	1·62	2·20	2·66	3·50	2·30	2·60	3·00	3·60	3·40	3·95	4·70	5·80
$\bar{t}_1+\bar{t}_2$, h	3·34	3·62	3·97	4·52	3·11	3·59	3·96	4·78	3·79	3·97	4·28	4·82	4·99	5·36	6·00	7·03
Output of fermented milk solids, g per l per h	24·0	22·2	20·2	17·7	32·2	27·8	25·2	20·9	36·9	35·2	32·7	29·1	40·1	37·4	33·4	28·5

RESULTS

The first-stage stirred fermenter was operated at pH 5·4, 5·6, 5·8 and 6·0 using reconstituted skim-milk containing 8 % solids. The steady-state hourly flow rates at each pH were averaged and divided by the volume of culture in the fermenter to give the dilution rate. The experiment was duplicated and the results were then plotted against the operating pH. A curve was hand-fitted to the data and from this were obtained the average dilution rates given in Table 1. The mean residence times for the first-stage fermenter ($\bar{t}_1$), given in Table 1, are the reciprocals of the dilution rates. This experimental plan was repeated with reconstituted milk containing 10, 14 and 20 % solids.

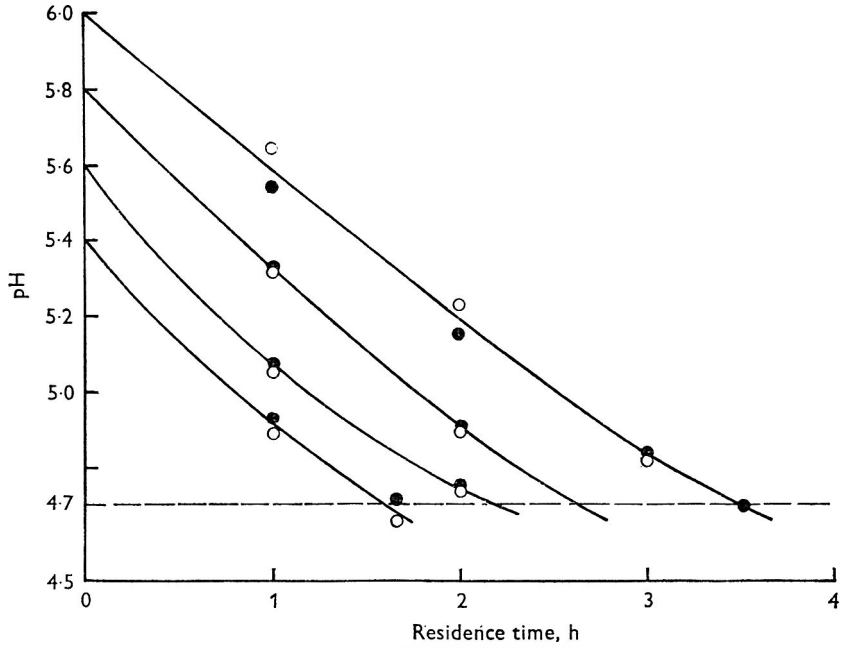

Fig. 2. Residence times in a simulated plug-flow fermenter. Reconstituted skim-milk (10 % solids). ○, Expt 1; ●, expt 2.

The method for the estimation of residence times for the second-stage fermentation has been described above. Fig. 2 shows the results obtained with reconstituting milk containing 10 % solids; these are typical of the results for other levels of milk solids. The estimates of residence times for the second-stage fermentation ($\bar{t}_2$) are given in Table 1. The total time in both fermenters ($\bar{t}_1+\bar{t}_2$) is also given in Table 1. The productivity (P) of the fermentation system has been defined as the rate of output of milk solids per unit volume and expressed in Table 1 as g milk solids per litre per hour.

$$P = \frac{S}{\bar{t}_1+\bar{t}_2},$$

where S = solids concentration in the reconstituted milk (l),
$\bar{t}_1$ = mean residence time in first-stage fermenter (h),
$\bar{t}_2$ = mean residence time in simulated second-stage fermenter (h).

The relationship between productivity and percentage solids in the reconstituted milk is shown in Fig. 3. The curves in Fig. 3 were hand fitted. At every level of milk solids the highest productivity was obtained when the first-stage fermenter was operated at pH 5·4. Table 1 shows that maximum productivity was obtained with reconstituted milk containing 20 % solids and operation of the first-stage fermenter at pH 5·4.

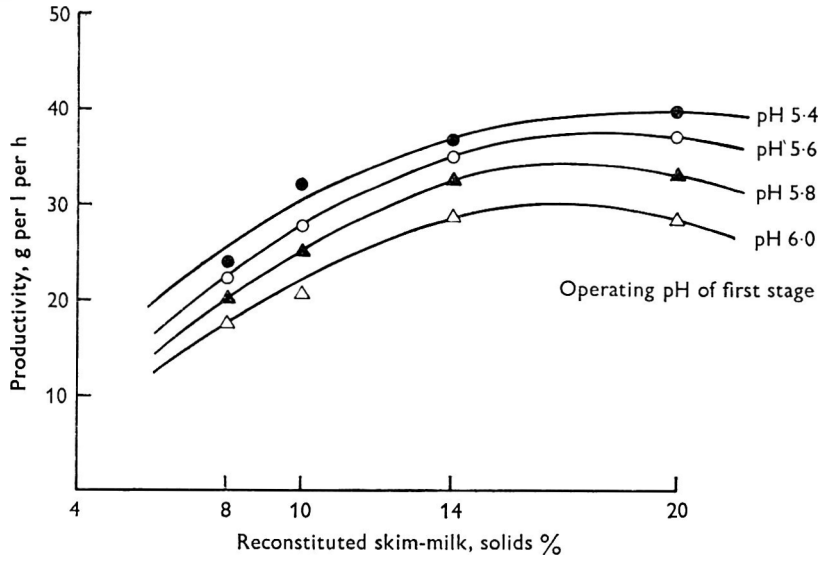

Fig. 3. Optimization for a sour curd cheese production unit, based on a two-stage continuous fermenter with a total volume of 1 l. First-stage operating pH: ●, 5·4; ○, 5·6; ▲, 5·8; △, 6·0.

DISCUSSION

The simple conclusion given above conceals a complex interaction of factors which require further elucidation for any realistic appraisal of these results in terms of dairy technology. Fermentation of milk depends on the growth of the lactic streptococci and the production of lactic acid, with a consequent drop in pH. In any milk fermentation, as the cell numbers increase, the specific growth rate is decreased due to an accumulation of hydrogen and lactate ions. Thus, growth of the bacteria gives an increased number of acid-producing units, but the resulting lactic acid and concomitant drop in pH both tend to suppress the rate of acid production by the individual bacteria. The growth and acid production of the lactic streptococci in the first- and second-stage fermenters can now be discussed in turn.

The dilution rates for the first-stage fermenter given in Table 1 are numerically equal to the specific growth rates at steady state in a continuous fermenter. Fig. 4 shows the relationship between the specific growth rate and the fermenter operating pH, for all concentrations of milk solids. A general trend of decreasing specific growth rate at the lower pHs is seen. Harvey (1965) reported that the optimum pH for growth of *Str. lactis* ML3 was 6·3. The results for the 14- and 20 %-solids conform to the pattern obtained by Harvey (1965). They also show a reduction in the growth rate in the 20 %-solids milk below that in the 14 %-solids milk, caused by the

inhibitory effect on growth of increasing lactate concentration (Bergère & Hermier, 1968). Table 2 shows the increased lactic acid concentration as the milk solids concentration is increased. The results from the 8%- and 10%-solids milks do not conform to the pattern obtained by Harvey (1965), and it appears unlikely that extrapolation of the specific growth rate versus pH curves, shown in Fig. 4, would show an optimum at pH 6·3. Linklater & Griffin (1971*b*) postulated an inhibition of growth

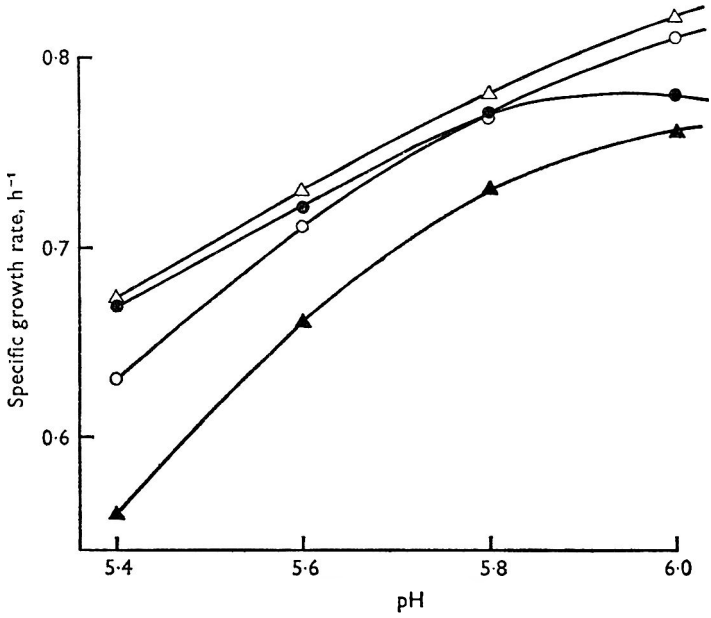

Fig. 4. The growth rate in the first-stage continuous fermenter. Skim-milk solids %: ▲, 8; ●, 10; △, 14; ○, 20.

Table 2. *Inter-relationships between pH and lactic acid concentration, at different milk solids concentrations*

Milk solids, %	8	10	14	20
	Lactic acid concentration, mM			
pH: 6·0	10	12	15	19
5·8	16	18	24	29
5·6	22	27	35	45
5·4	30	37	48	62
4·7	57	70	96	140

in their fermenter, caused by stirring the culture when bacterial numbers were below 7×10^8/ml. This would explain the low growth rates in the 8- and 10%-solids milks at pH 5·8 and 6·0, but not at pH 5·6 and 5·4 where bacterial numbers would exceed the threshold value mentioned above. G. R. Jago (personal communication) suggested that these anomalies can be explained by variation in the milk solids concentration. In the milk containing the highest level of solids the inhibitory compound, H_2O_2, is reduced by the higher milk protein concentrations, and there is correspondingly less

inhibition of bacterial growth. In the milk containing only 8% solids medium there is less protein and therefore growth inhibition is accentuated.

The mean residence times for the second stage of the fermentation were reduced as the operating pH of the first stage was lowered. This trend occurred with all concentrations of milk solids and is shown for the 10%-solids milk in Fig. 2. This reflects the lower proportion of the total lactic acid required to be produced in the second stage and the effect of increasing bacterial numbers entering the second stage. Fig. 2 shows how different combinations of the first- and second-stage fermentations reach the same final pH. Fig. 5 shows that with different concentrations of milk solids, and

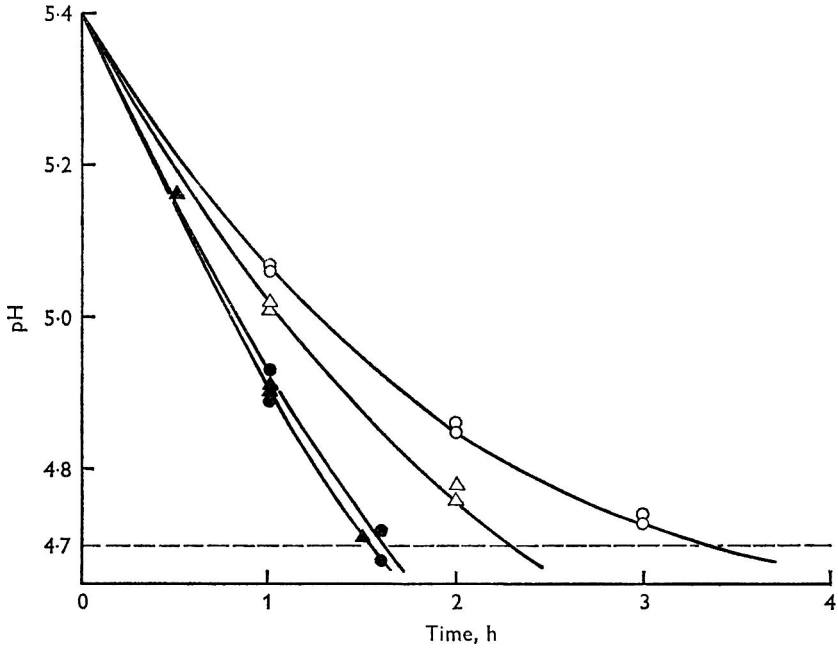

Fig. 5. Decrease of pH in a simulated second-stage fermenter, with the first-stage fermenter operating at pH 5·4. Skim-milk solids %: ▲, 8; ●, 10; △, 14; ○, 20.

at the same operating pH of the first-stage fermenter, there were substantial differences in the time required to reach pH 4·7. The rate of lactic acid production depends on the bacterial numbers and their individual growth rates, as has been shown by Luedeking & Piret (1959). Bacterial numbers increase in proportion to the increase in lactic acid concentration, Linklater & Griffin (1971*b*). However, the increased time required for the culture to reach pH 4·7 in the milk containing more milk solids can be attributed to the higher levels of lactate increasingly inhibiting the rate of bacterial acid production. This effect was most marked with the reconstituted milk containing 20% solids in which the bacterial growth was so slowed down, to the extent that it barely exceeded that for the 14%-solids milk (Table 1). At still higher levels of milk solids, growth would be inhibited even more, so that the rate of quarg production would decline. This has been taken into consideration in drawing Fig. 3, where the rate of production of fermented milk solids, and thus of quarg, reached a broad maximum at about 20% milk solids. Further work on the production of

quarg from reconstituted milk by continuous fermentation should therefore be based on approximately 20% -solids milk and operation of the first-stage fermenter at pH 5·4.

One reason for considering the use of continuous fermentation for the production of fermented dairy products is the increase in productivity as compared with batch fermentation. Table 1 shows that in a 2-stage continuous fermenter, reconstituted milk, containing 20% solids, had a total residence time of 4·99 h. This represents a rate of production 4·8 times that obtainable in 24 h in a batch fermenter filled once daily with the same reconstituted milk. This would appear to be commercially significant for large-scale quarg production where the introduction of continuous fermentation should bring the advantages of automatic process control.

We are grateful to the School of Biological Technology for providing laboratory facilities and for many stimulating discussions with the staff. It is a pleasure to acknowledge the constructive criticism of Mr J. Czulak and Mr E. G. Pont, Division of Dairy Research, C.S.I.R.O., in the preparation of this paper.

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The effect of ageing on heat-sterilized milk

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Summary. During 7 months' storage of heat-sterilized milk there were changes in the glycopeptides released by rennin (E.C. 3.4.4.3) action and soluble in 12% (w/v) trichloroacetic acid (TCA). The results showed a progressive decrease in the amount of carbohydrates attached to κ-casein. As these carbohydrates may contribute to the micelle-stabilizing power of κ-casein it was concluded that the loss of carbohydrates could be one factor in the development of gelation in heat-sterilized milks during storage.

Milks that have been subjected to heat-processing sometimes develop gelation or thickening during storage, but as yet there is little information on how or why these problems arise. Evidence of gelation and sedimentation of milk that had been subjected to an ultra-high-temperature (UHT) heat-sterilization treatment and then stored for up to 28 months was provided by Vojičić & Bačić (1970). Hostettler, Stein & Bruderer (1957) showed that in a thickened heat-sterilized milk there was a decrease in the relative concentration of the β-casein and a change in the electrophoretic pattern of κ-casein which was similar to that induced in milk by the action of rennet. An increase in the NPN soluble in the 5% TCA filtrate of milks heated for 30 min at temperatures between 55 and 95 °C and then allowed to gel has been observed by Fox, Holsinger, Posati & Pallansch (1967). These workers isolated 2 peptides from the TCA filtrate, which had similar properties to those of peptides released from milk by rennin. When isolated κ-casein was heated (Alais, Kiger & Jollès, 1967) a peptide was also released which was similar to that released by the action of rennin on κ-casein. It is possible, therefore, that the development of gelation in heat-sterilized milks may be due to a gradual break-down of κ-casein so that its micelle-stabilizing powers are destroyed in a similar way to those in the rennin coagulation of milk.

The peptides and glycopeptides that are released from κ-casein during rennin coagulation are soluble in TCA and so the enzyme action can be conveniently followed by determining the increase in the peptides soluble in the TCA filtrate of the milk. Consequently, if there is any break-down of κ-casein due to heat-treatment of the milk, there would probably be an increase in the peptides soluble in the TCA filtrate of the milk. There would also be a corresponding decrease in the amount of

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peptides released by rennin action. In the present experiments we have used this approach in an attempt to determine whether or not there is any break-down of κ-casein during storage of heat-sterilized milk.

EXPERIMENTAL

Bottled sterilized milk samples were supplied by Associated Dairies Ltd, Accrington. The milk had been subjected to UHT heat treatment (137 °C for ~3 s, preceded by holding at 80 °C for 7 min). As the filling was not an aseptic procedure there was a further 'in bottle' sterilization (40 min at not less than 90 °C, including 20 min at 110 °C during this period). All samples were taken from the bottle-filler at the same time to ensure that the milk came from the same bulk sample.

One batch of samples was stored at 4 °C and the other at 25 °C for the duration of the experiments. Visible coagulation of any milk sample was not apparent throughout the experiment, although some very slight sediment was observed in the samples at both temperatures within 2 months of processing.

The peptides and glycopeptides soluble in the 12% TCA filtrate of the milk were estimated. The increase in peptides and glycopeptides soluble in this filtrate after rennin action (0·8 ml of a solution containing 51 mg rennin/100 ml to 80 ml milk at 37 °C) was determined. The times of rennin action were 10, 20 and 40 min.

N was determined by the micro-Kjeldahl method and the carbohydrates attached to the glycopeptides were estimated by gas liquid chromatography, using the method of Sinkinson & Wheelock (1970).

Rennin was supplied by Sigma Chemical Co. Ltd, London, and stored in the deep-freeze for the duration of the experiments.

RESULTS AND DISCUSSION

It has already been shown that there is a marked reduction in the release, by the action of rennin, of non-carbohydrate-containing peptides soluble in 2% (w/v) TCA, immediately after the UHT processing (Hindle & Wheelock, 1970*a*). The present experiments have, therefore, been confined to the carbohydrate-containing peptides that are soluble in 12% TCA.

The effect of storage on the release of peptides and glycopeptides from heat-sterilized milk is shown in Fig. 1. There was a very gradual increase in the amount of peptides and glycopeptides, as shown by N, soluble in the 12% TCA filtrate of the sterilized milk stored at 25 °C, but there was no detectable change in the milks stored at 4 °C. At both storage temperatures the concentrations of 2-acetamido-2-deoxy-D-galactose attached to the peptides decreased and those for D-mannose-containing glycopeptides increased. There was little change in the concentrations of the other carbohydrates except for an increase in the D-galactose-containing glycopeptides of the milk stored at 25 °C. This information suggests that there may have been some loss of carbohydrates from the glycopeptides soluble in the 12% TCA filtrate of milk during storage of heat-sterilized milk. In addition, there may also have been a release of D-galactose- and D-mannose-containing glycopeptides during storage at 25 °C, similar to that observed in previous investigations (Hindle & Wheelock, 1970*b*) when

milk was heated for varying periods at 80 and 100 °C. Nevertheless, the results as a whole suggest that during storage there was no significant decomposition of κ-casein, with the release of glycopeptides similar to those released by rennin action. This conclusion is supported by the results for the second series of experiments (Fig. 2) which showed that there was no decrease in the amount of peptides, as estimated by N, released by rennin action. There was, however, a general trend showing a decrease in all the carbohydrates attached to these glycopeptides.

The amount of α_s-casein stabilized by κ-casein is reduced by treatment with neuraminidase, which removes the N-acetyl neuraminic acid from the molecule (Thompson & Pepper, 1962). Mackinlay & Wake (1965) showed that there was

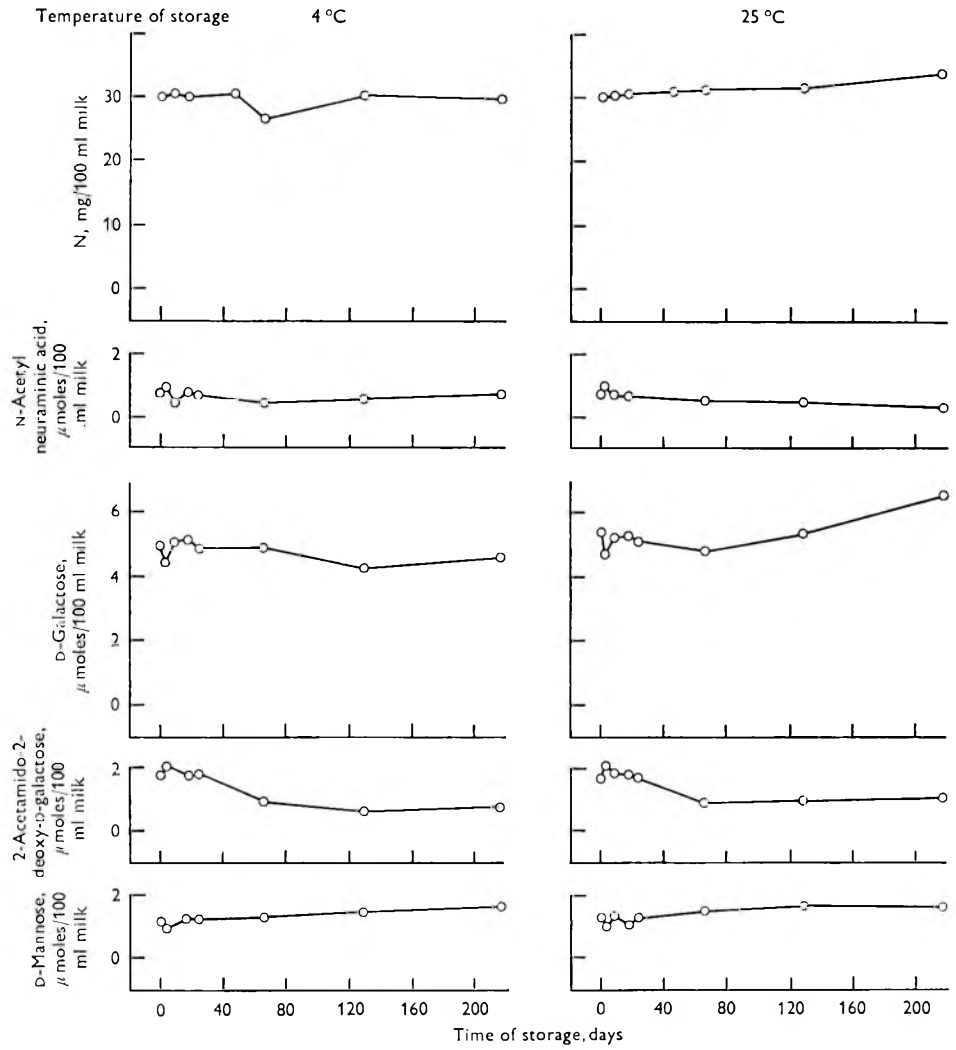

Fig. 1. The changes, on ageing, of N and of N-acetyl neuraminic acid, D-galactose, 2-acetamido-2-deoxy-D-galactose and D-mannose attached to glycopeptides, soluble in the 12% trichloroacetic acid filtrate of ultra-high-temperature sterilized milk. Values are the average of duplicate determinations.

slightly more α_s-casein stabilized by S-carboxymethyl-κ-casein which contained carbohydrate than by that which lacked carbohydrate.

From theoretical considerations of the amino acid composition of the caseins, Hill & Wake (1969) have concluded that the casein micelle may be regarded as a hydrophobic particle with a stabilizing hydrophilic coat. They suggest that this coat consists of the peptides and glycopeptides that are released from the κ-casein during the primary phase of rennin action. The inclusion of carbohydrates in this

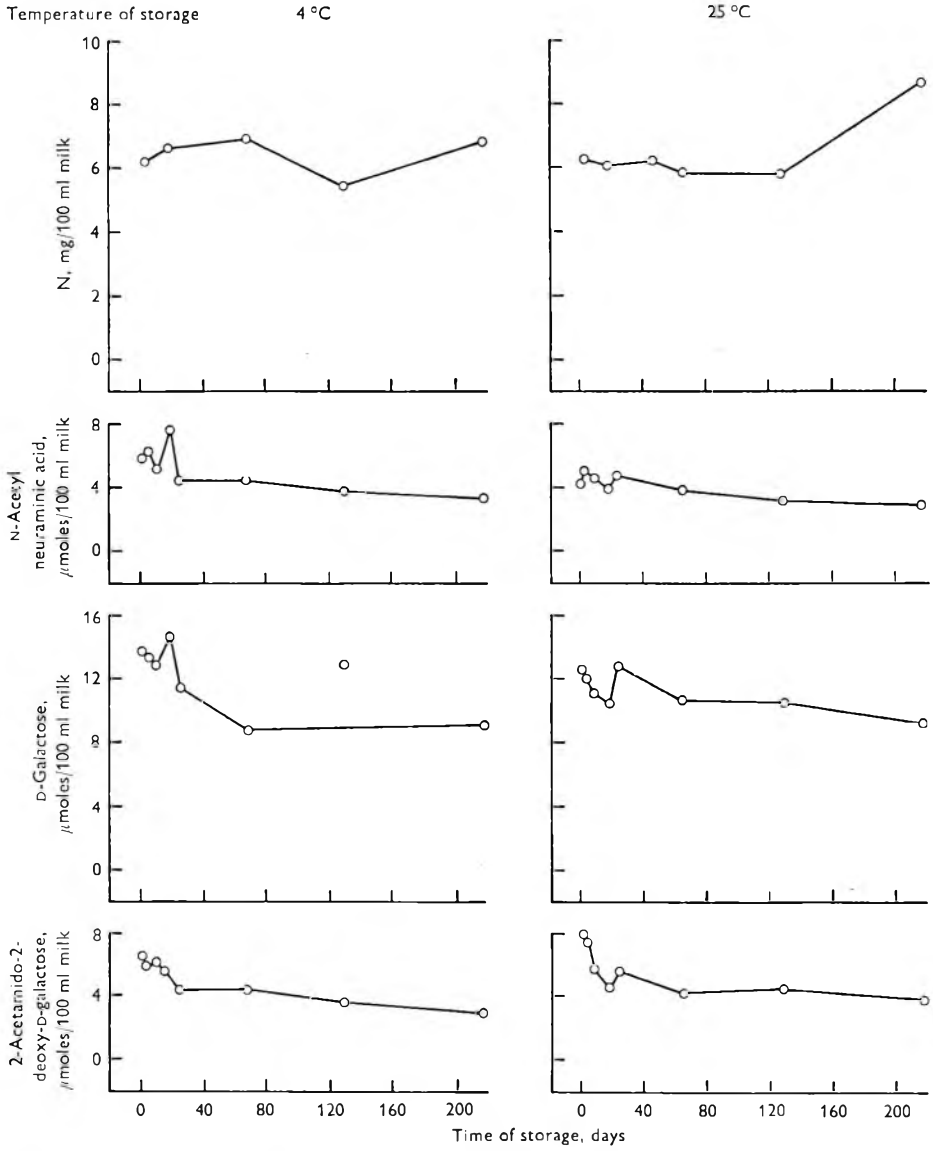

Fig. 2. The changes, on ageing, of N and of N-acetyl neuraminic acid, D-galactose and 2-acetamido-2-deoxy-D-galactose attached to the glycopeptides released by the action of rennin, and soluble in the 12 % trichloroacetic acid filtrate of ultra-high-temperature sterilized milk. Values are the average of 3 determinations at different times of rennin action after the maximum release of glycopeptides was observed.

hydrophilic part of the κ-casein amino acid skeleton would increase the frequency of hydroxyl groups and, therefore, the net negative charge. Consequently, the degree of stabilization (i.e. resistance to de-stabilization as opposed to the amount of α_s-casein stabilized) may increase with the carbohydrate content of κ-casein. If this is so, then the loss of carbohydrates from κ-casein during storage of heat-sterilized milk could contribute to the development of gelation. We consider that this development of gelation in heat-treated milk is probably due to a combination of factors which may include an alteration in the Ca^{2+} concentration and the release of peptides from the caseins in addition to any decrease in the micelle-stabilizing power of the κ-casein.

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With 2 plates

Density-gradient electrophoresis of native and of rennet-treated casein micelles

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(*Received* 26 *October* 1970)

Summary. Analytical electrophoresis of native and of rennet-treated casein micelles was carried out at 26 and at 5 °C in a sucrose density gradient in a medium of the same ionic composition as milk. Under normal circumstances, the micelles were negatively charged and showed little heterogeneity in the electric field, the fastest-moving ones having at 26 °C a mobility not more than 30 % greater than that of the slowest. Native micelles were more highly charged at 5 than at 26 °C; at each temperature, the mobility was approximately halved by rennet treatment. The results suggest that the effects of rennet treatment and of alterations in temperature on the tendency of the micelles to clot can be entirely explained as being caused by changes in charge.

The casein micelles of milk are spherical particles with diameters ranging between 40 and 280 nm (Nitschmann, 1949). They consist of the various caseins, probably arranged in an open network (Ribadeau Dumas & Garnier, 1970) held together by copolymers of basic calcium phosphate and citrate (McGann & Pyne, 1960). Although all of the components of the micelle are present in the milk serum in a soluble form, the 2 phases do not equilibrate significantly (Rose, 1968) and therefore, the large particles do not grow at the expense of the small ones. Thus, the whole system can be considered as a stable colloidal suspension in which the electrical repulsive forces due to the charge on the particles overcome the attractive forces between the particles, so forming a potential energy barrier which prevents sufficiently close approach for aggregation to occur.

Treatment of milk with rennin at room temperature leads to aggregation of the micelles to form a clot. The primary action of rennin is the cleavage of a highly acidic glycomacropeptide from κ-casein (Jollès, 1966) and clotting is not accompanied by an increase in bound calcium (Verma & Gehrke, 1957). Therefore, it would appear that treatment of a micelle with rennin causes it to become more basic. Since rennin-treated micelles aggregate at room temperature, this alteration of charge must be sufficient to allow the attractive forces between the micelles to overcome the repulsive ones. Hence, the native casein micelles must be negatively charged. This conclusion is in agreement with the results of electrophoresis of artificial micelles prepared by titrating aqueous casein suspensions to pH 6·6 with saturated $Ca(OH)_2$ (Hankinson & Briggs, 1941).

Although rennin-treated micelles aggregate at room temperature, they do not aggregate in the cold. Thus, at low temperatures, the attractive forces between rennin-treated micelles are insufficient to overcome the repulsive ones. The reason for this is still unclear. It is known (Payens, 1966) that most types of casein associations are endothermic, which suggests that the attractive forces between micelles may be reduced at low temperatures. On the other hand, the dissociation of caseins from micelles at low temperatures (Rose, 1968) might be expected to reduce the charge on the micelles and thus the repulsive forces. Therefore, these 2 opposite effects of reducing the temperature would be expected to partially offset one another.

In the present work, electrophoresis of casein micelle suspensions was carried out in a sucrose density gradient prepared in milk serum or milk dialysate at both 26 and 5 °C. The primary purpose was to confirm the sign of the charge on native and rennet-treated micelles and to obtain data on their homogeneity. Also, by making certain assumptions, the zeta-potentials of the micelles were calculated from their mobilities in the electric field. Thus, by use of the theory of colloid stability (Verwey & Overbeek, 1948), it was possible to estimate the repulsive forces between the micelles, which together with consideration of the conditions required for clotting, enabled the attractive forces to be estimated.

EXPERIMENTAL

Materials

Streptomycin sulphate and benzylpenicillin were products of Glaxo Laboratories Ltd, Greenford, Middlesex. Dextran 20 was obtained from Pharmacia, Uppsala, Sweden. The rennet used was a product for cheese-making from Chr. Hansen Ltd, London.

Density-gradient electrophoresis

Descending electrophoresis was carried out in modification of a Tiselius free boundary apparatus (Longsworth & MacInnes, 1939). The medium was milk serum or milk dialysate, the density gradient being formed in the cell by the addition of sucrose.

Preparation of milk dialysate. Water was dialysed at 4 °C for 24 h against 50 vol of fresh separated milk containing 0·1 % (v/v) chloroform, with gentle stirring (Davies & White, 1960). Benzylpenicillin and streptomycin sulphate (each at 50 μg/ml) were dissolved in the milk dialysate, which was then used immediately.

Preparation of milk serum. Benzylpenicillin and streptomycin sulphate (each at 50 μg/ml) were dissolved in fresh separated milk. This was then centrifuged at 75000g for 2 h at 0 °C unless otherwise indicated. The supernatant was poured off, filtered through Whatman No. 54 paper and used immediately.

Preparation of the density gradient. Since sucrose binds small amounts of Ca^{2+}, solution of sucrose in a Ca^{2+}-containing solution slightly reduces the Ca^{2+} concentration. It was necessary to correct for this, in the electrophoresis experiments, by adding a compensatory amount of $CaCl_2$ to the solutions used for forming the gradient. The amount of $CaCl_2$ required was determined for each experiment, the clotting time of milk with rennet at 30 °C being used as a measure of the Ca^{2+} concentration

(Cheeseman, 1962). The amount of $CaCl_2$ required to restore the clotting time of separated milk containing 20% (w/v) sucrose to that of the same milk without sucrose was determined. This was normally about 0·07 ml 1 M-$CaCl_2$/50 ml 20% sucrose in milk. The amount of $CaCl_2$ required for solutions containing 10, 15 and 30% (w/v) sucrose was calculated by simple proportion, since it was found that the amount of $CaCl_2$ required to restore the clotting time to the control level was proportional to sucrose concentration up to 30% sucrose. The amount of dextran used in preparation of the gradient had a negligible effect on the clotting time of milk.

The solutions used to prepare the density gradient consisted of milk serum or milk dialysate in which was dissolved sucrose at the desired concentration, sufficient $CaCl_2$ to compensate for Ca^{2+} binding by sucrose and 0·4% (w/v) Dextran 20. The last was added to reduce droplet sedimentation on insertion of the sample (Svensson, 1960).

Before the preparation of the gradient, the bottom section of the cell was filled with the solution containing 30% sucrose. This section was not connected with the vertical section until just before the start of the electrophoretic run. A density gradient was prepared in each limb of the vertical section; 0·35 ml portions of solutions containing 30, 20 and 10% sucrose were layered above one another and left to diffuse overnight in a room at the temperature required for the electrophoretic run. This method was reported by Brakke (1964) to give a gradient identical with that prepared by mixing chambers.

Preparation of casein micelle suspension and its insertion into the density gradient. Casein micelles were normally sedimented by centrifugation at 75000**g** for 2 h at 0 °C from the same milk sample as was used for preparation of the medium. They were resuspended in milk serum or milk dialysate at twice their original concentration by stirring overnight. Immediately before use this suspension was diluted 20-fold with medium containing 15% (w/v) sucrose and a compensatory amount of $CaCl_2$, to form the sample. Where renneted samples were needed, 10 ml of this mixture was treated with 0·2 ml rennet for 30 min at 26 °C or overnight at 5 °C – long enough to liberate maximal amounts of 12% (w/v)-trichloroacetic acid-soluble nitrogen.

A portion (0·06 ml) of the sample was injected into one limb of the density gradient at the isopycnic point (a level estimated as corresponding to about 20% sucrose) so as to form a narrow band. This was balanced by injection of a similar volume of 15% sucrose-containing solution into the other limb.

Electrophoretic run. The electrode compartments, containing Ag/AgCl electrodes, were filled with saturated KCl. The rest of the apparatus was filled with milk serum or milk dialysate and the compartments of the cell were then moved into the open position. A voltage of 135 V, giving a current of 9–13 mA, was applied between the electrodes for about 40 min.

The progress of the run was followed by taking photographs at 3–7 min intervals. Measurements were made from prints enlarged to 4 times the size of the cell.

Viscosity determinations

Viscosities and densities of solutions used for preparing the gradients were determined at the temperature of the electrophoretic run using an Ostwald viscometer and a pycnometer respectively.

Analysis of data

All calculations were made assuming a viscosity for the medium equal to that of the 20 % (w/v)-sucrose-containing solution and a field strength equal to the average across the electrophoretic cell (10 V/cm). Mobilities were determined by the least-squares method from the straight line obtained when the distance of the upper boundary from the top of the cell was plotted against time. Zeta-potentials were calculated from the equation, $\zeta = 4\pi\eta v/\epsilon$, where η is the viscosity of the medium, ϵ is the dielectric constant and v is the electrophoretic mobility of the particles (Abramson, Moyer & Gorin, 1942).

RESULTS

Viscosity determinations

The viscosities of the solutions used in preparing the density gradients are shown in Table 1. For the calculation of electrophoretic mobility, the viscosity used was that of the appropriate 20 %-sucrose-containing solution at the temperature of the experiment.

Table 1. *Viscosities of solutions used in preparing density gradients at* 26 *and* 5 *°C*

(All solutions contained 0·4 % (w/v) Dextran 20.)

Concentration of sucrose, %, w/v	Viscosity, cP
Milk serum, 26 °C	
10	1·50
20	2·16
30	3·21
Milk dialysate, 26 °C	
10	1·43
20	2·07
30	3·51
Milk dialysate, 5 °C	
10	2·83
20	4·26
30	6·03

Electrophoretic experiments

Native micelles at 26 *°C.* The initial experiments were designed to test the validity of the method used as well as to obtain data on the mobilities of micelles at 26 °C. For all these the micelles and milk serum were separated by centrifugation at 0–5 °C; milk dialysate was likewise prepared at 5 °C. The various experiments were designed to investigate (1) the use of a.c. rather than d.c. to determine the rate and direction of movement of the upper and lower boundaries of the micelle band under other than electrical forces, (2) the effects of doubling and halving the concentrations of $CaCl_2$ added to the sucrose solutions used for preparation of the gradient on the rates of movement and spreading of the boundary, and (3) the mobilities of casein micelles in milk serum and milk dialysate, so as to detect any influence of the serum proteins.

The progress of a casein micelle boundary in a density gradient in milk serum is illustrated by Plate 1 (*a*) and (*b*). The photographs also show the droplet sedimentation which obscures the bottom of the boundary. Hence mobilities were determined from the rate of movement of the upper boundary. In all cases, this was constant during the period of the experiment, as is shown in Fig. 1, indicating that any retardation caused by the increasing viscosity of the medium was balanced by acceleration

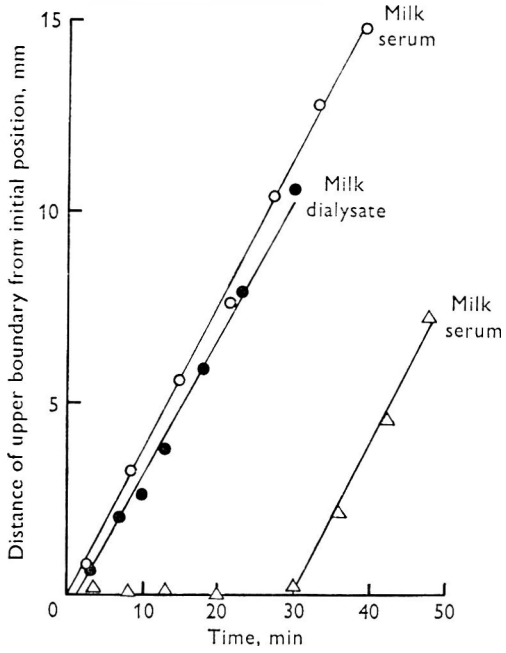

Fig. 1. The rates of movement of the upper boundaries of micelle bands in milk serum and milk dialysate at 26 °C. The points were taken from measurements on the prints of photographs taken at the various times after starting the current. The lines drawn are those calculated by the method of least squares. ○, Milk serum; ●, milk dialysate; △, milk serum, a.c. applied for 30 min, then switched to d.c.

caused by an increase in the field strength. Fig. 1 shows also that there was no significant movement of the upper boundary in the absence of an electrical force, indicating that there was no significant diffusion or sedimentation under the conditions of the experiment. When d.c. was applied, however, the boundary moved at the same rate as was found in experiments in which d.c. was applied from the start of the experiment. The mobilities calculated from experiments carried out under the various conditions, and the zeta-potentials derived from them, are shown in the first 7 lines of Table 2. This shows that doubling or halving the amount of $CaCl_2$ added to the solutions used for establishing the gradient, or replacement of milk serum by milk dialysate, only slightly affected the mobility of the upper boundary.

Further conclusions can be drawn from the rate of movement of the lower boundary in relation to the upper one. Under ideal circumstances this would indicate the extent to which the casein micelle population is heterogeneous in an electric field and enable determination of the mobilities of at least the fastest and slowest components. In the experiments described here the movement of the lower boundary in the electrical

field was obscured by the droplet sedimentation which occurred. However, it is possible to make some deductions. There was in all instances a continuous gradation between the fastest and slowest moving micelles, and in no case was the micelle band split into 2 or more bands. This indicates that the casein micelles were not divisible into definite classes on the basis of their mobilities. The extent of heterogeneity can

Table 2. *Mobilities and zeta-potentials of native and rennet-treated casein micelle upper boundaries at 26 and 5 °C*

(The calculations of both mobilities and zeta-potentials were made assuming that the initial conditions, a field strength of 10 V/cm and a medium viscosity equal to that of the appropriate 20 % (w/v)-sucrose-containing solution, obtained throughout the experiment. Mobilities were calculated from the rate of movement of the upper boundary of the micelle band, obtained by the method of least squares from measurements of the photographic prints.)

Medium	Mobility, $cm^2\ s^{-1}\ V^{-1} \times 10^4$	Zeta-potential, mV
	Native micelles, 26 °C	
Milk serum	$-0{\cdot}63 \pm 0{\cdot}01$	$-19{\cdot}1 \pm 0{\cdot}3$
Milk serum		
Alternating current	$0{\cdot}00 \pm 0{\cdot}01$	
Switched to d.c.	$-0{\cdot}67 \pm 0{\cdot}03$	$-20{\cdot}1 \pm 0{\cdot}9$
Milk serum with added $CaCl_2$ doubled	$-0{\cdot}62 \pm 0{\cdot}01$	$-18{\cdot}6 \pm 0{\cdot}3$
Milk serum with added $CaCl_2$ halved	$-0{\cdot}65 \pm 0{\cdot}01$	$-19{\cdot}6 \pm 0{\cdot}3$
Milk dialysate		
(1)	$-0{\cdot}60 \pm 0{\cdot}02$	$-17{\cdot}4 \pm 0{\cdot}6$
(2)	$-0{\cdot}60 \pm 0{\cdot}01$	$-17{\cdot}4 \pm 0{\cdot}3$
Milk serum and micelles prepared at 25–30 °C		
(1)	$-0{\cdot}48 \pm 0{\cdot}03$	$-14{\cdot}6 \pm 0{\cdot}9$
(2)	$-0{\cdot}38 \pm 0{\cdot}01$	$-11{\cdot}6 \pm 0{\cdot}3$
	Rennet-treated micelles, 26 °C	
Milk dialysate		
(1)	$-0{\cdot}27 \pm 0{\cdot}02$	$-7{\cdot}7 \pm 0{\cdot}6$
(2)	$-0{\cdot}35 \pm 0{\cdot}01$	$-10{\cdot}1 \pm 0{\cdot}3$
	Native micelles, 5 °C	
Milk dialysate	$-0{\cdot}45 \pm 0{\cdot}01$	$-26{\cdot}8 \pm 0{\cdot}6$
	Rennet-treated micelles, 5 °C	
Milk dialysate		
(1)	$-0{\cdot}18 \pm 0{\cdot}01$	$-10{\cdot}9 \pm 0{\cdot}6$
(2)	$-0{\cdot}23 \pm 0{\cdot}01$	$-13{\cdot}9 \pm 0{\cdot}6$

be estimated if it is assumed that the rate of extension of the bottom of the boundary by droplet sedimentation in the absence of an electrical force is the same as that in its presence. The rate of droplet sedimentation can be determined from an experiment in which d.c. was replaced by a.c., which is illustrated by Plate 1 (*c*) and (*d*). The rate of descent of the lower boundary compared with the upper one is shown in Fig. 2, which also shows similar data for experiments in which d.c. was applied. The rates of boundary spreading in milk serum and milk dialysate are shown in Table 3. The results indicate that the micelle population was only slightly heterogeneous in an electrical field.

