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S. J. ROWLAND, B.Sc. (Hons.), Ph.D., Reading, F.R.I.C.

# Obituary

## S. J. Rowland, B.Sc. (Hons), Ph.D., Reading, F.R.I.C.

Samuel John Rowland, who had been a member of the senior staff of the National Institute for Research in Dairying since 1939 and Head of the Chemistry Department from 1950 till his retirement in 1968, died on 12 April 1971 after a coronary thrombosis. Under his headship the Chemistry Department developed from a small analytical section to one of the foremost centres in the world for the advanced study of the composition of milk and of the influence of changes in the feeding, management and environment of the dairy cow on lactational efficiency. He served for 11 years on the Editorial Board of this Journal.

He was born in Yetminster, Dorset, on 15 January 1907. Both his father and his mother (who is still alive) came of West Country farming stock. Rowland entered Sexey's School in Bruton, Somerset, in 1920 with an Intermediate County Scholarship. At Sexey's he was fortunate to find unusually good facilities for scientific studies; this school has given, for 60 years or more, an early interest in science to a succession of boys who, like Rowland, have later achieved eminence in the scientific world. Its influence doubtless played a large part in deciding the young Rowland to take up a scientific career. He did well at school, becoming captain of one of the Houses.

In 1925 he entered the Honours School of Agricultural Chemistry, Reading, which had been recently established under the headship of Professor H. A. D. Neville, an outstanding and discriminating chief who played a large part in the development of the young University. Neville was much impressed by Rowland's ability and shortly after the latter's graduation with honours in 1929 took him on to his staff as lecturer in Agricultural Chemistry.

In his undergraduate days Rowland, tall and well-built, was a considerable athlete, representing his University in football, hockey, lawn tennis and athletics. He became Chairman of the University Athletics Club. For several years after graduation he maintained his skill on the tennis court, but with his increasing responsibilities, amongst which was his becoming official Resident of St Patrick's Hall, most of his other athletic activities had to go by the board.

He remained on Neville's staff for 10 years, during which his researches on the nitrogen compounds in milk resulted, *inter alia*, in a new and valuable method for their analysis. He received a Ph.D. in 1936. In the following year he married Joyce Lansdell of Long Stretton, Norwich, then an assistant librarian in the University.

Though an efficient University teacher, Rowland's real inclination was towards research, and a vacancy which occurred in 1939 at the N.I.R.D. for a 'Chemist and Research Analyst' greatly attracted him, particularly since the Institute was actively extending its research on the composition of milk. He applied for the post and was duly appointed. During the war years much of the duty of the 'Chemist and Research Analyst' was in providing analytical services for other departments, but some chemical research continued. In 1947, Rowland was given charge of an Analytical Section and in 1950 was appointed Head of the full Department of Chemistry, with staff and accommodation of more adequate size.

It was with milk proteins, their quantitative determination, their important industrial properties, including thermolability, and the causes of their variability that much of Rowland's work during a large part of his research life was concerned. His papers published between 1933 and 1939 were laboratory investigations in one or other of these fields. After his appointment to the N.I.R.D. in 1939, which occurred within a few weeks of the outbreak of the war, his interest in milk proteins did not diminish, though as research analyst to the whole Institute his work of necessity embraced a wider field.

One practical war-time outcome of his protein investigations was the disturbing finding that, especially during the winter months, an increasing proportion of milk of inferior protein content was reaching the market. During the war, and in the period immediately following it when the national diet was exceptionally dependent on milk, even a small loss of the nutritionally valuable milk protein was a serious matter. With his colleagues, he was able to provide cogent evidence pointing to the major reasons for this defect. In collaboration with Stephen Bartlett of the Dairy Husbandry department of the Institute he made the important and unexpected finding that it was a shortage of energy rather than a shortage of protein in the diet of the cow that reduced the protein content of her milk – a finding of obvious practical importance.

Amongst the many subjects concerned with lactational efficiency that were studied from 1939 onward by Rowland and colleagues from his own or other departments at Shinfield were the effect on the composition of cow's milk of carefully controlled alterations in the diet (an example is mentioned above) and of other experimental variables – iodinated protein, thyroxine and related compounds, oestrogens, corticotrophic hormones, pre-partum feeding, pre-partum milking, incomplete milking, fractional milking and novel fertilizer treatment of grassland. The fatty acid and other constituents of rumen contents in relation to dietary changes, the composition of silage made in different ways from different materials, the causes and control of hypomagnesaemia and grass tetany, the effect of mastitis on milk composition, and the freezing point of normal and abnormal milk, were also amongst the many questions whose chemical or analytical aspects were investigated by Rowland in collaboration with various colleagues.

His first major researches in the 1930s had been dependent on careful chemical analysis of a complex fluid of great nutritional and commercial significance. During the next 30 years of his working life, alongside his other research and departmental activities, he continued to improve, refine, and assess the real value of these analytical methods, so important to the dairy industry.

Increasing ill health, which after some 10 years was eventually the cause of his retirement at the age of 61, diminished his personal output of published work. That of his department did not suffer. He continued to keep in close touch not only with all the research his immediate colleagues were doing but also with any new work in dairy chemistry that was going on elsewhere. He was able to maintain, in his department, a reasonable balance between basic and applied research. He still had the responsibility for most of the analytical work required by other departments of the Institute, and whilst he was always helpful and cooperative he had to be convinced that the seemingly immoderate amount of analytical assistance that was sometimes called for was justified. He reacted most unfavourably to any work that he con-

sidered shoddy and he did not suffer fools gladly. On the other hand he gave much thought to the welfare, both scientific and personal, of his staff – which made for a happy and harmonious atmosphere in his department.

Rowland was very alert and perceptive, calm in discussion and clear in exposition – a well-balanced and experienced research worker whose ill health and death have been a real loss to dairy science.

His wife and his two sons, John Lansdell, A.R.I.B.A., and Alan David, M.A., M.I.C.E., survive him.

H. D. KAY

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*With 1 plate*



# The action of rennets on the caseins

## I. Rennin action on β-casein-B in solution

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(*Received* 26 *November* 1970)

Summary. A study of the hydrolysis of β-casein-B by crystalline rennin or rennet extract at pH 6·5, using a disk electrophoresis technique, showed that 3 bonds in β-casein are appreciably more sensitive than the others to rennin proteolysis, and that these bonds are probably located near the C-terminus of the protein. The most susceptible bond is hydrolysed, at 10°C, about 200 times faster than any other bond, whilst at 37°C it is hydrolysed 60 times faster. A study of the hydrolysis of this bond showed that its rate of hydrolysis at 37°C and pH 6·5 is decreased by either increased ionic strength or increased calcium ion concentration at constant ionic strength. Conformational changes in the substrate are probably responsible for these effects.

It is now well established (see the review by Lindqvist, 1963) that the action of the enzyme rennin in the rennet extract is to cleave a single methionyl-phenylalanine bond in κ-casein and thereby to cause the milk to coagulate.

Examination of the enzymic breakdown of components other than κ-casein has been relatively sparse. Lindqvist & Storgårds (1960) followed the hydrolysis of casein fractions with crystalline rennin by free boundary electrophoresis. They concluded that with β-casein the course of degradation of the protein was dependent upon the pH of the reaction mixture, with the maximum number of products produced in the pH range 4–5. Bakri (1968) attempted to use turbidimetric techniques to follow the rennin degradation of a number of casein fractions. He concluded from his measurements that after initial enhancement, further addition of calcium ions retarded the rate of rennin degradation of β-casein and that sodium ions caused complete inhibition of the proteolysis.

In the present investigation a quantitative disk gel electrophoresis technique was developed and used to investigate the early stages of the rennin hydrolysis of β-casein.

## EXPERIMENTAL

### *Materials*

*β-Casein-B.* This protein was prepared by the method of Lawrence & Creamer (1969) from the milk of twin cows typed as having only the B-variant of β-casein, using disk gel electrophoresis (Davis, 1964). The B-variant was chosen for study because the A variant is known to be heterogeneous (Peterson & Kopfler, 1966).

*Rennin.* Crystalline rennin from Pentex Inc., Kaukakee, Ill. 60901, U.S.A., was used.

*Rennet.* A commercial sample from the New Zealand Co-operative Rennet Company, Bridge Street, Eltham, was used without further purification.

All other chemicals used were of reagent grade and were used without further purification.

### *Method of following hydrolysis*

Stock β-casein solution (1·47 %, w/v) which had been stored frozen was dialysed against 0·01 M imidazole-HCl buffers containing the appropriate salts and then diluted as required with the dialysate. For investigating the overall course of the reaction diluted stock β-casein solution was used, after pH adjustment. The β-casein solution (0·1 % w/v, 10 ml) was brought to temperature in a water bath. Rennin solution, rennet or diluted rennet (0·1 ml) was then added, the mixture gently shaken and returned to the water bath. Samples (0·5 ml) were taken at pre-selected time intervals and the reaction quenched by mixing the sample with a solution of mercaptoethanol (0·1 %, v/v) in 8 M urea solution (4·5 ml). Each time interval was double the previous one. Normally the sampling time interval scale was 0·5, 1, 2, 4, 8, 15, 30 min, 1, 2 and 4 h, but in one experiment the sampling period was extended to up to 64 h.

Samples (100 μl) of the quenched reaction mixture were electrophoresed in random order, in duplicate (and interspersed with standard β-casein solutions) using the disk technique devised by Davis (1964) and Ornstein (1964). After electrophoresis, the gels were stained in freshly prepared Amido Black (0·2 %, w/v) dye solution (in 7 % (v/v) acetic acid) for at least 4 h and usually overnight. The excess dye was removed electrophoretically. The amount of dye absorbed by the protein in the gel was estimated using a Joyce Chromoscan fitted with a red filter. Each of the duplicate gels was scanned twice, the gel being rotated 90° between scans. The peak areas of the recorder traces were determined (*a*) by cutting out and weighing a copy of the trace, (*b*) by calculating the product of peak height and peak width at half height, or (*c*) by planimeter measurements. Where peaks overlapped to some extent – as, for example, with β-casein and the first degradation product – a proportion of the area of the doublet was assigned to each of the components in the ratio of their respective peak heights when methods (*a*) or (*c*) were used. Where only the loss of β-casein from the solution was being determined, peak heights were used, and the amount of β-casein in each sample was estimated from the standard line constructed using the peak height measurement from the standard β-casein solutions. For the breakdown products of β-casein it was not possible to obtain an absolute estimate of the relation between the densitometer peak area measurement and quantity of protein present. As a

consequence the amount of dye bound by each protein band had to be estimated. Calculations were made using an I.B.M. 1620 Mark 2 computer and a Hewlett Packard 9100A calculator.

### *Molecular weight determination*

A sample of partially hydrolysed β-casein (0·5 %, w/v) was centrifuged concurrently with a sample of β-casein (0·5 % w/v), at 5·75°C and 60,000 rev/min, in 4° single sector cells using a Beckman Model E ultracentrifuge.

## RESULTS

### *Standard curve*

A linear relationship was found to exist between the amount of β-casein electrophoresed and the peak area of the densitometer tracing (Fig. 1). However, special care had to be taken at all stages of the sampling, electrophoresis and densitometry, to obtain reproducible results.