The apparent heterogeneity was much greater when excess $CaCl_2$ was added to the gradient. The results were similar to those of a normal run for the first 15 min, but after that time a definite heterogeneity appeared with part of the band ascending in

the cell. Presumably, in this instance, the micelles took up variable amounts of additional Ca^{2+} which altered the net charge and thus the mobility.

In all the further experiments reported no attempt was made to determine the heterogeneity of the micelles; only the mobilities of the upper boundaries are reported.

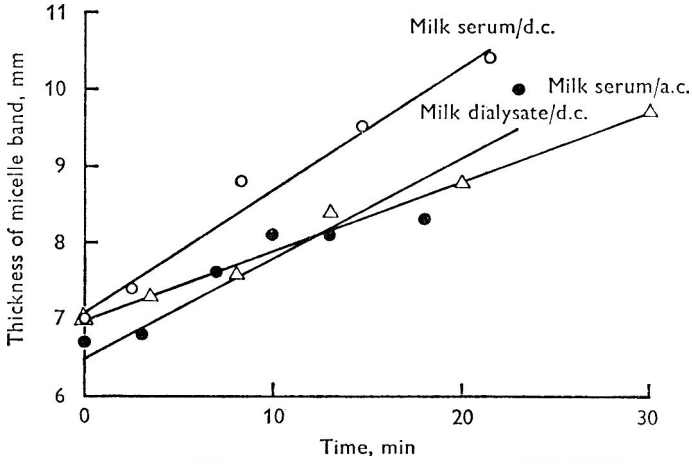

Fig. 2. Rates of descent of the lower boundary of the micelle band compared with the upper boundary when a.c. or d.c. was applied. The points were taken from measurements on prints of photographs taken at various times after starting the current. The lines drawn are those calculated by the method of least squares. ○, Milk serum, d.c.; ●, milk dialysate, d.c.; △, milk serum, a.c.

Table 3. *Heterogeneity of casein micelles in an electrical field*

(The rates of movement of the upper and lower boundaries were obtained from measurements on the photographic prints by the least squares method. It was assumed that the rates of movement when a.c. was applied gave measures of the basic rate under other than electrical forces and this could be subtracted from the corresponding values when d.c. was applied to give rates of movement due to the electrical field.)

Expt	Rate of movement of upper boundary, mm/min	Rate of movement of lower boundary, mm/min	Rate of movement of lower boundary due to electrical field, mm/min	Rate of movement fastest micelles/rate of movement of slowest
Milk serum, a.c.	$0{\cdot}00 \pm 0{\cdot}01$	$-0{\cdot}09 \pm 0{\cdot}01$		
Milk serum, d.c.	$-0{\cdot}38 \pm 0{\cdot}01$	$-0{\cdot}54 \pm 0{\cdot}01$	$-0{\cdot}45 \pm 0{\cdot}02$	$1{\cdot}18 \pm 0{\cdot}09$
Milk dialysate, d.c.	$-0{\cdot}36 \pm 0{\cdot}01$	$-0{\cdot}49 \pm 0{\cdot}02$	$-0{\cdot}40 \pm 0{\cdot}03$	$1{\cdot}11 \pm 0{\cdot}09$
Milk serum with added $CaCl_2$ halved	$-0{\cdot}37 \pm 0{\cdot}01$	$-0{\cdot}58 \pm 0{\cdot}03$	$-0{\cdot}49 \pm 0{\cdot}04$	$1{\cdot}32 \pm 0{\cdot}15$

Micelles and milk-serum prepared from uncooled milk. The cooling of milk below 20 °C is known to solubilize some of the casein from the micelles (Rose, 1968). Therefore, duplicate electrophoretic runs were made under conditions as near as possible to those pertaining in fresh milk. Casein micelles and milk serum were separated by centrifugation at 25–30 °C and all other parts of the experiment were carried out at

26 °C. As shown in Table 2, the casein micelle suspensions in these experiments had lower mobilities than those in which casein micelles were separated from the milk serum at 0–5 °C.

Rennet-treated micelles at 26 °C. In the dilute suspension used, rennet-treated micelles took 30–60 min to clot after the release of non-protein-N was complete. Thus, it was possible to determine the electrophoretic mobility of such micelles. Milk dialysate was used as the medium, since milk serum became turbid after rennet treatment and the micelle band became obscured. As shown in Table 2, the mobility of the micelles was approximately halved by the rennet treatment.

Native and rennet-treated micelles at 5 °C. Despite the increased viscosity gradient at 5 °C compared with that at 26° C, the rate of descent of the upper boundary of the casein micelle band was constant during the period of the electrophoretic run for both native and rennet-treated micelles. As shown in Plate 2 the extent of droplet sedimentation at the lower end of the boundary was much reduced compared with that at 26 °C. The mobilities and the zeta-potentials calculated from them are shown in Table 2. Although the mobilities were lower than those found in similar experiments at 26 °C, the zeta-potentials were higher. This is entirely a result of the increased viscosity of the sucrose solutions at the lower temperature.

DISCUSSION

Both native and rennet-treated casein micelles moved towards the anode on electrophoresis; hence they were negatively charged. In addition, provided the assumptions summarized below are justified, the micelles showed little heterogeneity in an electric field, the fastest components moving not more than 30% faster than the slowest ones.

The derivation of mobilities and zeta-potentials rests on the following assumptions: (1) that the extra $CaCl_2$ added to the solutions used for preparing the density gradient to compensate for the Ca^{2+} bound by sucrose did not alter the charge on the casein micelles; (2) that the presence of sucrose in the medium had no effect on the mobility other than that attributable to the increase in viscosity, and that the viscosity was unaltered by passage of the electric current; (3) that the retardation of the micelles due to the viscosity increase on descent in the gradient was balanced by an increase in field strength due to progressive reduction in conductivity as the sucrose concentration increased; (4) that the casein micelle behaved as a large smooth-surfaced particle. Assumption (1) would appear to be justified since only a very small concentration of $CaCl_2$ was used and doubling or halving this had no significant effect on the initial mobility of the micelle band. With regard to assumption (2), MacRitchie & Alexander (1961) have shown that sucrose has a small effect on the surface potential of protein films, possibly by modifying the protein structure due to removal of water. It is not clear what relevance this observation may have in the present instance and no direct data are available. The assumption, however, is a very important one, since the conclusions as to the effect of changes in temperature on the zeta-potentials depend entirely on estimates of the viscosities of the media. Assumption (3) has been verified since the rate of descent of the upper boundary of the micelle band, from which mobilities were calculated, was constant in all instances, an observation also made

by Brakke (1955) for viruses in a sucrose density gradient. This indicates that any retardation of the sample due to increasing viscosity of the medium was balanced by an increase in the field strength due to depletion of the ions of the medium in the heavy sucrose solutions (Svensson, 1960). The size of these effects can be estimated. The range over which the boundary moved varied between about 20 and 25 % (w/v) sucrose, over which the viscosity increased by about 25 %. The progressive increase in field strength must have been similar to this. Since these effects cancelled each other out, it would appear justified, in the calculations, to use the values for the viscosity of the medium and the field strength obtaining at the start of the electrophoretic run. Assumption (4) is made in the calculation of zeta-potentials. The equation used assumes a large non-conducting particle with a smooth surface. Its application to the casein micelle needs justification since recent evidence (Ribadeau Dumas & Garnier, 1970) indicates that the casein micelle is a spongy particle riddled with large pores, the liquid in which is in rapid equilibrium with the bulk liquid. Thus, the electrical double layer will extend into the pores and the contained ions being, in the main, of charge opposite to the charge on the micelle, will move in the opposite direction from the particle. Therefore, for present purposes, the micelle should be considered as an aggregate of small particles rather than as one large particle. The aggregate as a whole will not have a smooth surface, but, as the micelle is of conducting material, the lines of force are not restricted to passage through the ionic layer surrounding the particle, which offsets this difficulty. In addition, the equation applies only if the radius of curvature at all points on the surface is much greater than the thickness of the electrical double layer. The thickness of the double layer in milk, calculated as described by Abramson *et al.* (1942) from the ionic composition of milk ultrafiltrate (Jenness & Patton, 1959) is 1·05 nm at 26 °C. α-Chymotrypsin is an almost spherical protein of mol. wt 21 600, and of radius about 1 nm as calculated from the data given by Sober (1968). Since casein monomers have molecular weights close to that of α-chymotrypsin (Jollès, 1966) and the micelle probably consists of aggregates of these, it is probable that this restriction is satisfied.

The mobility and zeta-potential of native casein micelles at 26 °C, derived from the present results, were $-0{\cdot}43 \times 10^{-4}$ cm^2 s^{-1} V^{-1} and $-13{\cdot}1$ mV respectively. Hankinson & Briggs (1941), using artificial micelles prepared by titrating aqueous casein suspensions to pH 6·6 with saturated $Ca(OH)_2$ and adjusting to 2·5 % (w/v) casein, obtained a zeta-potential of $-13{\cdot}3$ mV in water at 30 °C. Payens (1966) reported a value for the zeta-potential of micelles of 8 mV, presumably negative, but no experimental data were given, derived from electro-osmotic experiments. Apart from the differences in materials and conditions, the figures are not strictly comparable because of differences in ionic strength of the media. Depending on the size and shape assumed for the casein micelle, the zeta-potential at zero ionic strength, a value which approximates to the potential at the surface of the particle, could be up to 50 times greater than that determined in a milk-salts medium. This is discussed further below.

The zeta-potential of native casein micelles, $-13{\cdot}1$ mV at ionic strength 0·086, is within the range reported for erythrocytes of various species, -7 to -21 mV at pH 7·4 and ionic strength 0·175 (Abramson *et al.* 1942). In addition, potato yellow dwarf virus has a mobility of about $-0{\cdot}12 \times 10^{-4}$ cm^2 s^{-1} V^{-1} in 15–20 % (w/v) sucrose at pH 7·4 and ionic strength 0·053 (Brakke, 1955), giving a zeta-potential of a

similar value. Since the zeta-potential, at a given ionic strength and in an aqueous medium, is directly proportional to the charge density (Abramson *et al.* 1942), these similarities suggest that these biological particles, at the pH value at which they are normally present in a biological system, tend to have a similar negative charge. This charge is quite low; it has been estimated that less than 1 % of the surface of an erythrocyte is occupied by charges (Abramson *et al.* 1942). It may be that the charge on such a particle is only just sufficient to overcome the attractive forces between the particles so that aggregation is prevented.

There was a gradation towards increasing negative charge in the order: both micelle preparation and electrophoresis at 25–30 °C, micelle preparation at 0° C and electrophoresis at 26 °C, and both micelle preparation and electrophoresis at 0–5° C. Thus, there was an increase in the negative charge of the micelles on cooling, which was not completely reversed by subsequent warming. This effect of temperature on the charge on the micelle provides an explanation of the 'Utrecht abnormality', which is characterized by decreased stability of milk so that it coagulates on boiling or sometimes even at room temperature. The abnormal milks have an unusually high content of calcium (Schipper & Mulder, 1962); therefore the micelles, under these conditions, may have a lower negative charge than normal. This negative charge would further decrease with increase in temperature, to the point at which the attractive forces between the micelles would be sufficient to overcome the repulsive forces, when flocculation would occur.

The question arises as to why the negative charge on the micelles increased on cooling. There are 3 known effects of cooling micelles. The first is to solubilize about 44 % of the β-casein, 38 % of the κ-casein and 4 % of the α_s-casein of the micelle (calculated from the data of Rose, 1969, and Ribadeau Dumas, 1968). Since all the caseins are negatively charged at the pH value of milk (Jollès, 1966), this would be expected to reduce the negative charge on the micelle. The second effect of cooling is to alter the pK values of ionizing groups. The only relevant groups are imidazole and phosphate which ionize near to pH 6·6. In both cases the pK values rise as the temperature falls (Sober, 1968), which would slightly reduce the negative charge on the micelles. Therefore, both these effects would alter the charge of the micelles in the direction of reduced negativity with fall in temperature. The third known effect of cooling is to induce conformational changes in β-casein resulting in the exposure of previously masked aromatic groups (Garnier, 1966). If charged groups become similarly exposed, their pK values may be altered, as has been observed with the tyrosine residues of ribonuclease (Shugar, 1952), which could result in an increase in the negative charge on the micelles. However, there may be additional, at present unknown, effects on the micelles, perhaps involving release of Ca^{2+} or uptake of phosphate. For instance, it seems likely that β- and κ-caseins are released from the micelles on cooling as their Ca-complexes. It is not possible to determine whether this would result in a net loss of positive charges from the micelle as there are insufficient data on the extent of Ca binding by individual caseins.

Predictably, rennet treatment reduced the mobility and zeta-potential of the micelles, reflecting a reduction in negative charge. The charge on an average micelle under the different conditions used has not been calculated because of uncertainty as to the assignment of size and shape. However, provided the micelle is not altered

significantly in size by changes in temperature and by rennet treatment, the negative charge is directly proportional to the zeta-potential (Abramson *et al.* 1942). Therefore, the higher zeta-potential for rennet-treated micelles at 5 °C than at 26 °C reflects a greater repulsive force between the micelles. The question whether this is sufficient to account for the reduced tendency to clot can be investigated by application of the theory of colloid stability (Verwey & Overbeek, 1948). According to this, the repulsive and attractive forces between the particles can be calculated and thus the potential energy curves for close approach of the micelles can be drawn.

These curves are obtained by plotting the sum of the electrical repulsive force, V_R, and the van der Waals–London attractive force, V_A, against the distance, d, between the particles. Since casein micelles are spherical (Nitschmann, 1949), the equations applicable to large spheres are used. If d is small, $V_A = -Ar/12d$, where r is the radius of the micelle and A is the van der Waals constant, having the dimensions of energy (Verwey & Overbeek, 1948), and $V_R = \epsilon r\psi_0^2 \ln(1+e^{-\kappa d})/2$, where ψ_0 is the potential at the surface of the particle, ϵ is the dielectric constant of the medium and κ is the reciprocal of the thickness of the electrical double layer (Derjaguin, 1940).

The selection of the value for A is empirical. Theoretical considerations suggest that it is of the order of 1×10^{-12} erg (Verwey & Overbeek, 1948), but the value determined by Albers & Overbeek (1960) from the stability of emulsions of water in benzene is $0{\cdot}4 \times 10^{-14}$ erg. However, in the present context the absolute value selected for A is unimportant since the interest lies in the comparison of the various potential energy curves. Changes in the value of A alter V_A only, which, in any case, is assumed to be the same for native and rennet-treated micelles both at 26 and 5 °C. In fact, since all other variables are assumed to be the same under the conditions of the experiments, the differences in the potential energy curves arise entirely from differences in ψ_0. However, the assignment of ψ_0 presents the major problem, since it cannot be directly deduced from the results.

ψ_0 approximates to the value of ζ at zero ionic strength (Abramson *et al.* 1942). For a large sphere in which $\kappa r \gg 1$ it can be derived from the equations given by Abramson *et al.* (1942) that, in the presence of salt, $\zeta = 4\pi\sigma/\kappa\epsilon$, where σ is the charge on the particle/cm^2 of surface, and in the absence of salt, $\zeta = 4\pi r\sigma/\epsilon$. Thus, if the value of κr is large, i.e. in concentrated salt solutions or when large particles are involved, ζ may be very much smaller than ψ_0. The radius of the average micelle is 53 nm, as calculated from the data of Nitschmann (1949). This would give a value for κr of about 50 for micelles in milk although actually the figure is probably much smaller than this since, as discussed above, the micelle cannot be considered as a rigid, non-conducting sphere. In the present work, a minimum value for ψ_0 is taken by assuming that it is equal to the value of ζ determined in the electrophoretic experiments. This has the effect of minimizing the differences between the micelles under the various conditions. Thus, the differences obtained in the potential energy curves can probably be considerably magnified to represent the correct situation.

The calculated potential energy curves for native and rennet-treated micelles in milk dialysate at 26 and 5 °C are shown in Fig. 3. For the purpose of calculation, the values taken for ζ were the average of duplicates given in Table 2 and it was assumed that $r = 53$ nm, $\kappa = 0{\cdot}95$ nm^{-1} at 26 °C and $0{\cdot}98$ nm^{-1} at 5 °C, and $A = 1 \times 10^{-14}$

erg. This value for A was selected to fit the known data on the stability of rennet-treated micelles. Verwey & Overbeek (1948) deduced that, as a first approximation, a value of at least $1kT$ for the maximum of the curve, V_{max}, is sufficient to prevent aggregation. If V_{max} for rennet-treated micelles at 26 °C is set at less than $1kT$, V_{max} for such micelles at 5 °C can be at least $2kT$. Hence, on this basis and selecting the minimum values for ψ_0, the effect of temperature on the stability of rennet-treated micelles can be explained entirely by charge differences.

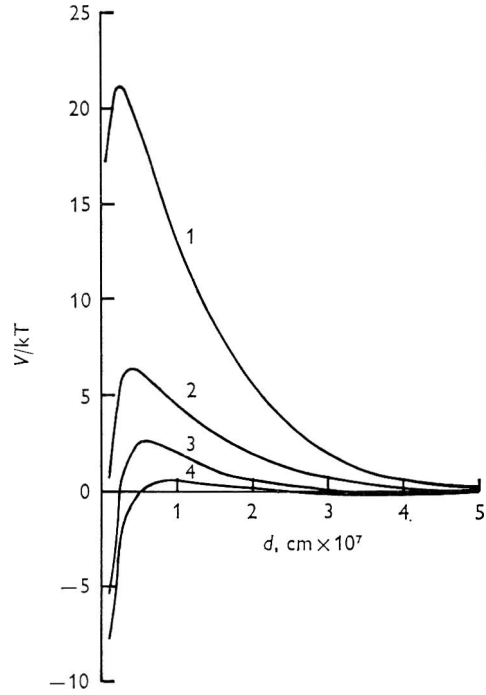

Fig. 3. Potential energy curves for casein micelles in milk dialysate. The potential energy, V, is the difference between the potential energies of repulsion and van der Waals–London attraction and is plotted against the inter-particle distance, d. Curve 1, native micelles at 5 °C; curve 2, native micelles at 26 °C; curve 3, rennet-treated micelles at 5 °C; curve 4, rennet-treated micelles at 26 °C.

However, Verwey & Overbeek (1948) have also reported a more refined analysis of the stability of colloidal systems taking into account the time required for coagulation of the particles. This makes use of the ratio, W, between the minimum time and the observed time for coagulation. The minimum time for coagulation $t = 3\eta/4kTn_0$, where n_0 is the concentration of the particles. From the particle weight of an average micelle (calculated from the data of Nitschmann, 1949) and its casein content (calculated from data reported by McMeekin & Groves, 1965) and the casein content of milk, it can be calculated that skim-milk contains about 7×10^{16} micelles/ml. Taking account of the dilution used in the electrophoretic experiments, this gives values for t of 5×10^{-5} s at 26 °C and 1×10^{-4} s at 5 °C. Since the actual clotting time observed at 26° C was about 60 min and that at 5 °C was greater than 20 h, the values for W are of the order of 10^8 at 26 °C and at least 10^9 at 5 °C. This means that V_{max} is required

to be of the order of about $20kT$ and at least $25kT$ for rennet-treated micelles at 26 and 5 °C respectively (Verwey & Overbeek, 1948). Such a value for V_{max} is not possible using $\psi_0 = \zeta$ whatever value is selected for A. However, it is clear that, if ψ_0 was assigned as some reasonable multiple of ζ, such values for V_{max} would readily be obtained. If ψ_0 was taken as 3ζ and A as 2×10^{-14} erg, V_{max} for rennet-treated micelles was $17kT$ at 26 °C and $41kT$ at 5 °C. Larger values for A and ψ_0 were not used because this led to greater differences between the values for V_{max}.

Within the limits of experimental accuracy and the theoretical assumptions made, the analysis given suggests that the differences in charge between native and rennet-treated micelles at 26 °C and between rennet-treated micelles at 26 and 5 °C are sufficient to account for their differences in stability to aggregation. In fact, it is probable that the charge effects are reinforced by differences in the attractive forces between micelles resulting from the increasing tendency of caseins to associate with increasing temperature (Payens, 1966). This aspect was not taken into consideration here, where the same value for A was used regardless of temperature.

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EXPLANATION OF PLATES

Photographs of casein micelle bands during electrophoresis.

PLATE 1

Native micelles in milk serum at 26 °C: (*a*) after 2 min; (*b*) after 15 min. Alternating current applied to native micelles in milk serum at 26 °C: (*c*) at start of run; (*d*) after 13 min.

PLATE 2

Native micelles in milk dialysate at 5 °C: (*a*) at start of run; (*b*) after 16 min. Rennet-treated micelles in milk dialysate at 5 °C: (*c*) at start of run; (*d*) after 16 min.

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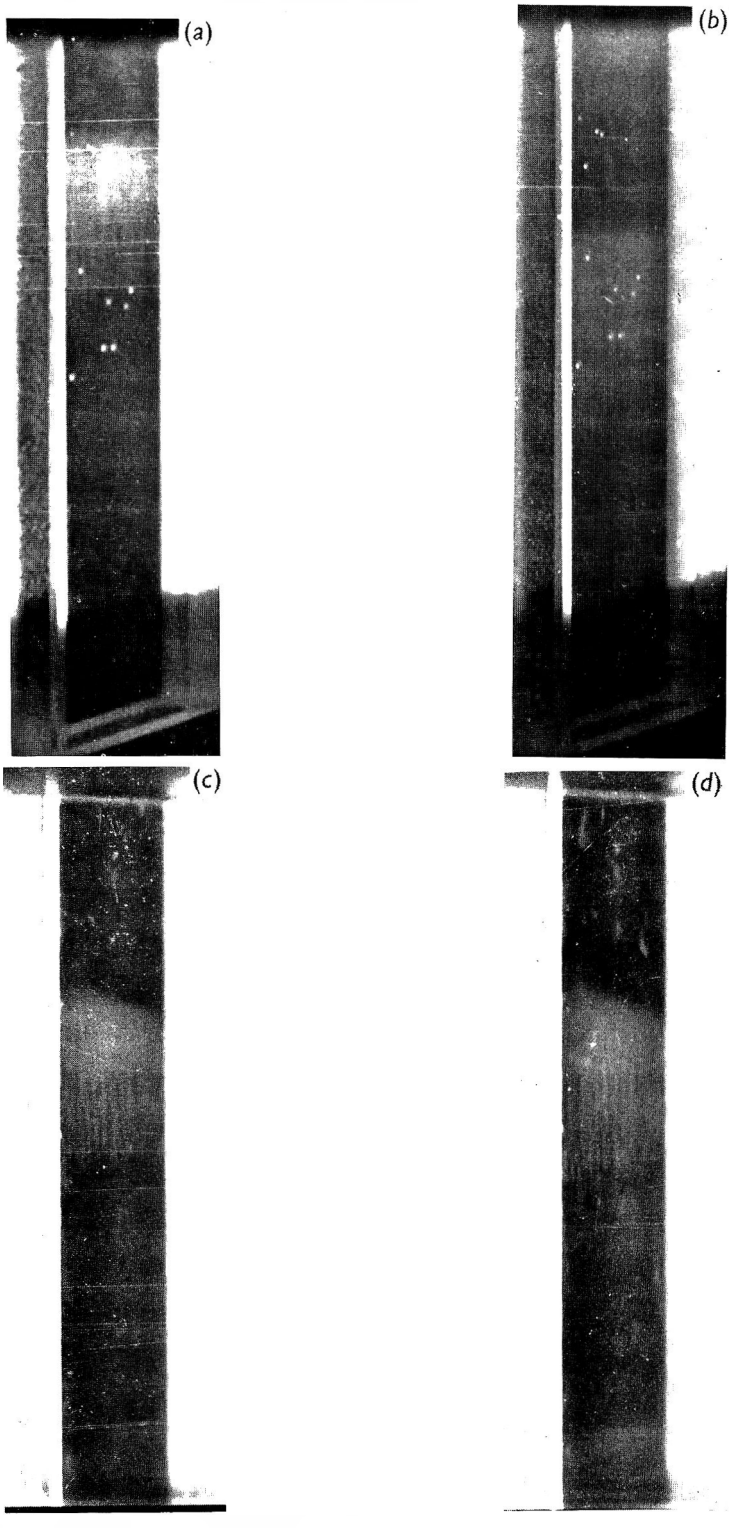

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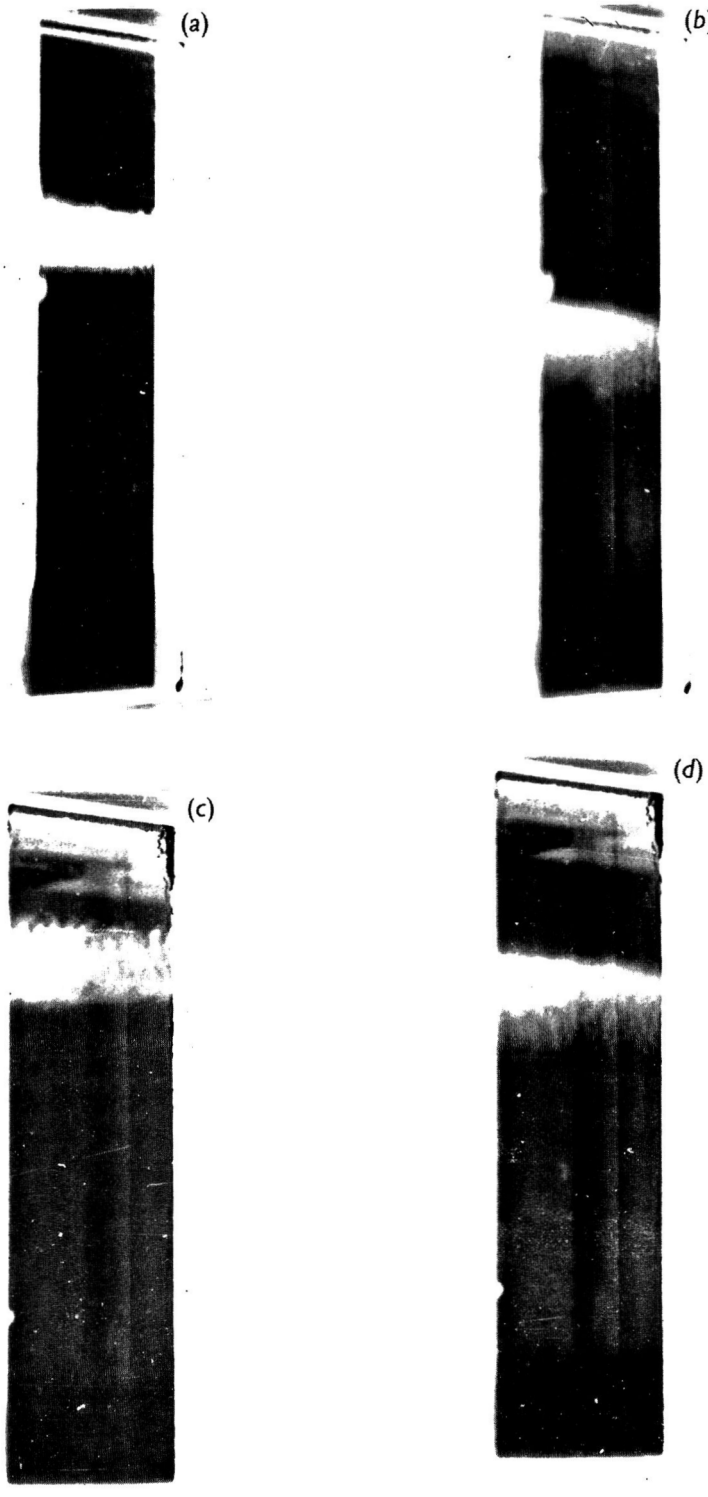
(a)
(b)
(c)
(d)

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With 4 plates

Influence of sodium chloride on the proteolysis of casein by rennet and by pepsin

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Summary. 1. Proteolysis of β-casein by rennin and by pepsin was completely inhibited in the presence of 10% NaCl and was very significantly reduced by 5% NaCl. The rate of proteolysis of α_s-casein was maximal in the presence of 5–10% NaCl. 2. The inhibitory effect of NaCl on the proteolysis of β-casein was independent of pH and incubation temperature. 3. The effectiveness of NaCl in controlling the development of bitter flavour in Cheddar cheese may be due to its inhibitory effect on the proteolysis of β-casein. 4. Rennin hydrolysates of β-casein were bitter in flavour whereas those of α_s-casein were not.

Rennin, in addition to being a very potent catalyst for the cleavage of a specific peptide bond in κ-casein, possesses very definite general proteolytic ability (Fish, 1957; Bang-Jensen, Foltmann & Rombauts, 1964; Lindqvist & Storgårds, 1959*b*, 1960, 1962; Fox, 1969, 1970). Cheese proteins undergo extensive proteolysis during cheese ripening (Schormuller, 1968), a process in which rennin is believed to play an important but not fully understood role. Two recent studies (Lindqvist & Storgårds, 1959*a*; Ledford, O'Sullivan & Nath, 1966) have shed some interesting light on the sequence of events occurring during the proteolysis of casein in hard and semi-hard cheeses (mostly Svecia and Cheddar). However, the results of these studies do not agree concerning the extent to which β-casein is degraded during ripening. Lindqvist & Storgårds (1959*a*), using free boundary electrophoresis, showed that β-casein undergoes very considerable degradation during ripening and have confirmed this in studies using isolated casein fractions (Lindqvist & Storgårds, 1959*b*, 1960, 1962). The data of Ledford *et al.* (1966) from experiments using polyacrylamide gel electrophoresis show that, in Cheddar cheese, β-casein undergoes very little proteolysis, while α_s-casein is extensively degraded. Ledford, Chen & Nath (1968) came to the same conclusion from studies on isolated casein fractions.

Fox (1969, 1970) has reported on the influence of pH, incubation temperature and degree of substrate aggregation on the proteolysis of casein by rennin and on the nature of the products. Compared with α_{s1}-casein, β-casein was shown to be much

more susceptible to proteolysis at low (2 °C) than at higher temperatures (32 °C). The temperature-dependence of the relative susceptibility of α_{s1}- and β_1-caseins to proteolysis was suggested as an explanation of the conflicting data on the extent of β-casein degradation during cheese ripening.

The proteolytic activity of rennin is influenced by NaCl (Stadhouders, 1962), greatest activity, as measured by increase in non-protein nitrogen (NPN), being observed in the presence of 5% NaCl at pH 5·2. Stadhouders also pointed out that cheeses with low NaCl levels were more prone to development of bitter flavour. This latter observation has been substantiated recently by Lawrence & Gilles (1969), who showed a very definite correlation between salt levels in cheese and the development of bitter flavour. Cheese containing a salt-in-moisture level of 4·9% rarely developed bitterness even when starters associated with bitter flavour development were used in its manufacture.

Gel electrophoresis is a very useful technique in the study of low level proteolysis. Accordingly, it was decided to investigate the influence of NaCl on casein proteolysis by rennin and pepsin using a gel electrophoretic method.

MATERIALS AND METHODS

Enzymes

Commercial Hansen's rennet extract at a rate of 1 μl/ml milk, or powdered porcine pepsin (1:2500, British Drug Houses Ltd) at a rate of 150 μg/ml milk, was used. Both of these enzyme levels coagulate milk at pH 6·6 in about 6 min at 32 °C.

Substrate

Isoelectric casein, prepared from bulk skim-milk, and washed several times with distilled water, was dissolved in 0·1 M phosphate buffer of pH 6·5 to give a final protein concentration of about 3%. During solution the pH was maintained at 6·5 by small additions of NaOH.

Sodium chloride was added to aliquots of sodium caseinate solution to give final concentrations of 0, 2, 5, 10, 15 and 20%. The pH of one half of each aliquot was adjusted to 6·0, and that of the other half to pH 5·2.

Proteolysis

Each of 12 samples (6 salt concentrations at 2 pH values) was subdivided into 4 aliquots. Two aliquots from each sample were treated with rennin and the other 2 with pepsin, at the above concentrations. One rennet-treated and one pepsin-treated aliquot were incubated at 4 °C for 4 days, and the remaining 2 aliquots were incubated at 32 °C for 1 day.

Samples to which no enzyme had been added and containing 0 and 20% NaCl served as controls at both temperatures.

All the samples were saturated with toluene as preservative.

After incubation, the samples were frozen at −10 °C until examined by electrophoresis.

Electrophoresis

Electrophoresis in vertical polyacrylamide gels was performed as described by Thompson, Kiddy, Johnston & Weinberg (1964).

Preparation of α_{s1}- and β-caseins

The residue remaining from the preparation of κ-casein from whole acid casein by the method of Zittle & Custer (1963) was fractionated into α_{s1}- and β-caseins by the method of Hipp, Groves, Custer & McMeekin (1952). Each of these latter caseins was essentially free of the other, as shown by electrophoresis on polyacrylamide gels.

RESULTS AND DISCUSSION

The results of a typical series of experiments are shown in Figs. 1–8 (Plates 1–4).

It is readily apparent from the electrophoretograms that proteolysis of both α_{s1}- and β-caseins by either rennin or pepsin was markedly influenced by the presence of NaCl.

The most interesting and perhaps the most significant result of these experiments was the rather selective inhibitory effect of NaCl on the proteolysis of the various casein components. The primary phase of rennin (or pepsin) action was not investigated here but the electrophoretograms show that both rennin and pepsin acted on κ-casein at all NaCl levels, although it would appear that κ-casein was not completely hydrolysed at the higher salt concentrations.

Proteolysis of β-casein by both enzymes was almost completely inhibited by 10% NaCl and was very considerably reduced in the presence of 5% NaCl at the temperatures and pH values used in these experiments. However, the nature of the proteolysis products arising from β-casein was not altered by the presence of sub-inhibitory levels of NaCl. The marked temperature-dependence of the relative susceptibilities of α_{s1}- and β-casein to proteolysis previously reported (Fox, 1969) was again apparent here (Figs 1, 3).

α_{s1}-Casein was optimally degraded by rennin in the presence of 5% NaCl and by pepsin at somewhat higher levels, 5–10% NaCl. A more heterogeneous mixture of polypeptides of mobilities intermediate between α_{s1}- and β-caseins was also produced in the 5–10% NaCl range than at higher or lower NaCl concentrations. Although higher salt concentrations (15 and 20%) had a considerable inhibitory effect on the proteolytic activity of rennin on α_{s1}-casein, 50% of the α_{s1}-casein was still hydrolysed by rennin in the presence of 20% NaCl under most conditions employed here. At 32 °C, the proteolytic activity of pepsin on α_{s1}-casein in 20% NaCl was only slightly reduced from that at optimum salt concentrations and was considerably greater than in the absence of NaCl (Figs 5, 6). However, at 4 °C, pepsin activity was considerably inhibited by such NaCl levels (Figs 7, 8). At both temperatures, pepsin produced a broader spectrum of polypeptides of high electrophoretic mobility ($v > \alpha_{s1}$-casein and presumably arising from α_{s1}-casein) at pH 5·2 in the presence of 15 and 20% NaCl than at lower salt levels. No such effect was apparent with rennin.

The inhibitory influence of NaCl on the proteolysis of β-casein is most likely due

to some alteration in the substrate (folding or aggregation) rather than the enzyme, because

(1) both enzymes were still quite active on α_{s1}-casein at NaCl levels which completely inhibited proteolysis of β-casein, and

(2) with β-casein the inhibitory effect was identical for both enzymes whereas with α_{s1}-casein the 2 enzymes were inhibited by different NaCl levels.

Salt-induced aggregation or folding would, like the temperature-induced folding (Fox, 1969), render it less accessible for proteolysis. A reduction in the attractive forces between enzyme and substrate at higher ionic strength may also be involved. The difference observed between the activity of the 2 enzymes on α_{s1}-casein at high NaCl levels was probably the result of differences in the salting-out effect of high ionic strength on the 2 enzymes rather than to any alteration of the substrate.

Stadhouders' (1962) demonstration that rennet produced NPN optimally from casein in the presence of 5% NaCl is interesting in the light of the present results. The electrophoretic methods employed here show that α_{s1}-casein was most extensively degraded in the presence of 5–10% NaCl but that the proteolysis of β-casein was very considerably inhibited by this level of NaCl. One might therefore expect a salt level lower than 5% to be optimal when proteolysis is measured by changes in NPN. As was initially pointed out by Lindqvist & Storgårds (1960) and confirmed by Fox (1969), rennin proteolysis of casein, and especially of β-casein, results in the production of large polypeptides that are still insoluble in 12% TCA. Thus, only the proteolysis of α_{s1}-casein is measured by the NPN method, which explains the compatibility of Stadhouders' results with those of the present study. This again demonstrates the superiority of electrophoretic methods over changes in NPN for the measurement of low-level proteolysis.

The rather selective inhibitory effect of NaCl on the proteolysis of β-casein may explain the lack of agreement between the results of Lindqvist & Storgårds (1959*a*) and those of Ledford *et al.* (1966) concerning the extent to which β-casein is degraded during cheese-ripening. Unfortunately, the salt content of the cheeses examined by the 2 groups of workers was not reported and it is possible that there were considerable differences in salt content, which would explain the lack of agreement. Such differences in salt content would be quite likely since Lindqvist & Storgårds (1959*a*) worked mostly with Svecia cheese and only to a limited extent with Cheddar, while Ledford *et al.* (1966) studied Cheddar exclusively. (Both groups of workers also investigated proteolysis in soft, surface-ripened cheese varieties but such cheeses are not being considered here.) Preliminary unpublished results of studies by the present authors on Cheddar cheese made using rennet or rennet substitutes show that β-casein is not substantially degraded during ripening, which agrees with the findings of Ledford *et al.* (1966). A study of casein degradation in cheese containing various levels of NaCl would be of considerable interest.

The present results suggest possible association between the inhibitory effect of NaCl on the proteolysis of β-casein and the effectiveness of NaCl in preventing the development of bitter flavour in Cheddar cheese. Czulak (1959) proposed that bitter flavour develops in cheese when the starter bacteria are deficient in peptidases capable of degrading the peptides produced by rennin, which therefore accumulate and result in bitter flavour. This postulate has been supported by others (Emmons,

McGugan, Elliott & Morse, 1962) but Lawrence & Gilles (1969) caution against its complete acceptance in view of work by Gordon & Speck (1965*a*, *b*) which showed that some starter bacteria are themselves capable of producing bitter peptides. Perhaps the peptides produced by rennin from β-casein are more bitter than those arising from α_{s1}-casein. If so, the partial or complete inhibition by NaCl of rennin proteolysis of β-casein would reduce or eliminate the build-up of bitter peptides in cheese made using peptidase-deficient starters and so control the development of bitterness.

Rennet hydrolysates of α_{s1}- and β-caseins, prepared in the absence of NaCl at 4 °C for 4 days at pH 5·2, were evaluated for bitterness by a taste panel consisting of 4 experienced people. Each panel member was given a coded sample of both control and hydrolysed α_{s1}- and β-caseins, and was asked to rank the samples in order of increasing bitterness. All the members agreed that α_{s1}-casein, both before and after proteolysis, had a mild, milky flavour and that β-casein had a slight bitter taste even before proteolysis which increased very markedly during proteolysis. It is therefore suggested that peptides arising from β-casein during rennin proteolysis are largely responsible for bitter flavour in cheese, and that the effectiveness of NaCl in preventing the development of bitterness is due to its inhibitory effect on the proteolysis of β-casein.

The question arises why peptides from β-casein should be more bitter than those from α_{s1}-casein. A recent report by Motaba, Nagayasu, Hayashi & Hata (1969) may provide the explanation. These workers isolated a number of bitter-tasting peptides from trypsin hydrolysates of casein and showed that they all contained both phenylalanine and proline. β-Casein contains twice as much proline and slightly more phenylalanine than α_{s1}-casein. Consequently, the probability of peptides containing proline and phenylalanine arising on proteolysis is much higher for β-casein than for α_{s1}-casein.

Loftus Hills (1970) announced that recent work at his laboratory had shown that the peptides responsible for bitterness in cheese contained a cyclized glutamate end-group. When this cyclized glutamate was removed the peptides lost their bitterness. More information is required on this point. In particular, it remains to be explained why rennin hydrolysates of β-casein were bitter while those of α_{s1}-casein were not, since α_{s1}- and β-casein contain approximately the same content of glutamic acid (about 40 residues/mole).

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Electrophoretograms of casein containing various levels of NaCl after proteolysis by rennet at pH 6·0 and 32 °C for 24 h. % NaCl: 1, Control 0 % (no rennet); 2, 0 %; 3, 2 %; 4, 5 %; 5, 10 %; 6, 15 %; 7, 20 %; 8, Control, 20 %.

Fig. 2. Electrophoretograms of casein containing various levels of NaCl after proteolysis by rennet at pH 5·2 and 32 °C for 24 h. % NaCl: 1, Control, 0 %; 2, 0 %; 3, 2 %; 4, 5 %; 5, 10 %; 6, 15 %; 7, 20 %; 8, Control, 20 %.

Plate 2

Fig. 3. Electrophoretograms of casein containing various levels of NaCl after proteolysis by rennet at pH 6·0 and 4 °C for 4 days. % NaCl: 1, Control, 0 %; 2, 0 %; 3, 2 %; 4, 5 %; 5, 10 %; 6, 15 %; 7, 20 %; 8, Control, 20 %.

Fig. 4. Electrophoretograms of casein containing various levels of NaCl after proteolysis by rennet at pH 5·2 and 4 °C for 4 days. % NaCl: 1, Control, 0 %; 2, 0 %; 3, 2 %; 4, 5 %; 5, 10 %; 6, 15 %; 7, 20 %; 8, Control, 20 %.

Plate 3

Fig. 5. Electrophoretograms of casein containing various levels of NaCl after proteolysis by pepsin at pH 6·0 and 32 °C for 24 h. % NaCl: 1, Control, 0 %; 2, 0 %; 3, 2 %; 4, 5 %; 5, 10 %; 6, 15 %; 7, 20 %; 8, Control, 20 %.

Fig. 6. Electrophoretograms of casein containing various levels of NaCl after proteolysis by pepsin at pH 5·2 and 32 °C for 24 h. % NaCl: 1, Control, 0 %; 2, 0 %; 3, 2 %; 4, 5 %; 5, 10 %; 6, 15 %; 7, 20 %; 8, Control, 20 %.

Plate 4

Fig. 7. Electrophoretograms of casein containing various levels of NaCl after proteolysis by pepsin at pH 6·0 and 4 °C for 4 days. % NaCl: 1, Control, 0 %; 2, 0 %; 3, 2 %; 4, 5 %; 5, 10 %; 6, 15 %; 7, 20 %; 8, Control, 20 %.

Fig. 8. Electrophoretograms of casein containing various levels of NaCl after proteolysis by pepsin at pH 5·2 and 4 °C for 4 days. % NaCl: 1, Control, 0 %; 2, 0 %; 3, 2 %; 4, 5 %; 5, 10 %; 6, 15 %; 7, 20 %; 8, Control, 20 %.