### *The overall reaction*

The sequential breakdown of β-casein (0·1 %, w/v) by rennet (2·2 %, v/v) was followed at 10°C and pH 6·5. The quantity of β-casein decreased as the reaction proceeded, and the quantity of a second, more mobile component (β-I) increased (Plate 1). After some time β-I slowly disappeared whilst 2 other components (β-II and β-III) appeared; β-II apparently being converted into β-III. The degradation

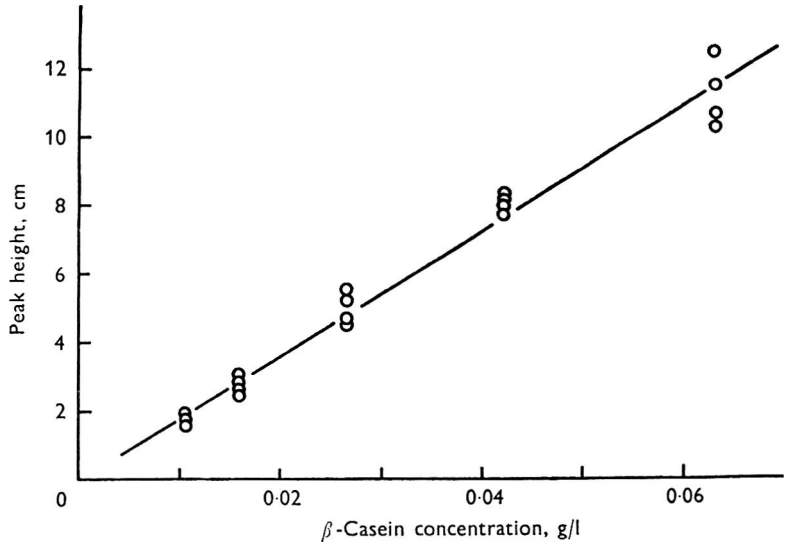


Fig. 1. A standard curve of peak heights of densitometer traces versus concentrations of β-casein solutions.

products have been labelled β-I, β-II and β-III in order of appearance. This was also in order of increasing mobility at pH 8·6. Although the β-III appeared somewhat diffuse it was not possible to resolve the bands further nor were any new bands apparent when larger samples were electrophoresed. The use of crystalline rennin gave the same results as those shown (Plate 1). A plot of averaged densitometer

tracing areas from the gels is shown in Fig. 2 and it indicated that the dye bound by $\beta$-I increased to 90% of that bound by $\beta$-casein, and that bound by $\beta$-III increased to over 60% of that bound by $\beta$-casein (Fig. 2). At no time did the total amount of dye absorption from all bands fall below 60% of that of the initial

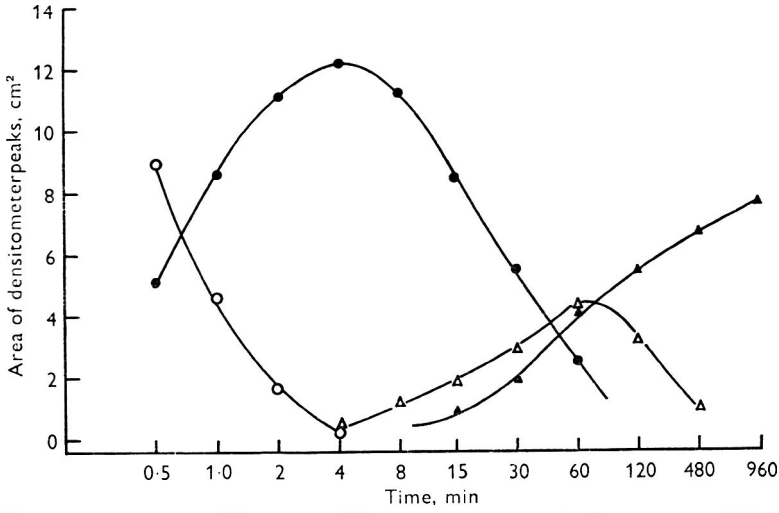


Fig. 2. Densitometer trace areas for each of the degradation products. ○, $\beta$-Casein; ●, $\beta$-I; △, $\beta$-II; ▲, $\beta$-III. The degradation was carried out at 37 °C and pH 6·5. At zero time the area for $\beta$-casein alone was 13·6 cm².

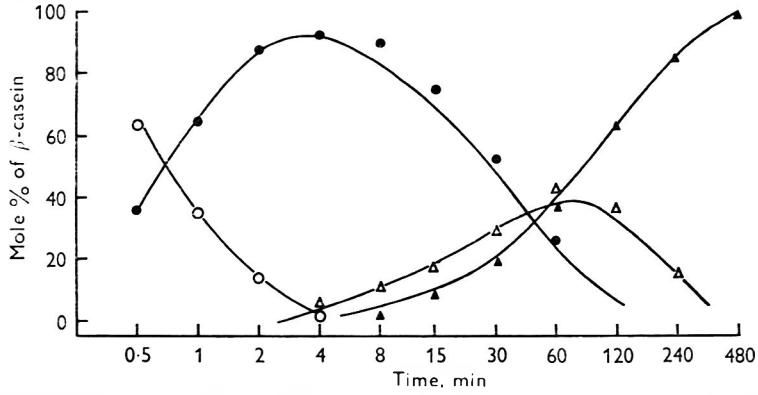


Fig. 3. Proportion of original $\beta$-casein in each of the degradation products formed at 37 °C and pH 6·5, as estimated by gel electrophoresis and quantitative densitometry, using the procedure outlined in the text. ○, $\beta$-Casein; ●, $\beta$-I; △, $\beta$-II; ▲, $\beta$-III. The continuous lines were calculated using the rate constants shown in Table 2.

$\beta$-casein, and consequently $\beta$-I, $\beta$-II and $\beta$-III were each likely to be major portions of the original $\beta$-casein (as indicated by dye binding). If $\beta$-II and $\beta$-III are each major fragments of $\beta$-casein, and $\beta$-III can apparently be derived from $\beta$-II ($\beta$-III increased whilst only $\beta$-II decreased, Fig. 2), then $\beta$-II and $\beta$-III could not be derived from $\beta$-I by the cleavage of a single bond.

To convert the experimental results of dye absorbed by each protein fraction into molar quantities of each component it was necessary to know the amount of dye absorbed per mole of each of the degradation products. From the data in Fig. 2,

β-I, β-II and β-III were estimated to bind 93, 70 and 64% of the dye bound by β-casein respectively. Using these factors the amounts of each peptide were calculated as percentages of the original β-casein. The changes with time at 37, 25 and 10°C are shown in Figs 3–5.

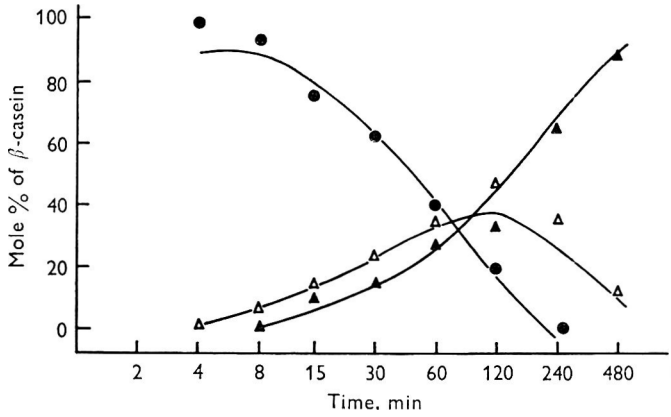


Fig. 4. Proportion of original β-casein in each of the degradation products formed at 25°C and pH 6·5, as estimated by gel electrophoresis and quantitative densitometry. ●, β-I; △, β-II; ▲, β-III. The continuous lines were calculated using the rate constants shown in Table 2.

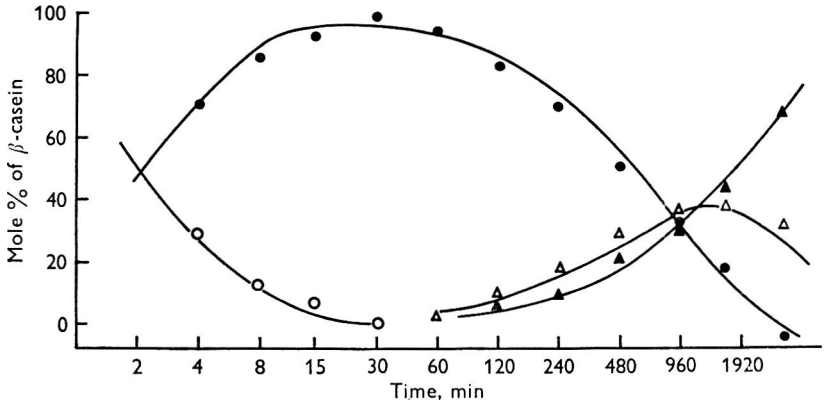


Fig. 5. Proportion of original β-casein in each of the degradation products formed at 10°C and pH 6·5, as estimated by gel electrophoresis and quantitative densitometry. ○, β-Casein; ●, β-I; △, β-II; ▲, β-III. The continuous lines were calculated using the rate constants shown in Table 2.

*Molecular weight of β-I*

Disk gel electrophoresis of the partially hydrolysed β-casein solution showed that it contained 80% β-I and 20% β-casein. Analysis of the ultracentrifugal analysis data showed that β-casein and the partially hydrolysed β-casein had sedimentation coefficients of 1·59 S and 1·46 S under the same conditions. If it is assumed that β-I is very little different from β-casein in its frictional factor and in its partial specific volume, then it can be shown that

$$\frac{\text{Mol. wt. } \beta\text{-casein}}{\text{Mol. wt. } \beta\text{-I}} = \frac{\text{sed. coef. } \beta\text{-casein}}{\text{sed. coef. } \beta\text{-I}}.$$

With a mol. wt. of 24100 for β-casein then the mol. wt. of β-I would have been 22100. Because the hydrolysed casein sample contained some β-cascin this was a maximum value, and consequently the small peptide split from β-casein had a mol. wt. of at least 2000.

*Substrate concentration variation*

The initial substrate concentration was altered over an 8-fold range from 0·25 to 1·94 g/l. The concentration of the rennet solution was maintained at 2 % (v/v) and the reaction was carried out as described above. A Lineweaver–Burk plot showed good linearity with both intercepts near zero. This indicated a large value of the Michaelis

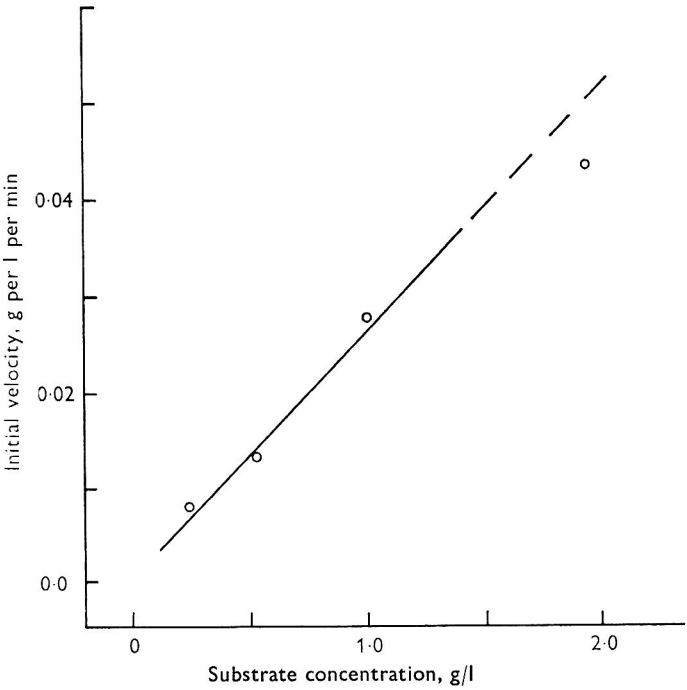


Fig. 6. A plot of initial reaction velocities, measured by the disappearance of β-casein from the reaction mixture using quantitative gel electrophoresis, versus original substrate (β-casein) concentrations.

constant $K_m$ and of the maximum reaction velocity $V$. The initial reaction velocity against substrate concentration was linear up to 1·0 g/l at a rennet concentration of 0·043 % (v/v) in the reaction solution (Fig. 6). In the range of conditions under investigation it appeared that the reaction velocity was limited by substrate concentration, i.e. at any given enzyme concentration the substrate was degraded according to first order kinetics.