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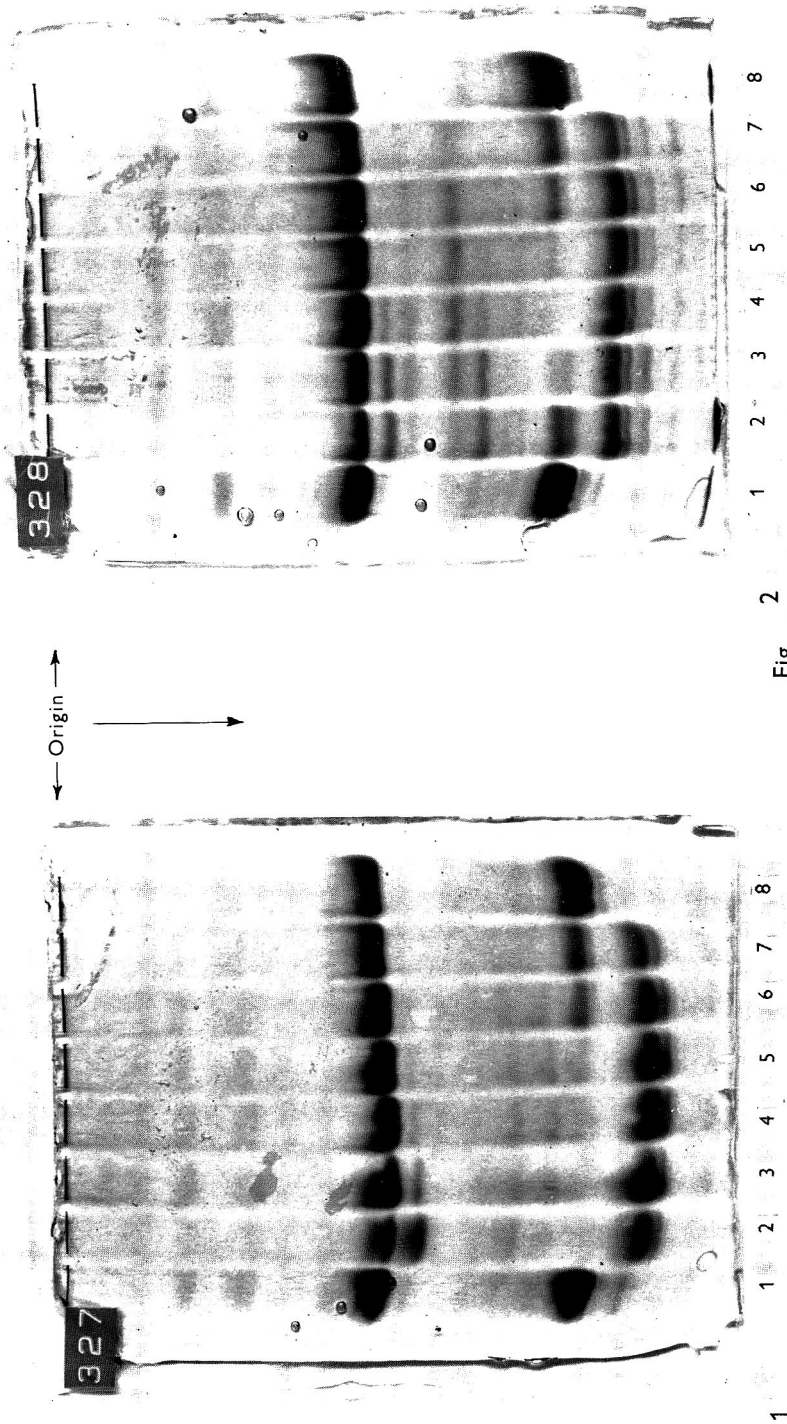

Fig. 2

Fig. 1

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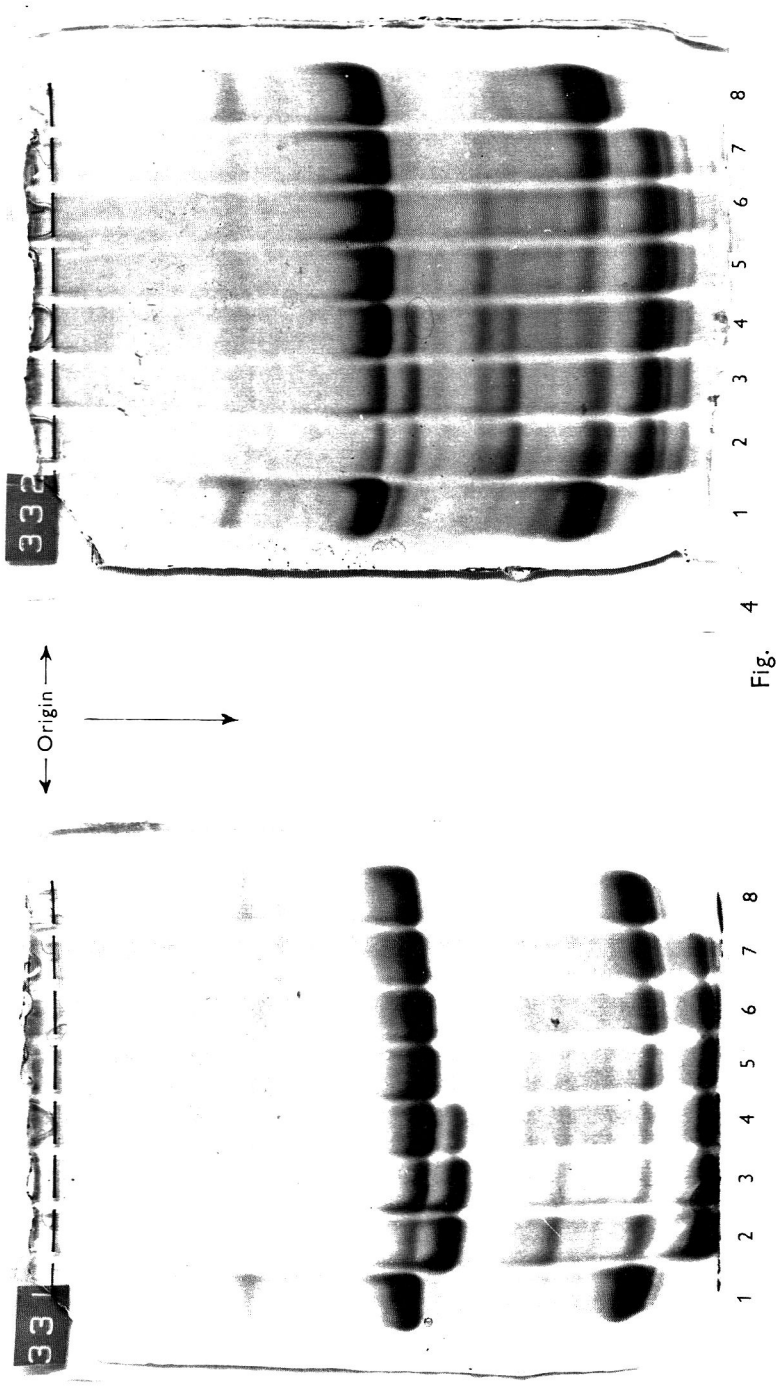

Fig. 4

Fig. 3

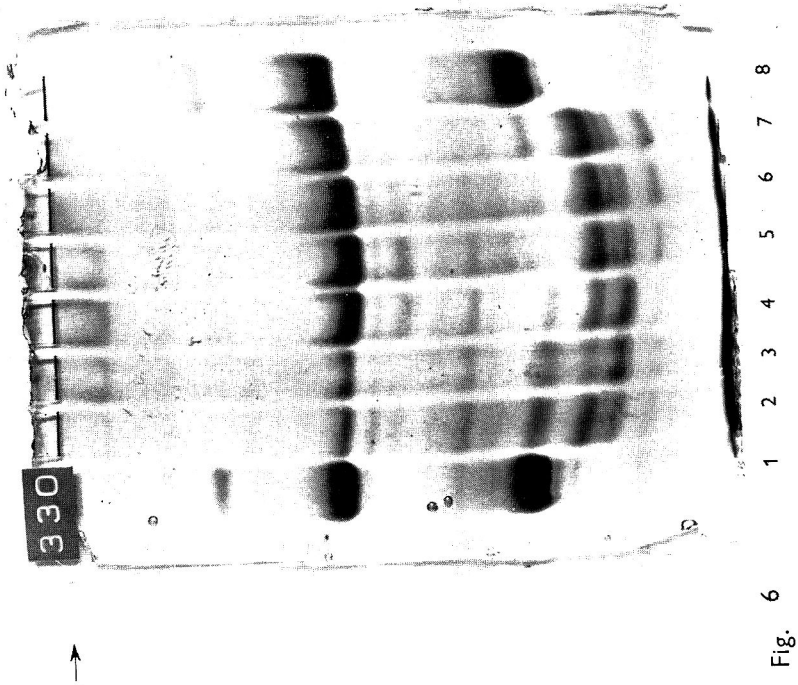

Fig. 6

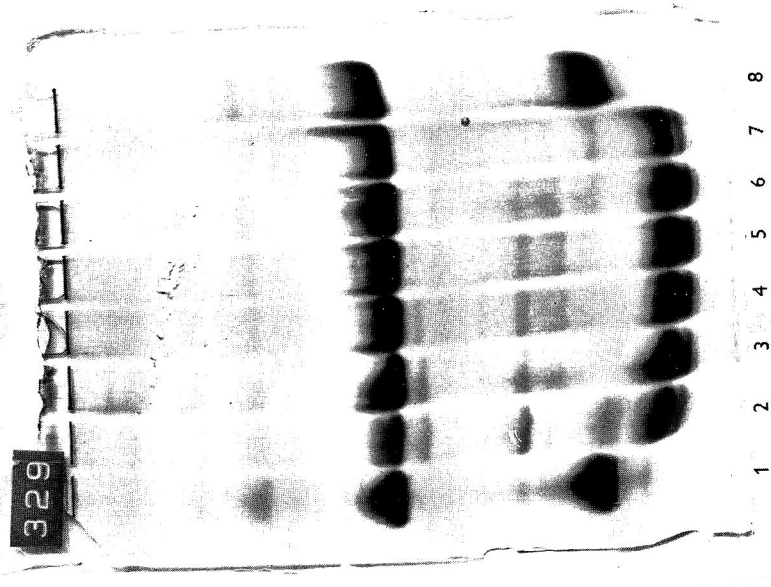

Fig. 5

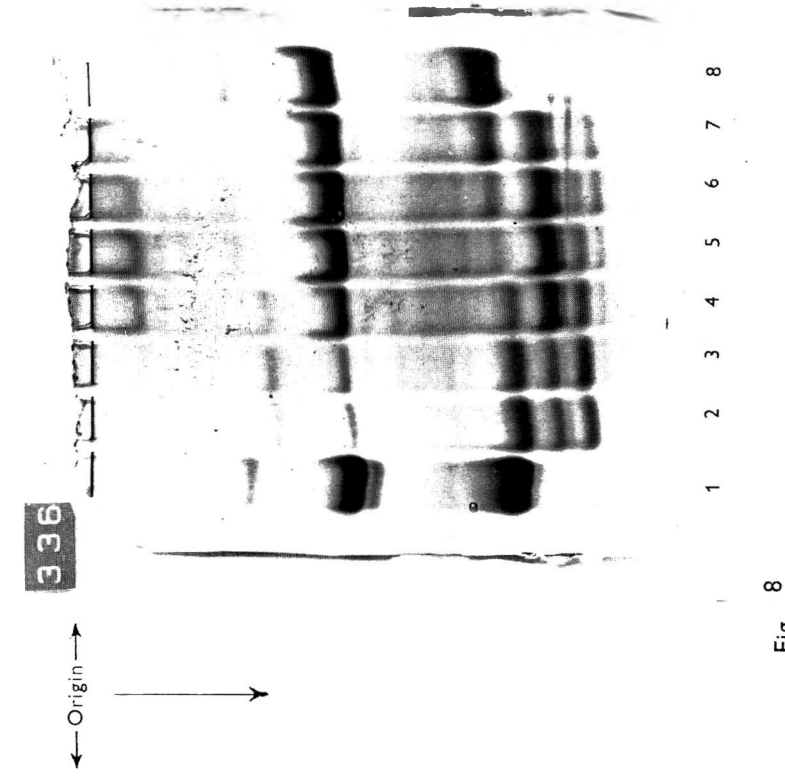

Fig. 8

Fig. 7

Bovine milk esterases

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SUMMARY. The type and distribution of esterases in milk has been investigated using selective inhibitors during normal assay procedures and during histochemical staining of polyacrylamide gels. Enzyme solutions were obtained from skim-milk by acid and alkali precipitation, followed by ammonium sulphate fractionation, ultra-filtration and Sephadex G-100 chromatography. The major type of esterase present was an aryl-esterase (E.C. 5.1.1.2) while a smaller amount of a choline-ester hydrolase (E.C. 3.1.1.7; 3.1.1.8) was detected. The significance of these findings is discussed.

Some of the biochemical processes involved in Cheddar cheese ripening are being studied in this laboratory at the present time, and the action and significance of milk esterases is one facet of this investigation.

The 2 types of hydrolases that were considered likely to contribute to fat hydrolysis in milk and milk products were certain of the esterases (E.C. 3.1.1.1, 2, 6, 7, 8) and lipases (E.C. 3.1.1.3). Bovine milk has been previously found to contain esterases (Forster, Bendixen & Montgomery, 1959), and the existence of milk lipase has been documented for some time (Herrington, 1954), but the significance of these milk enzymes in Cheddar cheese ripening has not been fully investigated.

It would be expected that if esterases were present in cheese, then the levels would be extremely low and difficult or impossible to measure by normal assay techniques (Scott, 1965). Some insight into their significance might be gained, however, by first examining the types and distribution of esterases present in milk. The present paper deals with some initial work on the properties of milk esterases as distinct from milk lipases.

MATERIALS AND METHODS

Milk collection

Whole-milk samples were collected and treated as indicated previously (Kitchen, Taylor & White, 1970). Skim-milk was utilized as the source of the enzymes.

Protein determination

Protein was determined by the method of Gornall, Bardawill & David (1949) using bovine serum albumin as a standard. The protein concentration of column effluents was estimated from the absorbance obtained at 280 nm (Warburg & Christian, 1942).

(I) *Gel chromatography*

Sephadex G-100 (Pharmacia, Uppsala) was swollen for 2 days at room temperature in 0·01 M tris/HCl buffer of pH 8·0. The swollen gel, in the form of a slurry, was poured into a 15 mm diam. glass column (Type K 15/90, Pharmacia, Uppsala) to a height of 80 cm. The column was equilibrated with the same buffer at 5 °C by passing 3–4 column volumes of buffer through the column. Fractions were collected automatically at suitable time intervals with an L.K.B. RadiRac fraction collector.

(II) *Polyacrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis was carried out as described by Davis (1964). A 7·5% gel was used and run for 2 h at 5 °C and 2 ma/gel.

Histochemical staining

Polyacrylamide gels were stained for esterase activity with α-naphthyl acetate using the method described by Holmes & Masters (1967), except that the incubation time was longer, being dependent on the amount of activity applied to the gel. After staining, the gel was washed in methanol–acetic acid–water solution(45:10:45 v/v), and scanned in a Joyce Loebl Chromoscan densitometer. Protein bands were located by staining the gels in 1% Amido black for 60 s, and de-staining was accomplished by continuous washings in 7% acetic acid.

Assay procedure

The method was based on that described by Zeller (1956). To 0·5 ml of 10^{-2} M phenyl acetate was added 0·01 M tris-HCl buffer of pH 8·0 and enzyme to a total volume of 5 ml.

The initial absorbance at 270 nm was read, and after 3 h incubation at 37 °C the final absorbance reading was taken. The rate of change in absorbance at 270 nm per min per ml of enzyme solution was calculated and converted into μmoles of phenol produced per min per ml. One unit of activity was defined as that amount of enzyme that would produce 1 μmole phenol/min under the specified conditions.

Enzyme preparations

Skim-milk at 5 °C was adjusted to pH 4·6 by slow addition of 1 N-HCl, with constant stirring. The casein precipitate was removed by centrifugation and the supernatant was adjusted immediately to pH 7·0 with 1 N-NaOH. Any insoluble material was again removed by centrifugation at 5 °C for 10 min at 6000 *g*. A 40–80% ammonium sulphate precipitate was prepared from this supernatant, and dissolved in 0·01 M tris-HCl buffer of pH 8·0 and dialysed exhaustively against the same buffer. The dialysed ammonium sulphate fraction was concentrated using a Diaflo XM-50 ultrafiltration membrane in a concentration cell. The concentrate was applied to a Sephadex G-100 column and eluted with 0·01 M tris-HCl buffer of pH 8·0.

In some instances, milk serum was prepared by acid precipitation of skim-milk as

described above, followed by dialysis and concentration on an XM-50 membrane. The concentrate was re-dialysed and any insoluble material was removed by centrifugation.

Chemicals used

Phenyl acetate was obtained from Eastman Organic Chemicals, Rochester, N.Y., U.S.A., and redistilled before use (b.p. 194–195 °C). All chemicals used for polyacrylamide gel electrophoresis were also obtained from Eastman.

p-Chloromercuribenzoate (p-CMB) was obtained from Calbiochem, Los Angeles, California, U.S.A., and eserine sulphate was obtained from the Sigma Chemical Co., St Louis, Missouri, U.S.A. Ammonium sulphate was a special enzyme grade (heavy metal free) obtained from Mann Research Chemicals, New York 6, N.Y., U.S.A. All other chemicals used were of the highest purity available.

RESULTS

The steps involved in preparing the active enzyme solution are summarized in Table 1. Recoveries are quoted as relative to milk serum, because an accurate measure of skim-milk activity was difficult to obtain. This procedure resulted in a 7-fold increase in the number of units/ml, but the specific activity only doubled.

Table 1. *Summary of enzyme preparation*

Fraction	Units/ml	Recovery, %	Specific activity $\times 10^{-3}$
Serum	0·042	100	2·6
Dial. 40–80 % as precipitate	0·095	50	4·8
XM-50 Conc.	0·280	30	5·0

Table 2. *Inhibition of concentrated ammonium sulphate fractionated serum*

Description	Inhibition, %
Control	0
10^{-3} M p-CMB	50
10^{-3} M Mg^{2+}	35
10^{-5} M La^{3+}	60
10^{-4} M EDTA	80

Enzyme solution was preincubated with inhibitor for 15 min at 25 °C, after which the activity remaining was determined as described in the text.

Table 2 shows the effect of inhibitors on the enzyme obtained from ammonium sulphate fractionation and XM-50 concentration of serum. p-Chloromercuribenzoate (p-CMB), La^{3+}, Mg^{2+} and EDTA all inhibited the enzyme in varying degrees, EDTA being the most active and causing 80 % inhibition.

Table 3 shows the degree of inhibition by eserine and p-CMB on the concentrated serum enzyme. p-CMB produced 84 % inhibition whereas eserine inhibited the activity by 9 %.

Fig. 1 indicates the effluent profile obtained after Sephadex G-100 chromatography of the XM-50 concentrate of the 40–80 % ammonium sulphate precipitate.

The enzyme activity was located in the first protein peak, while a great deal of protein was removed in the following peak. A considerable loss of activity apparently occurred during chromatography, because very little purification was achieved by the removal of the bulk of the protein in the second peak.

Table 3. *Inhibition of unfractionated concentrated serum*

Description	Inhibition, %
Control	0
$+4 \times 10^{-5}$ M eserine sulphate	9
$+10^{-3}$ M p-CMB	84

Enzyme solution was preincubated with inhibitor for 15 min at 25 °C, after which the activity remaining was determined as described in the text.

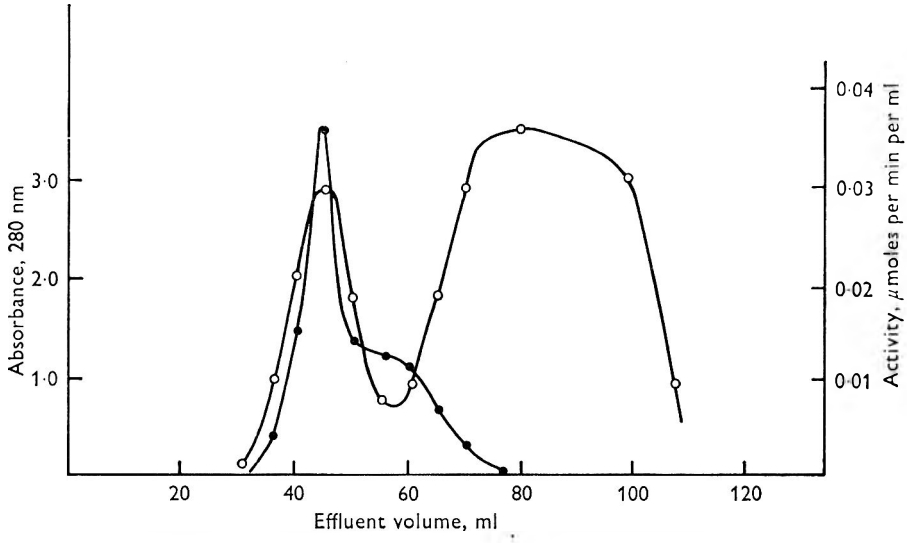

Fig. 1. Sephadex G-100 chromatography of XM-50 concentrate of 40–80 % ammonium sulphate precipitate. ○, Absorbance 280 nm; ●, activity μmoles per min per ml.

Fig. 2(*a*) represents a densitometer tracing of a gel, histochemically stained for esterase activity. The sample loaded onto the gel was obtained from the XM-50 concentrate of unfractionated serum. Two bands of activity were observed – one with a relative mobility (R.M., bromo phenol blue = 100 %) of 10 % and the other of 50 %.

Fig. 2(*b*) is the same as described for Fig. 2(*a*) but the gel was incubated in eserine (4×10^{-5} M) during staining with α-naphthyl acetate. The almost complete removal of the band with R.M. = 50 % can be seen. The other band, of R.M. 10 %, could not be removed by p-CMB, eserine or E-600, but it was removed if the sample was heated at 60 °C for 5 min before being applied to the gel.

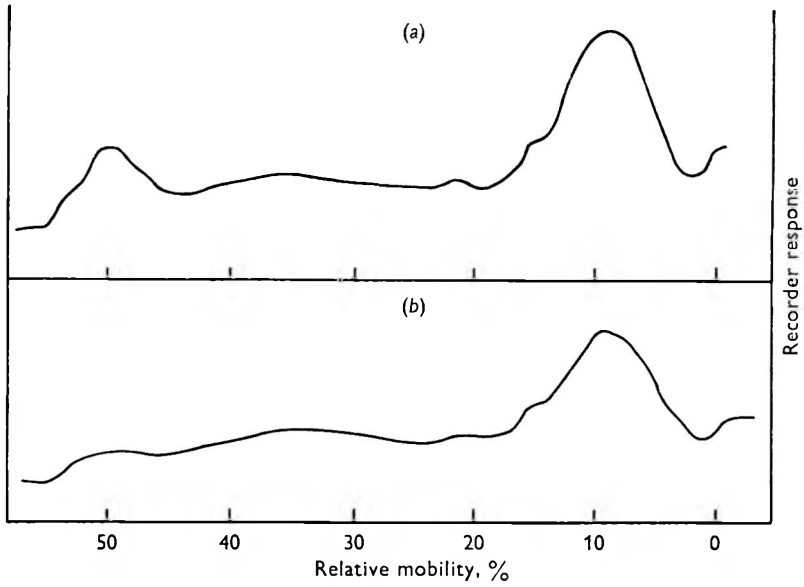

Fig. 2. (*a*) Densitometer tracing of gel stained for esterase activity. (*b*) Densitometer tracing of gel stained for esterase activity in the presence of eserine.

DISCUSSION

The presence of esterases in milk has been known for some time (Herrington, 1954). Previous workers (Forster *et al.* 1959) classified milk esterases into 3 classes – A, B, and C – depending on their substrate specificity and sensitivity towards organophosphate inhibitors (Augustinsson, 1958). Assay procedures have generally involved the use of manometric techniques, with all substrates being in the emulsion form. In the light of present definitions of lipases and esterases (International Union of Biochemistry, 1965), a more detailed investigation into the types and distribution of milk esterases seemed warranted.

The assay procedure described in the present paper resulted from many attempts to find a suitable substrate for the enzymes. The hydrolysis of ethyl acetate was monitored using a pH-stat, but there was poor agreement between duplicates, possibly due to the low level of activity towards this substrate. Hydrolysis of p-nitrophenylacetate appeared to give higher rates but most of the activity could be attributed to non-specific catalysis as described by Downey & Andrews (1965). The activity towards α-naphthyl acetate was even less than for the other substrates tested.

The activity towards phenyl acetate was found to be specific and reproducible. Also, reasonable activity was found towards indophenylacetate but a deeper investigation of this substrate was not undertaken. A problem with the phenyl acetate assay was that only small amounts of enzyme solution could be used in the procedure because larger amounts of protein (λmax. = 280 nm) interfered with the absorbance of free phenol produced (λmax = 270 nm, Erdos, Debay & Westerman, 1960).

The procedure outlined in Table 1 served to provide a more concentrated enzyme

solution but, in fact, very little purification of the activity was achieved. The effect of inhibitors on the most concentrated fraction indicated that the major type of esterase present was an arylesterase. This was inferred because of the similarity between the inhibitor properties of this enzyme and other mammalian arylesterases that have been studied (Erdos *et al.* 1960; Marton & Kalow, 1962), but incomplete inhibition by the agents tested indicated the presence of other esterases. Because some of the esterases present in milk might have been lost during the preparation procedure used, unfractionated serum was concentrated and the effect of inhibitors on this source was studied also. The results indicated that milk contained 84% arylesterase (p-CMB inhibition) and 10% cholinesterase (eserine inhibition). Similar results have been reported by Forster *et al.* (1959), and this distribution is consistent with that found in bovine blood plasma as well (Augustinsson, 1958).

A significant point with regard to the present study is that the results take into account the effect of lipase, as the substrates employed were not emulsified. This has not been the case in previous investigations which must therefore include a considerable contribution of extraneous activity. Again, as a certain amount of confusion exists in the literature in regard to esterase and lipase nomenclature (Okuda & Fujii, 1968), the recent proposals for esterase nomenclature as presented by Holmes & Masters (1967) have been adopted in the present investigation.

The elution pattern on Sephadex G-100 of the concentrated ammonium sulphate fraction of serum indicated that most of the activity was eluted at, or very close to, the void volume of the column, and inhibitor studies indicated that this peak was mainly arylesterase in nature. It appears from these results that milk arylesterase may have a higher molecular weight than other vertebrate esterases, which display mol. wts in the range 50000–70000 (Kingsbury & Masters, 1970). This view is supported by the weights obtained by Murphy & Downey (1970), which indicate that milk esterase has a mol. wt in excess of 500000. Other investigations have shown, however, that serum cholinesterases can exist in a variety of molecular weight forms (La Motta, Woronick & Reinfrank, 1970), and the same might be true of this arylesterase in milk.

Polyacrylamide gel electrophoresis of the concentrated unfractionated serum indicated that a small amount of the total esterase activity could be attributed to cholinesterase and this was consistent with the result obtained using inhibitors during spectrophotometric assay.

This initial investigation then has provided some insight into the nature and distribution of milk esterases. An important property in relation to the presence of milk esterases in Cheddar cheese is that the esterases are soluble at pH 4·6. It is unlikely, therefore, that any high proportion of the milk esterases would be present in the curd, although, as some mechanical occlusion of whey proteins occurs during cheese-making, some enzymes would be present in the final pressed cheese. Over the lengthy period of ripening this small amount of enzyme might still be significant in relation to flavour development.

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The chemical composition and physical properties of fractions of milk fat obtained by a commercial fractionation process

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Summary. Samples of liquid and solid fractions obtained by a commercial process from anhydrous milk fat of softening point (s.p.) 33·5–34 °C had s.p. values of 22–23 °C and 36–38 °C respectively.

Determinations of fatty acid composition by GLC showed that unsaturated and short chain fatty acids were present in increased concentration in the liquid fraction (average 37·8 and 12·4 % as compared with 35·1 and 10·8 % in the original milk fat) and long chain saturated acids in the solid fraction (average 57·8 as compared with 53·8 %). There was some concentration of carotene and vitamin A, and to a lesser extent of cholesterol, in the liquid fraction.

Crystallization and melting curves determined by a differential scanning calorimeter (DSC) showed that while the liquid fraction was completely melted at 25 °C, the solid fraction contained an increased proportion of fat melting between 30 and 40 °C. In addition to these high melting triglycerides the solid fraction also contained some 65 % of fat in the liquid phase at the original crystallization temperature of 25 °C.

The physical properties of butter and other products consisting mainly of milk fat are dependent on the chemical composition of the fat.

A limited variation can be made by altering processing conditions, e.g. in cream treatment, to modify the crystallization of the fat. Some further extension is possible by making use of seasonal variations in milk fat composition and selecting the fat accordingly. The range of properties possible is, however, still very limited in comparison with that available with competing products based on vegetable oil.

More extensive alterations to the chemical composition of milk fat can be made by special methods of feeding dairy cows (Scott *et al.* 1970) but these are unlikely to be economically feasible.

Modification of milk fat by chemical methods such as interesterification (deMan, 1961*a*; Richardson, 1968) and hydrogenation (Yoncoskie, Holsinger, Posati & Pallansch, 1969) destroys the natural flavours and causes loss of many of the desirable characteristics of milk fat.

A more promising process is the separation of milk fat into portions of different melting ranges by fractional crystallization either from solution in an organic solvent (Brown, 1941; Morse & Jack, 1949) or from the melted fat (deMan, 1968*a*). The latter would be preferable for use in the dairy industry as flavour problems from solvent residues would be avoided.

The transfer of this process from a laboratory to a commercial scale was facilitated by the development by the Alfa Laval Co. of a process described by Fjaervoll (1970). In this process, mixing the fat with an aqueous solution allows the use of an ordinary centrifugal separator without deposition of fat crystals in the separator bowl. A pilot plant based on this principle has been further developed and modified at this Institute. As full details are to be published elsewhere only a brief description is given here. The object of the present paper is to describe the chemical and physical properties of typical milk fat fractions obtained by this process.

EXPERIMENTAL

Materials

Commercial anhydrous milk fat was obtained from the New Zealand Dairy Board plant in Auckland where it was produced from commercial butter, 2 lots of which (A and B) were made in February and one (C) in March 1970, i.e. the fat was late summer and early autumn milk fat.

In the fractionation process the milk fat was crystallized by holding overnight at 25 °C (sample A) or 26 °C (samples B and C). The partly crystallized milk fat was then mixed with water, containing a wetting agent and an electrolyte, at the same temperature as the fat and passed through a centrifugal separator which discharged the liquid fat fraction and an aqueous phase containing suspended crystals of solid fat. The aqueous mixture was then heated to liquify the fat and passed through a second separator in which the higher melting fat fraction was separated from the aqueous phase. The 2 fat fractions were then washed and dehydrated.

The relative yields of liquid and solid fractions ranged from 40:60 (sample A) to 54:46 (sample C).

A high melting glyceride fraction (HMGF) was separated on a laboratory scale from the solid fraction of milk fat A by fractional crystallization from acetone at 24 °C according to the method of Patton & Keeney (1958).

Methods of analysis

Milk fat and its fractions were analysed as follows:

(1) *General analysis*

Saponification values, iodine values, Reichert values and refractive indices were determined according to British Standards Institution (1961). Softening points were measured by Dolby's modification (1961) of Barnicoat's method (1944).

Carotene and vitamin A were estimated essentially as described by Thompson, Ganguly & Kon (1949).

'Total colour' was estimated by dissolving 2 g of fat in petrol ether, making up to a final volume of 25 ml and measuring the yellow colour in a spectrophotometer at 450 nm. Since the true carotene was measured under similar conditions the same factor was used to convert the optical density to a 'carotene' figure. Cholesterol was estimated after saponification of the fat and extraction with ether, by the intensity of colour (at 650 nm) given in the Liebermann–Burchard reaction as described by Lampert (1930).

(2) *Estimation of fatty acids*

Milk fat fractions were methylated with methanol containing 14% (w/v) of boron trifluoride according to Van Wijngaarden's modification (1967) of the procedure of Metcalfe, Schmitz & Pelka (1966). The methyl esters were separated by gas–liquid chromatography using a Varian Aerograph 1520 Chromatograph fitted with a flame ionization detector and a matrix temperature programmer. A column (8 ft × $\frac{1}{8}$ in. i.d.) packed with 12% diethylene glycol succinate on acid-washed D.M.C.S. treated Chromosorb W (60–80 mesh) was used. Nitrogen flow rate was 25 ml/min and the chromatograph was programmed from 40 to 60 °C at 4 degC/min and then to 175 °C at 6 degC/min. The identity of the methyl esters was established from the logarithmic values of the retention volumes (Hawke, Hansen & Shorland, 1959) and the proportions of esters present were determined by measuring peak areas by height × width at half-height. Linearity of the detector was tested by chromatography of standard methyl ester mixtures purchased from Applied Science Laboratories Inc. (Pa., U.S.A.).

The *trans* fatty acid contents of the 18-C monoene acids were estimated by gas–liquid chromatography after the separation of the methyl esters of the fatty acids into saturated, *cis*-monoene, *trans*-monoene, diene and triene fractions, by thin-layer chromatography on Silica-gel G (E. Merck AG) impregnated with 20% silver nitrate. Chromoplates were prepared by the method of Lees & De Muria (1962) and developed in benzene. Compounds were detected under u.v. light after spraying with 0·2% 2′,7′-dichlorofluorescein in 95% ethanol (Mangold & Mallins, 1960). The *trans*- and *cis*-monoene bands were then scraped off the chromatoplates into centrifuge tubes and the esters extracted from the Silica-gel G 3 times with 5 ml portions of chloroform–methanol (2:1, w/v). This solvent was removed *in vacuo*. The methyl esters were then dissolved in 0·2 ml hexane and subjected to gas–liquid chromatography.

(3) *Thermal analysis*

(*a*) *Calibration.* Thermal analysis of the milk fat fractions was performed using a Perkin–Elmer differential scanning calorimeter (DSC-1 B). The DSC was calibrated for temperature read-out using high purity melting point standards (Thermal Analysis Newsletter No. 5, Perkin–Elmer Corp.). Under the operating conditions used in the present work, the correction (real temperature minus recorded temperature) for Figs 1 and 3 varied from +2 °C at −60 °C (recorded temperature) to −4 °C at +50 °C. The correction for Fig. 2 varied from +5·4 °C at −60 °C to −0·6 °C at +50 °C, while the correction for Fig. 4 was approximately constant at −6·7 °C. In this paper unless otherwise stated, corrected temperatures only are reported. The reproducibility of the programme temperature was found to have a standard deviation of approximately 0·2 °C so that corresponding peak temperatures of duplicate thermograms generally agreed within 0·4 degC.

(*b*) *Procedure.* Perkin–Elmer hermetically sealed sample pans were used because preliminary trials indicated that milk fat samples tended to creep out of the normal sample pans by capillary action along the side seams.

Milk fat fractions were completely melted at 60 °C and 4–9 mg of sample was

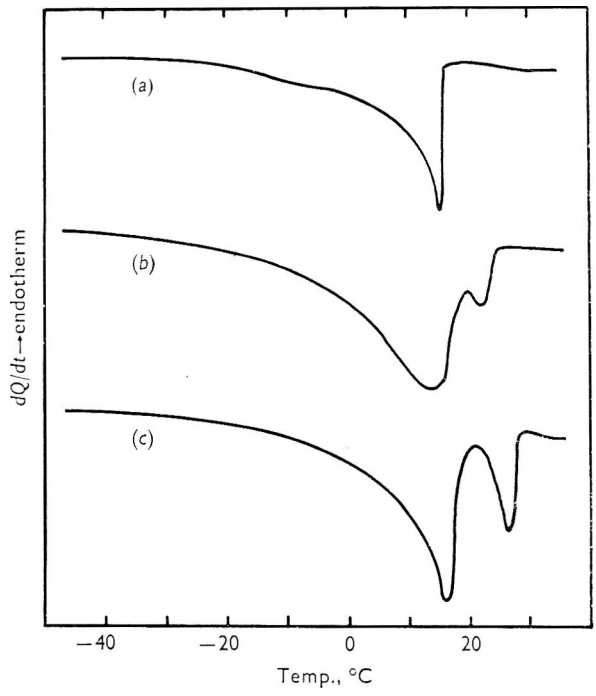

Fig. 1. The crystallization thermogram of milk fat A and its solid and liquid fractions. *a*, Liquid fraction; *b*, original milk fat; *c*, solid fraction.

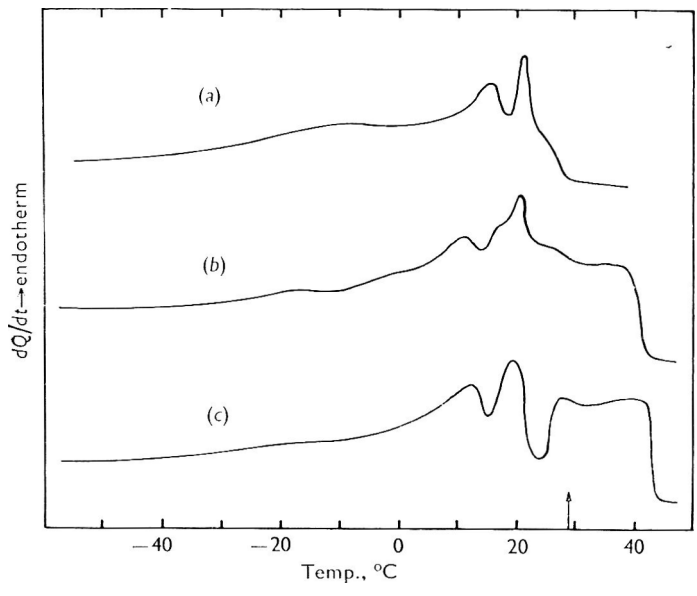

Fig. 2. The melting thermograms of milk fat A and its solid and liquid fractions. The arrow on the *X*-axis indicates the crystallization temperature in the original fractionation. *a*, Liquid fraction; *b*, original milk fat; *c*, solid fraction.

quickly transferred to the sample pan using a glass capillary. To ensure representative sampling the entire procedure was carried out while the fat was completely liquid. The sample pans were then sealed and loaded into the instrument and aluminium covers were placed over the sample holders. The instrument was held isothermally for 15 min at 60 °C to erase previous thermal history, and a cooling thermogram was then recorded down to −60 °C at a rate of 8 degC/min. The instrument was then held at this temperature for 5 min prior to recording a heating thermogram at a rate of 8 degC/min up to 60 °C. Duplicate readings were always obtained on a fresh sample taken at a later date.

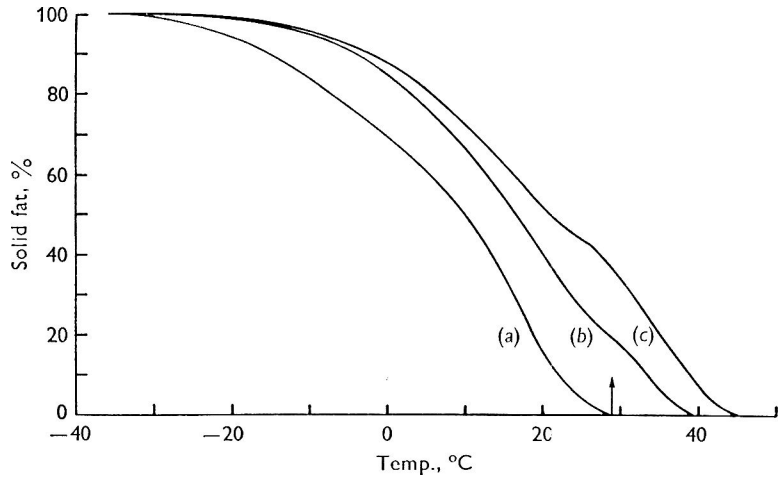

Fig. 3. The variation in solid fat content with temperature for milk fat A and its solid and liquid fractions. The arrow on the X-axis indicates the crystallization temperature in the original fractionation. *a*, Liquid fraction; *b*, original milk fat; *c*, solid fraction.

(*c*) *DSC measurement of solid fat content.* To compare the liquid and solid fractions with the original milk fat on a quantitative basis, a normalized integral curve was constructed for each melting thermogram. Such curves give a useful relative measure of the proportion of solid fat present in a fat mixture at a given temperature (Sherbon, 1963; Yoncoskie *et al.* 1969). The curves were derived by drawing a straight baseline between the upper and lower melting limits, calculating the total area between the DSC trace and the baseline, and then measuring the partial areas corresponding to different temperatures. The solid fat content at temperature T is then given by the ratio of the partial area above temperature T to the total area. The main difficulty in this procedure is the selection of the lower melting limit because the initial slope of the melting curve is quite small. However, this does not greatly affect the accuracy of the integration because only a thin wedge of very small area is involved. The precision of the measurement is good: duplicated curves showed a standard deviation of approximately one absolute percentage unit at any temperature.

RESULTS

The chemical properties of the orginal fats and their fractions are shown in Tables 1 and 2. The softening points showed differences of 14–15 °C between the liquid and

solid fractions. As anticipated, the iodine, saponification and Reichert values were higher in the liquid than in the solid fractions (Table 1). Refractive indices, however, showed negligible differences between fractions. Both vitamin A and carotene tended to concentrate in the liquid fractions as did cholesterol though to a lesser degree (Table 2).

Table 1. *Softening points, saponification, iodine and Reichert values and refractive indices of the original milk fats and their liquid and solid fractions*

	Milk fat A			Milk fat B			Milk fat C		
Property	Liquid	Original	Solid	Liquid	Original	Solid	Liquid	Original	Solid
Softening point, °C	22·6	33·8	36·2	22·7	33·8	37·5	22·9	33·4	37·6
Iodine value	41·6	39·1	36·3	41·9	38·8	36·9	40·7	38·7	36·2
Saponification value	228·1	226·2	225·8	228·4	226·3	224·8	230·6	228·4	226·3
Reichert value	28·93	26·24	25·19	28·66	26·07	24·37	30·03	27·61	25·28
Refractive index, n^{40}	1·4550	1·4550	1·4548	1·4550	1·4550	1·4548	1·4548	1·4548	1·4548

Table 2. *The vitamin A, carotene, total colour and cholesterol contents of original milk fats and liquid and solid fractions*

	Milk fat A			Milk fat B			Milk fat C		
Property	Liquid	Original	Solid	Liquid	Original	Solid	Liquid	Original	Solid
Vitamin A, μg/g fat	9·8	8·4	6·6	10·0	9·2	8·2	9·6	8·6	7·7
Carotene, μg/g fat	7·5	6·8	5·7	7·6	7·0	6·7	8·5	7·7	7·2
Total colour, as 'carotene', μg/g fat	9·0	8·8	7·3	9·0	8·5	7·5	11·0	10·5	8·5
Cholesterol, mg/100 g fat	250	240	220	250	230	230	260	250	220

The fatty acid compositions of the original milk fats and the solid and liquid fractions are shown in Table 3. In addition to the fatty acids reported in Table 3, traces of saturated acids $C_{13\ 0}$, $C_{17:0}$, $C_{19:0}$ and $C_{20:0}$, monoene acids $C_{12:1}$ and $C_{17:1}$, and branched chain acids $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{17:0}$, $C_{18:0}$, were detected on the chromatograms. Differences in fatty acid composition were not marked but short chain saturated fatty acids ($C_{4:0}$–$C_{12:0}$) were found to concentrate in the liquid fraction while long chain saturated fatty acids, especially palmitic ($C_{16:0}$) and stearic ($C_{18:0}$) acids, concentrated in the solid fraction. Unsaturated fatty acids were more concentrated in the liquid fraction, although elaidic acid ($C_{18:1}$ *trans*) showed the reverse tendency. Table 4 shows the proportions of 3 groups of fatty acids in the milk fats and in their solid and liquid fractions. It will be seen that the proportion of $C_{18:1}$ *trans* in the total unsaturated fatty acids was considerably higher in the solid than in the liquid fractions.

The high melting glyceride fraction (HMGF) obtained from milk fat A by crystallization from acetone comprised approximately 25% of the solid fraction. In comparison with the entire solid fraction the proportion of saturated acids in the HMGF was very high while the proportions of short chain and unsaturated fatty acids (mainly $C_{18:1}$ *trans*) were low (see Table 5).

The crystallization and melting thermograms for milk fat A and the solid and liquid fractions are shown in Figs 1 and 2 respectively. The thermograms all have

sharp upper temperature limits but the lower temperature limits were less well defined. As expected the thermograms for the original milk fat were intermediate between those for the solid and liquid fractions.

Table 3. *The fatty acid composition (% methyl ester) of original milk fats and their liquid and solid fractions*

Fatty acid	Milk fat A			Milk fat B			Milk fat C		
	Liquid	Original	Solid	Liquid	Original	Solid	Liquid	Original	Solid
4:0	2·5	2·0	1·4	1·9	1·7	1·8	2·4	1·9	1·3
6:0	2·0	1·8	1·3	1·8	1·6	1·5	2·3	1·9	1·5
8:0	1·3	1·1	0·9	1·2	1·1	1·0	1·6	1·3	1·1
10:0	2·9	2·6	2·2	2·8	2·6	2·6	3·7	3·4	2·9
11:0*	0·3	0·2	0·2	0·2	0·3	0·2	0·3	0·3	0·3
12:0	3·0	2·8	1·5	3·2	2·6	3·1	3·8	4·2	3·4
14:0	10·1	10·3	10·8	10·6	9·5	10·9	10·8	11·9	10·9
14:1†	2·0	1·7	1·7	2·2	1·9	2·1	1·9	2·0	1·8
15:0	1·1	1·0	1·2	1·2	1·0	1·2	1·1	1·1	1·1
16:0	26·2	27·5	29·7	26·4	27·9	30·0	25·1	28·0	29·0
16:1	1·7	1·0	1·0	1·7	1·3	1·0	1·3	1·2	1·0
18:0	12·5	14·9	17·0	11·9	15·1	15·3	12·4	13·2	16·2
18:1 *cis*	26·6	25·0	21·9	27·1	24·7	21·0	23·9	20·3	19·5
18:1 *trans*	4·4	5·6	6·7	3·8	5·9	6·0	5·0	5·7	6·0
18:2	1·3	0·9	0·8	1·4	1·0	0·8	1·6	1·5	1·6
18:3	2·1	1·6	1·7	2·6	1·8	1·5	2·8	2·1	2·4

* Includes $C_{10:1}$ fatty acid.
† Includes $C_{15:0}$ branched chain and $C_{14:2}$ fatty acids.

Table 4. *Fatty acid composition expressed as various fatty acid groups (% of total methyl esters) in the milk fats and their liquid and solid fractions*

Class of fatty acid group	Milk fat A			Milk fat B			Milk fat C		
	Liquid	Original	Solid	Liquid	Original	Solid	Liquid	Original	Solid
Short chain saturated acids, $C_{4:0}$–$C_{12:0}$	12·0	10·5	7·5	11·1	8·9	10·2	14·1	13·0	10·5
Medium and long chain saturated acids, $C_{14:0}$–$C_{18:0}$	49·9	53·7	58·7	50·1	53·5	57·4	49·4	54·2	57·2
Unsaturated fatty acids	38·1	35·8	33·8	38·8	36·6	32·4	36·5	32·8	32·3
$C_{18:1}$ *trans* as % total unsaturated acids	11·6	15·7	19·8	9·5	16·1	18·5	13·7	17·4	18·6

The crystallization thermogram of the liquid fraction, Fig. 1(*a*), showed one skewed peak with a temperature at peak maximum (t.p.m.) of 14·2 °C. The crystallization thermogram for the original milk fat A, Fig. 1(*b*), had 2 peaks; a skewed main peak (t.p.m. = 13·8 °C) and a small, partly resolved 'shoulder' peak (t.p.m. = 21·4 °C). The solid fraction crystallization thermogram, Fig. 1(*c*), had a skewed main peak (t.p.m. = 14·8 °C), and a large, high temperature peak (t.p.m. = 25·7 °C) which were almost completely resolved.

The melting thermogram for the liquid fraction (Fig. 2(*a*)) showed 2 partially resolved main peaks (t.p.m. = 21·1 and 17·4 °C) and had an upper melting limit of 25·2 °C. The melting thermogram for the original fat (Fig. 2(*b*)) also showed 2 peaks

(t.p.m. = 8·1 and 16·0 °C) but there was a 'shoulder-plateau' which was not present in the liquid fraction thermogram. The upper melting limit was 37·6 °C. The melting thermogram for the solid fraction (Fig. 2(*c*)) was similar to that of the original fat except that the high-temperature melting plateau was more pronounced and was well separated from the lower temperature peaks (t.p.m. = 8·7 and 16·2 °C). The upper temperature limit was 41·4 °C. The thermograms for milk fat fractions B and C closely paralleled the above and corresponding peak temperatures agreed within 1 degC.

Table 5. *The fatty acid composition (% methyl ester) of the HMGF isolated from the solid fraction of milk fat A by acetone crystallization*

Fatty acid	Methyl ester, %
6:0	Trace
8:0	Trace
10:0	0·2
12:0	2·8
14:0	8·9
14:1	0·2
15:0	1·5
16:0	42·5
Unidentified	0·8
17:0	1·5
18:0	33·4
18:1 *cis*	2·2
18:1 *trans*	4·5
18:2	0·5
19:0	0·5
20:0	0·5

The variations in solid fat content with temperature for milk fat A and the solid and liquid fractions are shown in Fig. 3. As expected, the integral curves demonstrate that at any temperature the solid fat content increased in the order: liquid, original, solid, although all 3 curves converged towards a lower melting limit of approximately −30 °C.

The solid fraction at the temperature used for crystallization in the fractionation process contained approximately 65 % liquid phase. In view of this contamination it is not surprising that the difference in chemical composition between the solid and liquid fractions was comparatively small.

The integral curves (Fig. 3) for the liquid fraction and original fat were almost smooth while that for the solid fraction showed a clear discontinuity corresponding to the 'melting gap' in the thermogram (Fig. 2*c*). Table 6 shows the variation in solid content with temperature for all fat fractions from runs A, B and C.

The softening points of the milk fats and fractions (Table 1) all corresponded to the temperatures at which the solid fat content of the sample was 2 % or less.

The crystallization and melting thermograms for the high melting glyceride fraction (HMGF) isolated from solid fraction A by crystallization from acetone (Fig. 4) showed the typical monotropic polymorphic transitions associated with simple glycerides such as tristearin (Chapman, 1965; Lutton & Fehl, 1970). After rapid cooling at 16 degC/min (Fig. 4(*a*)) the HMGF when heated at 16 degC/min gave the thermogram shown in Fig. 4(*c*). The HMGF apparently crystallized from

the quickly cooled melt in an unstable form. On heating, this unstable form melted (Fig. 4(*c*), low temperature endotherm) but the resultant liquid was supercooled so that crystallization occurred immediately with formation of a stable form (Fig. 4(*c*) exotherm). The overlapping melting and recrystallization processes were probably accompanied by a direct transformation of the unstable to the stable form. As the temperature increased further, the stable form melted (Fig. 4(*c*), high temperature endotherm). Re-crystallization of the quickly cooled HMGF by holding for 5 min above the melting range of the unstable form but below that of the stable form con-

Table 6. *Percentage of solid fat in original milk fats and fractions at various temperatures*

Temp.,	Milk fat A			Milk fat B			Milk fat C		
°C	Liquid	Original	Solid	Liquid	Original	Solid	Liquid	Original	Solid
0	63·1	79·5	84·7	64·4	80·2	84·5	64·1	80·3	84·0
5	52·0	69·3	76·1	54·6	70·1	75·9	53·3	70·8	75·8
10	38·7	58·6	64·5	39·4	58·6	65·8	36·3	59·7	65·7
15	21·4	43·8	54·9	22·8	43·2	54·1	19·6	45·1	55·3
20	5·2	30·4	46·5	4·9	30·5	46·0	4·0	31·0	48·2
25	0	19·2	36·1	0	19·6	36·7	0	19·7	39·3
30	0	9·0	23·3	0	9·3	24·2	0	9·1	26·6
35	0	0	9·4	0	0	10·3	0	0	12·6

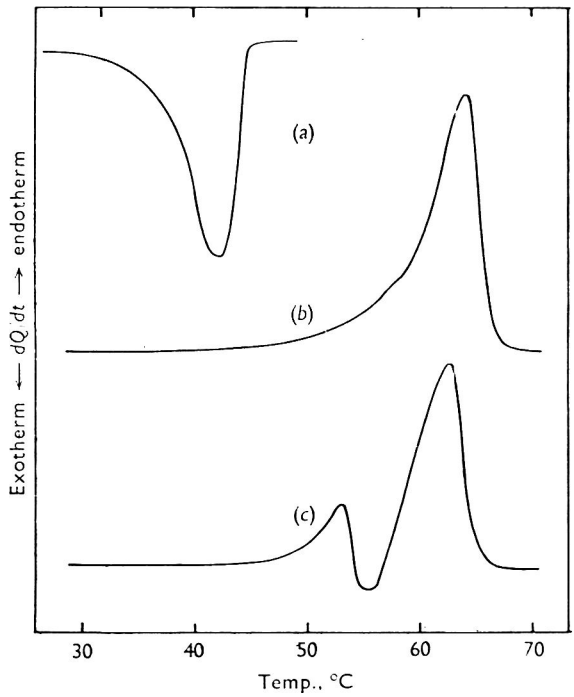

Fig. 4. The crystallization and melting thermograms of the high-melting glyceride fraction (HMGF). *a*, The crystallization thermogram (16 degC/min); *b*, the melting thermogram of the tempered HMGF (16 degC/min); *c*, the melting thermogram of the untempered HMGF (16 degC/min).

verted all the sample to the stable form. The melting thermogram for the HMGF tempered in this way is shown in Fig. 4(*b*). The corrected peak temperature of 57·3 °C agrees well with the upper melting limit of 57·8 °C reported by Sherbon (1963) for a similar HMGF.

DISCUSSION

The results presented in this paper demonstrate that fractionation of milk fat in a commercial plant can produce marked differences in the physical properties of the milk fat fractions. In view of the 14–15 °C difference in softening points between the solid and liquid fractions the differences in chemical properties were, however, less than might have been expected.

The higher iodine values in the liquid compared with the solid fractions are consistent with the differences in fatty acid composition (see Tables 3 and 4). Comparable results have been reported by Fjaervoll (1970) for milk fat fractionated under similar conditions.

In view of the high correlation between iodine value and refractive index in milk fat (McDowell & Creamer, 1970; Reil, 1962) the slight differences, if any, between the refractive indices of the original milk fat and the solid and liquid fractions appear surprising. McDowell & Creamer (1970) also showed, however, that there is a high negative correlation between the refractive index and the Reichert value. Thus, the expected increase in refractive index in the liquid fraction, due to increased proportions of unsaturated fatty acids, is largely compensated for by the opposite effect of the increased proportions of lower chain (C_4–C_{10}) fatty acids.

The levels of vitamin A and carotene in the original milk fats (Table 2) were typical of those for New Zealand summer and autumn samples (McDowell & McDowall, 1953). Both tended to be concentrated in the liquid fractions. Cholesterol showed the same tendency but to a lesser extent.

The fatty acid composition of the milk fat fractions was similar to that reported by deMan (1968*a*) who used pressure filtration to remove crystallized fat formed at 30, 27 and 24 °C and by Fjaervoll (1970) who used the system described in the present paper. Grouping of fatty acids into classes as in Table 4 showed that short chain and unsaturated fatty acids were concentrated in the liquid fraction and the medium and long chain saturated acids in the solid fraction.

The fatty acid composition of the HMGF (Table 5) differed quite considerably from that of the solid fraction, especially in the proportions of palmitic, stearic and oleic acids. Similar results for a HMGF have been reported by deMan (1968*b*) and by Sherbon & Coulter (1966).

The content of *trans*-octadecenoic acid in the original milk fats (see Table 3) was within the range reported by Cornwell, Backderf, Wilson & Brown (1953), Antilla (1966) and deMan (1968*b*). The *trans*-isomers are reported by Hay & Morrison (1970) to consist mainly of the $\Delta 11$ isomer (MP 44 °C) while the *cis*-isomer is mainly the $\Delta 9$ isomer (MP 10·5 °C). In general, the melting points of *trans*-monoene triglycerides are almost as high as those of the corresponding trisaturated glycerides (Hartman, 1958). This accounts for the occurrence of a higher proportion of *trans*-octadecenoic acid in the solid than in the liquid fractions and a still higher proportion in the HMGF. The latter observation is in agreement with the finding of deMan (1968*b*)

that, of 14% of unsaturated fatty acids in the HMGF of milk fat, 80% were in the *trans* form.

The major differences between the thermograms of the liquid, solid and original milk fats are readily explained by the removal of triglycerides melting above 25°C from the liquid fraction and the concentration of these in the solid fraction which, however, still contains a considerable proportion of triglycerides melting below 25°C. The explanation of more detailed differences requires reference to the formation of solid solutions in fat crystallization (Mulder, 1953; Sherbon & Coulter, 1966). The solid fraction consists of a solid phase contaminated with occluded liquid phase and may therefore be considered to be derived from the original milk fat by addition of this solid phase. The appearance of the melting gap in the solid fraction thermogram (Fig. 2(*c*)) was probably the result of the incorporation of some intermediate-melting (16–24°C) glycerides in solid solution with this additional solid phase. DeMan (1961*b*) found a similar solid solution effect on adding a high melting glyceride fraction to normal milk fat. As a result of the re-crystallization occurring during the commercial fractionation process, the liquid fraction probably contained dissolved glycerides which, in the original milk fat, would melt above the fractionating temperature. The presence of these dissolved glycerides is demonstrated in the melting thermogram (Fig. 2(*a*)) because the 2 peak temperatures of the liquid fraction were higher than those of the untreated milk fat (Fig. 2*c*). Such an increase in melting point is the typical effect of the solid solution of a high melting fat in a low melting one (Hannewijk, Haighton & Hendrikse, 1964). The composition of the glycerides melting below about 0 degC is unaffected by the presence or absence of the higher melting glycerides (Hannewijk *et al.* 1964) so that the original milk fat and both the fat fractions have approximately the same lower melting limit and the solid-fat content curves converge towards this limit.

The shapes of the melting and crystallization thermograms differ because the crystallization of plastic fats is mainly dependent on kinetic effects such as supersaturation, supercooling and diffusion, whereas the dynamic melting of fats is largely independent of such solution effects (Hannewijk *et al.* 1964). Supersaturation effects were mainly responsible for the precipitation of the high melting glycerides of the solid fraction (Fig. 1*c*) at a much higher temperature than those of the untreated milk fat (Fig. 1*b*). The crystallization peaks for the fat fractions were sharper than those of the untreated fat (Fig. 1). It has been found (Norris & Taylor, 1970, unpublished) that chromatography of milk fat on a silicic acid column to separate the triglycerides from other lipid material resulted in much sharper peaks in the crystallization thermogram of the triglycerides although the position of the peaks and the shape of the melting thermograms were unaltered compared with the original milk fat. This effect was probably due to the removal of polar compounds. It is likely that the fat fractions contain less polar compounds than the untreated fat because of the more effective washing in the fractionation process.

The HMGF probably accounted for the major proportion of the solid fat present in the solid fraction A at the crystallization temperature of 25°C. However, the high melting temperature of the HMGF suggests that it precipitates from acetone solution independently of the intermediate melting glycerides which, in the DSC, co-precipitate with the high melting glycerides of the solid fraction. A major difference between

the thermal analysis of the HMGF and the commercial milk fat fractions was the detection of polymorphic transitions in the former but not in the latter. The minima in the melting thermograms of the commercial fat fractions (Fig. 2) were not associated with polymorphic exothermic transitions superimposed upon normal melting endotherms because tempering (recrystallization) of these fats in the DSC did not result in significant changes in these minima. This confirms the thermal analysis work of Sherbon (1963) who was unable to detect polymorphic transitions in milk fat and milk fat fractions isolated from the melt.

The main source of error in the determination of the solid fat content involves the implicit assumption that the heat of fusion of triglycerides and triglyceride mixtures is a constant. In general, however, the heat of fusion shows a positive correlation with melting point (Sherbon, 1963). Calculations using Sherbon's data to estimate the magnitude of this error resulted in corrected solid fat contents for the solid fractions which were lower than those given in Table 6 by approximately 10% in the temperature range 10–35 °C compared with 5% for the original milk fats and 1% for the liquid fractions. The uncorrected results, however, are still valid for comparative purposes. Since the solid fat content is the most important parameter determining the consistency of a plastic fat, curves such as Fig. 3 can give a general indication of the suitability of a fat for a particular purpose.

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Properties of aseptically packed UHT milk: casein modification during storage and studies with model systems

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Summary. Storage of aseptically packed UHT milk produced changes in the electrophoretic pattern of the milk caseins when the milk was stored at ambient or higher temperatures. Lower temperature storage at 4 °C did not give rise to these changes.

The alterations in electrophoretic properties of the caseins appeared to be due to the action of carbonyl compounds, produced by a Maillard type of reaction, which led to changes in the charge of the protein together with some degree of polymerization. These conclusions have been drawn from results obtained on model systems of casein–lactose subjected to various heat treatments, and on casein and milk treated with acetaldehyde.

Changes in the sensitivity to calcium ions of individual caseins, whole casein and milk that had been subjected to various heat treatments or to treatment with acetaldehyde showed that all these different treatments gave rise to modified casein which, in general, became less sensitive to calcium. κ-Casein when treated alone rapidly lost its ability to protect α_{s1}-casein from precipitation by calcium ions, while α_{s1}-casein treated alone only gradually became more soluble in the presence of calcium. Thus, on treating whole casein there was evidence for a stability minimum when the protective ability of the κ-casein has been destroyed without a compensating gain in the stability of the α_{s1}-casein.

The importance of these changes in relation to the stability of UHT milks has not yet been elucidated but the results indicate that cross linking between protein chains and changes in calcium sensitivity occur during long-term storage.

The ultra-high-temperature (UHT) treatment for milk sterilization coupled with aseptic packaging is nowadays a commercially viable method of obtaining a product capable of remaining in good condition for several months of storage without the necessity of refrigeration. The types of UHT plant and the effects of processing milk in this way have been comprehensively reviewed (Burton, 1969). The results of such processing on the protein components of milk appear to be largely confined to partial denaturation of the whey proteins and some aggregation of these and the caseins (Burton, 1969; Morr, 1969). Although effects on the nutritional value of the milk and on the amino acid content appear to be minimal, it has been reported that following UHT processing there is an increase in the amount of sugar bound to α-casein (Albonico, Prati, Resmini & Zanini, 1966) and Burton (1969) suggests that this

indicates that a preliminary stage of the Maillard reaction may have occurred (Maillard, 1912).

In addition to these consequences of the UHT process itself, further changes occur during storage. These also have been reviewed by Burton (1969) and include a gradual deterioration in the milk flavour and increases in non-protein nitrogen and viscosity (Swartling, 1967). The formation of a sediment has been reported by several workers and we have also observed that on prolonged standing there was a partial physical separation of the phases which appeared to be readily reversed by agitation. In practice, the ultimate shelf life of UHT milk is finally decided by the onset of gelation which occurs quite suddenly but is preceded by a more gradual development of instability, as judged by the alcohol precipitation test or the effects of heat or low pH on the milk system. Among the chemical changes which occurred during storage of UHT milk some of the most readily apparent were changes in the patterns given by the caseins on examination by starch gel electrophoresis (G. C. Cheeseman, unpublished observations). A further study of these changes has been undertaken and the results are now presented.

MATERIALS AND METHODS

Milk and casein samples

UHT milk in aluminium-lined 1-pint Tetrapaks was obtained from a local retail store. Samples which were stored at 4 and 30 °C were from the same batch.

'Whole casein' was prepared by adjusting the pH of milk to 4·6 by addition of 1 N-HCl. The precipitate was filtered off, redissolved in a volume of distilled water equal to the volume of milk taken by adjusting the pH to 7·0 with 1 N-NaOH, and reprecipitated at pH 4·6. This procedure was repeated twice more. Small traces of lipid coprecipitate were removed by chloroform–methanol extraction (Folch, Lees & Sloane Stanley, 1957). The casein was air-dried at room temperature.

Partially purified α_s-casein was prepared according to Hipp, Groves, Custer & McMeekin (1952). β-Casein (Aschaffenburg, 1963) and κ-casein (Zittle & Custer, 1963) were also prepared in a moderately pure state. These were all prepared from the fresh raw milk of an individual cow genetically typed α_{s1}-casein A, β-casein A^1A^2 and κ-casein A. The final precipitation and drying stages of all these methods were omitted and instead the individual caseins were further purified by chromatography on a DEAE cellulose column. The concentrations of the final solutions obtained above were calculated from the absorbance at 280 nm and aliquots of the casein solutions containing 6–7 g protein in a volume of not more than 70 ml were applied to a DEAE cellulose column (7·5 × 27 cm) made up in 0·02 M tris–HCl buffer of pH 7·0 containing 10^{-4} M EDTA and 6·6 M urea. The column was then eluted with stepwise increases in NaCl concentration in tris–HCl–urea buffer. Non-absorbed impurities were washed through with 1·5 l salt-free buffer which was followed by 1·5 l buffer containing 0·05 M-NaCl, 1·5 l containing 0·075 M-NaCl, 1·5 l containing 0·1 M-NaCl, 1·5 l containing 0·175 M-NaCl and 1·5 l containing 0·25 M-NaCl. The column effluent was collected in 5-ml fractions and the optical density of each fraction at 280 nm recorded. κ-Casein was eluted in 0·075 M-NaCl, β-casein in 0·1 M-NaCl and α_{s1}-casein in 0·175 M-NaCl. The peak fractions containing the pure individual caseins were

pooled, dialysed throughly against distilled water to remove urea and salts, concentrated against Carbowax 20-M (Union Carbide Co.), further dialysed against distilled water and stored frozen. Final concentrations were about 7–9 %. Examination of the purified caseins by starch-gel electrophoresis revealed that the β- and α_{s1}-caseins migrated as single bands and that the κ-casein migrated as a single series of bands such as would be expected from components with different sialic acid content (Alais & Jollès, 1961; Armstrong, Mackinlay, Hill & Wake, 1967). Hence all preparations were essentially pure within the limits of detection of impurities by this procedure.

Calcium sensitivities

For calcium sensitivity measurements an approximately 3 % solution of the protein under investigation was made up in 0·1 M sodium acetate buffer of pH 6·5. Portions (0·5 ml) were placed in a series of centrifuge tubes and 0·1 M-$CaCl_2$ in 0·1 M-sodium acetate buffer of pH 6·5 was added in graded volumes from zero to 4·5 ml. The content of each tube was made up to 5·0 ml by addition of an appropriate volume of the buffer solution. The tubes were then warmed at 30 °C for 30 min and centrifuged at 2000*g* for 20 min. Portions (1 ml) of supernatant were diluted with 4 ml water or, if this resulted in turbid solutions, with 4 ml 6·6 M urea. Optical densities at 280 nm were measured and expressed as a percentage of the reading obtained when no $CaCl_2$ was added.

Analytical methods

Whatman DE-52 macrogranular anion-exchange cellulose was used for the DEAE cellulose column chromatography. Before use the material was recycled through the acid and base forms by washing sequentially with 0·5 N-HCl, water, 0·5 N-NaOH and water. The washed cellulose powder was then suspended in 6·6 M urea made up in 0·02 M tris–HCl buffer of pH 7·0 containing 10^{-4} M-EDTA and washed with this solvent until the pH was constant at pH 7·0, and then packed into a 2×30 cm column. The column was washed with tris–HCl–urea buffer for a minimum of 24 h to allow the cellulose to pack down. The sample was then applied in a total volume of not more than 5 ml and rinsed onto the column with 50 ml tris–HCl–urea buffer before the application of a salt gradient varying from zero to 0·25 M-NaCl in the tris–HCl–urea buffer.

Sephadex G-100 was used for gel filtration, the column dimensions being $2{\cdot}0 \times 40{\cdot}0$ cm. The solvent used for swelling the Sephadex, making up the column and all subsequent washings and elutions was 0·02 M-tris–HCl buffer of pH 7·0 containing 6·6 M-urea and 10^{-4} M-EDTA. For some experiments 0·03 M-2-mercaptoethanol was added to this buffer. Samples were applied in a volume of 2·0 ml and 3·0-ml fractions were collected.

Sedimentation velocity runs were conducted with a Beckman Spinco Model E centrifuge using a type An-D rotor fitted with single-sector cells with aluminium centre pieces and run at 42040 rev/min and at 24 °C. All sedimentation coefficients (s-values) are uncorrected.

Starch-gel electrophoresis (SGE) was routinely carried out using gels made up in 6·6 M-urea containing 2-mercaptoethanol (Aschaffenburg & Michalak, 1968). All

chemicals used were of Analytical Reagent grade and were obtained from BDH Ltd, Poole, England. Glass-distilled water was used in the preparation of all the solutions.

RESULTS

Properties of caseins isolated from UHT milk

Earlier observations (G. C. Cheeseman, unpublished) of diffuse streaky patterns obtained on the SGE examination of caseins obtained from UHT milk after long-term storage at 30 °C have been confirmed on several occasions during the current investigation. Typical results are shown in Plate 1 (*a*) and it may be seen that whereas

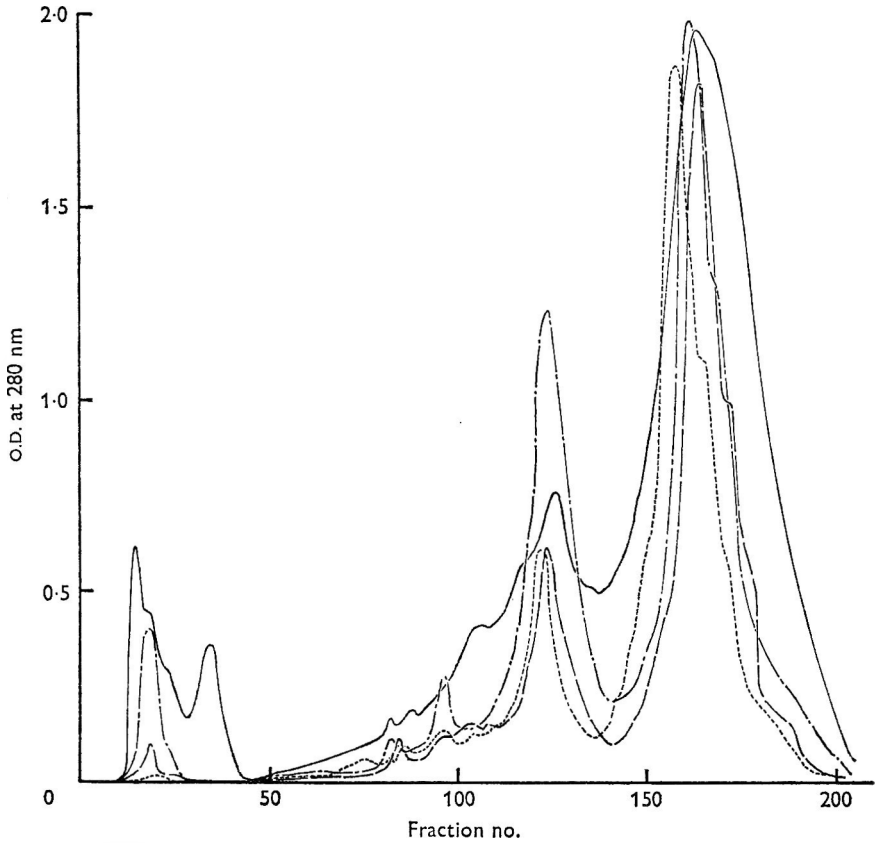

Fig. 1. DEAE-cellulose column (2 × 30 cm) chromatography of lipid-extracted whole casein from various milk samples. The solvent was 6·6 M urea in 0·02 M-tris–HCl buffer of pH 7·0 containing 10^{-4} M-EDTA with an NaCl gradient of 0–0·25 M (see text for details). ——, UHT milk stored at 30 °C for 1 year; – –, fresh UHT milk; - - - -, UHT milk stored at 4 °C for 1 year; — — —, fresh raw milk. Each fraction was of 5 ml. Fractions 10–40 correspond to unabsorbed or only weakly absorbed material, fractions 80–100 to κ-casein, fractions 115–135 to β-casein and fractions 150–190 to α_{s1}-casein.

acid-precipitated 'whole caseins' from fresh UHT milk or from UHT milk which has been stored at 4 °C for 12 months gave sharply defined protein bands, samples from UHT milk stored at 30 °C for 12 months did not. It may also be seen from Plate 1 (*a*) that the patterns obtained when the sample papers were removed after 30 min

running time were identical to those obtained when the sample papers were not removed. Thus, the streaky patterns did not arise as a result of the formation of aggregates or complexes from which the individual components are only gradually released into the gel matrix.

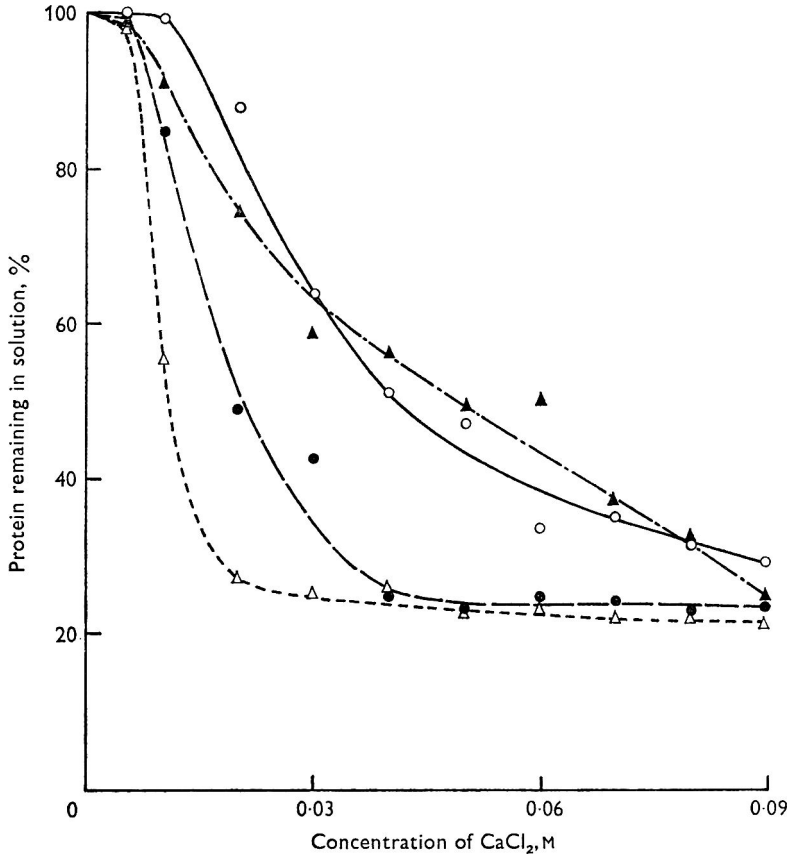

Fig. 2. Solubility of lipid-extracted whole caseins (3·5 %, w/v) from various milk samples in the presence of calcium ions. ○—○, Fresh raw milk; ●—●, fresh UHT milk; △. . . .△, UHT milk stored at 30 °C for 1 year; ▲·–·▲, UHT milk stored at 4 °C for 1 year. Similar results were obtained in 3 other experiments.

Extensive dialysis at 4 °C of casein samples against several changes of 0·02 M-tris–HCl buffer of pH 8·6 containing 0·025 M-EDTA showed that removal of metal ions did not alter the SGE patterns. Likewise, solvent extraction of the whole casein preparations to remove any accompanying lipid and to dissociate any lipoprotein material did not affect the SGE patterns to any observable extent. Examination by thin-layer chromatography (Storry & Tuckley, 1967) of the small quantities of lipid extracted showed that in all cases it resembled the lipid content of whole milk and consisted predominantly of saturated triglycerides. Although there was some evidence for a slightly larger content of polar lipoprotein-like material, this was similar with all the casein samples and the results did not indicate significant interaction between protein and any specific lipid component during storage.

The observed loss of resolution of casein bands on SGE following storage at 30 °C has a counterpart in the results of DEAE-cellulose chromatography. In the absence of 2-mercaptoethanol small quantities of κ-casein adhered strongly to the DEAE-cellulose and were not eluted with the main κ-casein peak at the expected position in the salt gradient (Thompson, 1966) but contaminated later fractions. It was considered undesirable to include a further factor such as 2-mercaptoethanol into the model system and for comparative purposes this phenomenon can be ignored. Fig. 1 shows that whereas whole casein from fresh raw milk, fresh UHT milk and UHT milk stored at 4 °C for 12 months all gave elution profiles showing a clear separation of α_{s1}-, β- and κ-caseins, with material isolated from UHT milk stored at 30 °C for 12 months the separation was not so distinct. Thus, although the main regions were still well resolved there was evidence of a general 'smearing' of the peaks with more overlap so that, for example, the trough between the peak of β-casein elution and the peak of α_{s1}-casein elution was not so deep as with the other preparations. Solvent extraction of lipid from the casein samples prior to DEAE-cellulose chromatography had no observable influence on the subsequent elution profile except for removal of traces of material eluted in the region just before the κ-casein region.

Measurements of the stability of solutions of whole casein prepared from the various milks in the presence of calcium ions (Fig. 2) showed that prolonged storage of UHT milk at 30 °C gave rise to a product that was more sensitive to the presence of calcium than was casein prepared from fresh UHT milk. Casein from fresh raw milk possessed a slightly greater stability than that from fresh UHT milk, but by contrast casein prepared from UHT milk stored for 12 months at 4 °C exhibited a stability to calcium ions equal to or slightly greater than casein from fresh raw milk.

The most obvious difference between solvent-extracted whole casein prepared from UHT milk that had been stored at 30 °C for 12 months, and similar preparations from other milks including UHT milk stored at 4 °C for 12 months, was that the former possessed a marked brown coloration. This led us to suspect that the Maillard reaction might be implicated in some of the observed changes.

Studies on model systems

The Maillard reaction involves chemical reactions between carbohydrates and proteins so it seemed logical to study initially the behaviour of a simplified system consisting of casein and lactose in a suitable buffer solution rather than the highly complex environment of milk itself. The first model system used consisted of whole casein (25 mg/ml) and lactose (50 mg/ml) in either 0·1 M sodium phosphate buffer of pH 6·8 or 0·1 M sodium acetate buffer of pH 6·5. These concentrations of casein and lactose were similar to those generally found in cow's milk.

Heating of such a model system on a boiling water bath led, after about 1 h, to the development of a clearly visible brown coloration which became progressively darker with time. Withdrawal of samples after various time intervals and examination by SGE showed a 'streaky' pattern developed which after about 2 h qualitatively resembled the pattern given by caseins prepared from UHT milk stored at 30 °C for 12 months. On longer heating, a slow-moving densely staining band developed, clearly visible after 5 h heating, and all traces of the initial casein bands were lost.

There was some evidence for an increase in the proportion of material not penetrating the gel after long heating times. A control sample consisting only of casein in buffer at the same concentration showed (Plate 2) that although in the absence of lactose similar changes in the gel pattern occurred, the time scale was quite different and the changes were not so rapid. The fast-moving bands immediately ahead of the α_{s_1}-band, apparent in slots 8–14 of Plate 2, are artifacts caused by migration of buffer salts in the discontinuous buffer system used in the procedure of Aschaffenburg & Michalak (1968) and may be observed if fresh buffer is not used in the apparatus. The absence of any true fast-moving protein component was demonstrated by re-running the same samples using the gel and buffer systems of Neelin, Rose & Tessier (1962), which gives a somewhat slower migration rate and no salt band.

Table 1. *Variation in sedimentation coefficient values* (S_{obs}) *of a casein–lactose mixture on heating at* 100 °*C*

Heating time, h	S_{obs}	Proportion exhibiting S_{obs} value, %
0	9·7	100
1	8·8	100
1·5	8·9	100
2	8·8	100
3·5	6·4	100
5	3·5	91·0
	16·2	9·0
7	3·4	72·5
	16·7	27·5
10	3·5	61·7
	18·6	38·3
0	0·8*	100
7	1·1*	72·5
	6·1*	27·5

* Runs conducted in 6·6 M-urea.

Sedimentation velocity runs showed that initially in the casein–lactose model system the casein exists in a multimeric form with a sedimentation constant (S_{obs} value) of 9·7 at a casein concentration of 0·4 % in 0·1 M-sodium phosphate of pH 6·8. Portions of the casein–lactose solution were withdrawn for ultracentrifuge runs after various times of heating at 100 °C and diluted with buffer to give a protein content of 0·4 %. The results are shown in Table 1 and it may be seen that the initial multimeric state was gradually broken down until after 3·5 h S_{obs} was 6·4. On further heating 2 peaks were observable in the ultracentrifuge: a small component of S_{obs} 3·5 and a much larger component of S_{obs} 16–18. As the time of heating was prolonged the proportion and perhaps also to some extent the size of the larger component increased. Two runs conducted in 6·6 M urea after 0- and 7-h heating suggested that this solvent dissociated the initial casein multimer to the monomeric state but had little effect on the polymers formed on heating, thus demonstrating that the polymerization was most probably the result of covalent bond formation.

In addition to a study of SGE patterns from heating of the model casein–lactose

system the behaviour on DEAE-cellulose chromatography was also examined. The general trends are clearly demonstrated in Fig. 3, from which it may be seen that from the unheated control system sharp, well-separated peaks of the individual casein components were eluted. Heating similar volumes (3 ml) of the same solutions of the casein–lactose solution portions for 1, 2·5 and 7 h caused a progressive diminution in peak heights, and loss of resolution analogous to that observed in caseins from UHT milk stored at 30 °C for 12 months (Fig. 1). There was also a progressive increase in the amount of material passing straight through the column and not being

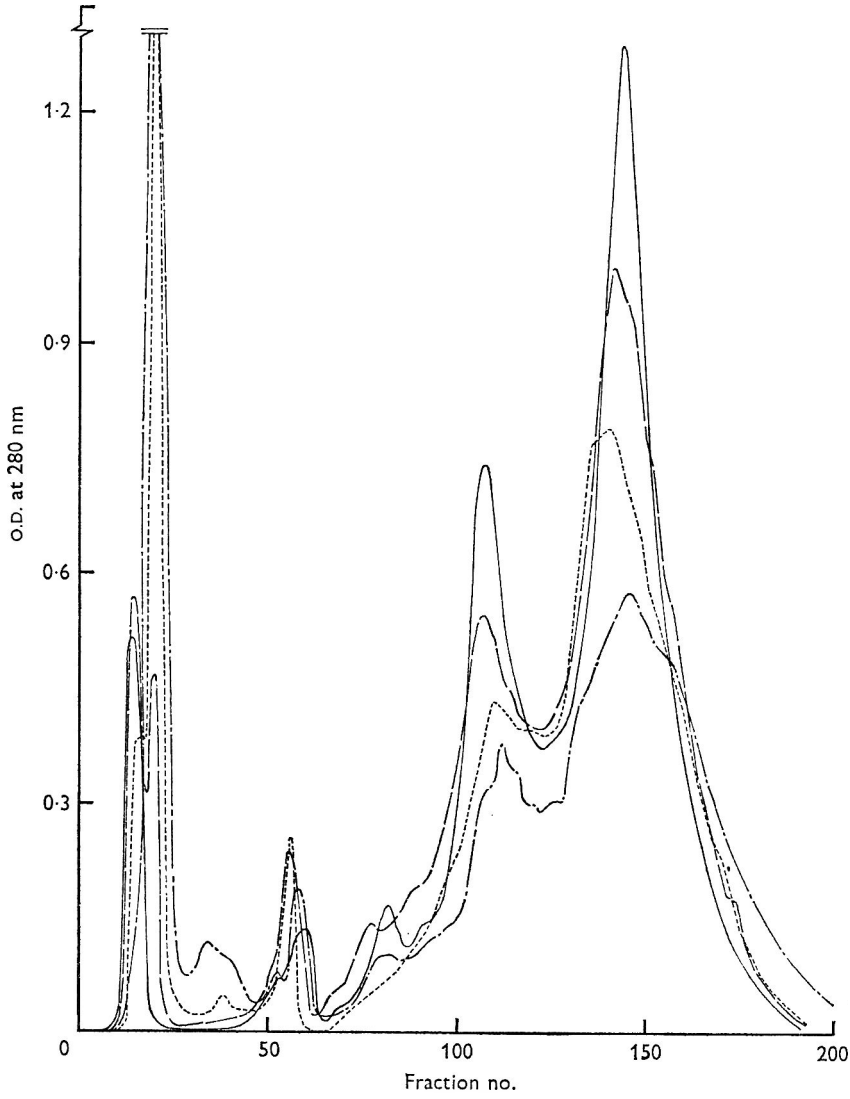

Fig. 3. DEAE-cellulose column (2 × 30 cm) chromatography of lipid-extracted whole casein heated for different times at 100 °C with lactose. ——, Control without heating; – –, after 1 h heating; - - - -, after 2·5 h heating; – — –, after 7 h heating. Each fraction was of 5 ml. Fractions 10–45 correspond to unabsorbed or only weakly absorbed material, fractions 50–90 to κ-casein-like material, fractions 100–120 to β-casein and fractions 130–180 to α_{s1}-casein.

absorbed onto the DEAE-cellulose. After 2·5- and 7-h heating this unabsorbed material was also brown coloured, although much of the most highly coloured material was absorbed strongly to the ion-exchanger and remained as a broad zone at the top of the column throughout the application of the salt gradient.

As might be expected, the sensitivity of the casein–lactose model system to the presence of calcium ions at pH 6·5 diminished with increasing time of heating at 100 °C, until after about 5 h the addition of $CaCl_2$ to a final concentration of 0·1 M was virtually without effect on the system. This loss of calcium sensitivity appeared to be most rapid within the first 15 min of heating although changes in the casein pattern on SGE were minimal in this time and no brown products of the Maillard reaction were visible. Similar experiments, carried out in an identical manner but omitting lactose, showed that under these conditions the effect of heat alone on the casein was not so marked although it was still considerable. Within the first 30 min there was some loss of calcium sensitivity which appeared to be followed by a slight recovery of sensitivity. Although the effect was not large it gave rise to a small sensitivity maximum after about 1·5-h heating, followed by a progressive slow loss of sensitivity on further heating.

When samples of α_{s1}-, β- or κ-casein were heated in 0·1 M sodium phosphate buffer of pH 6·8, either alone or with added lactose and at concentrations similar to those occurring in milk, and examined by SGE after suitable time intervals, there was no apparent difference in the rates at which the different casein bands became more diffuse, but in all cases the presence of lactose accelerated the process. After 7 h at 100 °C β-casein heated in the presence of lactose gave a broad, slow-moving band of mobility similar to or slightly lower than that obtained (Plate 2) on prolonged heating of whole casein in the presence of lactose. All 3 caseins heated alone, and α_{s1}- and κ-casein heated with lactose, gave long streaky patterns of much greater mobility. Although the rennin-sensitive bond in κ-casein is known to be exposed and susceptible to attack under a variety of conditions, there was no evidence for the formation of para-κ-casein-like material in any of these experiments; indeed the electrophoretic band, due to small quantities of para-κ-casein resulting from a slight spontaneous decomposition of the κ-casein which was generally present, became diffuse at much the same rate as the other casein bands and was no longer observable after about 1 h at 100 °C.

Purified α_{s1}-casein was very sensitive to the presence of calcium ions, less than 3 % of the initial amount (24·5 mg/ml) remaining in solution as judged by the optical density of the supernatant at 280 nm following addition of $CaCl_2$ to the level of 0·02 M or greater at pH 6·5. After heating at 100 °C for 6 h this figure was about 16 % and increased to 28 % if lactose (49 mg/ml) was also present, suggesting that heating alone caused some loss of sensitivity to calcium and that this was accelerated in the presence of lactose. The addition of κ-casein (6·15 mg/ml) protected α_{s1}-casein and α_{s1}-casein heated with or without lactose from calcium ions, there being little precipitation of protein until $CaCl_2$ levels reached at least 0·075 M. Prior heating of the κ-casein at 100 °C for 6 h, either with or without lactose, completely destroyed this protecting ability.

The addition of 0·1 ml acetaldehyde to 3·0 ml of whole casein solution (25 mg/ml) at 18 °C led to changes in the SGE patterns (Plate 3) that closely resembled those

observed previously (Plate 2) on heating casein with lactose. As the reaction proceeded the individual casein bands became less distinct and a blurred streaky pattern developed. After 2–3 h, a marked brown coloration was apparent and a broad slow-moving band was formed. This possessed a mobility similar to that observed on heating the casein–lactose system for 5 h or more (Plate 2). There was evidence that a considerable proportion of protein-containing material failed to penetrate the starch gel following long incubation times with acetaldehyde. This may suggest a greater degree of polymerization than in the heated casein–lactose system. Further evidence for polymerization was gained from the preliminary results of Sephadex G-100 gel chromatography, in which casein treated with the same levels of acetaldehyde for 6 h at 20 °C was completely excluded from the Sephadex beads and was eluted at the void volume. Material from the casein–lactose system after 7 h heating at 100 °C was also excluded but the peak was broader, suggesting that not all the

Table 2. *Variation in sedimentation coefficient values* (S_{obs}) *of casein on treatment with acetaldehyde*

Incubation time, h	S_{obs}
0	11·7
1	11·4
2	10·0
3	9·4
4·5	10·7
6	9·9
8	11·4
24	10·0
0	0·6*
24	5·6*

* Runs conducted in 6·6 M-urea.

molecules were completely excluded and that polymerization might not be so extensive and the product more heterogeneous. Under these chromatographic conditions untreated whole casein behaved quite differently, splitting into 2 peaks when 2-mercaptoethanol was not present, with κ-casein being largely excluded and α_{s1}- and β-casein both being retarded, and giving a single retarded peak when 2-mercaptoethanol was present.