*Ionic variations*

The effect of variations in the ionic composition of the medium was assessed by measurement of the rate of hydrolysis of the most susceptible bond of β-casein. In a preliminary experiment it took 4 times the rennet concentration for equal reaction

ates when the solution was made 0·04 M in KCl over and above the minimum ionic trength (substrate adjusted to pH 6·50 and then dialysed against water); the conribution from the added rennet was about 0·0001. Thus, it is clear that ionic strength vas an important factor in determining the rate of reaction. Precautions were thereore taken to ensure, in experiments designed to test whether calcium has an effect

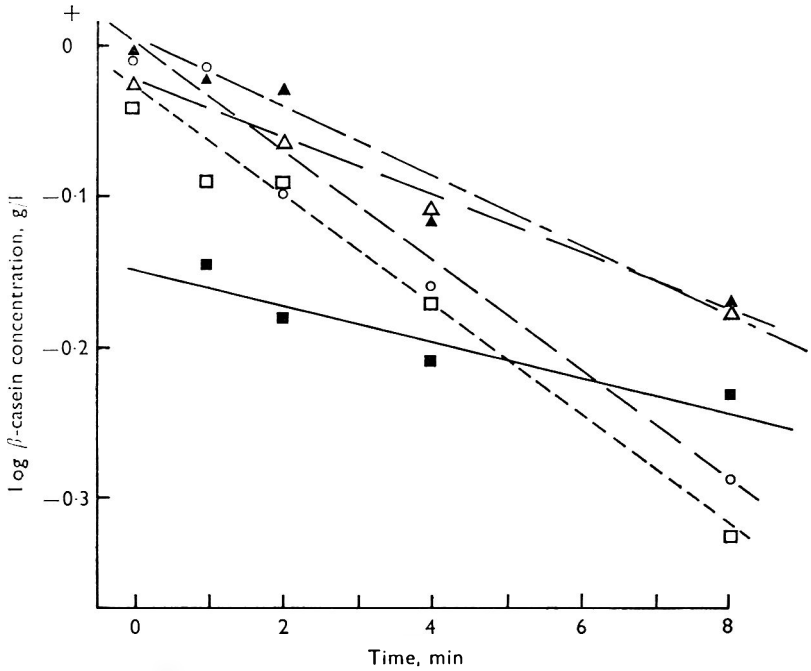


Fig. 7. Plots of the natural logarithm of β-casein concentration in the reaction mixture, determined by quantitative gel electrophoresis, versus time showing the effect of ionic variations. All reaction mixtures were the same concentration of β-casein (1·0 g/l) and rennet (0·044%). Measurements were made on solutions having pH values of 6·50 (○), 3·94 (△) and 6·12 (▲) and on solutions at constant pH (6·49) but containing additional citrate (□) or calcium (■), the concentration of potassium chloride being adjusted to maintain constant ionic strength.

'able 1. *The rate of hydrolysis of β-casein at 37°C and at a total ionic strength of 0·04*

| pH | Added potassium chloride, molar | Calcium ion concentration, molar | Total citrate added, molar | Rate constant, $min^{-1}$ |
|---|---|---|---|---|
| 6·50 | 0·03 | — | — | 0·038 |
| 6·12 | 0·03 | — | — | 0·020 |
| 6·94 | 0·03 | — | — | 0·025 |
| 6·48 | 0·025 | — | 0·00083 | 0·037 |
| 6·49 | 0·020 | 0·0033 | — | 0·014 |

n reaction rates, that the ionic strength of the medium was not altered unduly. The mount of calcium that can be added to a β-casein solution is limited because the '-casein readily precipitates in the presence of calcium. The amount of calcium used 'as just insufficient to cause precipitation (0·033 M). In another experiment a small

amount (0·0008 M) of tri-potassium citrate was incorporated into the solution as a polyvalent metal ion chelating agent.

By plotting the natural logarithm of the concentration of the unreacted $\beta$-casein versus time, a line of slope -k was obtained for each of the experiments (Fig. 7, Table 1).

It can be seen (Fig. 7) that while the increased calcium ion concentration depressed the rate of reaction, increases in citrate concentration at constant ionic strength did not alter it perceptibly. Both increase and decrease in the pH decreased the rate of reaction.

It was clear from the results (Table 1) that ionic strength was a very important parameter. The method used to vary the pH of the solutions in estimating the effect of pH in the range 6·12–6·94 was to dialyse the casein solution against an imidazole buffer adjusted to the required pH. Unfortunately, the lower the pH the higher the ionic strength of the buffer, and consequently it was not possible to say whether the decrease in rate in going from pH 6·50 to 6·12 was caused by the lower pH *per se* or by the concomitant increase in ionic strength. A significant decrease in rate was obtained by increasing the pH to 6·94, and might have been greater if the ionic strength had been maintained constant. Hence, our results were not at variance with the concept of maximum rennin activity at a low pH.

### *A model for the calculations*

Because of the difficulty in deriving analytical equations for the breakdown of $\beta$-casein by rennin, or any other similar reaction involving a sequential degradation, a simple model of the reaction was set up and the various parameters adjusted until the calculated degradation curves fitted the experimental data.

Enzyme reactions are normally considered to consist of several steps, one such reaction pathway being:

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EP \xrightarrow{k_3} E+P,$$

where $E$ is the enzyme, $S$ is the substrate, $ES$ is the enzyme substrate complex, $EP$ the enzyme product complex, and $P$ the product(s) (Bernhard & Gutfreund, 1970). (Many complications, e.g. competitive inhibition by product, are not represented in this scheme.) At constant total enzyme concentration and if $k_1$ and $k_{-1}$ are both large and $k_2$ is small (the rate determining step), then the breakdown of $S$ to $P$ would appear to be first order in $S$.

When a number of bonds are being broken simultaneously, provided that the various $k_1$ and $k_{-1}$ are large, and the alteration of the substrate does not change any of the $k_1$ to $k_{-1}$ ratios through some conformational change, then each bond will be observed to be broken by first-order kinetics. Some further assumptions must be made because of the particular system being dealt with, namely (i) that each of the electrophoretic bands corresponds to a distinct species and (ii) that only one observable product arises from each scission. Four possible degradation schemes are shown (Fig. 8). Schemes A and D give rise to only 3 products whilst schemes B and C give rise to 4 products. A is not easily distinguishable from D because bond (i) was cleaved so much more rapidly than (ii) or (iii) in our particular study. If the rate constant for

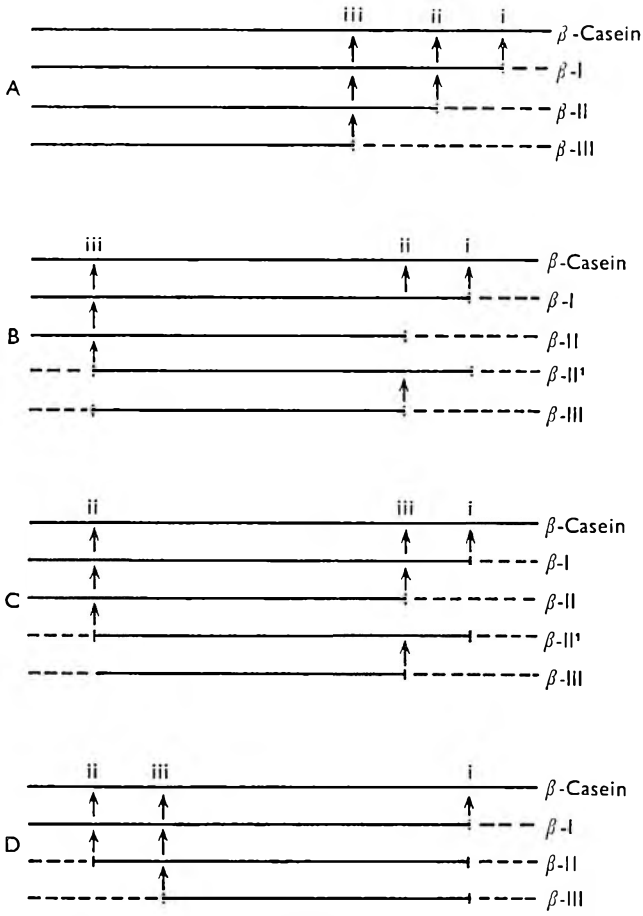


Fig. 8. A representation of the possible pathways of the specific degradation of β-casein by rennin. The horizontal lines represent the β-casein polypeptide chain, the points i, ii and iii represent the positions and order of the bonds attacked by the enzyme and β-casein, β-III represent the species released.

the cleavage of bond (i) is $k$i, bond (ii) is $k$ii and bond (iii) is $k$iii, the following equations apply to scheme A or D:

$$\begin{aligned} d\beta &= -\beta(k\text{i}+k\text{ii}+k\text{iii})\,dt, \\ d\beta\text{-I} &= [\beta k\text{i}-\beta\text{-I}(k\text{ii}+k\text{iii})]\,dt, \\ d\beta\text{-II} &= [(\beta+\beta\text{-I})\,k\text{ii}-\beta\text{-II}\,k\text{iii}]\,dt, \\ d\beta\text{-III} &= (\beta+\beta\text{-I}+\beta\text{-II})\,k\text{iii}\,dt. \end{aligned}$$

Further breakdown, although known to occur, is of a non-specific nature, as is shown by the result that no new electrophoretic bands could be seen after extended reaction, and it was therefore neglected. The above equations were used for the calculation of the concentration changes over a short time interval (initially 0·005 min). The concentrations of β-III, β-II, β-I and β-casein were then calculated, in that order. After 100 cycles of this calculation the concentrations were displayed, the time interval doubled and the calculation cycle repeated. (The first 2 time intervals, normally

0–0·5 and 0·5–1·0 min, were kept the same.) Table 2 shows the first-order rate constants finally used in the calculation of the curves that best fit the experimental data (Figs 3–5).

Table 2. *Rate constants, $min^{-1} \times 10^4$, used to calculate the continuous lines in Figs 3–5*

| Temp., °C | *k*i | *k*ii | *k*iii |
|---|---|---|---|
| 10 | 2500 | 9 | 4 |
| 25 | 5000 | 100 | 50 |
| 37 | 10000 | 160 | 84 |

## DISCUSSION

### *The overall reaction*

The initial degradation of β-casein-B under the influence of rennin action at pH 6·5 is essentially the same as that reported by other workers for β-casein in whole casein (Fox, 1969*a*; Ledford, Chen & Nath, 1968; Mickelsen & Fish, 1970). The only major study on the degradation of purified β-casein has been made by Lindqvist & Storgårds (1960), who used free-boundary electrophoresis to investigate the reaction. They purified their material by fractional precipitation and checked purity by free boundary electrophoresis. The existence of genetic variants of casein was not widely known at that time and for these reasons it seems unlikely that their material contained only a single protein. However, their use of free-boundary electrophoresis would give a much better idea of the distribution of *all* of the casein fragments, whereas the technique used in this study reveals only the fragments that are negatively charged at high pH and are precipitated with the Amido Black dye. Considering these differences it would have been surprising if the present results had been the same as those obtained by Lindqvist & Storgårds.

The relative sizes and shapes of the species β-I, β-II and β-III are not known with certainty. From the ultracentrifugation data the mol. wt. of β-I is most probably close to that of β-casein. If the degradation scheme outlined earlier is correct, then β-II is smaller than β-I, and β-III is smaller than either of them. Because of the relatively high dye-binding capacity of β-III we assume that most of the positively charged groups (at pH 2–3) are retained, and because of the greater mobility of β-II and β-III, then either they are smaller than β-I, or they are more negatively charged at pH 9. From these considerations it seems likely that β-II is derived from β-I by removal of a peptide segment with perhaps 10–20 % of the basic groups and the same number of acidic groups. The number of charged groups removed in the conversion of β-II into β-III is probably about the same, because the dye binding capacity of β-II and β-III seem similar, indicating similar net charge at low pH, while the mobility at higher pH is greater.