For measurements of the calcium sensitivity of a casein solution following acetaldehyde treatment, 1·9 g whole casein was dissolved in 60 ml 0·1 M-sodium acetate buffer of pH 6·5. Acetaldehyde (3·0 ml) was added and the mixture kept at 18 °C. After suitable time intervals portions were withdrawn for calcium sensitivity assay. The results were very similar to those obtained on heating casein alone. A rapid loss of sensitivity to calcium within a few minutes was followed by a slight recovery of sensitivity, which was greatest after 1·5–2 h. A further very gradual loss of sensitivity continued after longer reaction times. Other experiments using individual purified α_{s1}-casein or κ-casein in concentrations similar to those occurring in milk and in the above system, clearly showed that κ-casein lost the ability to protect α_{s1}-casein from precipitation by calcium ions within less than 2 h incubation with acetaldehyde. The effect of acetaldehyde on α_{s1}-casein itself was slow, only a small loss of sensitivity to calcium being observed over a 7-h period.

Sedimentation coefficients given by the casein plus acetaldehyde mixture at the above concentrations following various lengths of time of incubation at 20 °C are shown in Table 2. It may be seen that in an aqueous buffer medium the observed sedimentation constants (S_{obs}) remained virtually unchanged throughout the experiment and only a single species was observable at any stage. Preliminary runs conducted in 6·6 M urea, however, showed that while the initial casein aggregate was broken down to the monomeric form, after 24 h reaction with acetaldehyde this solvent caused comparatively little dissociation, especially if it is taken into account that the S_{obs} values are uncorrected for the solvent density so that S_{obs} = 5·6 in the urea solvent would probably be equivalent to S_{obs} of about 10 in buffer.

The addition of 0·05 M-sodium metabisulphite to the casein–lactose model system prior to heating at 100 °C in order to remove carbonyl intermediates formed during the heating led to marked changes in the resulting SGE patterns. The rate of blurring of the individual bands was somewhat diminished although not eliminated, and within 7 h of heating at 100 °C there was no evidence for the formation of the broad, slow-moving band which developed after about 5 h (Plate 2) when sodium metabisulphite was absent. The development of a brown coloration was inhibited by the metabisulphite and, in general, the results resembled those for the heated control samples of Plate 2 in which no lactose was present, both in the overall appearance of the protein bands and in the rate at which the various bands became more diffuse.

Studies on milk

The studies on model systems have been extended to an examination of fresh skim-milk. A milk sample was centrifuged for 1 h at 100000*g* to separate micellar from soluble casein. The soluble casein in the supernatant was concentrated 5-fold by dialysis against Carbowax 20 M. A further portion of this supernatant was adjusted to pH 4·6 and the precipitated caseins filtered off. Samples of the centrifuged micellar casein pellet were resuspended either in the casein-free milk readjusted to pH 6·7 or in distilled water and together with the concentrated soluble casein solution were then dialysed for 48 h against a large volume (50-fold excess) of the original milk to equilibrate concentrations of low molecular weight components. Heating of these solutions at 100 °C for various times showed that, as judged from SGE patterns, there was no apparent difference in the rates of reaction, and that the protein bands of soluble casein or micellar casein in either milk serum or H_2O became diffuse at a similar rate to those or an untreated control sample of the original milk.

SGE patterns of heated homogenized fresh milk (Plate 4) showed distinct differences from those of the heated casein–lactose model system. The various protein bands became diffuse in a similar manner and a brown coloration developed, but even after 7 h at 100 °C there was no formation of the slow-moving band observed (Plate 2) in the model system after 4–5 h heating. The addition of sodium metabisulphite to a concentration of 0·05 M to milk prior to heating inhibited the browning reaction but had little effect on the SGE patterns (Plate 4) except for a slight reduction in the proportion of material present in the slower-moving regions of the streaky patterns given after long heating times. Addition of 25 μl acetaldehyde to 0·75 ml samples of fresh homogenized whole milk at room temperature, however, led to

browning and SGE patterns (Plate 1*b*) resembling those of the heated milk samples very closely, although in this case after incubation times of about 2 h or more there was some coagulation and a large amount of material failed to penetrate the gel.

DISCUSSION

The changes that occur in the electrophoretic patterns given by the caseins isolated from UHT milk following prolonged storage were striking and at 30 °C preceded the phenomenon of gelation. In fact none of the milk samples used in this investigation had reached the stage of gelation when the casein preparations were made.

The overall appearance of the SGE patterns suggests that at 30 °C there may be a random modification of various groups on the polypeptide chains. It may be envisaged that processes occur which are similar to those taking place in dried-milk powders and casein–glucose mixtures during storage at elevated temperatures (Henry, Kon, Lea & White, 1948; Lea, 1948; Lea & Hannan, 1950*a*, *b*, *c*). Such processes also occur in solution (e.g. Mohammad, Fraenkel-Conrat & Olcott, 1949).

The results of the present work clearly showed that the observed streaky patterns from UHT milk stored at 30 °C, or from the caseins isolated from it, may be reproduced by the application either of heat or of acetaldehyde to fresh milk or to casein plus lactose. The finding that the changes produced in SGE patterns by heating a casein–lactose mixture were largely inhibited by addition of sodium metabisulphite provided further evidence for the involvement of carbonyl compounds in the production of these changes. Although the initial stages of the Maillard reaction consist of a condensation of sugar reducing groups with the ϵ-NH_2 groups of lysine residues the later stages are less well defined but are known to give rise to many carbonyl compounds. Several aldehydes and ketones have in fact been isolated from heated casein–lactose systems (Ferretti, Flanagan & Ruth, 1970), dried whole-milk powders (Parks & Patton, 1961), sterilized milks (Patel, Calbert, Morgan & Strong, 1962), and many other heated similar materials (see Reynolds, 1965).

The participation of charged groups in various stages of the Maillard reaction might be expected to lead to a somewhat random number of modifications to the molecules of a single protein component resulting in a population of molecules with differing net charge. This would lead to ill-defined or streaky gel patterns of the kind that were observed experimentally. A further factor which would lead to changes apparent on SGE is the polymerization which is known to occur during the later stages of the Maillard reaction. This probably also proceeds via carbonyl intermediates, as simple aldehydes are well known to be capable of cross-linking polypeptide chains (Fraenkel-Conrat & Olcott, 1948*a*, *b*; Fraenkel-Conrat & Mecham, 1949; French & Edsall, 1945; Mohammad, Olcott & Fraenkel-Conrat, 1949).

The present work demonstrated that the heating of a casein–lactose mixture or the addition of acetaldehyde to casein also leads to polymerization. Sedimentation velocity studies on the casein–lactose system revealed that on heating the initial casein aggregate of close to 10 *S* was gradually dissociated. As heating was continued 2 peaks became apparent, one (16–18*S*) corresponding to higher molecular weight material than the other (3·5*S*). The 16–18*S* peak increased in proportion to the 3·5*S* peak as heating continued. When studied in 6·6 M-urea 2 peaks were still present, the

smaller molecular weight material probably being of about the same size as monomeric caseins. These results were consistent with the breakdown of the casein aggregates, possibly as far as to the monomeric state, which was subsequently followed by covalent bond formation and polymerization. Bond formation between fragments of casein aggregates as well as within individual aggregates or fragments may also occur as the ultracentrifuge peaks were broad and far from monodisperse.

In contrast to this behaviour the addition of acetaldehyde to casein resulted in sedimentation properties which remained virtually unchanged for 24 h. Covalent bonding was occurring, however, since while urea dissociated the untreated material to the monomeric state it caused no dissociation after 24 h reaction with acetaldehyde. Thus, clearly, acetaldehyde formed bonds within the aggregate particles, linking the individual polypeptide chains together covalently, but did not form bonds between aggregate particles.

Dialysis against EDTA demonstrated that removal of metal ions from UHT milk which had been stored at 30 °C did not alter the streaky SGE patterns. The results of analysis of the small amounts of lipid coprecipitated with the caseins did not indicate the concentration of any specific component such as might be expected if the formation of lipoprotein or lipid–protein complexes was an important factor in the development of these patterns. The calcium sensitivity measurements showed that the effect of heat on purified κ-casein was to completely destroy its ability to protect α_{s1}-casein from precipitation when calcium ions were added to the system. This was in broad agreement with earlier investigations (Zittle, 1961; Alais, Kiger & Jollès, 1967), although in the present work the heating conditions were much more severe. The effect of heat on α_{s1}-casein was to enhance its stability towards addition of calcium ions. There appeared to be a considerable difference between the rate at which α_{s1}-casein gained stability to calcium ions and the more rapid loss of protecting ability of κ-casein, so that on heating whole casein one might expect a stability minimum when the protecting ability of κ-casein was diminished without a compensating gain in stability by the α_{s1}-casein. Some evidence for this was found, since a rapid loss of sensitivity to calcium was followed by a slight recovery of sensitivity which could have been due to the differential destruction of the protective power of the κ-casein, but the effect was small. In the presence of lactose, when the above effects on the individual caseins were accelerated, no evidence for a stability minimum was apparent on heating whole casein.

A very similar situation pertains in the acetaldehyde treatment of caseins in that the protective ability of κ-casein was rapidly destroyed whereas α_{s1}-casein only slowly lost its sensitivity to calcium ions. With whole casein there was a rapid decrease in sensitivity, as with the heated system, but then there was quite a marked recovery of sensitivity reaching a maximum after 1·5–2 h which might reflect the difference in the rates of reaction of κ- and α_{s1}-caseins, although this suggestion is clearly an oversimplification of the process actually occurring.

The properties of caseins from various milk samples were in broad agreement with the above findings. Casein from fresh UHT milk stored at 4 °C for 1 or 2 years was less sensitive to calcium than that from fresh UHT milk. This would suggest that the UHT process itself gives rise to some instability, but that storage at 4 °C results in a loss of calcium sensitivity as does brief heating or acetaldehyde treatment in the

model system. Material isolated from UHT milk stored at 30 °C for 1 year was the most sensitive to calcium ions and this may suggest that at this temperature the reaction has progressed a little further towards the state corresponding to the recovery of sensitivity noted after about 1·5 h heating of casein or acetaldehyde treatment. Caution should be exercised in extrapolating results in the model systems to milk iself, however, because although it is well known that increasing instability precedes the phenomenon of milk gelation there is no definite evidence that calcium sensitivity is related directly to gelation, although it may provide a valid measure of the stability of the milk system.

Small amounts of many carbonyl compounds, including acetaldehyde, are known to be formed during the UHT process itself (Scanlan, Lindsay, Libbey & Day, 1968; Kirk, Hedrick & Stine, 1967) and may have minor significance in the development of subtle alterations in the SGE patterns given by the protein components of UHT milk. Many of these carbonyl compounds are highly reactive, so the present demonstration that UHT milk stored at 4 °C did not exhibit major changes in the protein SGE bands even after a year or more of storage suggested that these products of the UHT process do not play a dominant role in the development of the observed SGE changes. The calcium sensitivity measurements showed that UHT treatment did give rise to some loss of stability of the isolated caseins in the presence of calcium, so it appeared probable that this type of heat treatment may have some influence on the stability of the resulting product. Of far greater importance, however, was the demonstration that a Maillard type of reaction continued to take place during storage of UHT milk at 30 °C. Such a reaction would give rise to a steady supply of carbonyl compounds throughout the storage period. Furthermore, it has been shown in model systems that the Maillard reaction was capable of causing polymerization and of modifying casein components in such a way that their behaviour on SGE, DEAE-cellulose chromatography, Sephadex G-100 and on treatment with $CaCl_2$ was altered in a manner similar to the changes induced in the caseins by storage of the milk at 30 °C for a year. Further work is now in progress to elucidate what changes actually take place at the molecular level and how these may lead to interactions between the individual casein components which may be capable of giving rise to instability in the milk system and also adversely affecting nutritional properties.

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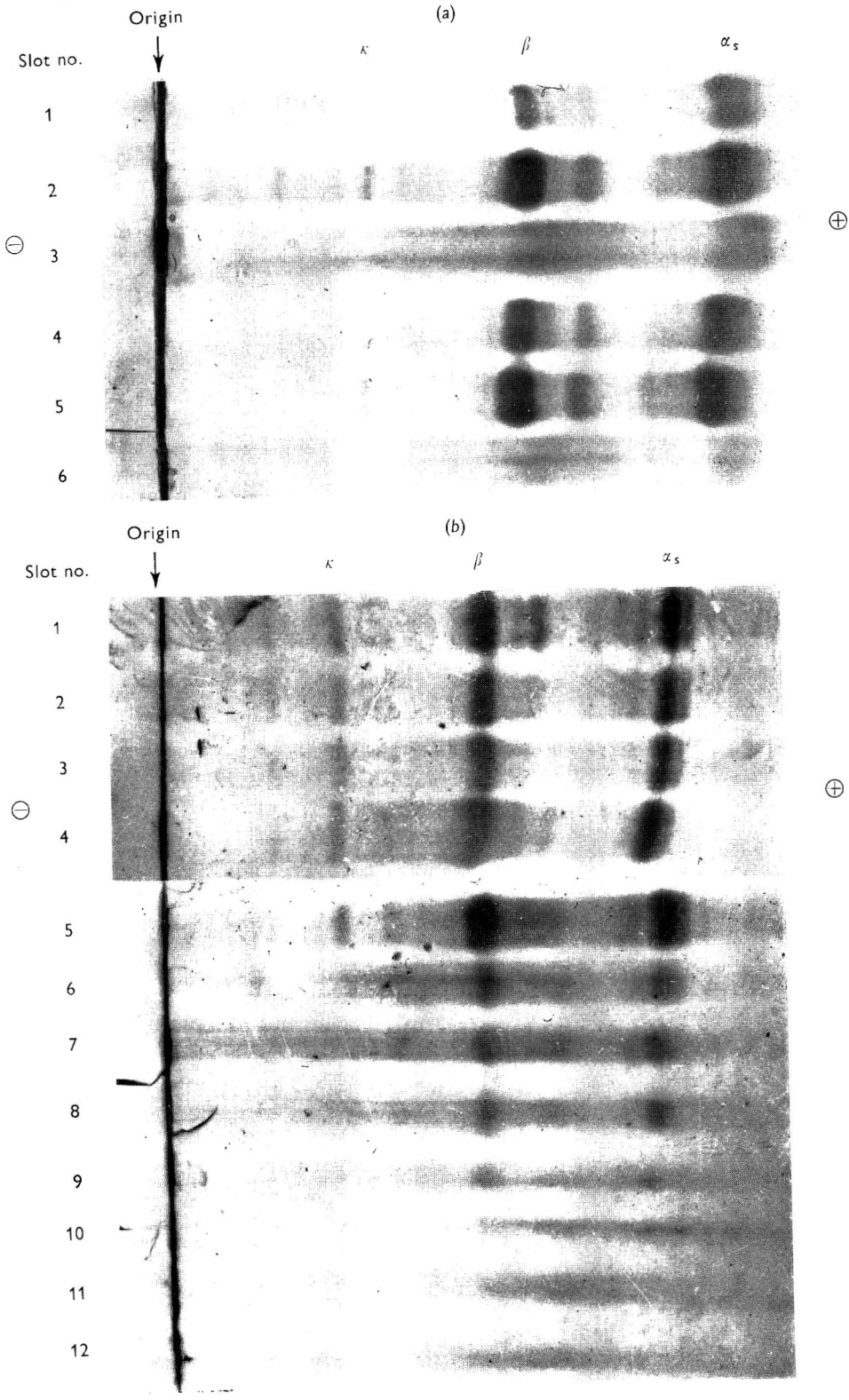
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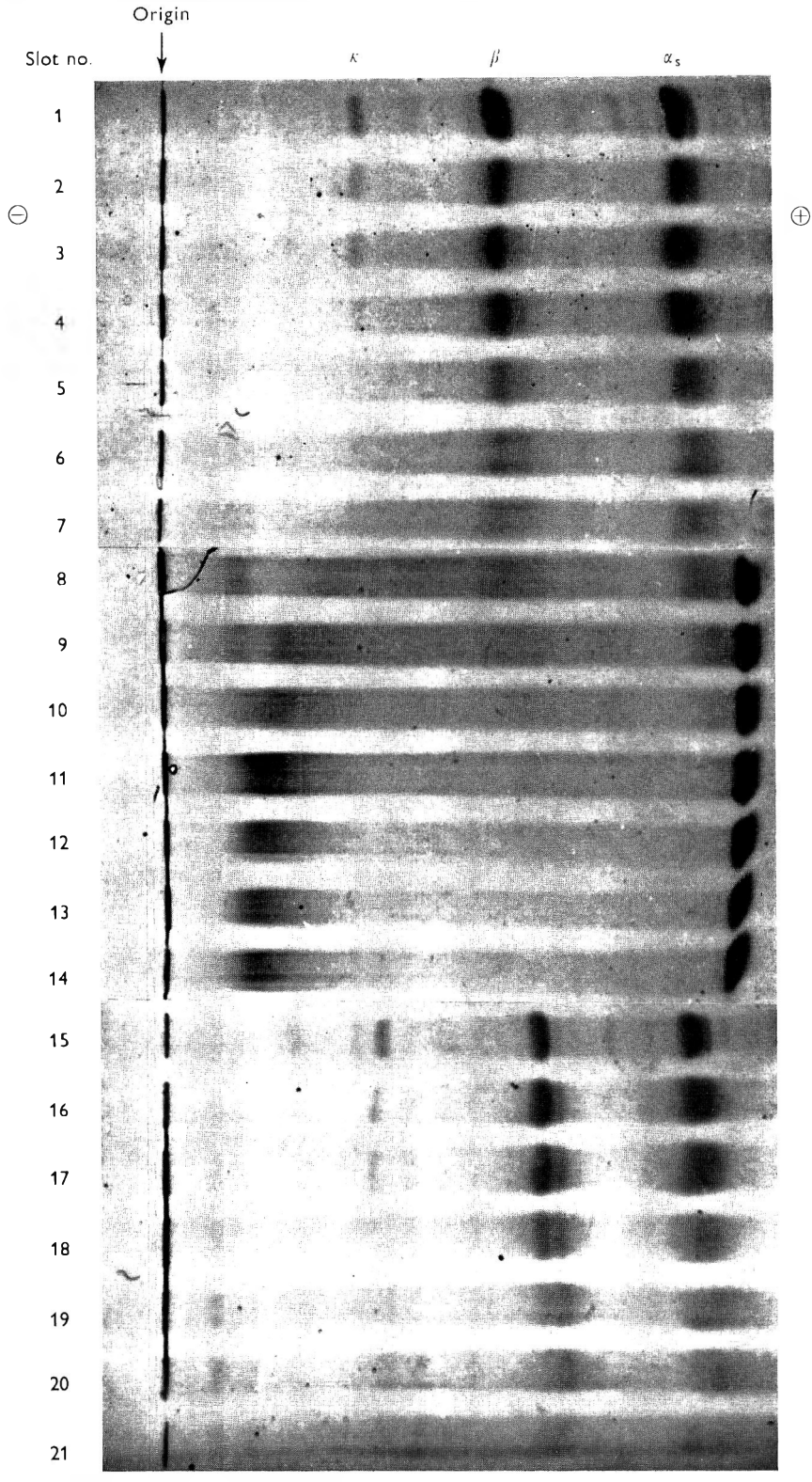

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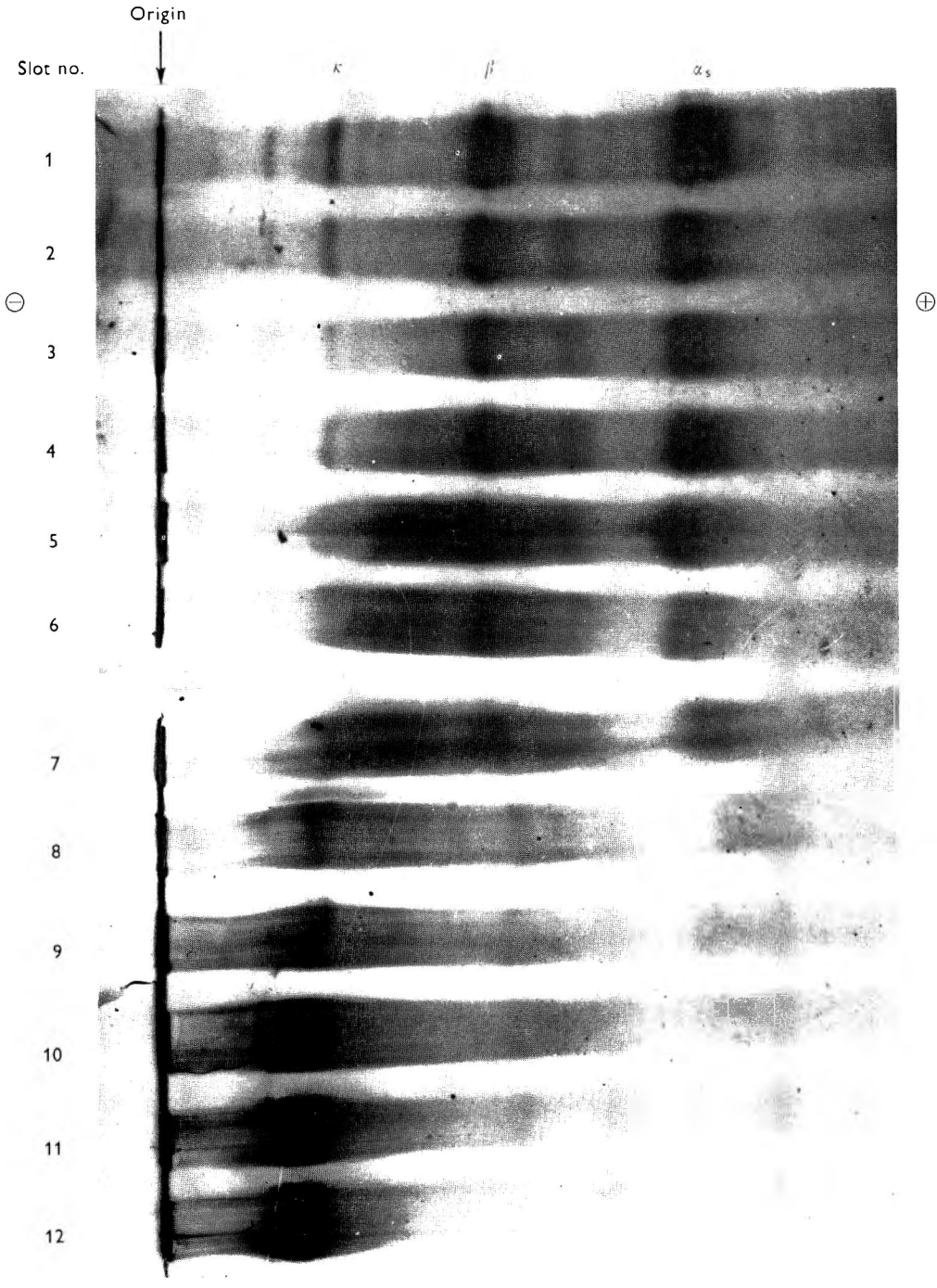

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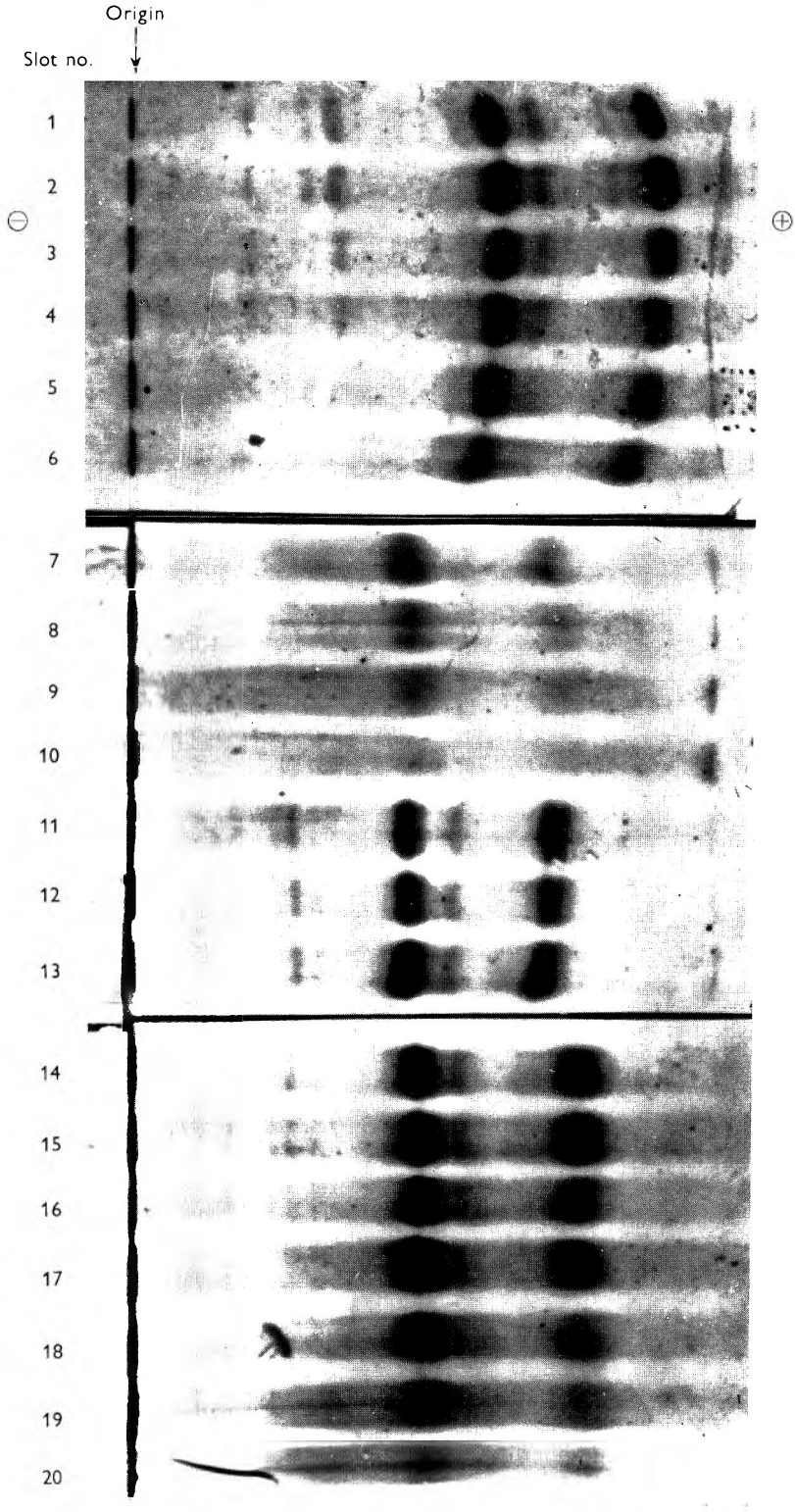

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EXPLANATION OF PLATES

Plate 1. (*a*) SGE patterns of caseins isolated from various milk samples. Slots: 1, UHT milk stored at 4 °C for 1 year; 2, fresh UHT milk; 3, UHT milk stored at 30 °C for 1 year; 4, as 1, but sample paper removed after 30 min; 5, as 2, but sample paper removed after 30 min; 6, as 3, but sample paper removed after 30 min. (*b*) The effect of acetaldehyde (23 μl) at room temperature on the SGE patterns given by fresh homogenized whole milk (0·75 ml). Lengths of reaction time in hours: slot 1, zero; 2, 0·25; 3, 0·5; 4, 0·75; 5, 1·0; 6, 1·5; 7, 2·0; 8, 2·5; 9, 3·0; 10, 4·5; 11, 5·5; 12, 7·0.

Plate 2. SGE patterns of whole casein heated at 100 °C in 0·1 M-sodium phosphate buffer pH 6·8. Protein solutions were 3 % by weight and lactose was added to 6 % by weight. Heating times in hours: slot 1, zero; 2, 0·25; 3, 0·5; 4, 0·75; 5, 1·0; 6, 1·5; 7, 2·0; 8, 3·0; 9, 4·0; 10, 5·0; 11, 6·5; 12, 8·0; 13, 10·0; 14, 12·0; 15, zero; 16, 0·5; 17, 1·0; 18, 2·0; 19, 5·0; 20, 8·0; 21, 12·0. Slots 15–21 were controls containing casein only with no added lactose. The strong, fast-moving band on the centre gel is an artifact caused by migrating buffer salts.

Plate 3. SGE patterns of whole casein in 0·1 M-sodium phosphate buffer pH 6·8 (3·0 ml, containing 3 % protein by weight) mixed with acetaldehyde (0·1 ml) and allowed to stand at room temperature. Reaction times in hours: slot 1, zero; 2, 0·25; 3, 0·5; 4, 0·75; 5, 1·0; 6, 1·25; 7, 1·5; 8, 2·0; 9, 2·5; 10, 3·0; 11, 5·0; 12, 7·0.

Plate 4. SGE patterns showing the effect of adding sodium metabisulphite (50 mM) to fresh homogenized whole milk prior to heating at 100 °C. Heating alone with no added $Na_2S_2O_5$ for various times in hours: slot 1, zero; 2, 0·25; 3, 0·5; 4, 0·75; 5, 1·0; 6, 1·5; 7, 2·0; 8, 3·0; 9, 5·0; 10, 7·0. Heating in the presence of $Na_2S_2O_5$ for various times in hours: slot 11, zero; 12, 0·25; 13, 0·5; 14, 0·75; 15, 1·0; 16, 1·5; 17, 2·0; 18, 3·0; 19, 5·0; 20, 7·0.

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Factors affecting the concentration of vitamins in milk

III. Effect of season and solar radiation on the vitamin D potency of butter

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Summary. Between August and May – the butter-making season in New Zealand – the average vitamin D content of the butterfat varied between 10 and 64 i.u./100 g and during the corresponding months in Great Britain (February–November) the values were between 15 and 39 i.u./100 g. At the beginning and end of the season the values in both countries were similarly low but in mid-season New Zealand values were up to twice those for Great Britain. Within each country the vitamin D values were highly correlated with hours of sunshine and, for both countries, with total radiation, which includes the diffuse radiation in 'skyshine' and also allows for variation in sunshine intensity. The higher content of vitamin D in New Zealand butter in mid-season was attributed to a higher level of total radiation.

Thompson, Henry & Kon (1964) determined the vitamin D activity of New Zealand butters made in one creamery during the butter-making season, August 1958 to April 1959. They also determined the activity in milk fat obtained from 14 depots in Great Britain between January 1958 and December 1959. Late autumn and early spring values for New Zealand butter were little higher than those found in Great Britain, but the summer values were considerably higher. Thompson *et al.* (1964) suggested that the difference in latitude, and correspondingly increased intensity of sunshine, in New Zealand could account for these higher potencies. The present paper reports the vitamin D potency of New Zealand butter made in 4 widely separated creameries, one of them being the same as was reported on by Thompson *et al.* (1964).

The relation between the amount of solar radiation in the 2 countries and the vitamin D levels in the milk fat was investigated.

EXPERIMENTAL

Climatological data

For New Zealand, sunshine records were taken from the *New Zealand Gazette* using the data provided by the meteorological stations nearest to each creamery. For the

North Wairao district the data were abstracted from the records of stations at Kerikeri, Waipoua Forest and Dargaville, whilst for the Waikato district (Te Awamutu) records obtained at Rukuhai, Arapuni, Te Awamutu and Te Kuiti were used. For the Westland district of the South Island, the records were made from Hokitika aerodrome, and for the most southerly factory from Invercargill aerodrome. For Great Britain, the sunshine records collected by Thompson *et al.* (1964) were used.

Butter samples

Throughout the butter-producing areas of New Zealand the Jersey breed of cows is predominant. Table 1 shows details of the creameries, which were widely separated geographically. North Wairao and Te Awamutu are in the North Island in large

Table 1. *Creameries in New Zealand supplying butter samples*

Creamery no.	Place	Latitude	Butter-making season	Butter produced in 1965, ton
1358	North Wairao	36° S	1963–4	6358
1880	Te Awamutu	38° S	1963–4	13650
1880	Te Awamutu	38° S	1965–6	
145	Hokitika	42° S	1964–5	1007
336	Invercargill	46° S	1965–6	507

butter-producing areas from which most of the butter is exported, and Hokitika and Invercargill are in the South Island where butter production is on a smaller scale and none is exported. Each butter sample, weighing about 4 lb, was taken from one churning of about 2 tons of butter during 1 day's production in mid-month. As the samples were collected they were cold-stored in New Zealand until all those representing one season's production were obtained. They were then shipped under refrigeration to Great Britain and stored in a deep freeze at − 30 °C until required for analysis.

Analytical methods

Some samples of butter were stored in the deep freeze for as long as 40 months. We consider it unlikely that there was any change in the vitamin D content during this prolonged storage, as Bechtel & Hoppert (1936) found that vitamin D was stable in butter during storage for 30 months at 0 °C.

All the butters were saponified and the non-saponifiable residue, dissolved in arachis oil, was biologically assayed for vitamin D. The method of assay was based on that of Henry & Thompson (1954). As standards, suitable dilutions in arachis oil of the 2nd International Standard 1949 (WHO Expert Committee on Biological Standardization, 1950) for vitamin D_3 (a solution of vitamin D_3, irradiated 7-dehydrocholesterol, in vegetable oil containing 25 μg (1000 i.u.) in 1 g) were used.

For the samples collected in 1963–4 four dose levels of standard and 2 levels of each of 2 test preparations were allocated to 8 litter-mate rats from each of 10 litters. For the samples collected in 1964–5 and 1965–6 the assay design was modified and the standard and a single test preparation were each given at 3 levels with doses being allocated to 6 litter-mates from each of 6 different litters. Statistical analysis of the individual assays and the combination of potency estimates followed the methods described by Finney (1964).

RESULTS

Effects of season and latitude

Table 2 shows for each creamery in New Zealand the date of manufacture of the butter sample and its vitamin D activity. For each creamery the higher vitamin D values were found in the summer months, showing a relationship between vitamin D concentration and season. Monthly vitamin D contents, obtained after combining estimates for the different creameries (Table 2), are shown in Fig. 1 together with the average monthly values for milk fat produced in Great Britain (Table 4). The low values found for spring butter samples from New Zealand, 10–13 i.u./100 g fat, were similar to those of 15 i.u./100 g for milk fat produced in Great Britain for the corresponding months (Thompson *et al.* 1964), but during the summer period the New Zealand butter values of 61–64 i.u./100 g fat were much higher than those of 35–39 i.u./100 g for milk fat produced in Great Britain.

Table 2. *The vitamin D content, with* 95% *confidence limits, of butterfat from* 4 *creameries in New Zealand* (*i.u.*/100 *g fat*)

Month	North Wairao	Te Awamutu		Hokitika	Invercargill	Combined
	1963–4	1963–4	1965–6	1964–5	1965–6	estimate
Aug.	11 (9, 13)	9 (7, 11)	—	—	—	10
Sept.	15 (11, 18)	11 (8, 14)	—	—	—	13
Oct.	45 (35, 58)	43 (33, 55)	20 (7, 34)	—	—	36
Nov.	44 (34, 56)	41 (31, 52)	32 (20, 44)	41 (33, 49)	33 (24, 45)	38
Dec.	80 (63, 103)	62 (48, 79)	65 (42, 93)	47 (36, 62)	64 (51, 83)	61
Jan.	77 (63, 96)	58 (47, 72)	63 (42, 89)	54 (40, 70)	—	63
Feb.	82 (64, 105)	61 (47, 77)	65 (42, 93)	81 (57, 114)	43 (33, 56)	64
Mar.	64 (48, 90)	46 (37, 60)	52 (39, 66)	38 (16, 66)	38 (24, 69)	47
Apr.	34 (27, 46)	25 (19, 32)	32 (24, 41)	13 (7, 19)	26 (19, 34)	26
May	—	—	22 (14, 31)	16 (9, 23)	—	19

For most of the butter-making season the vitamin D content of New Zealand butter was substantially higher than that of the corresponding British product. This difference may be largely ascribed to the differences in the latitudes of the 2 countries. There was also some evidence of an effect of latitude within New Zealand (Table 2), as the vitamin D content of the butters made in 1963–4 was greater for North Wairao, latitude 36 °S, than for Te Awamutu, latitude 38° S, and in 1965–6 greater for Te Awamutu, 38° S, than for Invercargill, latitude 46° S. Moreover, in both seasons these differences in vitamin D which appear to be related to latitude were greatest in summer, between December and March at the height of the butter-making season.

Effects of sunshine and radiation

Fig. 1 also shows average sunshine values for each month. A close relationship between hours of sunshine and vitamin D concentration is indicated, and the correlation coefficients were 0·93 for New Zealand and 0·86 for Great Britain. However, from April to September in Great Britain and for the corresponding period of October to March in New Zealand, although the daily sunshine recorded in the 2 countries was similar, the New Zealand butterfat contained far more vitamin D.

A better estimate of the energy available for vitamin D formation may be that of total incoming radiation in which allowance is made not only for the duration of the period of sunshine but also for its intensity, and for skyshine. The use of radiometers together with sunshine recorders at several of the locations in New Zealand and Great Britain has permitted the development of empirical relationships which have now been used to derive values for total radiation from the sunshine records of this investigation. The total radiation in Great Britain is distinctly less than that in New Zealand (Fig. 1), and this difference could account for much of the difference in vitamin D levels in summer.

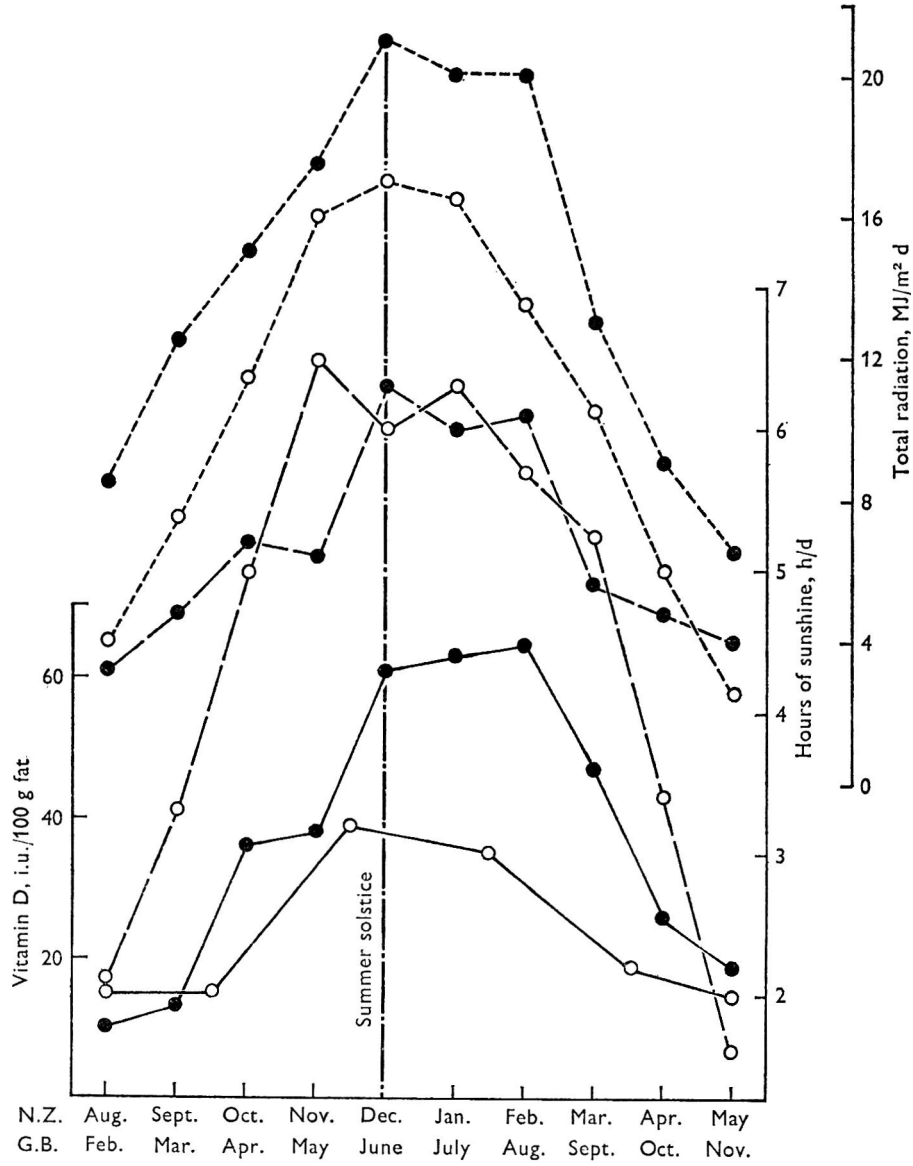

Fig. 1. Content of vitamin D in milk fat, sunshine and total radiation for New Zealand (●) and for Great Britain (○). ——, Vitamin D; – –, sunshine; - - - -, total radiation.

However, somewhat lower vitamin D values occurred in the months preceding the summer solstice than in those after it (Fig. 1). This suggested a type of hysteresis or time lag and, to compensate for it, an empirical correction was applied. The radiation ratings were calculated as the average daily total radiation for the 30 days before sampling plus half of that for the previous 30 days. Allowing for the fact that

Table 3. *Radiation ratings appropriate to the New Zealand creameries; ratings calculated as the mean daily radiation for the* 30 *days before butter sampling plus half of that for the preceding* 30 *days* (MJ/m^2 *d*)

		Te Awamutu				
Month	North Wairao 1963–4	1963–4	1965–6	Hokitika 1964–5	Invercargill 1965–6	Mean
Aug.	9·0	10·0	—	—	—	9·5
Sept.	11·5	12·0	—	—	—	12·0
Oct.	19·5	18·0	16·5	—	—	18·0
Nov.	23·0	21·0	23·0	22·0	23·0	22·5
Dec.	28·5	27·0	28·0	25·0	25·5	27·0
Jan.	31·5	32·0	29·5	27·0	—	30·0
Feb.	32·0	32·5	29·5	27·0	26·5	29·5
Mar.	26·0	26·0	27·5	23·0	21·5	25·0
Apr.	15·5	20·5	21·5	17·5	12·5	17·5
May	—	—	14·5	12·5	—	13·5

Table 4. *Vitamin D content of milkfat from* 14 *creameries in Great Britain* (*from Thompson* et al. 1964) *and the radiation rating for* 1958 *and* 1959

Months	Vitamin D, i.u./100 g fat	Radiation rating, MJ/m^2 d
Jan.–Feb.	15	3·0
Mar.–Apr.	15	9·0
May–June	39	20·5
July–Aug.	35	23·0
Sept.–Oct.	19	16·0
Nov.–Dec.	15	6·0

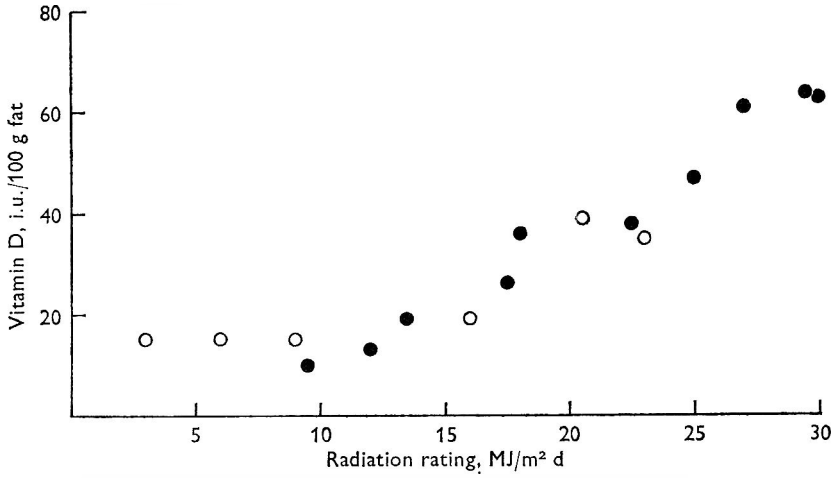

Fig. 2. Relationship between the radiation ratings and vitamin D content of milk fat. Ratings are calculated as the average daily total radiation for the 30 days before sampling plus half of that for the preceding 30 days. ●, New Zealand; ○, Great Britain.

the sunshine records were not a precise measure of the sunshine experienced by contributing herds and for inaccuracies in deriving radiation values from the sunshine records, it was estimated that the errors in radiation ratings calculated for individual samples were about 15%.

The derived radiation ratings for the New Zealand butters are in Table 3 and those for milk fat from Great Britain in Table 4, together with the corresponding vitamin D values. Pooling the results for the different creameries, the relationship between monthly vitamin content (Tables 2, 4) and radiation rating (Tables 3, 4) is shown in Fig. 2. The correlation coefficients between vitamin D content and radiation rating were 0·98 for the New Zealand butter and 0·88 for the milk fat produced in Great Britain. Fig. 2 also shows that the results for Great Britain are consistent with those for New Zealand and that a linear relationship exists between vitamin D content and radiation rating except, possibly, at low radiation levels. The low radiation ratings of 3·0 and 6·0 MJ/m^2 d in Great Britain were for November–February, when the cow's vitamin D intake could have been largely contributed by the diet.

DISCUSSION

Although light converts the plant sterol ergosterol into ergocalciferol, vitamin D_2 (Rosenheim & Webster, 1927), the vitamin is not present in actively growing pasture but is formed in senescent materials such as hay. Campion, Henry, Kon & Mackintosh (1937) found that freshly mown grass when fed to cows did not contribute to an increase in vitamin D in summer milk, whereas hay did to a limited extent. Windaus, Lettré & Schenck (1935) found that light converts the animal sterol 7-dehydrocholesterol into cholecalciferol (vitamin D_3). Bechtel & Hoppert (1936) suggested that solar radiation acting on the cow's skin was responsible for increases in the vitamin D content of milk. Other workers, including Campion *et al.* (1937) and Henry & Kon (1942), also found a correlation between sunshine and the vitamin D content in milk. Thus it seems that solar radiation is of primary importance for the presence of vitamin D in milk. Other factors which could influence the vitamin D level are breed, stage of lactation and ambient temperature. Thompson *et al.* (1964) found that Jersey milk fat contained somewhat more vitamin D than Friesian milk fat but Hartman & Dryden (1965), from a comprehensive review of the literature, concluded that there is little breed difference. Colostrum is somewhat richer in vitamin D than is mature milk, but after day 5 the level remains fairly constant (Henry & Kon, 1937). No studies have been reported on the effect of ambient temperature.

Effect of radiation and season

In New Zealand and in Great Britain the vitamin D content of butter was less in the months before than in the months after the summer solstice, suggesting a lag in response. It is not known how rapidly the level of vitamin D in milk responds to change in the level of radiation, but it is known that there is more erythemal (sunburn) energy in the period around the autumnal equinox than the period around the vernal equinox (Luckiesh, 1946). Erythema can be used as a measure of the radiation available for vitamin D formation as both are caused by radiation in the same narrow range of the solar spectrum. We have shown that the higher levels of

vitamin D in autumn (March–May) than in the corresponding early spring (August–September) in New Zealand are due in part to the higher total radiation level. A further contributory cause may be a lag due to stores accumulated in the cow's body during the high summer, which Wallis (1938) suggested take only 2–4 months to deplete as the vitamin D levels in butterfat are distinctly higher for autumn than for spring months of the same total radiation. Yet another factor could be the intake of vitamin D from autumn pasture containing a proportion of old and dried herbage. The vitamin levels found in samples from Great Britain taken during conditions of low radiation intensity (Fig. 2) were probably due to the vitamin D intake from hay in winter months.

Effect of latitude

Since solar energy at sea level is dependent on solar altitude it varies not only with time of day and month of the year but also with latitude. Decrease in solar altitude reduces the radiation intensity but the effect of this is partly offset by the increased daylength in the summer months. It would be expected therefore that vitamin D formation in the cow's skin would vary with these factors. New Zealand lies between latitudes 36 and 46° S and Great Britain between 50 and 56° N – which is sufficient to explain why butter produced in New Zealand contains for the greater part of the season almost twice as much vitamin D as that produced in Great Britain (Fig. 1). There is evidence for an effect of latitude even within New Zealand, as the vitamin D content of butter from the 2 creameries in the North Island was somewhat higher than that found in butter from the 2 South Island creameries. From the ratios of antirachitic effectiveness of sunlight at different latitudes (Abrams, 1952) the potency of butter in Great Britain would be expected to be 60 % of that in New Zealand, and this expectation was in reasonable agreement with our actual findings. However, the findings of an apparent relationship between latitude and antirachitic effect may not be altogether well founded, since for Indian butter the vitamin D values were not much higher than those for British summer butter (Henry & Kon, 1954). It is possible that there is a level above which further irradiation causes no further increase in vitamin D. It is, in fact, known that intense irradiation has a destructive effect on vitamin D.

The effect of radiation, direct and diffuse

Luckiesh (1946) has pointed out that the ultraviolet component of diffuse radiation (skyshine) has generally been ignored or neglected. He found that the erythemal and antirachitic energy from the sky alone on clear days was greater than that from the sun, even at high solar altitudes, and that the ultraviolet energy in skyshine does not diminish with decreasing altitude of the sun as much as does that in sunshine. In earlier work the contribution from skyshine appears to have been consistently neglected. Abrams (1952) used only the direct (solar) radiation in his calculations, which showed that a 1000-lb beast receives sufficient radiation to form 4500 i.u. of vitamin D daily in summer at latitude 50° N. This value differs little from a cow's vitamin D intake (4000 i.u.) from hay in winter (Henry, Kon, Thompson, McCallum & Stewart, 1958), from which milk fat containing 15 i.u./100 g is produced (Thompson *et al.* 1964). However, Tables 2 and 4 suggest that milk fat contains up to 5 times more

vitamin D in summer than in winter, and therefore either Abrams's (1952) estimates are too low or the transfer to milk of vitamin D produced by daylight acting on the skin is more efficient than the transfer through the digestive processes.

In conclusion, it is clear that the vitamin D potency of milk fat was related to sunshine hours. A better correlation was obtained with total radiation, which takes into account variation in the intensity of sunshine (direct radiation) and the contribution from skyshine (diffuse radiation).

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A double-bed process for the removal of cationic fission products from milk

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Summary. A process for the removal of cationic fission products from milk is described in which no prior acidification is necessary. The milk is treated at 40 °C by 2 beds of suitably charged cation exchange resins, one of which is of the carboxylic acid type and the other of the sulphonic acid type. On the first bed all the metallic cations of the milk are replaced by K^+ and H^+, and on the second the ionic composition is restored. During processing the pH is reduced from 6·8 to approximately 6·2 and is restored to 6·8 by the addition of alkali metal carbonates to the milk after treatment.

On a pilot plant of capacity 1600 l (30 resin bed volumes) per 5-h day it was found that the radiochemical efficiency was approximately 80 % for ^{85}Sr and 97 % for ^{137}Cs.

The process caused some losses of vitamins, especially of thiamine, and an increase in the lead, copper and iron contents all of which, however, were at an acceptable level. Other changes in chemical composition were negligible and enzymic clotting time remained unchanged. Flavour and appearance of the product were satisfactory and it was satisfactorily both roller- and spray-dried. The process is discussed with reference to an earlier method in which prior acidification is necessary. It is concluded that the double-bed process is somewhat more efficient for the removal of fission products and that a full-scale plant would be little or no more expensive to erect and only slightly more expensive to operate.

A process for the removal of cationic fission products from milk was patented in 1962 by Murthy, Campbell, Mazurovsky & Edmondson, and plants based on this process have been set up both in America (Edmondson *et al.* 1962; Producer's Creamery Company, 1965) and in Britain (Glascock, Hall, Suffolk & Bryant, 1968). The method consists essentially of the passage of milk, acidified with citric acid to pH 5·2–5·3, through a cation exchange resin of the sulphonic acid type charged with a suitable mixture of the ions of Ca, K, Na and Mg. The ionic composition of the resin is chosen so that little or no change in the ionic composition of the milk occurs except that 96–98 % of the ^{90}Sr and about 75 % of the ^{137}Cs are removed. Milk treated in the pilot plant set up in Britain has been subjected to analysis (Glascock & Bryant, 1968) and to tests of its nutritional quality (Braude, Glascock, Newport & Porter, 1969). Although the analysis revealed no change in composition likely to prove deleterious

to the consumer, it was concluded from experiments with baby pigs that the nutritional value of the milk was adversely affected by treatment in the plant. This adverse effect was shown by a higher mortality in the animals given treated milk than in those given untreated milk, although post-mortem findings in both groups revealed no specific cause of death. These results were not in agreement with those of American workers (Isaaks *et al.* 1967), who, however, gave their animals a much smaller ration which, as is suggested in the paper by Braude *et al.* (1969), may have masked the effect observed in our laboratory.

In the absence of any indication as to the precise nature of the changes which render the milk harmful to baby pigs it was thought possible that the change of pH from neutral to 5·25 and back again could produce changes in the proteins to which baby animals are sensitive. Although the effect observed in baby pigs might not occur in human infants, this possibility clearly cannot be investigated. It was therefore decided to study the removal of radioactive cations by a method involving minimum change to the natural pH of the milk during treatment.

Acidification is an important feature of the method patented by Murthy *et al.* (1962), for at normal pH, resins of the sulphonic acid type, such as Dowex-50 or Zeo-Karb 225 which are specified in the patent, will remove only about 50% of contaminating radiostrontium. Resins of the carboxylic acid type, however, have a very high affinity for the alkaline earth metals. In 1958 Fisons Ltd patented a method for the manufacture of a cheese starter in which the Ca of milk was exchanged for K by passage at normal pH through a carboxylic-acid-type resin charged partly with K and partly with H ions.

Preliminary experiments on a laboratory column with a resin of this type showed that, as expected, it was very efficient in the removal of ^{85}Sr from milk labelled in vitro. When the resin was charged, however, with a mixture of ions in such concentrations that no other change in the ionic content of the milk would be expected, the efficiency of removal of ^{85}Sr was very low. It was therefore necessary to devise a system in which a carboxylic-acid-type resin charged with a mixture of K and H ions could be used for the removal of radioactive cations and a sulphonic-acid-type resin in the mixed ionic form could be used for the restoration of the ionic composition of the milk. The present paper describes the development of the process and the chemical properties of the milk after treatment in a pilot plant.

MATERIALS AND METHODS

Milk at 4–5 °C was obtained direct from the Institute farm. Radioactive strontium and caesium (^{85}Sr and ^{137}Cs) were obtained from the Radiochemical Centre, Amersham, Bucks. Hydrochloric and nitric acids were technical grade. Potassium hydroxide was 'Puriss' flake (L.R.B. Pearce Ltd, London, W.C.1) and the chlorides of Ca, Na, K and Mg were the cheapest available industrial grades.

Introduction of ^{85}Sr and ^{137}Cs into milk

In vitro

For the determination of the efficiency of removal of nuclides introduced in vitro, milk containing ^{85}Sr or ^{137}Cs at a concentration of 0·25–0·3 μCi/l was used. For work with laboratory columns, the required amount of the nuclide was added to the whole of the milk to be treated and allowed to equilibrate at 4–5 °C for 72 h (^{85}Sr) or overnight (^{137}Cs). For work on the pilot plant the nuclide was allowed to equilibrate for these times with 20 l of milk which was then well mixed with the whole of the batch to be treated.

In vivo

Two experiments were performed on the pilot plant with milk containing ^{85}Sr introduced in vivo. In the first, the milk was obtained from the Institute for Research on Animal Diseases, Compton, Berkshire, where an experiment was being carried out on the retention of the nuclide by lactating cows. The milk was collected over 5 days from 3 cows that had been injected intravenously with a total of 400 μCi of $^{85}SrCl_2$ and was preserved by the addition of 0·1 % (v/v) of commercial formalin. This milk was diluted to 1800 l with normal milk containing the same concentration of formalin. The radioactivity of a 450-ml sample of the mixture was 17·5 c.p.s.

In the second experiment milk containing more radioactivity was used and formalin was omitted. A lactating Friesian cow received 5 mCi of $^{85}SrCl_2$ and the 40 l of milk collected during the next 48 h was diluted to 1800 l with normal milk. The radioactivity of a 450-ml sample of this mixture was 245 c.p.s.

Analytical methods

Radioactivity of milk samples was measured as previously described (Glascock & Bryant, 1968) by counting in a specially designed beaker of capacity 450 ml which fitted over the sodium iodide crystal of a scintillation counter.

The methods of chemical analysis of the milk were all as described previously (Glascock & Bryant, 1968) with the following exceptions. In experiments on the variation of mineral content with the volume of milk treated, calcium was determined by the method of Sedlaček & Dušek (1966), which gives a sharper end-point than the method previously used. Thiamine was measured with *Lactobacillus fermenti* as described by Bánhidi (1958). Pantothenic acid was assayed with *Lactobacillus arabinosus* in the medium of Roberts & Snell (1946), modified by the omission of pantothenate and inclusion of riboflavin, and by the substitution of Bacto vitamin-free casitone for the charcoal-treated digest. Vitamin A and α-tocopherol were determined as described by Ford, Porter, Thompson, Toothill & Edwards-Webb (1969) and ascorbic acid was determined as described by Toothill, Thompson & Edwards-Webb (1970). Copper, lead and iron were determined by atomic absorption spectroscopy, antimony by pulse polarography and arsenic spectrophotometrically by the molybdenum blue method.

ION-EXCHANGE COLUMNS

Resins

The resins used were Amberlite IR-50 (Rohm and Haas Ltd, Independence Mall West, Philadelphia, Pa. 19105) and Zeo-Karb 225 grade SRC-13 (Permutit Company Ltd, Gunnersbury Ave., London W. 4), both of particle size 16–52 mesh. These resins are respectively a cross-linked polymethacrylic acid and a sulphonated polystyrene with 8% cross-linkages.

Laboratory columns

Milk was fed by gravity through glass columns of 25 mm diam. containing the ion-exchange resin. In later experiments the bed of Amberlite IR-50 was fitted with a constant-temperature water-jacket. The volume of the bed of Amberlite IR-50 was always 100 ml (20 cm deep) but the volume of the bed of Zeo-Karb 225 was varied according to the experiment.

Pilot plant

Two columns were used, each constructed of borosilicate glass tubing supplied by Q.V.F. Ltd, Stoke-on-Trent, and fitted with stainless-steel gauze and end-covers as described by Glascock *et al.* (1968). The column containing the Amberlite IR-50 was 310 cm high and 22 cm in diam. and that containing the Zeo-Karb 225 was 155 cm in diam. The remainder of the plant was as previously described by Glascock *et al.* (1968) except that neither the delay tank nor any provision for adjustment of pH was necessary, and that the columns were connected together with plastic hose of a quality suitable for use in food manufacture.

PRE-TREATMENT OF MILK

In preliminary experiments with laboratory columns packed with Amberlite IR-50 it was found that approximately 10% of the fat was removed from milk during passage through the resin. This loss was reduced to 2·5% or less by prior homogenization, and in all other experiments therefore, both in the laboratory and on the pilot plant, all milk was first homogenized at a pressure of $1{\cdot}7 \times 10^4$ kN/m^2 (170 atm) and then pasteurized by the HTST process.

REMOVAL OF RADIOSTRONTIUM FROM MILK WITH LABORATORY COLUMNS

Here, and throughout the work described below, all resin bed volumes (r.b.v.) are expressed relative to the bed of Amberlite IR-50 unless otherwise stated.

In previous work (Glascock & Bryant, 1968) a flow rate of 0·125 r.b.v./min had been used. For preliminary experiments in the present work, however, a somewhat smaller flow rate of 0·1 r.b.v./min was used, and the effect on radiochemical efficiency of other flow rates was investigated at a later stage in the work.

Amberlite IR-50 in potassium–hydrogen form

Radiostrontium would be expected to be removed from milk together with Ca and with about the same efficiency. In experiments with the bed of Amberlite IR-50

charged with K and H ions, its efficiency with respect to radiostrontium was therefore inferred from measurements of calcium in the effluent milk. When a bed of Zeo-Karb 225 was added for the purpose of restoring the ionic composition of the milk, the efficiency of the whole system was, of course, measured by the use of milk containing ^{85}Sr.

Effect of potassium content of the Amberlite IR-50 on the efficiency of removal of calcium

Milk was passed through beds of Amberlite IR-50 charged with different proportions of K and H ions ranging from 28 to 100 % K, and the concentration of calcium in the effluent milk was measured. Fig. 1 (*a*) shows that with increasing ratio of K to H ions the efficiency of removal of calcium increased and the breakthrough point occurred later. The pH of the treated milk also increased with the proportion of K on the resin (Table 1). Thus, when passed through a resin wholly in the K form, the effluent milk had an initial pH of 9·5 which diminished as the resin became depleted in K ions.

Table 1. *Effect of K content of Amberlite IR-50 on the pH of milk before and after treatment by Zeo-Karb 225 (mixed ionic form) at* 20 °C.

(Samples taken at 10 r.b.v. with respect to the Amberlite IR-50 resin bed. pH of untreated milk = 6·73.)

	pH	
K in resin, %	After treatment by Amberlite IR-50	After treatment by Zeo-Karb 225
100·0	9·50	6·67
64·5	7·28	6·29
37·7	6·83	6·11
28·8	6·52	5·96
13·2	6·08	5·85

Restoration of ionic composition: effect of composition of first bed on performance of second bed

It was found that with increasing ratios of K : H on the Amberlite IR-50 and hence with increasing pH of the milk entering the Zeo-Karb 225, the second bed, when charged with the ions of Ca, K, Na and Mg, became increasingly clogged with a white solid. This was found to consist of a mixture of calcium phosphate, fat and protein. The loss of phosphate is illustrated in Fig. 1 (*d*), where the phosphate recovered in all the milk collected from the second column after the passage of 12 r.b.v. is plotted against the K content of the first bed. It will be seen that at all K : H ratios on the Amberlite IR-50 some loss of phosphate occurred on the Zeo-Karb 225. Although the removal of some calcium phosphate from the milk might be considered desirable if it were intended for infant feeding, the loss which could be tolerated in this work was limited by the inconvenience caused by clogging of the resin bed. Experiments showed that the maximum ratio of K : H ions on the Amberlite IR-50 which permitted the process to be operated satisfactorily was 35 : 65. The loss of phosphate from the milk was then 9–10 % (Fig. 1 *d*) and did not result in sufficient deposition of calcium phosphate to clog the bed of Zeo-Karb 225. The pH of the milk leaving this bed was,

however, somewhat lower than normal (6·1–6·2) for, as shown in Table 1, milk of unchanged pH could be obtained from the double-bed system only if the first bed were wholly in the K form. It was later found that loss of phosphate on the pilot plant was less than on laboratory columns and that the K content of the Amberlite IR-50 could be increased to 40 ions % without causing the second bed to become clogged.

Effect of relative volumes of resin beds

In order to determine the optimum ratio of the volumes of the 2 resin beds for satisfactory restoration of the cationic composition of the milk, the ratio of the volume

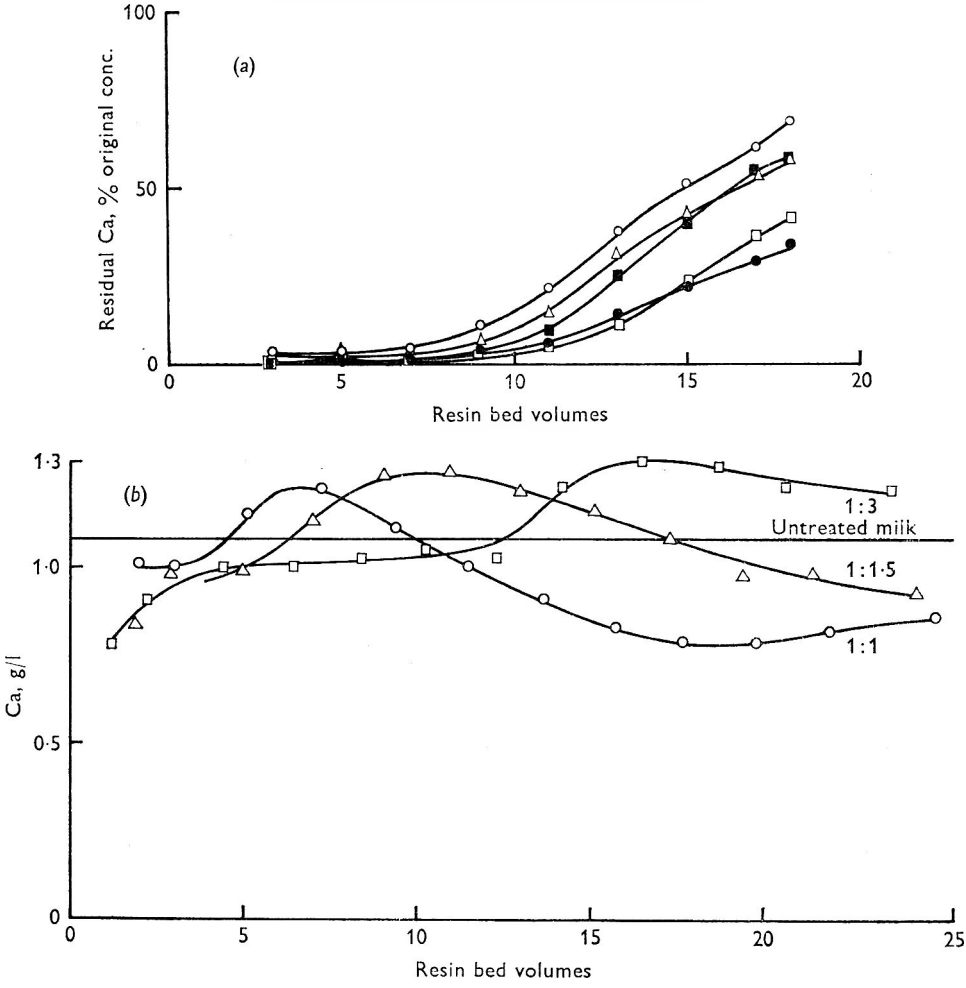

Fig. 1. Experiments with laboratory columns. (*a*) Concentration of Ca in milk after passage through Amberlite IR-50 containing different K:H ratios. K (ions %) on resin: ○, 28; △, 37; ■, 47; □, 70; ●, 100. (*b*) Concentration of Ca in milk after passage through both Amberlite IR-50 (K:H = 40:60) and Zeo-Karb 225 (mixed ionic form) in different ratios by volume as indicated on curves (first to second bed). (*c*) Concentration of Ca and of ^{85}Sr (introduced in vitro) in milk after passage through Amberlite IR-50 (K:H = 40:60) and Zeo-Karb 225 (mixed ionic form). ▽, ^{85}Sr and ●, Ca in effluent from first bed only. ○, △, ▲, ■, □, ^{85}Sr in effluent from second bed at volume ratios of 1:1, 1:1·5, 1:2, 1:2·5 and 1:3. (*d*) Variation in recovery of phosphate with K content of Amberlite IR-50 after passage of milk through both resin beds.

of the bed of Amberlite IR-50 to that of the bed of Zeo-Karb 225 was varied by steps of half a unit from 1 : 1 to 1 : 3. The Amberlite IR-50 was in the K/H form (K, 40 ions %) and the Zeo-Karb 225 was charged by treatment with a solution of the ions of Ca, K, Na and Mg in the molar proportions (%), 46·7, 27·6, 19·5 and 6·2. The milk contained ^{85}Sr introduced in vitro, and the extent to which the cationic composition of the milk had been restored was inferred, as a first approximation, from its Ca content.

It was found that at no ratio of the volumes of the resin beds did the Ca content of

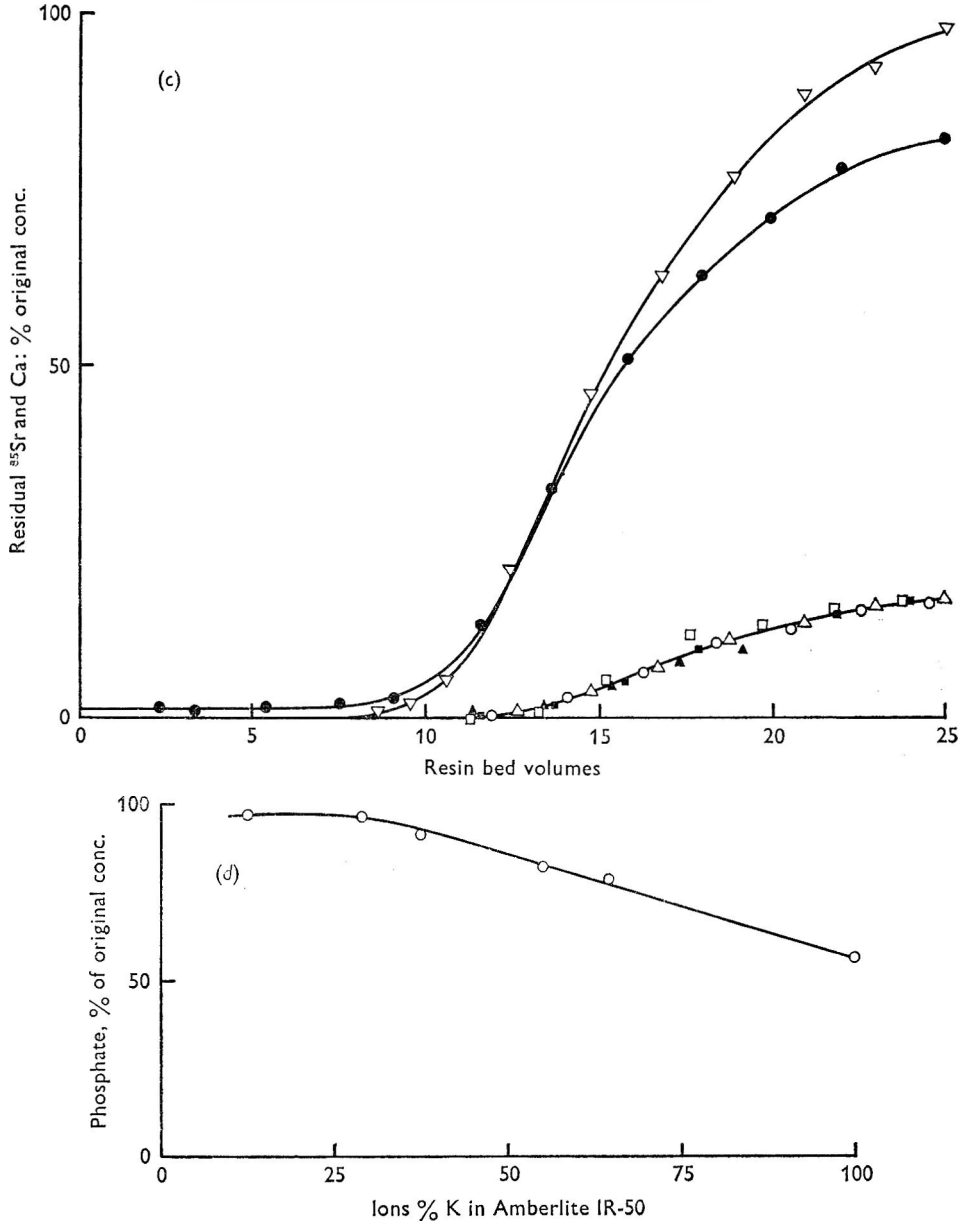

Fig. 1. For legend see opposite page.

the effluent milk remain constant throughout the experiment. It passed through a maximum early in the experiment and then declined again. The effect of increasing the bed volume ratio was to delay this maximum and slightly to increase the Ca content of the whole batch of treated milk. This is illustrated in Fig. 1 (*b*), where the results for bed volume ratios of 1:1, 1:1·5 and 1:3 are given. It was considered that fluctuations in the Ca content with the bed volume ratio of 1:1 were too great but that little advantage would result from the use of a ratio greater than 1:1·5.

Radiochemical efficiency

The efficiency of removal of ^{85}Sr introduced in vitro and of Ca by the Amberlite IR-50 alone is given in Fig. 1 (*c*), which shows that the efficiencies of removal of the

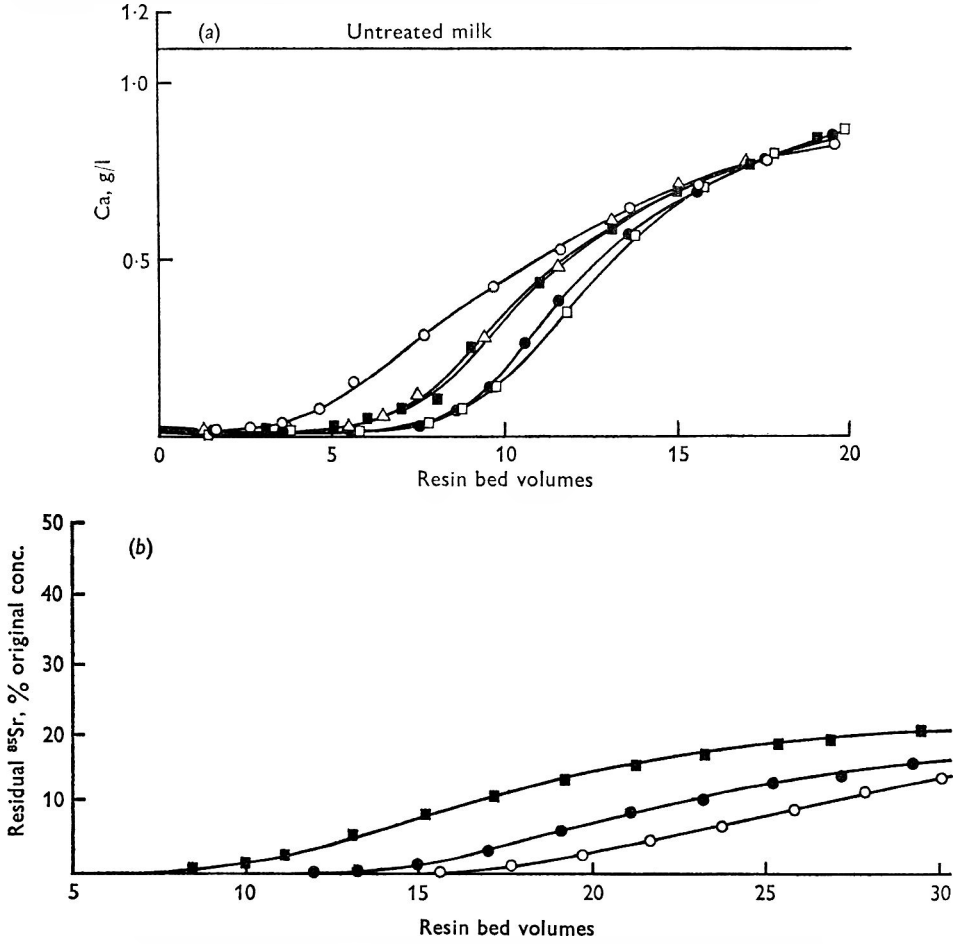

Fig. 2. (*a*) Concentration of Ca in milk after passage at different temperatures through Amberlite IR-50 (K:H = 30:70). ○, 5 °C; △, 20 °C; ■, 25 °C; ●, 30 °C; □, 40 °C. (*b*) Concentration of ^{85}Sr (introduced in vitro) after passage through double-bed system at different flow rates. Amberlite IR-50, K:H = 40:60; Zeo-Karb 225, mixed ionic form. Flow rates (r.b.v./min): ○, 0·05; ●. 0·1; ■, 0·2. (*c*) Concentration of Ca in milk after passage through beds of Amberlite IR-50 (K:H = 40:60) of different geometry. Diameter and height: ●, 30 and 48 cm; ○, 15·5 and 149 cm; ■, 2·5 and 20 cm. (*d*). Concentration of Ca in milk after passage through beds of Amberlite IR-50 (K:H = 40:60) on laboratory and pilot plant columns. △, Laboratory column, diam. 2·5 cm, bed depth 20 cm; ●, plant column, diam. 22 cm, bed depth 140 cm.

2 elements were similar but not identical. Although the removal of radiostrontium had fallen to zero by the time 24 r.b.v. of milk had passed through the resin the mean efficiency for the whole batch was then 53 %.

The efficiency of removal of ^{85}Sr by the bed of Zeo-Karb 225 in the mixed ionic form was such that the whole process had an efficiency of 95 %. The lower curve in Fig. 1 (*c*) also shows that the efficiency of removal of radiostrontium was independent of the ratio of the volumes of the 2 resin beds. Results for all ratios of bed volume have been plotted and all lie on the same curve.

Effect of temperature

The breakthrough point of Ca in milk flowing through the Amberlite IR-50 became progressively later as the temperature was raised to 30 °C, but with little change above that temperature (Fig. 2*a*). These experiments were carried out on a resin charged (K) to only 30 ions %. Although this resulted in an earlier breakthrough at all temperatures than would have been obtained with a higher mole fraction of K, there is no reason to suppose that a different conclusion as to the effect of temperature would have been reached.

As Easterly, Harris, Bunce & Edmondson (1963) had found that the efficiency of

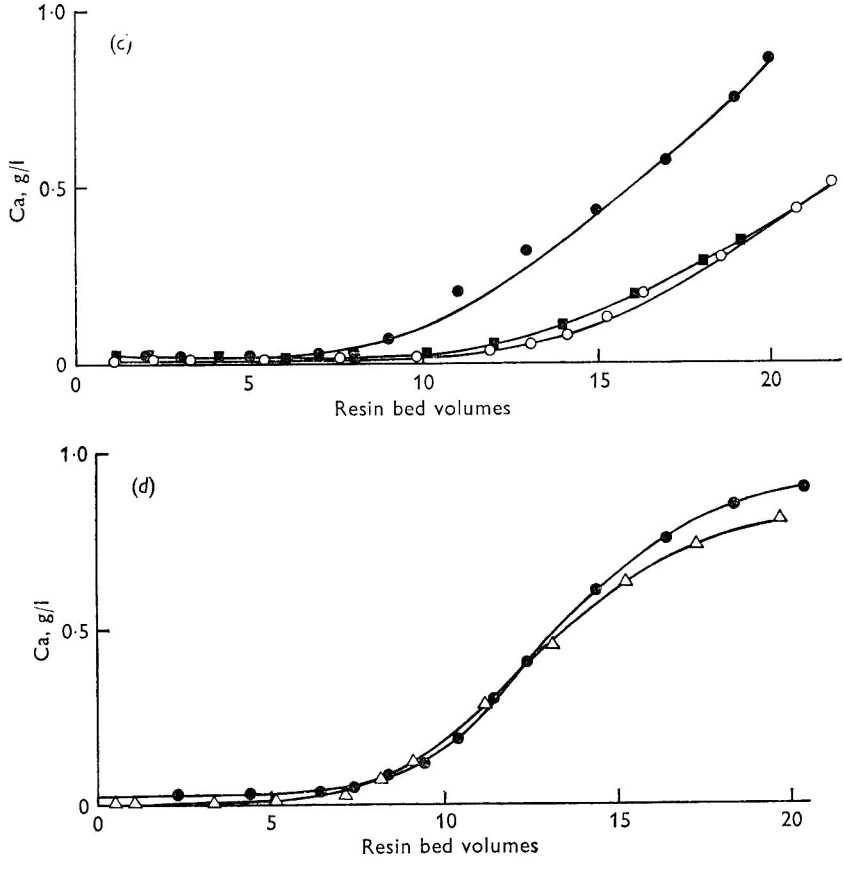

Fig. 2. For legend see opposite page.

removal of radiostrontium from milk at normal pH by a sulphonic acid-type resin in the mixed ionic form also increased with temperature up to 40 °C, it was decided to use this temperature for double-bed systems.

Effect of flow rate

Fig. 2(*b*) shows that between 0·05 and 0·2 r.b.v./min the slower the flow rate of milk the later the breakthrough of ^{85}Sr and the greater the average efficiency of removal. It was, however, decided to use 0·1 r.b.v./min in the double-bed process as a slower flow rate produced only a slight improvement in efficiency and would have entailed inconvenient modifications in the existing plant.

Restoration of the cationic composition and adjustment of pH

When the Ca content of the milk was restored exactly to what it had been before treatment, there was a net deficiency of other cations which must have been due at least in part to the presence of the H ions responsible for the reduction in pH. The relative proportions of the ions on the resin bed were therefore adjusted so as to produce the least possible alterations in the Ca and Mg content of 30 r.b.v. of treated milk. This confined the deficiency in metallic cations to Na and K which could easily be added in the form of soluble compounds when the pH was finally adjusted. The composition of the solution required for charging the resin bed was found by experiment to be, per l: $CaCl_2.2H_2O$, 72·2 g; KCl, 23·1 g; NaCl, 8·5 g; $MgCl_2.6H_2O$, 15·1 g.

As one of the objects of developing the present method was to avoid large changes of pH, even local and temporary, alkali metal bicarbonates or carbonates were preferred for final adjustment of pH. Bicarbonates were found, however, to give a saline taste to the milk and a mixture of K and Na carbonates was therefore used. The solution which produced the milk of ionic composition and pH nearest to that found before treatment was a mixture of equal volumes of 1·2 N-Na_2CO_3 and 0·8 N-K_2CO_3 added at the rate of 11–12 ml/l.

THE PILOT PLANT

Preliminary experiments

Preliminary investigations into the optimum operating conditions were commenced before the investigations with laboratory columns were complete and in particular before the influence of temperature upon the efficiency of the Amberlite IR-50 was known. For this reason the earlier experiments were carried out at a variable temperature, depending upon how long the milk had been stored since pasteurization. Another characteristic of the Amberlite IR-50 that also was not at first appreciated was that after conversion to the K/H form by treatment with KOH the distribution of K was not uniform throughout the bed (Fig. 3). Once this fact was realized some of the experiments on the efficiency of the Amberlite IR-50 were carried out with the resin fully converted to the K form. Complications arising from the necessity of taking samples at different depths were then avoided. In later experiments, the ionic capacity of the whole bed was measured by first converting it completely to the H form and then determining the total amount of KOH required to convert it back to the K form. Once the total ionic capacity was known, any desired mean K:H ratio could be obtained by treating the bed with the calculated amount of KOH.

Effect of column geometry

In order to ascertain whether the geometry of the resin bed could influence its efficiency, resin fully converted to the K form was used to compare the removal of Ca by beds 30 cm in diam. and 48 cm deep (about 34 l resin), 15·5 cm in diam. and 149 cm deep (about 28 l resin) and 2·5 cm in diam. and 20 cm deep (about 100 ml resin). The results of these experiments are shown in Fig. 2(*c*). The shorter wider bed

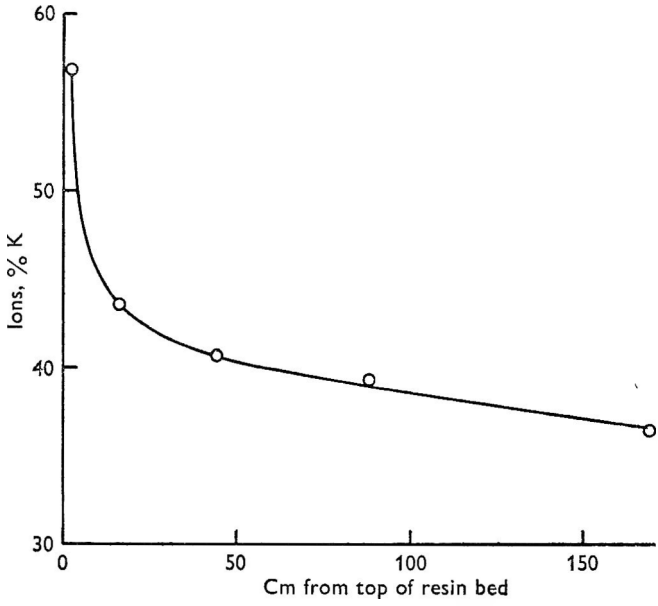

Fig. 3. Variation of K concentration with depth in bed of Amberlite IR-50 after charging to mean K:H ratio of 40:60.

was the least efficient but the deeper narrower bed had about the same efficiency as the laboratory bed. It thus appeared that in scaling up the process the bed height as well as diameter would have to be increased and would need to be about 140 cm. A glass column of diam. 22 cm was installed which, filled to a depth of 140 cm, contained 53 l of resin. Its efficiency for the removal of Ca from milk was found to be about the same as that of a standard laboratory bed (Fig. 2*d*).

Final operational procedure

The plant as finally operated and tested included 2 glass columns. The first was of diam. 22 cm and height 300 cm and contained 53 l Amberlite IR-50 charged with the ions of K and H in the ratio 40:60. The second was of diam. 30 cm and height 200 cm and contained 79 l of Zeo-Karb 225 that had been previously treated with 2000 l (25 r.b.v.) of a solution containing per l: $CaCl_2.2H_2O$, 72·2 g; KCl, 23·1 g; NaCl, 8·5 g; $MgCl_2.6H_2O$, 15·1 g. Each resin bed about half-filled the column.

Homogenized milk (1600 l) at 40 °C was pumped through the plant at 0·1 r.b.v./min with respect to the first resin bed and the pH finally adjusted with the mixture of sodium and potassium carbonates already described. In most of the experiments the

milk was stored at 40 °C for up to 5 h after pasteurization. Bacterial growth during this period was prevented by the addition of penicillin at a concentration of 1 unit/ml. Heat-exchangers were subsequently installed by means of which the milk previously cooled to 8–9 °C after pasteurization was heated to 40–42 °C immediately before application to the columns and cooled to about 10 °C immediately after. As the mean time of residence on the columns was only 23 min no excessive bacterial growth was observed in any experiment. After all the milk had been treated the resin beds were cleaned and regenerated as summarized in Table 2.

Table 2. *Procedure for washing and regenerating resin beds (all by upflow unless otherwise stated.)*

Amberlite IR-50		Zeo-Karb 225	
1. Cold water from mains	30 min	1. Cold water from mains	30 min
2. Circulate hot (75 °C) alkaline detergent + 100 ppm available chlorine. Drain	20 min	2. Circulate hot (75 °C) alkaline detergent + 100 ppm. available chlorine. Drain	20 min
3. Cold water from mains	10 min	3. Cold water from mains	10 min
4. Drain and circulate 110 l of 1·3 N-HCl	20 min	4. Drain and circulate 270 l 0·5 N-HNO_3 at 75 °C	20 min
5. Drain, rinse for a few minutes with cold water and drain. Circulate calculated quantity of KOH dissolved in about 100 l water till uptake is complete	About 45 min	5. Drain, rinse and treat with 25 r.b.v. (2000 l) of regenerant solution + 50 ppm. available chlorine at 0·2 r.b.v./min by downflow	About 125 min

Radiochemical results

Removal of ^{85}Sr introduced in vitro

Results of 5 expts with milk containing ^{85}Sr introduced in vitro are shown in Fig. 4. In all these experiments penicillin had been added to the milk. The efficiency of removal of ^{85}Sr was fairly consistent, except for expt 3 whose low efficiency was due to an early breakthrough on the first stage, the reason for which is unknown.

The average overall efficiencies of removal of the nuclide were 87·4, 89·8 and 93·2 % on 30, 25 and 30 r.b.v. respectively. This compares with 97·1 and 97·5 % for 30 and 25 r.b.v. that were obtained by the method of Murthy *et al.* (1962) (Glascock & Bryant, 1968). The average amount treated for a removal of 90 % was 24 r.b.v.

After the installation of heat exchangers one experiment only was carried out on milk containing ^{85}Sr and ^{137}Cs introduced in vitro. The efficiency of removal of the ^{85}Sr in this experiment was 87 % for 30 r.b.v. confirming that the antibiotic had had no effect on efficiency in the earlier experiments.