It is known that in the *N*-terminal tryptic peptide of β-casein, containing 24 amino acids and having a mol. wt. of ~3000, there are 4 phosphoserine residues (Peterson, Nauman & McMeekin, 1958), 3 of them contiguous (Manson & Annan, 1970), and also 7 glutamic acid and 2 arginine residues giving a net negative charge of 12 for the peptide at pH 9. From other evidence (Mills, 1970) it is likely that in the conversion

of β-casein to β-I a C-terminal peptide is removed. The only schemes outlined (Fig. 8) that fit the data (Figs 3–5) are A and D, where conversion of β-I to β-II or to β-III involve removal from only one end of the molecule. Because of the high concentration of acidic groups near the *N*-terminus and the mobilities of β-II and β-III it is very likely that β-III includes the *N*-terminal sequence, (Fig. 8A). Further work should clarify this point.

The results obtained by Lindqvist & Storgårds (1960) indicate that the course as well as the rate of the reaction is markedly influenced by the pH of the solution (or coagulum). By non-protein-nitrogen release, maximum activity was between pH 4 and 5, with equal but lower activities at pH 2, 3 and 6. The electrophoretic patterns indicate that at the lower pH values (less than 4) the degradation is much less specific. This finding is supported by Fox (1969*a*), who showed, using gel electrophoresis, that at lower pH values (less than 4) more products were formed – particularly from $\alpha_{s1}$-casein – by the action of rennet on whole casein. At pH 6·5, at which we conducted most of our experiments, the reaction was highly specific, with one bond being broken much faster than any other.

From published polyacrylamide gel electrophoresis patterns showing protein breakdown in cheese (Ledford, O'Sullivan & Nath, 1966), it can be seen that in no case does the β-casein show the same pattern of breakdown as is shown in solution, whereas from studies showing the breakdown of whole casein in solution the same pattern of breakdown as we describe is clearly discernible in the early stages of breakdown (e.g. Fox, 1969*b*). (The products which we label β-II and β-III are unfortunately obscured by the $\alpha_{s1}$-casein bands in the alkaline polyacrylamide electrophoresis of whole casein and are only clearly seen in systems using purified casein fractions.) The reason for the differences between breakdown in cheese and in solution is not clear, and warrants further investigation.

It has been shown from optical rotatory dispersion measurements (Garnier, 1966; Herskovits, 1966) and hydrodynamic measurements (Noelken & Reibstein, 1968) that β-casein in solution undergoes temperature-dependent conformational changes. At low temperature (5°C) it appears to form a structure with a certain amount of poly-proline helix whilst at higher temperatures (40°C) this has largely disappeared. This is no doubt related to the well-known temperature-dependent insolubility of β-casein in the presence of sufficient calcium near neutral pH. Our results show that the relative rates of cleavage of the first and second bond alter with temperature, and that at 37°C and pH 6·5 the addition of just insufficient calcium to cause precipitation, reduces the rate of cleavage of the first bond. It has also been shown by Fox (1969*a*), from measurements on the degradation of whole casein at pH 6·0 and at temperatures of 4, 10, 21 and 32°C, that the ratio of $\alpha_{s1}$-casein to β-casein degradation rates (the most susceptible bond in each case) increases with temperature. Our results (Table 2) show that the ratio of the rate of cleavage of the second (ii) or third (iii) bond to the rate of cleavage of the first (i) bond of β-casein also increases with temperature, the greatest change occurring in the rates of cleavage of the bonds (ii) and (iii) between 10 and 25°C. It seems likely that the high-temperature conformation of β-casein is relatively less susceptible to attack at bond (i), whereas the reverse is true for bonds (ii) and (iii). The influence of calcium must be very similar in that the calcium protein complex has a conformation that is not as readily hydrolysed at bond

(i). It might be expected that the complexing of calcium to the protein would cause the total protein charge at pH 6·5 to fall from $\sim -11$ to $\sim -3$ (Waugh & Creamer, unpublished results). This would be expected to increase the rate of reaction, in the same way that lowered pH does, and consequently more than substrate charge must be involved.

The authors are grateful to Dr R. M. Dolby and Dr R. C. Lawrence for helpful and stimulating discussion, Miss D. M. Handley for skilled technical assistance, and the Applied Biochemistry Division, D.S.I.R., Palmerston North, for the use of the Joyce Chromoscan densitometer.

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## EXPLANATION OF PLATE

A set of disk polyacrylamide gels showing the degradation, with time, of $\beta$-casein by rennet at 10°C and pH 6·5. Samples were taken from the reaction mixture at doubling time intervals. The times (min) correspond to those shown in Fig. 5.

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## SYMBOLS AND ABBREVIATIONS

The symbols and abbreviations used are those of British Standard 1991: Part 1: 1967, *Letter Symbols, Signs and Abbreviations*.

## DESCRIPTIONS OF SOLUTIONS

Normality and molarity should be indicated thus: N-HCl, 0·1 M-$NaH_2PO_4$. The term '%' means g/100 g solution. For ml/100 ml solution the term '% (v/v)' should be used and for g/100 ml solution the correct abbreviation is '% (w/v)'.