Removal of ^{85}Sr introduced in vivo

In the first experiment with milk containing ^{85}Sr introduced in vivo the only measurements made were of total radioactivity in the milk before and after treatment of 30 r.b.v. These showed that the efficiency of the removal was only 78 % as

compared with a mean value of 87 % for ^{85}Sr introduced in vitro. As the milk had been preserved with formalin, which might conceivably have had an effect on efficiency, a second experiment was carried out on milk containing no formalin and about 14 times as much ^{85}Sr. This permitted the establishment of curves of specific radioactivity of the effluent from the 2 resin beds (Fig. 4). These curves were similar to those obtained with milk containing the nuclide introduced in vitro, except that the concentration in the effluent from the first bed rose at 22 r.b.v. to 106 % of its original value. The reason for this is unknown. The efficiencies of removal by

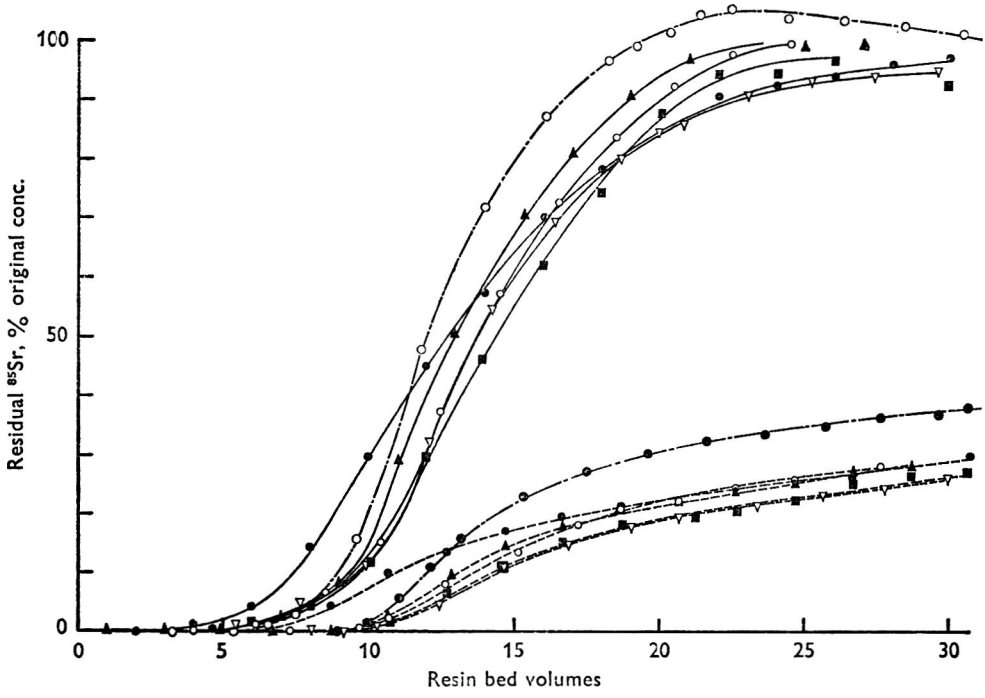

Fig. 4. Concentration of ^{85}Sr in milk after passage through first and second beds of pilot plant. First bed, Amberlite IR-50 K:H = 40:60. Second bed, Zeo-Karb 225 mixed ionic form. Temperature 40 °C. Upper curves, effluent from first bed. Lower curves, effluent from second bed. Uppermost curves in each group (○·–·○ and ●·–·●) ^{85}Sr introduced in vivo. All other curves, replicate experiments with ^{85}Sr introduced in vitro.

the whole system were 89, 84 and 81 % for 20, 25 and 30 r.b.v. respectively, as compared with 95·2, 89·8 and 87·4 % for ^{85}Sr introduced in vitro. Although the value for 30 r.b.v. was a little higher than that obtained in the first experiment where the milk had been preserved with formalin, there is no reason to suppose that the formalin had in fact affected the efficiency of removal. It therefore appears that environmental ^{90}Sr, which is also introduced in vivo, would be removed from 30 r.b.v. of milk with an efficiency of about 80 %.

Removal of ^{137}Cs

Only one detailed experiment was carried out on the removal of ^{137}Cs, the results of which are shown in Fig. 5. The nuclide was introduced in vitro and the milk con-

tained penicillin. In spite of the early breakthrough (5 r.b.v.) on the first stage which was followed by a rise in concentration in the eluate to nearly 150 % of the original concentration, the efficiency of removal of the nuclide by the second stage remained high for the whole of the 30 r.b.v. treated. The overall efficiency was 96·4 %. In the experiment with milk containing both nuclides and passed through heat exchangers the overall efficiency of removal of ^{137}Cs was 98·7 %, thus confirming, as for ^{85}Sr, that penicillin has no effect on the removal of this nuclide. No experiments were carried out on the removal of ^{137}Cs introduced in vivo because the element does not enter into organic combination and would therefore be removed with the same efficiency whether introduced in vitro or in vivo.

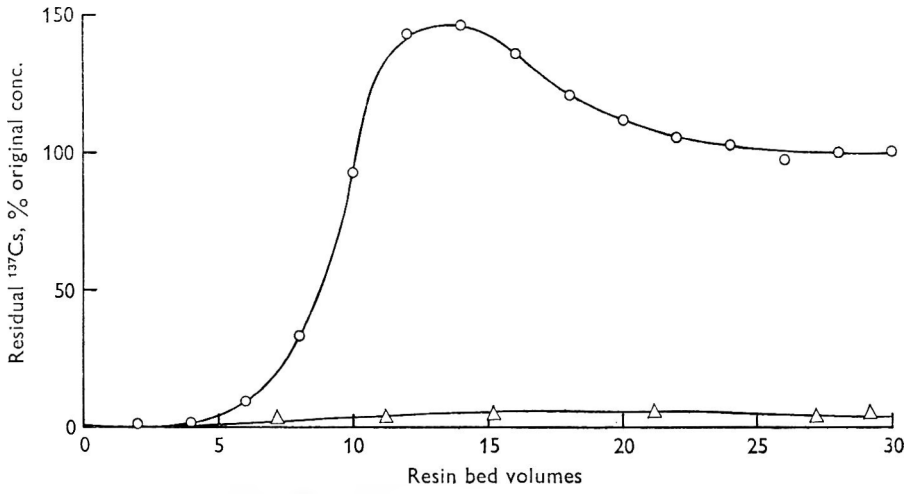

Fig. 5. Concentration of ^{137}Cs introduced in vitro into milk after passage through first and second beds of pilot plant. First bed, Amberlite IR-50 K:H = 40:60. Second bed, Zeo-Karb 225 mixed ionic form. Temperature 40 °C. Upper curve, effluent from first bed. Lower curve, effluent from second bed.

Composition and quality of processed milk

Principal constituents

Table 3 shows the concentration of the principal constituents of treated and untreated milk, together with enzymic clotting time. The treatment in the plant produced very little change in composition and most of the small differences observed are probably within the range of analytical error. It is to be noted in particular that no loss of total solids, fat or SNF, was observed. Although there were small losses amounting to about 10 % of K and Mg neither total nor soluble Ca was affected. There was also no appreciable loss of either total or soluble phosphate, although up to 10 % was expected from experiments on laboratory columns. The negligible effect on soluble Ca and phosphate is in contrast with that of the process of Murthy *et al.* (1962), in which a considerable increase in both occurred when citric acid was used for lowering the pH before passage through the resin. This negligible effect on soluble Ca in the present work is reflected in an unchanged enzymic clotting time which, again, was much increased in the process of Murthy *et al.* (Glascock & Bryant, 1968).

Fig. 6 shows variations in the concentration of Ca, K, Na and Mg during processing. The curves of concentration of these ions in the milk after treatment by the first stage only are also given as being of some interest.

Table 3. *Analysis of plant-treated milk*

Observation	Control	Treated milk
Added water, %	—	1·125
Total nitrogen, mg/100 g	507	496
Total solids, %	12·22	12·11
Fat, %	3·50	3·50
S.N.F., %	8·72	8·61
Chloride, mg/100 g	94·8	91·0
Citrate, mg/100 g	177	174
K, mg/100 ml	144	132
Na, mg/100 ml	62·5	60·9
Mg, mg/100 ml	12·6	11·0
Total Ca, mg/100 g	114	113
Soluble Ca, mg/100 g	35·9	37·4
Total P, mg/100 g	94·8	92·3
Soluble P, mg/100 g	37·2	34·9
Rennet clotting time	21·5 min	20·0 min

Lead, copper, arsenic, antimony and iron

Table 4 shows that treatment of the milk in the pilot plant produced a 6-fold increase in the concentration of lead which, however, was still below the legal limit for 'milk-based beverages' (1 ppm, Statutory Instruments, 1961). The slightly increased copper content was still within the normal range and the small amount of iron present was of little importance. The concentrations of arsenic and antimony were unchanged.

Vitamins

Table 5 shows that the loss of vitamins which occurred as a result of treatment by the double-bed process was similar both in type and magnitude to that resulting from the process of Murthy *et al.* (1962) (Glascock & Bryant, 1968). The greatest losses were of thiamine, 67%; nicotinic acid, 34%; riboflavin, 14%; vitamin B_6, 12% and vitamin B_{12}, 11%. Losses of fat-soluble vitamins were negligible. It is of interest to note that the loss of nicotinic acid which occurred in the process of Murthy *et al.* (1962) also occurs in the double-bed process.

Appearance and flavour

The appearance of the homogenized milk was affected neither by passage through the resin beds nor by the subsequent adjustment of pH with alkali metal carbonates. This suggests the production of less physico-chemical changes in the milk than resulted from the process of Murthy *et al.* (1962), where neutralization with KOH caused the milk to become noticeably more yellow (Glascock & Bryant, 1968).

To determine the effect of processing on the flavour of the milk, a test was carried out with the assistance of 17 members of the Institute staff. Participants were asked to decide which of 3 samples of treated and/or untreated milk were indistinguishable from a fourth sample. Nine subjects correctly grouped their 4 samples. The probability of such a result arising by chance was only 0·0002. Nevertheless, when asked

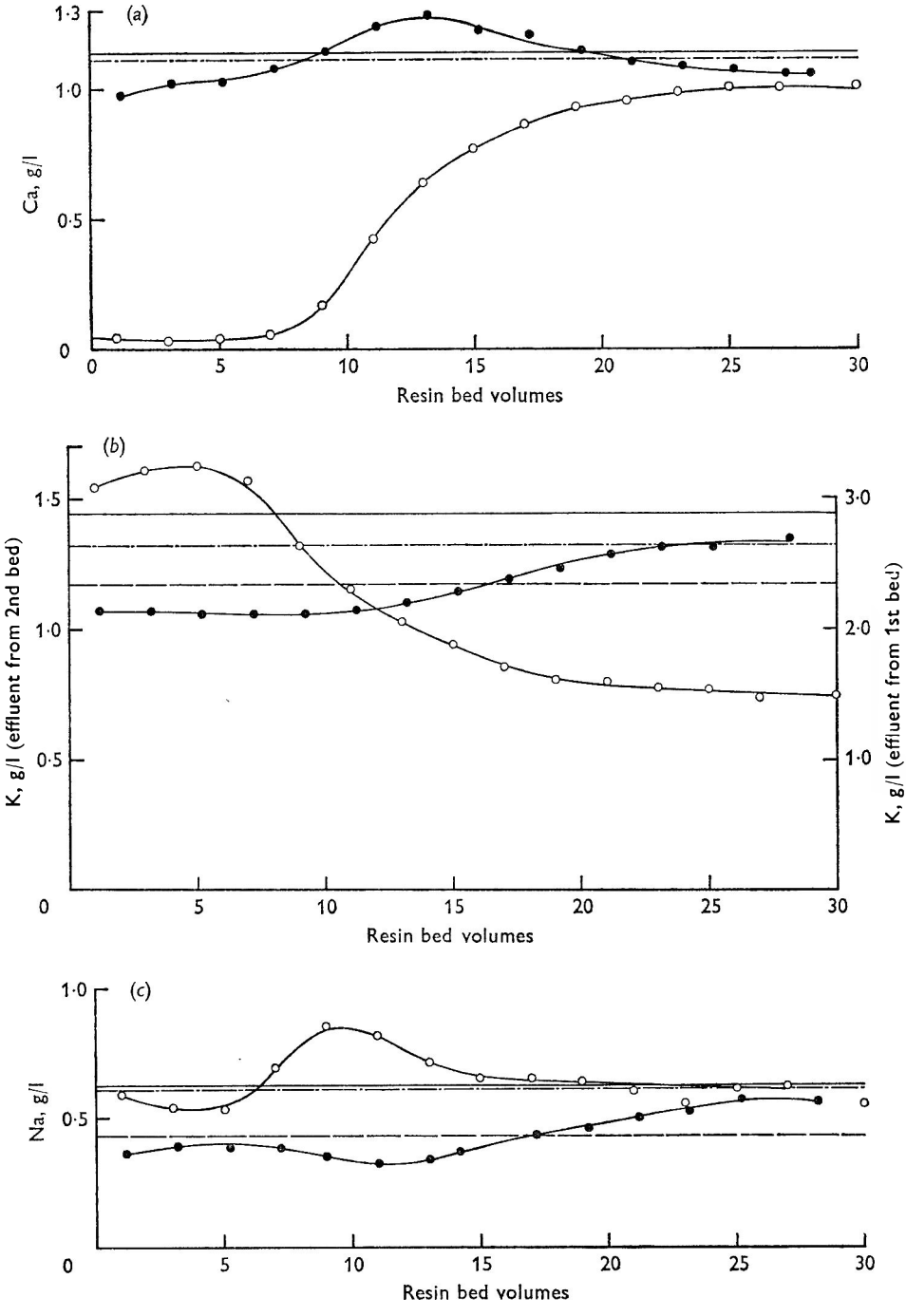

Fig. 6. Curves of concentration of cations in milk after passage through first and second resin beds of pilot plant at 40 °C. First bed, Amberlite IR-50 K:H = 40:60. Second bed, Zeo-Karb 225 mixed ionic form. (*a*) Ca, (*b*) K, (*c*) Na, (*d*) Mg. ○, Effluent from first bed; ●, effluent from second bed. Horizontal lines: ——, concentration in milk before treatment; – –, concentration after passage through both beds; –·–, concentration after adjustment of pH with K_2CO_3 + Na_2CO_3.

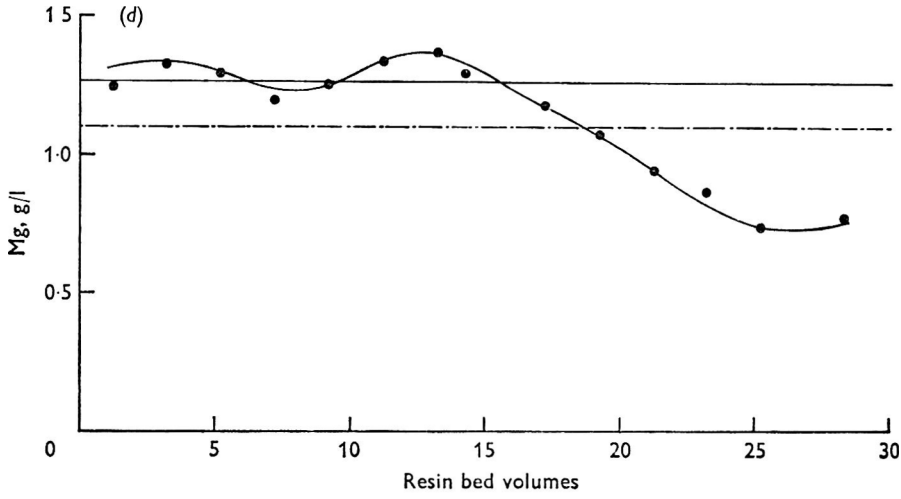

Fig. 6. For legend see opposite page.

Table 4. *Effect of processing on the content of some metals*

Metal	Concentration, mg/l	
	Untreated milk	Treated milk
Lead	0·016	0·095
Copper	0·11	0·16
Arsenic	0·004	0·002
Antimony	< 0·004	< 0·004
Iron	0·51	1·23

Table 5. *Effect of processing on the vitamin content of milk*

Vitamin	Concentration, mg/l	
	Before treatment	After treatment
Riboflavin	1·3	1·1
Pantothenic Acid	3·3	3·1
Vitamin B_{12}	0·0035	0·0031
Vitamin B_6	0·41	0·36
Biotin	0·019	0·018
Nicotinic Acid	1·05	0·81
Folic Acid	0·05	0·06
Thiamine	0·42	0·16
Vitamin C	15	14
Vitamin A	0·40	0·38
Carotene	0·35	0·35
α-Tocopherol	0·96	0·95
Xanthophyll	0·04	0·04

to state their preference, 9 preferred the treated milk, 6 preferred the untreated milk and 2 were undecided. It is concluded therefore that, although the process causes a change in the flavour of the milk, this change is by no means unacceptable.

Spray and roller drying

Milk treated by this process for nation-wide use would almost certainly be distributed in the dried form. A sample of the treated milk was therefore dried on pilot

plant equipment at the National College of Food Technology, Weybridge. It was first concentrated to about half its original volume by means of a natural circulation evaporator operating at 62 °C and at a pressure of 8·4–8·8 kN/m^2 (63–66 mmHg). During this process the milk frothed more than does normal milk, suggesting that difficulty might perhaps have been experienced with a climbing film evaporator. Although this frothing may have been due to release of CO_2 formed when the pH was adjusted, the pH of the milk did not rise during this part of the process.

Some of the concentrated milk was then dried on a 23-cm roller heated to 127 °C. Again, frothing occurred, but to a much smaller extent than during concentration, and caused no difficulty. The concentrated milk was also successfully spray-dried at an inlet temperature of 170 °C. When roller- or spray-dried milk was reconstituted only a trace of free oil was found to be present.

Removal of nuclides from resin beds

Amberlite IR-50

Circulation of acid through the first bed removed 99·5 % or more of both ^{85}Sr and ^{137}Cs, as would be expected from the known properties of carboxylic acid-type resins.

Zeo-Karb 225

Treatment of the resin bed with HNO_3 and then with 25 r.b.v. (with respect to the bed of Zeo-Karb 225) of regenerating solution removed 97–98 % of the ^{85}Sr and 99–100 % of the ^{137}Cs. During treatment with regenerating solution, however, the concentration of ^{85}Sr in the eluate was found to reach a maximum after the passage of about 7·5 r.b.v. This suggests a different distribution of ^{85}Sr on the bed from that obtained in the method of Murthy *et al.* (1962), in which the maximum concentration occurs in the first few r.b.v. of the eluate and then diminishes smoothly through regeneration. In that method it was found convenient to collect the eluate in 4 equal parts and to re-use all but the first quarter, which contained about 85 % of the total ^{85}Sr eluted. In the present process, more ^{85}Sr was removed in the second quarter of the eluate than in the first, and the first 2 quarters together contained 72 % of the total. It would thus be feasible to re-use only half of the regenerating solution.

Because of this finding, the distribution in the eluate of ^{137}Cs was examined in less detail. Only 6 % of the total ^{137}Cs eluted was, however, found in the second half of the eluate, which thus presents no obstacle to its re-use.

DISCUSSION

In general, the double-bed process described in this paper appears to be comparable in efficiency, cost and convenience of operation with that of Murthy *et al.* (1962). A study of this process in our laboratory has been described in a previous paper (Glascock & Bryant, 1968). Although a decrease in the pH of the milk during treatment by the double-bed process was not altogether avoided, it was so small (0·6 unit) as to be unlikely to cause any change in the properties of the milk proteins. Furthermore, the change in chemical composition was less than that produced in the process of Murthy *et al.* (1962). In particular, large increases in enzymic clotting time and in the citrate and K contents did not occur. There were some losses of vitamins,

especially of thiamine, but these occurred to about the same extent as in the process of Murthy *et al.* and could easily be made good. All other changes in chemical composition were within the limits of normal variation for untreated milk.

The overall efficiency of removal of ^{85}Sr from milk by the double-bed process (80 % when introduced in vivo) was lower than that obtained by the process of Murthy *et al.* (96 %). Against this must be set the much higher efficiency of removal of ^{137}Cs which was 96 % as compared with 73 %. The efficiency of removal by the Zeo-Karb 225 of ^{85}Sr in the effluent from the bed of Amberlite IR-50 was higher than expected. The efficiency of Zeo-Karb 225 in the mixed ionic form for the removal of radiostrontium from milk at neutral pH is known to be only about 50 % (Glascock & Bryant, 1968). As the bed of Amberlite IR-50 had removed 53 % of the ^{85}Sr in the milk, not more than half of the residual 47 % was expected to be removed by the second stage of the double-bed process. The proportion removed was in fact 86 % which suggests that the Amberlite IR-50 had converted bound strontium to the free ionic form even after its capacity to remove it had been exhausted. The unexpectedly high efficiency of removal of ^{137}Cs can be only partly explained by the effect on the Zeo-Karb 225 of the small increase of pH in the effluent from the first bed. Glascock & Bryant (1968) found that at pH 6·8 a similar bed would remove 86 % of ^{137}Cs instead of the 73 % removed at pH 5·25. As all the ^{137}Cs of milk is already in the free ionic form there can be no question of its conversion from the bound to the free state by the first resin bed and the high overall efficiency of removal therefore remains unexplained.

The difference between the 2 processes in their efficiency of removal from milk of the 2 main contaminating radionuclides must be evaluated in the light of conditions likely to prevail if it should ever be necessary to operate the process on a nation-wide scale. The dose to bone marrow and endosteum from ingested ^{137}Cs is about the same as that from ^{90}Sr (Medical Research Council, 1966), and in addition ^{137}Cs irradiates the rest of the body, and ^{90}Sr does not. Furthermore, under circumstances of high fallout, the ratio of ^{137}Cs to ^{90}Sr in milk is also high. For example, in 1964, when the concentrations of these nuclides in milk were at a maximum, this ratio was about 5:1 (Agricultural Research Council, 1970), and under such circumstances it might be at least as important to remove the one nuclide as the other. Calculations show that after the treatment of 30 r.b.v., 23 % of 'total radioactivity' (i.e. $^{90}Sr + ^{137}Cs$) would be left in milk contaminated to the 1964 level and treated by the method of Murthy *et al.* (1962), as compared with only 7 % when treated by the double-bed method. Although it is scarcely admissible to add together the hazards due to the 2 nuclides, this calculation does, however, suggest that the double-bed method is the more efficient for the decontamination of milk.

The equipment required for the operation of the double-bed process on a pilot plant at full commercial scale differs in some important respects from that used in the process of Murthy *et al.* (1962). For regular daily use 2 pairs of resin columns would be necessary, one pair being washed and regenerated while the other is in use. These 2 extra columns, together with the heat exchangers and homogenizer, which are not used in the other process, would take up extra space and add to the cost. Furthermore, resin regeneration is somewhat more complicated and larger quantities of chemicals are used, especially on the bed of Zeo-Karb 225. On the other hand, the 2 metering pumps required for the addition of acid and alkali and the 2 sets of in-line

pH monitoring equipment which are used in the process of Murthy *et al.* (1962) are now dispensed with and their absence makes the plant simpler to operate.

It is not yet known whether the product of the double-bed process is nutritionally superior to that of the process of Murthy *et al.* (1962). Experiments on baby animals are, however, in progress.

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Reviews of the progress of dairy science

Section A. Physiology. Hormonal control of lactogenesis

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CONTENTS

INTRODUCTION

A bibliographical review on the hormonal control of lactogenesis may well seem superfluous less than 2 years after a symposium, the proceedings of which were subsequently published (Reynolds & Folley, 1969), in which all aspects of milk secretion at parturition were reviewed and discussed. However, it seemed of interest to review not only the nature of the hormones which control milk secretion but also the relations which arise between plasma concentrations of pituitary, adrenal, and ovarian hormones, as well as the time when milk secretion first begins, which in many

species is well before parturition. It is in fact known that the mammary gland of ruminants is swollen with an abundant secretion well before the end of pregnancy, and indeed pre-partum milking has even been recommended. There is, however, no reason why synthesis of all the constituents of milk should begin simultaneously. Fat globules are present in the epithelial cells from the earliest stages of pregnancy in the rabbit (Bousquet, Fléchon & Denamur, 1969) and the rat (Murad, 1970), while the development of the Golgi apparatus, which is responsible for the secretion of most of the milk proteins, takes place much later. Nevertheless, in the course of examining the mammary gland for the compounds which require a functioning Golgi apparatus (lactose, caseins, α-lactalbumin and β-lactoglobulin) secretory activity has been detected on the 20th day in the rat (Shinde, Ôta & Yokoyama, 1965; Kuhn & Lowenstein, 1967; Kuhn, 1968, 1969*a*), on the 13th day in the mouse (Lockwood, Turkington & Topper, 1966), on the 90–100th day in the ewe (Denamur, 1965), and on the 21–22nd day in the rabbit (Denamur, 1963*a*, 1965). Recent observations on the rabbit by electron microscopy (Bousquet *et al.* 1969) have confirmed results obtained biochemically, and it seems reasonable to enquire whether precocious secretion is related to changes in plasma hormone levels.

On the other hand, this review will not discuss (*a*) the central nervous control of the secretion of those hormones concerned with lactogenesis, (*b*) those aspects of secretory mechanisms recently discussed by Bargmann & Welsch (1969), Hollman (1969), Bousquet *et al.* (1969) and Wooding, Peaker & Linzell (1970), or (*c*) the cellular changes caused by lactogenic hormones (Denamur, 1969*b*, *c*; Topper, 1970).

PART I. LACTOGENIC HORMONES

A. LACTOGENESIS IN ENDOCRINE-DEPRIVED ANIMALS AND IN ORGAN CULTURE

Comparative aspects of the hormonal control of lactogenesis have been discussed recently (Denamur, 1969*a*). The present survey is therefore limited to the more important findings on the endocrinology of experimental lactogenesis.

Minimum requirement: rabbit (1 *hormone in vivo*, 2 *in vitro*)

Ovine or bovine prolactin is the minimum requirement for lactogenesis in pseudo-pregnant rabbits deprived of adrenals and ovaries (Cowie & Watson, 1966) or of pituitary or pituitary, adrenals and ovaries (Denamur, 1965, 1969*a*; Denamur & Delouis, 1971*a*). Restoration of lactation may also be produced by the administration of prolactin to rabbits hypophysectomized during lactation (Cowie, Hartmann & Turvey, 1969).

A combination of prolactin and insulin added to cultures of mammary tissues from intact or hypophysectomized pseudopregnant rabbits results in secretory activity which may be detected by histological examination (Delouis, Bousquet & Denamur, 1971). Prolactin obtained from a culture of rabbit pituitary tissue has the same effect as ovine prolactin on mammary explants. These in vitro experiments have recently been complemented by a demonstration of lactose synthesis from [^{14}C]glucose under the influence of prolactin and insulin (Delouis & Denamur, 1971). It appears that cortisol (hydrocortisone) is not essential, but that it increases the output of [^{14}C]-

lactose when added to prolactin and insulin; this is in accordance with the significant secretion, judged by histological criteria, observed in rabbit mammary explants when prolactin and insulin with cortisol or corticosterone were added to the medium (Barnawell, 1965; Forsyth, 1970; Brumby & Forsyth, 1969; Delouis *et al.* 1971). A convincing demonstration that prolactin and insulin in combination are lactogenic in the absence of any corticosteroid is still lacking. Experiments are in progress to determine whether the synthesis of [^{14}C]lactose from [^{14}C]glucose can be stimulated by prolactin and insulin in mammary explants from adrenalectomized rabbits.

Prolactin is not the only peptide hormone with lactogenic effects in the intact rabbit. In the pseudopregnant rabbit, milk secretion may be induced not only by prolactin but also by human chorionic somatomammotrophin (HCS) (Josimovich & MacLaren, 1962; Friesen, 1965, 1966), by human growth hormone (HGH) (Chadwick, Folley & Gemzell, 1961; Chadwick & Folley, 1963; Forsyth, Folley & Chadwick, 1965), or by monkey growth hormone (Forsyth, 1965). HGH is also lactogenic in rabbits hypophysectomized during lactation (Cowie *et al.* 1969). However, there is no information on the ability of HGH or HCS to induce milk secretion in the rabbit deprived of pituitary, adrenals and ovaries, though this might be expected, since the combination HGH + corticosterone + insulin is at least as effective as prolactin + corticosterone + insulin in mammary explants from pseudopregnant rabbits (Rivera, Forsyth & Folley, 1967; Forsyth, 1970). It would be interesting to know what HCS or HGH can do in the absence of corticosteroids.

Minimum requirement: small mammals (2 *hormones in vivo*, 3 *in vitro*)

Complexes including prolactin

Mouse. Nandi (1958, 1959) and Nandi & Bern (1961) have experimented in vivo on animals deprived of pituitary, adrenals and ovaries, and Elias (1959, 1961), Elias & Rivera (1959), Rivera & Bern (1961), Lasfargues (1962), Rivera (1964*a*, *b*), Juergens, Stockdale, Topper & Elias (1965) and Wellings, Cooper & Rivera (1966) have studied organ cultures. All strains of mice respond to prolactin, but the degree of response is characteristic of the strain (see also Nagasawa, Yanai, Iwahashi, Fujimoto & Kuretani, 1967). Cortisol is much more active in vivo than is corticosterone, the principal natural glucocorticoid of the mouse, but deoxycorticosterone with prolactin has no lactogenic effect in vitro (Rivera, 1964*b*).

The following were the minimum concentrations in the culture medium for the stimulation of secretion in mammary explants (Turkington, Juergens & Topper, 1967): aldosterone, 10^{-9}M; cortisol, 10^{-8}M; corticosterone, 10^{-7}M; at a concentration of 3×10^{-7}M, aldosterone, cortisol, cortisone, prednisolone and 21-deoxycortisol were very effective, but corticosterone had little effect below $1{\cdot}5 \times 10^{-5}$M. Rivera (1964*b*) had previously shown that aldosterone, cortisol and corticosterone have equal potencies at concentrations of 1–5 μg/ml.

Rat. The injection of prolactin and a glucocorticoid into rats deprived of pituitary (Lyons, Li & Johnson, 1958; Gale, Taleisnik, Friedman & McCann, 1961; Denamur & Gaye, 1968) or of pituitary, adrenals and ovaries (Bintarningsih, Lyons, Johnson & Li, 1958; Lyons *et al.* 1958; Cowie & Lyons, 1959) induces milk secretion, as does the injection of a glucocorticoid into hypophysectomized rats with

a pituitary graft under the renal capsule (Ahmad & Lyons, 1966) or into rats with the pituitary isolated from the hypothalamus (Gale *et al.* 1961).

Growth hormone is synergistic with prolactin in vivo (Lyons *et al.* 1958) but not in vitro (Barnawell, 1965). There is some indication that glucocorticoids, especially the principal one elaborated by a species, were effective, but the concentration must be 10 times that of aldosterone (Barnawell, 1965).

Hamster. Bitch. Guinea-pig. Recent work has been confined to mammary explants (Barnawell, 1965, 1967). Growth hormone (GH) does not increase the response to prolactin of explants of bitch or hamster mammary glands. The secretory response of guinea-pig explants is very limited with all combinations studied.

Complexes including ovine or bovine growth hormone

In vivo studies have been carried out on mice deprived of pituitary, adrenals and ovaries (Nandi, 1961; Nandi & Bern, 1961). Different strains may be ranked in order of decreasing sensitivity to GH: C3H $\approx$ DBA > BALB/c > C3Hf = C57BL > A; strain A is virtually insensitive to GH. Strain sensitivity to GH stems from genetic differences in the mammary tissue and cannot be explained by differences in hormone metabolism (Nandi, 1961).

Rivera (1964*a*, 1966) has demonstrated with mammary explants that strain C3H is more sensitive, and strain A less sensitive, to bovine GH.

Since all the strains are receptive to prolactin with adrenal steroid or with adrenal steroid and insulin, it is of particular interest to compare the lactogenic potency of ovine prolactin with those of different preparations of ovine, bovine or porcine GH. It appears that the efficacy of these growth hormones, even for the most sensitive (C3H) strain, reaches only 50 % (ovine), 50 % (bovine) and 20 % (porcine) of the potency of prolactin (Rivera *et al.* 1967). However, ovine and bovine GH have specific effects on mammary explants from C3H mice in that they affect DNA synthesis at lower concentrations (4×10^{-9} M) than does insulin (4×10^{-8} M). On the other hand, considerably more mammary cells are stimulated by insulin than by GH (Turkington, 1968*a*).

Complexes including human or monkey growth hormone

A preparation of HGH (HS 612 A prepared by Wilhelmi) together with an adrenal steroid (1 μg/ml aldosterone or corticosterone) and insulin is more lactogenic in mouse strains C3H and A than is ovine prolactin under the same conditions (Rivera *et al.* 1967). All other preparations of human or monkey GH are equal or superior to prolactin in their effect on C3H mice. Rivera (1969) and Rivera & Cummins (1970) have also shown that human GH produces functional changes in mouse mammary explants identical to those produced by prolactin.

Complexes including human chorionic somatomammotrophin

In mammary explants from strain A mice, milk secretion is induced by HCS with an adrenal steroid and insulin comparable to that induced by equal concentrations (5 μg/ml) of prolactin with cortisol or corticosterone and insulin (Turkington & Topper, 1966; Turkington, 1968*b* – synthesis of casein, α-lactalbumin and

β-lactoglobulin measured; Forsyth, 1967*a* – histological examination). For strain C3H the minimum effective concentration of HCS, about 0·3 μg/ml, is similar to that of prolactin (Turkington & Topper, 1966), but for optimum stimulation the requirement per ml is 2 μg HCS or 1 μg prolactin (Forsyth, 1967*a*).

Complexes including rodent placental hormone

Such complexes are lactogenic in the rat and mouse (Averill, Ray & Lyons, 1950; Ray, Averill, Lyons & Johnson, 1955; Matthies, 1965, 1967, 1968; Shani, Zanbelman, Khazen & Sulman, 1970; Kohmoto & Bern, 1968, 1970: cultures of rat or mouse placental tissue were used in conjunction with mammary explants from the same animals). The placental hormone is active in combination with prednisolone when in contact with the mammary gland of rats deprived of pituitary, adrenals and ovaries, and has no GH activity (Matthies, 1967). It has been called rat chorionic mammotrophin (RCM) (Matthies, 1967) and is found in trophoblast extracts or in blood serum on the 12th day of pregnancy (Matthies, 1967; Cohen & Gala, 1969). Mammogenic activity of placental extracts has also been demonstrated in the rat by Desjardins, Paape & Tucker (1968) and Kinzey (1968, 1970), and in the mouse by Cerruti & Lyons (1960).

Minimum requirement: small ruminants
(more than 2 hormones in vivo, 3 in vitro)

The combination prolactin + GH + adrenal steroid is the minimum for milk secretion after pituitary ablation in the goat (Cowie, Knaggs & Tindal, 1964). The combination prolactin + adrenal steroid is not effective in the hypophysectomized ewe (Denamur, 1965, 1969*a*; Denamur, Delouis & Cannata, 1971). The addition of thyroxine and GH results in rapid secretion of significant quantities of milk, which is in agreement with the stimulatory properties of GH on milk production in the ewe (Denamur & Martinet, 1970).

Discussion and conclusions

Variations between species in minimum requirement

Prolactin in vivo or prolactin and insulin in vitro are constituents of the hormone complex which can induce milk secretion in all the species and strains studied. At present, the rabbit appears to require only one hormone in vivo, the mouse, rat, guinea-pig, hamster and bitch to require at least two, and small ruminants to require several. The initiation of synthesis of characteristic constituents of milk by mammary tissue having a developed lobule-alveolar system appears, therefore, to be under the control of hormonal factors varying in number from species to species.

Such differences may perhaps be explained by the fact that the mammary tissues of different species do not have the same degree of alveolar development when subjected to experimental lactogenic stimulus, nor have they experienced in the course of their development from the foetus the same hormonal stimuli or conditioning which would cause a specific programming of the mammary cells.

Small ruminants. The complexity of the hormonal association and the long intervals in hypophysectomized animals between the administration of hormones and the

lactogenic response may well indicate hormonal requirements for redevelopment of the mammary gland which has substantially regressed after hypophysectomy, as well as specific requirements for lactogenesis. Experiments are under way, using explants of ewe mammary tissue, to distinguish between mammogenic and lactogenic effects by sequential addition of the various hormones implicated in vivo. In any case the hormonal requirement for mammogenesis in the goat is just as complex as is that in the rat or mouse (Cowie, Tindal & Yokoyama, 1966). It is also significant that the lactogenic complex in the rat includes progesterone as well as prolactin, cortisol and insulin when the foetal mammary gland is studied at a very early stage (Ceriani, 1970*a*, *b*).

Rabbit. The apparent simplicity of the lactogenic complex, which has not yet been unequivocally demonstrated, does not exclude the possibility of a differentiating action of glucocorticoids before the second half of pseudopregnancy.

It therefore seems premature, before the role and the moment of intervention of each constituent of the lactogenic complex has been more clearly elucidated, to conclude that milk secretion is induced by a complex of hormones varying in number from species to species.

Variations between genotypes of a species

The secretory response of the different mouse strains to the combinations GH + adrenal steroid and prolactin + adrenal steroid is characteristic for each strain; the extreme case is that of strain A which is insensitive to GH. The sensitivity of a genotype to a particular combination of hormones increases with the number of pregnancies (Rivera, 1964*a*), though in C3H mice lactogenesis can be induced equally well by prolactin, ovine or bovine GH, HGH, or HCS, in conjunction with a mineralocorticoid or with natural or synthetic glucocorticoids. Furthermore, HCS and HGH may be substituted for prolactin in the rabbit. Cellular phenomena as complex as lactogenesis may then be triggered off by different hormonal components acting in similar molar concentrations on the same target organ. It is therefore logical to enquire whether a common steric configuration, or amino acid sequences common to the different hormones, may explain their common lactogenic property.

These problems have been discussed by Forsyth (1968), Li (1968), Denamur (1969*a*) and Sherwood (1969). Also the complete amino acid sequence of ovine prolactin has been determined (Li, Dixon, Schmidt, Pankov & Lo, 1969; Li, Dixon, Lo, Pankov & Schmidt, 1969), and a comparison with the sequences in HGH (Li, Liu & Dixon, 1966; Li, Dixon & Liu, 1969; Li, Dixon, Lo, Pankov & Schmidt, 1969; Li, Dixon, Schmidt, Pankov & Lo, 1969) reveals a close similarity between the C-terminal sequences. HCS and HGH have the same sequence of 12 amino acids at the C terminal (Sherwood, 1969). Dellacha, Santomé & Paladini (1968) and Santomé, Dellacha & Paladini (1968) have further observed that many sequences are common to human and bovine GH. Lastly, ovine prolactin and bovine GH have analogous conformations (Aloj & Edelhoch, 1970).

Lactogenic peptides in woman and other primates

HCS is found in high concentrations in the plasma of the pregnant woman (see references in Forsyth, 1967*b*) and must play an important part in mammogenesis and

lactogenesis, as is shown particularly by the stimulation in hypophysectomized primates of a transitory secretion after parturition (Agate, 1952; Kaplan, 1961). HGH also has lactogenic activity (Lyons, Li, Ahmad & Rice-Wray, 1968; Forsyth, 1968, 1970) and its administration increases milk secretion in women suffering from hypogalactia (Lyons *et al.* 1968). Moreover, isolation and purification of a primate prolactin lacking GH activity has not been effected (Greenwood, 1967; Forsyth, 1968, 1969). However, 3 arguments may be added to those of Lyons (1969) concerning the variability of mammogenic and somatotrophic activity of preparations of HGH, and they are not consistent with an important lactogenic role for HGH.

(1) Clinical and experimental observations show that the concentrations of HGH in the plasma of lactating women or those with galactorrhoea is small (Greenwood, Hunter & Klopper, 1964; Board, 1968; Roth, Gorden & Bates, 1968; Spellacy, Carlson & Schade, 1968; Benjamin, Casper & Kolodny, 1969; Forsyth, 1969). Moreover, lactation occurs in the absence of HGH in ateliotic dwarf women (Rimoin *et al.* 1968) or after pituitary stalk section (Eckles, Ehni & Kirschbaum, 1958), a procedure which diminishes the secretion of HGH (Antony *et al.* 1969).

It appears therefore that the lactogenic activity of the plasma of lactating women cannot be explained by its GH content (Pasteels, 1967; Forsyth, 1969).

(2) The 'prolactin' and 'growth hormone' activities are secreted as distinct molecular entities by primate pituitaries in organ culture (Pasteels, Brauman & Brauman, 1963; Brauman, Brauman & Pasteels, 1964; Pasteels, 1967, 1969; Nicoll, Parsons, Fiorindo, Nichols & Sakuma, 1970). These findings are not, however, confirmed by the results of Solomon, Grant, Burr, Kaplan & Grumbach (1969) obtained with cultures of pituitary cells.

(3) Primate pituitaries contain GH- and prolactin-secreting cells which differ in stain affinities (Herlant & Pasteels, 1967; Goluboff & Ezrin, 1969), in ultrastructure as revealed by the electron microscope (Peake, McKeel, Jarett & Daughaday, 1969), and in fixation of fluorescent antibodies (Herlant & Pasteels, 1967; Herbert & Hayashida, 1970).

All these experimental findings suggest that prolactin also exists in primates and affects mammary secretion.

Insulin in organ culture

The mammogenic properties of insulin, and the necessity for its presence in organ cultures to enable prolactin or more complex combinations to be lactogenic, have been analysed and reviewed by Topper (1968, 1970) and Denamur (1969*a*).

There are also interesting discussions on the action of insulin on the mammary gland in the publications of Leader & Barry (1969), El Darwish & Rivera (1970), Mayne & Barry (1970) and Takizawa, Furth & Furth (1970), but the problem of cellular modifications induced by lactogenic hormones is outside the scope of this review.

Lactogenic complexes in rat and mouse

The minimum hormonal requirement for lactogenesis in rat and mouse is prolactin or GH in conjunction with an adrenal steroid. Lactogenesis produced by prolactin with prednisolone is, however, augmented by GH in the rat deprived of pituitary,

adrenals and ovaries (Lyons *et al.* 1958). Likewise the combination prolactin + GH + glucocorticoid (1 mg corticosterone or 125 μg cortisol per day) is much more active than GH or prolactin with a glucocorticoid in mice deprived of pituitary, adrenals and ovaries (Nandi, 1958; Nandi & Bern, 1961; Wellings & Nandi, 1968 – ultrastructure criteria) or in mouse mammary explants (Wellings *et al.* 1966; Wellings, 1969). Moreover, these authors have shown that to obtain optimum lactogenesis in vivo a strict relationship must be observed between the quantities of prolactin (1 mg) and GH (0·1 mg). In particular it seems likely that such combinations, allowing the organism to utilize the smallest concentrations of each hormone, correspond to normal conditions of lactogenesis. However, Nandi & Bern (1961) have not found any benefit from the addition in vitro to this combination of aldosterone (8–40 μg), thyroxine (2 μg) or insulin (0·1 i.u.).

B. LACTOGENESIS IN PREGNANCY

Adrenal steroids

Injections

Injections of glucocorticoids can induce precocious milk secretion in the rat (Talwalker, Nicoll & Meites, 1961 – 2 mg cortisol daily from the 9th to the 13th day; Meunier, 1962 – 10 mg cortisone daily from 1st to 8th and 12th to 19th day; Fan Kuo-Yee, 1964 – 15 mg cortisone daily from 12th to 18th day), in the mouse (Nandi & Bern, 1961), in the cow (Tucker & Meites, 1965, 15 mg 9α-fluoroprednisolone acetate daily for 6–7 days half-way through the 5th and 7th months of pregnancy), in the ewe (Delouis & Denamur, 1967 – 25 and 50 mg cortisol daily from 100th to 120th or 120th to 140th day of pregnancy), in the rabbit (Talwalker *et al.* 1961 – 15 mg cortisol daily from 16th to 19th day; Meites, Hopkins & Talwalker, 1963 – 15 mg cortisol daily from 15th to 18th day; Denamur, 1965; and Denamur & Delouis, 1971*b* – 1·95, 3·75, 7·5 and 15 mg cortisol daily at 4 stages of pregnancy). The degree of milk secretion induced depends on the stage of pregnancy during which the glucocorticoid is injected. Thus the lactogenic response is negligible before the 100th day in the sheep (Delouis & Denamur, 1967) or the cow (Tucker & Meites, 1965), and small doses of cortisol have little effect during the first two thirds of pregnancy in the rabbit (Denamur & Delouis, 1971*b*).

Lactogenesis in the rat may be accompanied by a slight increase in total DNA content of the mammary gland (Kumaresan, Anderson & Turner, 1967), but Ferreri & Griffith (1969) observed no such change, nor did Delouis & Denamur (1967) in the ewe. An increase in RNA is more clearly produced by glucocorticoids (Kumaresan *et al.* 1967), especially if the hormone is administered at the end of pregnancy to rats (Ferreri & Griffith, 1969) or to ewes (Delouis & Denamur, 1967).

These findings suggest that the secretion of glucocorticoids may represent a limiting factor of importance in the appearance of milk secretion during pregnancy in the cow, ewe, rat, mouse and rabbit, but recent results of Davis & Liu (1969) have not confirmed earlier observations on the rat. In fact, cortisol given on days 8–12, 14–15, 14–18 (2 mg/day) or on days 8–12 (6 mg/day) did not induce the secretion of protein having immunological and electrophoretic properties comparable to those of rat casein. Moreover mammary concentrations of RNA and the incorporation of ^{32}P

into the ribosomes were not increased by cortisol. The authors concluded that sub-optimal secretion of adrenal steroids was not responsible for the absence of milk secretion during pregnancy in the rat.

Adrenalectomy

The specific role of the adrenals in the reproductive process is very poorly understood, even in the rat (see, for example, the contradictory results of Anderson & Turner, 1963, and Thoman, Sproul, Seeler & Levine, 1970). There seems, however, to be no doubt that adrenalectomy in the rat after the 11th day does not prevent normal completion of pregnancy (Mayer, 1957). This is explained by the intervention of the foeto-placental complex, which maintains a minimum level of plasma corticosterone in adrenalectomized mothers (Kamoun & Stutinsky, 1968; Kamoun, 1970). Nevertheless, adrenalectomy on the 14th (Davis & Liu, 1969) or 16th (Meunier, 1962) day of pregnancy in the rat inhibits subsequent secretory activity and expansion of the mammary alveoli by a secretory fluid (Meunier, 1962), and considerably diminishes the secretion of casein-like material and the increase in mammary RNA after ovariectomy on the 15th day (Davis & Liu, 1969). Cortisol, corticosterone (Davis & Liu, 1969) or cortisone (Meunier, 1962) restore normal milk secretion in rats deprived of adrenals or adrenals and ovaries, which confirms that glucocorticoids are essential for lactogenesis in this species.

Maternal glucocorticoids

Secretion of maternal glucocorticoids during the period of lactogenesis will be considered in turn in each of the 4 species in which the period is fairly well known.

Rat. Plasma corticosterone increases at the end of pregnancy (Saba & Hoet, 1963; Milković & Milković, 1963; Kamoun, Mialhe-Voloss & Stutinsky, 1965; Voogt, Sar & Meites, 1969), at least in relation to the values during the first 14 days, which are 60 % below those for virgin rats (Gala & Westphal, 1965*a*). However, although Kamoun *et al.* (1965) observed a significant increase in concentration of plasma corticosterone between the 19th and 21st days (which is in good agreement with the appearance of lactose in mammary extracts on the 19th or 20th day), Voogt *et al.* (1969) found that significant concentrations appeared only during parturition. Raised values for plasma corticosterone at the end of pregnancy must reflect an increased secretion of the hormone, since its metabolic clearance rate does not change (Kamoun, 1970).

The free corticosterone fraction in plasma is the physiologically active form of the corticoid (Slaunwhite, Lockie, Back & Sandberg, 1962). It is in equilibrium with the fraction bound to plasma proteins – albumin and a specific globulin called transcortin or corticosteroid-binding globulin (CBG) (Slaunwhite & Sandberg, 1959; Daughaday, 1958). The binding capacity of rat plasma increases after administration of thyroxine (Labrie, Pelletier & Fortier, 1966; Gala & Westphal, 1966*a*), of oestrogens or of progesterone (Gala & Westphal, 1965*b*); it is depressed by glucocorticoids and by testosterone (Gala & Westphal, 1965*b*, 1966*b*; Seal & Doe, 1965).

During the course of pregnancy complex interactions in some of these hormonal factors come into play. Pregnancy in the rat does not increase the total binding capacity of CBG (Gala & Westphal, 1965*a*; Koch, Mialhe-Voloss & Stutinsky, 1967;

Koch, 1969), perhaps because of a loss of stimulatory properties on the part of thyroxine (Koch, Mialhe-Voloss, Lutz & Stutinsky, 1968). Values for albumin–corticosterone binding are, however, significantly lower at the end of pregnancy (Koch *et al.* 1967), resulting in an increase in free corticosterone from the 20th day (Koch *et al.* 1967; Gala & Westphal, 1967); this corresponds to the first signs of milk secretion.

Ewe. The concentration of plasma cortisol is very low (compared with woman, guinea-pig, rat, etc.) and does not change during the first 120 days of pregnancy (Paterson & Hills, 1967; Paterson & Harrison, 1968). During the last 2 weeks of pregnancy, Paterson & Harrison (1967, 1968) even observed a decrease in the cortisol concentration, which resulted from an increase in metabolic clearance rate and not a diminution in rate of secretion. There is little CBG in the ewe (Lindner, 1964) and it does not increase during pregnancy (Lindner, 1964; Paterson & Hills, 1967). Despite low plasma concentrations, 58 % of the cortisol is bound to CBG, 20 % to albumin, and 20 % is free (Paterson & Hills, 1967).

Thus, in contrast to the rat, there appears to be no precise relation between the plasma level of cortisol and lactogenesis towards the 90–100th day of pregnancy.

Rabbit. Milk secretion is initiated at about the 21st day of pregnancy, at which time the concentrations of free glucocorticoids reach values as high as during lactation (Gala & Westphal, 1967). Plasma concentrations of cortisol and corticosterone are on the contrary at their lowest about the 27–28th day, and CBG rises considerably at the end of pregnancy. Interpretation of results with the rabbit may, however, be complicated by the fact that the affinities of cortisol, corticosterone and progesterone for serum globulin do not follow the normal rule of polarity, namely interaction between protein and steroid varies inversely with the number of polar groups of the steroid (Westphal, 1967).

Mouse. Plasma corticosterone increases considerably from the 12th to the 18th day of pregnancy (Gala & Westphal, 1967). Despite a considerable increase in CBG activity during this period, the concentration of free corticosterone rises steadily, which accords with the physiological role of glucocorticoids in initiating the secretory phenomena which are visible from the 13th day (Lockwood *et al.* 1966).

A similar rise in the level of circulating glucocorticoids during pregnancy has been observed in woman (Gemzell, 1953; Assali, Garst & Voskian, 1955), in the guinea-pig (Gala & Westphal, 1967; Rosenthal, Slaunwhite & Sandberg, 1969), and in the bitch (Seal & Doe, 1963, 1966), but not in the monkey (Wolf & Bowman, 1966). Moreover, in woman, guinea-pig and bitch, plasma protein-binding capacity becomes more important. As a result of relative variations in these 2 factors the concentration of free glucocorticoids rises strongly in the guinea-pig between the 50th and 60th days (Gala & Westphal, 1967). In domesticated animals – cow, goat, sow – plasma concentrations of glucocorticoids are low (Lindner, 1964; Saba, 1964; Heap, Holzbauer & Newport, 1966; Heitzman, Adams & Hunter, 1970), but there appear to be no studies on the rate of secretion of these hormones during pregnancy.

Altogether these experiments show that in many species the adrenal cortex becomes more active at the end of pregnancy. Recent research therefore confirms earlier findings based on indirect criteria for measuring adrenocortical activity, such as weight of thymus, liver glycogen, adrenal levels of vitamin C and cholesterol, adrenal

lipids, weight and histological appearance of adrenals (see references in Bearn, Gould & Jones, 1960; Poulton & Reece, 1957; Pepper, 1961; Ito & Hoshino, 1962; Anderson & Turner, 1962; Ito, Hoshino & Sawauchi, 1964; Kamoun, 1970).

Foetal glucocorticoids

The corticoids secreted by the foetal adrenal glands have been identified in woman (Stark, Gyévai, Szalay & Ács, 1965 – cortisol, cortisone, corticosterone), in the ewe (Chester-Jones, Jarrett, Vinson & Potter, 1964 – cortisol and corticosterone; Alexander *et al.* 1968 – cortisol, corticosterone, aldosterone), in the cow (Chouraqui & Weniger, 1969*a* – cortisol, corticosterone, 11-dehydrocorticosterone), in the rabbit (Chouraqui & Weniger, 1969*b* – cortisone, cortisol, aldosterone), and in the bitch (Jackson & Piasecki, 1969 – cortisol and corticosterone). Also the secretion of corticosterone has been studied in the rat foetus (Milkovic & Milkovic, 1962; Kamoun, Mialhe-Voloss & Stutinsky, 1964; Roos, 1967; Holt & Oliver, 1968), and that of cortisol in the guinea-pig embryo (Hoar, 1965). Metabolic pathways for the synthesis of steroids in the foetal adrenals of the lamb have been recently studied and found to differ from those in the adult ewe (Anderson, Pierrepoint, Jones, Griffiths & Turnbull, 1970).

Can foetal secretions contribute to lactogenesis in the maternal mammary gland? In the rat, the foetal adrenals at 21–22 days excrete as much corticosterone as do the adrenals of the mature animal (Milkovic & Milkovic, 1962) and the corticotrophic activity of the foetal pituitary is at a maximum between the 15th and 20th days of pregnancy (Cohen, 1963), i.e. the stage of lactogenesis. Corticosterone secreted by the foetus can moreover pass into the maternal circulation from the 18th day (Kamoun & Stutinsky, 1968 – adrenalectomized rats) and blood levels of corticosterone in the pregnant rat fall by only 50 % after adrenalectomy at the end of pregnancy (Milkovic & Milkovic, 1963). It is therefore possible that in this species secretions from the foetal adrenals may act on the maternal mammary glands, but further work is necessary to support this hypothesis.

Pregnancy in the ewe is prolonged by foetal hypophysectomy (Liggins, Kennedy & Holm, 1967; Comline, Silver & Silver, 1970) or adrenalectomy (Drost & Holm, 1968; Van Rensburg, 1967). Premature delivery, on the other hand, is obtained by infusion into the foetus of ACTH, dexamethasone or cortisol (Van Rensburg, 1967; Halliday & Buttle, 1968; Liggins, 1968, 1969*a*, *b*). Deoxycorticosterone or corticosterone infused into the foetus or ACTH, cortisol or oestradiol-17β injected into the mother do not initiate parturition. Lastly, progesterone injected into the ewe does not block the action of dexamethasone administered to the foetus, nor does it prolong pregnancy beyond the normal period in the ewe that is not receiving glucocorticoids (Bengtsson & Schofield, 1963). Parturition appears to be triggered when the foetal adrenals reach a certain development (Comline & Silver, 1961; Comline *et al.* 1970; Liggins, 1968, 1969*b*), resulting 5 or 6 days before parturition in increased secretion of glucocorticoids (Bassett & Thorburn, 1969). However, the rise in concentration of glucocorticoids in the foetal plasma occurs well after the appearance of mammary secretion, and has no repercussions on plasma concentrations in the ewe (Bassett & Thorburn, 1969). One cannot, however, exclude entirely the possibility that foetal glucocorticoids affect the maternal mammary gland since Drost & Holm (1968)

observed that the mammary gland did not develop normally after adrenalectomy of the foetus on the 110th day of pregnancy, even if the pregnancy continued.

Prolactin

Exogenous prolactin

Talwalker *et al.* (1961) have demonstrated that 30 i.u. prolactin daily from the 9th to the 13th day of pregnancy in the rat do not produce any milk secretion. Nandi & Bern (1961) obtained similar negative results with mice. Injection into the ewe of considerable quantities of prolactin (1200 i.u./day) caused only limited secretion in a minority of treated animals (4 out of 9); it had no significant quantitative effect on mammary DNA or on the RNA/DNA ratio (Delouis & Denamur, 1967). On the other hand, prolactin is very clearly lactogenic and mammogenic in pregnant rabbits (Denamur, 1963*b*). The cellular hyperplasia and hypertrophy which develop and also the induction of secretory activity in the last third of normal pregnancy (Denamur, 1963*a*) may all be controlled from the beginning of the pregnancy through the action of this hormone. Meites, Hopkins & Talwalker (1963) similarly obtained milk secretion in the rabbit by the administration of 110 i.u./day of prolactin from the 15th to 19th day of pregnancy.

Endogenous prolactin

Plasma levels of prolactin in the rat, after being comparatively high on the 2nd and 3rd days of pregnancy, remain low until the 20th day and then rise significantly on the 22nd day (Amenomori, Chen & Meites, 1970). In the ewe the plasma level falls from the 1st to the 5th month (Arai & Lee, 1967). In these 2 species, as also in the goat (Bryant, Greenwood & Linzell, 1968; Brumby & Forsyth, 1969), plasma prolactin reaches very high levels in the hours following parturition.

Thus, lactogenesis occurs in the ewe at the beginning of a phase of greater excretion of oestrogens in the urine (see Part II), but plasma concentrations of prolactin remain low and constant. These low levels, however, can only be considered indicative of the rate of pituitary secretion of prolactin if the metabolic clearance rate has not risen after the 70–90th day of pregnancy. Although measurement of the half-life of prolactin depends on the dose of exogenous hormone administered (van der Gugten & Kwa, 1970), it is interesting that Grosvenor (1967) should have found enormous variation with stage of development in the rat. The half-life is 10–12 min for ovine prolactin in the ovariectomized female and 3 min in the lactating female. Therefore studies on the rate of prolactin secretion are necessary to elucidate its importance for normal lactogenesis in the ewe.

In the rat 2 aspects of prolactin secretion during pregnancy call for comment.

(1) Increases in plasma oestrogens and prolactin are well correlated with the appearance of mammary secretion. The increase in oestrogens (Yoshinaga, Hawkins & Stocker, 1969), detectable from the 18th day, clearly precedes that of prolactin. This is in agreement with the stimulatory effect of injected oestrogens on the release of prolactin from the rat pituitary (see the review of Meites & Nicoll, 1966), and on the blood concentration of prolactin (Kwa & Verhofstad, 1967*b*; Kwa, van der Gugten & Verhofstad, 1969; Chen & Meites, 1970; van der Gugten, Sala & Kwa, 1970). Plasma prolactin is also at a higher level at pro-oestrus (Niswender, Chen, Midgley, Meites &

Ellis, 1969) or oestrus (Kwa & Verhofstad, 1967*a*, *b*; Amenomori *et al.* 1970), while the prolactin content of the pituitary reaches its maximum during these phases of the cycle (Sinha & Tucker, 1969) and its minimum during di-oestrus (Sar & Meites, 1967) or after ovariectomy (Baker, Clark & Hunter, 1963; MacLeod, Abad & Eidson, 1969). Furthermore, oestrogen reduces the activity in the hypothalamus of the prolactin inhibiting factor (PIF), directly increases pituitary release of prolactin (see reviews of Meites, Nicoll & Talwalker, 1963, Meites & Nicoll, 1966, and Rivera & Kahn, 1970), and stimulates prolactin synthesis in vitro (Baker *et al.* 1963; Catt & Moffat, 1965, 1967; Jones, Fisher, Lewis & Vanderlaan, 1965; Baker & Zanotti, 1966; Kahn & Baker, 1966; MacLeod *et al.* 1969). Finally, implantation of oestrogens in the median eminence increases the prolactin content of the pituitary (Ramirez & McCann, 1964; Nagasawa, Chen & Meites, 1969) and the plasma concentration of prolactin (Nagasawa *et al.* 1969).

The important secretion of prolactin at the end of pregnancy corresponds to the fall in blood levels of progesterone (see Part II). During pregnancy, progesterone partially inhibits the stimulatory effect of oestrogen on prolactin synthesis (Meites, 1966; Meites & Nicoll, 1966), although the administration of large doses of progesterone raises the blood and pituitary contents of prolactin (Chen & Meites, 1970), induces pseudopregnancy (Alloiteau & Vignal, 1958), slows down mammary involution after the litter is weaned (Rothchild, 1965), and diminishes the PIF activity of the hypothalamus (Pasteels & Ectors, 1967; Sar & Meites, 1968). However, 4 mg/day of progesterone, if injected in the presence of oestrogens, significantly inhibits the rise in plasma prolactin produced by 1 μg oestradiol (Chen & Meites, 1970). It seems likely therefore that the fall in progesterone and rise in oestrogens in the plasma are the factors responsible for the important secretion of prolactin observed at the end of pregnancy in the rat.

(2) Plasma and pituitary concentrations of prolactin (Meites, 1966; Amenomori *et al.* 1970) show little increase before the 20th day of pregnancy. As we have just seen, it is possible that the oestrogen–progesterone equilibrium during pregnancy restrains prolactin synthesis by the pituitary, but one may equally well question whether the RCM activity of the rat placenta does not come into play from the 12th day to control pituitary activity. This hormone has luteotrophic, mammogenic and lactogenic properties just like prolactin, and it has recently been shown that plasma prolactin controls its own secretion by acting at the level of the hypothalamus on the PIF activity (Clemens & Meites, 1968). In fact, prolactin implants in the median eminence of the rat diminish the prolactin content of the pituitary (Clemens & Meites, 1968) or the blood (Niswender *et al.* 1969), shorten pseudopregnancy (Chen, Voogt & Meites, 1968), alter the normal course of pregnancy (Clemens, Sar & Meites, 1969*a*), inhibit milk secretion (Clemens, Sar & Meites, 1969*b*) and induce regression of the mammary gland (Clemens & Meites, 1968; Mishkinsky, Nir & Sulman, 1969) and of the corpus luteum (Clemens & Meites, 1968). Furthermore, when grafted into rats, pituitary tumours that secrete large quantities of prolactin diminish the prolactin content of the host pituitary (MacLeod, Smith & DeWitt, 1966; MacLeod, DeWitt & Smith, 1968; Chen, Minaguchi & Meites, 1967) and prolactin synthesis (MacLeod & Abad, 1968). Similarly, isografts of pituitaries in the rat (Welsch, Negro-Vilar & Meites, 1968) or the mouse (MacLeod, 1970) lower the prolactin content of the host pituitary

and retard synthesis. The stimulatory effect of oestrogens on prolactin synthesis persists despite the presence of pituitary tumours (MacLeod *et al.* 1969), but the raised level of circulating prolactin seems to modify the threshold for oestrogen action (Welsch, Sar, Clemens & Meites, 1968). Using an ingenious method Averill 1969) has demonstrated the existence of a short feed-back of prolactin on pituitary secretion: pituitary grafts placed in the hypophysiotrophic zone of the hypothalamus contained numerous prolactin-containing cells, as Flament-Durand (1965) had suggested earlier, but they also prevented the formation of the corpus luteum of pregnancy which is normally stimulated by mating.

Pituitary growth hormone

Very little work has been done using radio-immunology on changes in plasma concentrations of GH during pregnancy and lactogenesis. In the rat, Schalch & Reichlin (1966) have shown that neither pregnancy nor lactation affects the level of GH in the blood, but that very important changes occur after hypophysectomy (Schalch & Reichlin, 1966; Frohman & Bernardis, 1968), in the presence of some pituitary tumours (Peake, Mariz & Daughaday, 1968; Frohman, Bernardis & Kant, 1968), or after destruction (Frohman & Bernardis, 1968) or electric stimulation (Frohman *et al.* 1968; Frohman & Bernardis, 1970) of the ventro-median nuclei. Even less is known of the ewe (Wallace & Bassett, 1970), for which plasma concentrations during pregnancy appear to be small (Bassett, Thornburn & Wallace, 1970) and slightly lower during lactation than are those in the lamb. In woman, normal or reduced levels of GH have been found in the blood during pregnancy (Ehrlich & Randle, 1961; Glick, Roth, Yalow & Berson, 1963; Kaplan & Grumbach, 1965*a*, *b*; Board, 1968; Grumbach, Kaplan, Sciarra & Burr, 1968; Spellacy & Buhi, 1969); these are surely overestimated values because of the cross-reaction which occurs between HGH and HCS, and they contrast with the high concentrations in the foetal circulation (Grumbach *et al.* 1968). Different criteria (hypoglycaemia, arginine infusion) used during pregnancy to estimate the capacity of the pituitary to secrete GH indicate a clear hyposecretion at the end of pregnancy (Spellacy, 1967; Yen, Samaan & Pearson, 1967; Mintz, Stock, Finster & Taylor, 1968; Grumbach *et al.* 1968; Tyson, Rabinowitz & Merimee, 1968). Lastly, in the early part of lactation HCS is no longer detectable and the low plasma concentrations of HGH (see Part I and Spellacy & Buhi, 1969) indicate feeble secretory capacity in the pituitary (Katz, Grumbach & Kaplan, 1968).

All these results suggest that GH is of little importance for lactogenesis in the rat and in woman. In the ewe, data are too scanty to be correlated with secretory activity (Wallace & Bassett, 1970; Bassett *et al.* 1970). It is, however, important to notice that fasting (Machlin *et al.* 1968; Trenkle, 1967, 1970) or the infusion of short-chain fatty acids (Hertelendy, Machlin & Kipnis, 1969) does not affect GH secretion in the ewe, but that it is diminished by infusion of adrenaline (Hertelendy *et al.* 1969; Wallace & Bassett, 1970), increased after infusion of insulin (Wallace & Bassett, 1970), and is not affected by hyperglycaemia (Wallace & Bassett, 1970).

Regulation of GH secretion in the ewe is therefore different from that reported in woman (Glick & Goldsmith 1968; Baylis *et al.* 1968). It is therefore possible that the development of plasma GH is also different in the 2 species during pregnancy.

Human chorionic somatomammotrophin

Forsyth (1967*b*) and Grumbach *et al.* (1968) have produced excellent reviews of the principal findings on the development of HCS secretion during pregnancy in woman. This hormone, which is detectable in the mother's plasma towards the 6th week of pregnancy, appears in considerable concentrations by the time of parturition. Thus at term, placental secretion of HCS amounts to 290 mg/day (Beck & Daughaday, 1967) or 1·03–1·78 g/day (Kaplan, Gurpide, Sciarra & Grumbach, 1968), which agrees with the significant incorporation of [^{3}H]leucine into HCS (Suwa & Friesen, 1969*a*, *b*; Friesen, Suwa & Pare, 1969).

Elaboration of HCS is peculiar in that it is independent of any circadian rhythm and is not affected by blood concentrations of glucose or insulin (Grumbach *et al.* 1968). It is an indication of good placental functioning during pregnancy (Genazzani, Aubert, Casoli, Fioretti & Felber, 1969).

While the precise period of lactogenesis is not known for woman, it is very likely that HCS plays a physiologically essential role in the evolution of the mammary gland during pregnancy. It is therefore of interest that Gusdon, Leake, VanDyke & Atkins (1970) have detected in the bitch, the sow, the mare and the ewe placental proteins having some of the immunological properties of HCS.

HCS has somatotrophic properties which are perhaps responsible for the slight secretion of HGH during pregnancy. It is in fact known that injections of GH diminish the GH content of the pituitary in the rat (Kruhlich & McCann, 1966; Muller & Pecile, 1966) and inhibit GH secretion in the monkey (Sakuma & Knobil, 1970). Likewise hypothalamic implants of GH (Katz, Molitch & McCann, 1969) have a negative effect on the GH content of the pituitary, and pituitary tumours secreting GH diminish greatly the GH content of host pituitaries (MacLeod *et al.* 1966; Peake *et al.* 1968), as well as reducing synthesis of the hormone (MacLeod *et al.* 1968).

PART II. CONTROL OF LACTOGENESIS BY OVARIAN STEROIDS

PROGESTERONE

Exogenous progesterone

Since the work of Meites & Sgouris (1953, 1954) on the rabbit, there have been a few experiments in vivo on the rat and the rabbit, and in vitro on mammary explants from the bitch and the mouse.

In vivo experiments

Rat. Experimental lactogenesis in the rat has been produced by ovariectomy or hysterectomy in the middle of pregnancy (Liu & Davis, 1967), or during the 2 or 3 days preceding parturition (Shinde *et al.* 1965; Yokoyama, Shinde & Ôta, 1969; Kuhn, 1969*a*). Lactose appears in the mammary gland 12 h (Kuhn, 1969*a*) to 48 h (Shinde *et al.* 1965; Yokoyama *et al.* 1969) after ovariectomy at the end of pregnancy, and proteins having immunological or electrophoretic properties of rat caseins are detectable at the end of 24 h after ovariectomy in mid-pregnancy (Liu & Davis,

1967). Similarly, hysterectomy leads to lactose formation, but after a slightly longer delay than occurs after ovariectomy (Kuhn, 1969*a*; Yokoyama *et al.* 1969). This difference may probably be explained by the extremely rapid fall in plasma progesterone after ovariectomy, while the half-life of placental luteotrophic hormone is sufficiently long to retard this decrease (Kuhn, 1969*a*).

In contrast to the results of ovariectomy and hysterectomy, daily injections of up to 8 mg progesterone decrease the amount of lactose formed after the 19th day of normal pregnancy (Kuhn, 1969*a*), and inhibit its appearance 24 h after ovariectomy or 34·5 h after hysterectomy (Kuhn, 1969*a*; Yokoyama *et al.* 1969), even if the ovariectomized rat receives 25–50 i.u. prolactin (Kuhn, 1969*a*).

Small doses of progesterone (3 mg/day) maintain pregnancy without preventing lactose formation (Yokoyama *et al.* 1969), which makes it clear that small changes in progesterone secretion at the end of pregnancy are compatible with the maintenance of pregnancy and the initiation of milk secretion.

Rabbit. Progesterone administered daily to pregnant rabbits decreases the amount of lactose detectable in the mammary gland on the 24th day and retards the incorporation of [^{14}C]glucose into lactose (Denamur & Delouis, 1971*b*). However, in the pseudopregnant ovariectomized rabbit, progesterone (0·4, 1, 2·5, or 6·25 mg/day) does not block the lactogenic activity of 12·5 i.u. prolactin when injected twice daily (Denamur, Delouis & Gaye, 1970).

In vivo experiments on the rat and the rabbit suggest therefore the existence of a negative correlation between lactogenesis and plasma levels of progesterone. It is nevertheless important that if an increased concentration of plasma progesterone retards the time of appearance of milk secretion, continued injection of progesterone cannot prevent lactogenesis (Kuhn, 1969*a*; Deis, 1968; Denamur & Delouis, 1971*b*). This latter observation is in accordance with the absence of any inhibitory effect of progesterone on galactopoiesis in the rat (Folley, 1942) and the high values for plasma progesterone at some stage of lactation in the rat (Tomogane, Ôta & Yokoyama, 1969).

Mouse. The administration of progesterone (1 mg/day) to C3H mice 1–2 days before parturition is expected prevents the increase in α-lactalbumin synthesis which normally occurs soon after parturition (Turkington & Hill, 1969). Galactosyltransferase activity is not affected by progesterone.

In vitro experiments

Milk secretion induced in bitch mammary explants by the combination prolactin + cortisol + insulin is strongly inhibited by the addition of 2 μg/ml progesterone (Barnawell, 1967 – histological examination). Using mammary explants from pregnant C3H mice, Turkington & Hill (1969) demonstrated that progesterone in certain concentrations (4×10^{-6} M) blocks the induction of α-lactalbumin synthesis by the combinations prolactin + cortisol + insulin and HCS + cortisol + insulin. The specificity of galactosyltransferase for its acceptor substrate is modified by α-lactalbumin, which causes the enzyme to synthesize lactose. Larger doses of progesterone (5×10^{-4} M) reduce the synthesis of galactosyltransferase but do not affect the formation of caseins.

These experiments are particularly interesting in that they demonstrate the existence of a selective inhibitory effect of progesterone on the elaboration of different

milk proteins or of proteins (protein A) participating in the synthesis of constituents of milk.

Progesterone, then, has a general inhibitory effect on lactogenesis. However, a stimulatory effect has at times been observed. Barnawell (1967) showed that 0·01 μg/ml of progesterone increased milk secretion induced by the combination prolactin (0·1 μg/ml) + cortisol (5 μg/ml) + insulin (5 μg/ml) in mammary explants from the bitch. Further, observations of Ceriani (1970*a*, *b*) are particularly suggestive in this respect. Using histological and biochemical (synthesis of casein-like material) criteria, Ceriani studied the nature of the hormone combination which could induce differentiation of and secretion in the embryonic mammary glands of the rat. He ascertained that the combination prolactin + cortisol + insulin + progesterone was the most effective in inducing synthesis of casein-like material (superior to prolactin + cortisol + insulin). The product gave 3 bands on electrophoresis on polyacrylamide gel, but only 1 band after the prolactin + cortisol + insulin treatment. Morphological observations confirm the biochemical findings. Thus, prolactin and cortisol increase the synthesis of the kind of casein produced under the influence of insulin, and progesterone in the presence of prolactin + cortisol + insulin induces the synthesis of normal casein. It is, however, possible (see Part I) that progesterone acts mainly on the formation of differentiated mammary cells which become sensitive to the lactogenic action of the combination prolactin + cortisol + insulin.

Endogenous progesterone

The point during pregnancy when milk secretion begins is fairly well established for the rat, mouse, ewe and rabbit (see Introduction). If progesterone plays an important part in lactogenesis in some species, as the findings assembled in the preceding part suggest, plasma concentrations of the hormone ought to diminish shortly before lactogenesis. A comparison of changes in these 2 factors in the rat and rabbit shows that they are in fact negatively correlated.

Thus in the rat, plasma progesterone in ovarian or peripheral venous blood has been determined during pregnancy by a number of workers (Eto, Masuda, Suzuki & Hosi, 1962; Telegdy & Endröczi, 1963; Fajer & Barraclough, 1967; Grota & Eik-Nes, 1967; Feder, Goy & Resko, 1967; Lindner, Sass & Morris, 1964; Hashimoto, Henricks, Anderson & Melampy, 1968; Siiteri, Tippit, Yates & Porter, 1968; Wiest, Kidwell & Balogh, 1968; Hashimoto & Wiest, 1969). It appears that after reaching a maximum about the 15th day, progesterone concentrations fall during the days of pregnancy preceding synthesis of lactose (Shinde *et al.* 1965; Denamur, 1965; Kuhn & Lowenstein, 1967; Kuhn, 1968, 1969*a*; Yokoyama *et al.* 1969) and phosphoproteins (Yokoyama *et al.* 1969). Furthermore, concentrations of 20α-hydroxy-4-pregnan-3-one rise considerably just before parturition under the increasing activity of ovarian 20α-hydroxysteroid dehydrogenase (Wiest *et al.* 1968; Hashimoto & Wiest, 1969; Kuhn & Briley, 1970), which appears to regulate ovarian secretion of progesterone.

Similarly in the rabbit, plasma progesterone concentrations (Mikhail, Noall & Allen, 1961; Hilliard, Hayward & Sawyer, 1964; Hilliard, Spies & Sawyer, 1968; Okano, Matsumoto, Kotoh, Endo & Seki, 1966) are at a maximum on the 15–20th days of pregnancy, and their decline precedes the appearance of lactose synthesis on the 21–22nd day (Denamur, 1963*a*, 1965; Denamur & Delouis, 1971*b*).

On the other hand, in the primiparous ewe, lactose synthesis occurs between the 90th and 100th day, while progesterone concentration in the ovarian vein (Edgar & Ronaldson, 1958) is relatively constant during this stage of pregnancy, or even rises substantially between the 50th and 121st day (Harrison & Heap, 1968). In the peripheral blood, progesterone concentrations vary little (Short & Moore, 1959; Lindner *et al.* 1964), or rise between the 50th and 130th days (Bassett, Oxborrow, Smith & Thorburn, 1969), and in any case their diminution before parturition does not appear to be invariable. Thus in this species, when ovariectomy after the 50th day does not interrupt pregnancy (Denamur & Martinet, 1955), the placenta must secrete most of the peripheral progesterone (Ainsworth & Ryan, 1967; Linzell & Heap, 1968 – 5–7 times as much as the ovary; Ronaldson, 1969) necessary for the maintenance of pregnancy. However, in the ewe, plasma concentrations are much lower than in the rat or the rabbit, and there is no clear correlation with lactogenesis. Study of the possible relation between plasma progesterone and lactogenesis is more difficult in the mouse, since Forbes & Hooker (1957) have only determined the progesterone-like activity of the blood.

Plasma progesterone has also been studied in several other species for which the precise moment of lactogenesis is not yet well established. It is interesting to note that, in species needing a corpus luteum for the maintenance of pregnancy, peripheral concentrations of progesterone tend to fall before parturition – in the cow (Erb, Estergreen, Gomes, Plotka & Frost, 1968; Hunter *et al.* 1970; Pope, Gupta & Munro, 1969; Stabenfeldt, Osburn & Ewing, 1970), in the sow (Masuda, Anderson, Henricks & Melampy, 1967), in the goat (Heap & Linzell, 1966) and in the hamster (Lukaszewska & Greenwald, 1970); this agrees with results obtained with the rat and the rabbit. (To some authors, e.g. Hunter *et al.* 1970, oestrogen–progesterone relations seem of more importance than the absolute value for plasma progesterone.) In contrast, ovarian secretions are not essential in the last 6 months of pregnancy in woman (Llauró, Runnebaum & Zander, 1968; Yannone, McCurdy & Goldfien, 1968; see also the review by Van der Molen & Aakvaag, 1967), in the monkey (Neill, Johansson & Knobil, 1969) and in the guinea-pig (Heap & Deanesly, 1966; Heap, Perry & Rowlands, 1967; Feder, Resko & Goy, 1968), and progesterone concentrations in the peripheral blood remain very high until the last stages of pregnancy.

Moreover, the placenta of all species studied is able to synthesize progesterone (Deane, Rubin, Driks, Lobel & Leipsner, 1962; Bloch & Newman, 1966; Botte, Materazzi & Chieffi, 1966; Davies, Davenport, Norris & Rennie, 1966; Ainsworth & Ryan, 1967; Ferguson & Christie, 1967), even that of the rabbit (Matsumoto *et al.* 1969). The relative importance of the ovary and placenta as sources of progesterone seems, however, to determine the development of plasma concentrations during the last stages of pregnancy, in which lactogenesis occurs.

In conclusion, a very clear negative correlation between plasma progesterone and lactogenesis has been demonstrated in the rat and the rabbit. The same is probably true for other species in which progesterone secretion by the placenta is of small importance, but such a generalization for all species can hardly be put forward on the basis of present knowledge. It is also necessary in interpreting changes in plasma concentrations of progesterone to take into consideration the metabolic clearance rate (Heap & Deansley, 1967) and the uptake of progesterone by the mammary gland.

Progesterone metabolites

The mammary gland of the pregnant goat can synthesize small quantities of progesterone (Heap, Linzell & Slotin, 1969; Slotin, Heap, Christiansen & Linzell, 1970), and circulating progesterone is strongly taken up and retained by the mammary gland of the pregnant rat (Lawson & Pearlman, 1964), rabbit (Chatterton, Chatterton & Hellman, 1969; Chatterton, 1970), goat (Heap & Linzell, 1966 – 20 % of ovarian secretion) and ewe (Linzell & Heap, 1968).

However, unlike oestradiol-17β (Puca & Bresciani, 1969), progesterone is metabolized by mammary cells of the rat (Lawson & Pearlman, 1964) and the rabbit (Chatterton *et al.* 1969), which limits its concentration at the target organ. In the rabbit, the principal metabolite produced by the mammary gland isolated at the end of pregnancy is 20α-hydroxy-4-pregnen-3-one arising through the action of 20α-hydroxysteroid dehydrogenase in the mammary gland (the concentration of the metabolite in the veins of the mammary gland is 5 times that in the artery). In the goat, 3β-hydroxy-5α-pregnan-20-one is an important metabolite of progesterone produced in the mammary glands (Chatterton, 1970). Also numerous steroids arising from the metabolism of progesterone by the liver, kidneys, brain and uterus (Dorfman & Ungar, 1965; Wichmann, 1967; Chatterton, 1970) are strongly taken up by the mammary gland.

Which particular hormone sets off the mechanisms at the level of the mammary cell which retard the initiation of milk secretion? Experiments on mouse mammary explants (Turkington & Hill, 1969) show that progesterone or a metabolite formed by the mammary cells may be active at the cellular level. In contrast to progesterone, 20α-hydroxy-4-pregnen-3-one is totally incapable of retarding lactogenesis in the ovariectomized pregnant rat (Kuhn, 1969*b*), and does not seem to be the compound responsible. It would be particularly interesting to know the lactogenic properties of 3β-hydroxy-5β-pregnan-20-one because of its strong retention by the mammary gland and the hypothesis of Chatterton (1970) that this compound lowers the receptor threshold of mammary epithelial cells to mammogenic pituitary hormones and insulin.

OESTROGENS

Exogenous oestrogens

There seems to be only a limited amount of work on the use of exogenous oestrogens for lactogenesis. In the ovariectomized pseudopregnant rabbit, oestrone (50, 100, 250, 625 μg/day) enables the induction of milk secretion by 12·5 i.u. prolactin administered twice daily (Denamur *et al.* 1970), which is in agreement with earlier results of Meites & Sgouris (1953, 1954). In mammary explants from C3H mice, Turkington & Hill (1969) observed that oestradiol-17β (2×10^{-10}–2×10^{-8} M) did not inhibit the synthesis of α-lactalbumin, as does progesterone. Moreover, mammary explants from the bitch which were treated with the combination prolactin + cortisol + insulin secreted milk which was not affected by doses of oestradiol-17β varying between 0·0001 and 1 μg/ml medium (Barnawell, 1967). Lastly, oestradiol has no stimulatory effect on the synthesis of casein-like material in rat foetal

mammary glands treated with the combination prolactin + aldosterone + insulin with or without progesterone (Ceriani, 1970*a*, *b*).

If oestradiol alone appears not to inhibit lactogenesis in the majority of these studies, it can induce synthesis in the rat of caseins, α-lactalbumin (Turkington & Riddle, 1969) and galactosyltransferase (McGuire, 1969; Turkington & Riddle, 1969) in mammary carcinomas R 3230 AC. It seems that following neoplastic transformation the regulation of genetic potential may be realized by new hormonal influences.

Endogenous oestrogens

Yoshinaga *et al.* (1969) observed that during the first week of pregnancy oestrogen secretion shows a 2-phase increase on the 3rd and 4th days (the same was observed by Shaikh & Abraham (1969) in pseudopregnancy when oestrogen was measured by radio-immunoassay), followed by a period in which plasma concentrations are negligible. From days 14 to 15 the secretion of oestrogens becomes more important, reaching very high values between the 18th day and parturition. It disappears entirely 24 h after delivery. The appearance of lactose synthesis on the 19–20th day occurs therefore during a phase of growing oestrogen secretion.

In the ewe there appear to be only urinary determinations of oestrogens (Fèvre & Rombauts, 1966), although the significance of these estimations is limited by the fact that 90 % of oestrogen excretion in sheep is in the faeces (Terqui, Rombauts & Fèvre, 1968). Measured in this way, oestrogen secretion grows from the 70th and particularly from the 90th day, before lactose synthesis begins (90–100th day). Concentrations of oestrone, oestradiol-17β and oestradiol-17α in foetal plasma increase similarly from the 95th day (Findlay & Cox, 1970).

Where oestrogen secretion has been measured in other species, the time of lactogenesis is not known with any precision. It appears, however, that plasma or urinary concentrations of oestrogens rise steadily during pregnancy in the monkey (Hopper & Tullner, 1967), from the 10–12th week in woman (see the review by O'Donnell & Preedy, 1967), and at the end of pregnancy in the cow (Hunter *et al.* 1970), the sow (Rombauts, 1962; Fèvre, Léglise & Rombauts, 1968) and the rabbit (Schofield, 1957).

The concept, well established for woman (Diczfalusy, 1969), of foeto-placental synthesis of oestrogen (the placenta synthesizes them from 19-C but not from 21-C precursors, according to Ainsworth, Daenen & Ryan, 1969) may be extended to the ewe (Ainsworth & Ryan, 1966; Davies, Ryan & Petro, 1970; Pierrepoint, Anderson, Griffiths & Turnbull, 1970; urinary excretion of oestrogens remains high after ovariectomy according to Fèvre, 1967, the cow (Ainsworth & Ryan, 1966; Pierrepoint, Anderson, Griffiths & Turnbull, 1969), the mare (Ainsworth & Ryan, 1966), the sow (Ainsworth & Ryan, 1966; Drane & Saba, 1967; Fèvre *et al.* 1968; Fèvre, 1970) and the monkey (Davies *et al.* 1970). The quantitative relations between foetal and maternal adrenal precursors in vivo are not yet known.

In short, whatever may be the origin and the mechanisms of intervention of oestrogens at the end of pregnancy (effects on the secretions of the anterior and posterior pituitary and the adrenals, or local effects on the mammary gland) lactogenesis seems to appear in a number of species at the time when the secretion of oestrogens increases. The importance of the physiological role of oestrogens is underlined by the finding of Abdul-Karim, Nesbitt & Prior (1966) that the administration

of an oestrogen antagonist (MER 25) for the last 3 weeks of pregnancy in the ewe almost completely inhibited milk secretion despite apparently normal mammary development.

OESTROGEN–PROGESTERONE COMPLEXES

Denamur *et al.* (1970) have shown in the ovariectomized pseudopregnant rabbit that certain oestrogen–progesterone combinations can inhibit milk secretion initiated by 12·5 i.u. prolactin twice daily. Since the same effect may be observed after ovariectomy and hypophysectomy, the inhibitory activity of oestrogen–progesterone combinations operates at least in part at the level of the mammary cells. Barnawell (1967) has observed the same inhibitory effect on mammary explants from the bitch.

CONCLUSIONS

Many theories of the hormonal mechanisms involved in lactogenesis have been assembled and discussed in a number of articles, of which the most recent are by Folley (1961), Nandi & Bern (1961), Cowie (1966, 1969), Meites (1966), Gala & Westphal (1967), Barnawell (1967). In contrast to this review, those authors have been concerned with the hormonal changes responsible for the abundant secretion of milk which develops (but is not initiated) at parturition or immediately afterwards. They distinguish 4 essentials.

(1) Ovarian steroids play an important part at different levels during pregnancy. Oestrogen–progesterone equilibria during pregnancy make the mammary gland resistant to the stimulatory effects of lactogenic hormones. They inhibit secretion of prolactin in the pituitary (Meites, 1966) and also probably the other constituents of the lactogenic hormone complex (Folley, 1961). At parturition the fall in plasma progesterone, together with the temporary maintenance of high levels of oestrogens in the blood, make the mammary gland receptive to pituitary hormones, as well as stimulating their secretion.

(2) One constituent of the lactogenic hormone complex is lacking during pregnancy. Nandi & Bern (1961), Meites (1966) and Gala & Westphal (1967) have suggested that adrenal steroids constitute the factor limiting the initiation of milk secretion during pregnancy. At parturition an increase in the secretion of adrenal steroids or in their biological activity completes the stimulatory hormone combination, and makes it lactogenic without requiring any change in the secretion of prolactin.

(3) Oxytocin is liberated in considerable quantities at parturition and may stimulate prolactin secretion (Bryant *et al.* 1968).

(4) Soon after delivery, suckling or milking stimulates the synthesis and release of anterior and posterior pituitary hormones (see the reviews of Tindal, 1967; Tindal & Knaggs, 1970; Sar & Meites, 1969; Voogt *et al.* 1969). The role of milking stimuli in the development of secretory phenomena has been well demonstrated recently by Cowie, Knaggs, Tindal & Turvey (1968), but the lactogenic properties of oxytocin during pregnancy of the ewe cannot be explained by an increased secretion of prolactin (Delouis & Denamur, 1967).

This bibliographical review confirms the importance of the ovarian and adrenal steroids even if one tries to link the first measurable manifestations of secretory activity with changes in other plasma hormones. At the same time, the review brings out the fact that during pregnancy there are great differences between species in the placental hormones and in the production of pituitary, adrenal and ovarian secretions. It would therefore be foolhardy to attempt an integrated theory of the hormonal mechanisms controlling lactogenesis during pregnancy.

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