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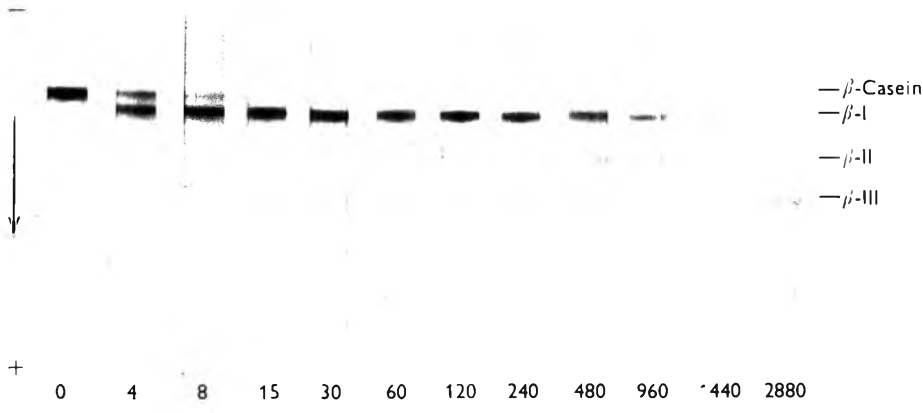
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# Seasonal variations in the viscosity and adhesive strength of casein from the milk of individual cows. II*

By C. R. SOUTHWARD and R. M. DOLBY
*New Zealand Dairy Research Institute, Palmerston North, New Zealand*

(*Received* 30 *December* 1970)

Summary. The viscosity and adhesive strength of casein from the milk of 6 individual cows were determined on samples collected regularly over a period of 21 months. Three of the cows calved in autumn and 3 in spring. The viscosity of the casein from 5 of the cows showed a decline during lactation that was independent of the date of calving. It was therefore concluded that the decline in casein viscosity may be attributed to lactational rather than seasonal effects.

In an earlier paper (Southward & Dolby, 1968) the seasonal decline in viscosity and increase in adhesive strength of caseins from the milk of individual cows was described. It was suggested that these trends could be attributed to lactational rather than seasonal effects. In an attempt to confirm this theory, a further trial was undertaken in which casein was prepared from samples of milk collected regularly from both spring- and autumn-calving cows.

The present paper describes the lactational variation in viscosity of casein and of a casein–clay paper-coating mix, and in adhesive strength of casein obtained from the milk of 6 individual cows.

## EXPERIMENTAL

### *Experimental design*

Six cows were chosen from an experimental herd of Friesian cows which produced milk for town supply. Three cows had calved in the autumn (April–May) and 3 in the following spring (July–September) before the trial was commenced.

For sampling purposes, the cows were divided into 3 pairs. As it was not possible to make more than 2 casein preparations in one day, 1 pair of cows was sampled on each of 3 successive days at the beginning of each week (Table 1).

### *Preparation of casein*

Morning milk from each cow was separated and a 1-gal sample of the resulting skim-milk was used for preparation of casein.

The casein was prepared as previously described (Southward & Dolby, 1968) except that from August 1967 the casein was dried in a laboratory fluid bed drier (Glatt type TR 2).

* Part I. *J. Dairy Res.* (1968), **35**, 25.

Table 1. *Details of cows supplying milk for trial*

| Sampling order | Cow no. | 1966 Calving date | 1966 Type | 1967 Calving date | 1967 Type | 1968 Calving date | 1968 Type |
|---|---|---|---|---|---|---|---|
| 1 | 2 | 13 Aug. | Spring | 5 Aug. | Spring | — | — |
| | 7 | 24 Apr. | Autumn | 28 July | Spring | — | — |
| 2 | 19 | 22 Apr. | Autumn | 2 May | Autumn | 9 Apr. | Autumn |
| | *21 | 13 Aug. | Spring | — | — | — | — |
| | 62 | — | — | 22 Apr. | Autumn | 20 Apr. | Autumn |
| 3 | 47 | 13 Aug. | Spring | 27 Sept. | Spring | — | — |
| | 87 | 24 Apr. | Autumn | 28 Apr. | Autumn | 8 May | Autumn |

* Approximately 4 months after commencement of the trial, cow 21 was culled from the herd and cow 62 was chosen to replace it. Data from cow 21 is accordingly not presented.

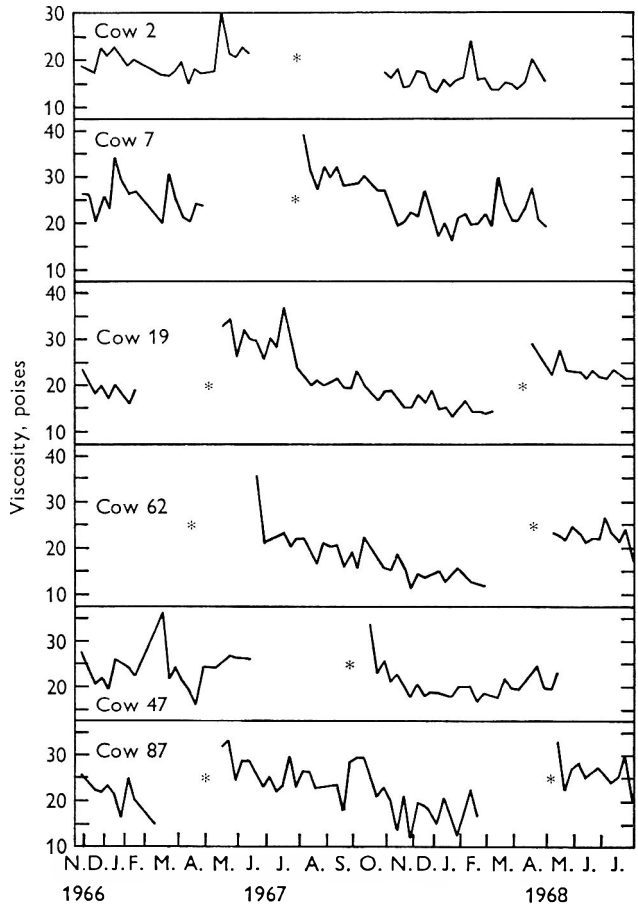


Fig. 1. Variation in the viscosity of caseins from individual cows during a lactation. Values (in poises) are for a 15% (w/w) solution of casein in ammonia, measured at a shear rate of 50 $s^{-1}$ at 25°C. * Calving date.

*Physical properties of casein in paper coating*

Laboratory paper coating and measurement of the viscosity of casein and of its adhesive strength on coated paper were carried out as previously described (Southward & Dolby, 1968). The paper-coating mix was placed in a water bath maintained at 25°C. After a suitable period for equilibration (30 min) the viscosity was measured with a Ferranti concentric cylinder viscometer at a shear rate of 50 $s^{-1}$. The standard error of the viscosity determination on a given coating mix was of the order of 25 cP.

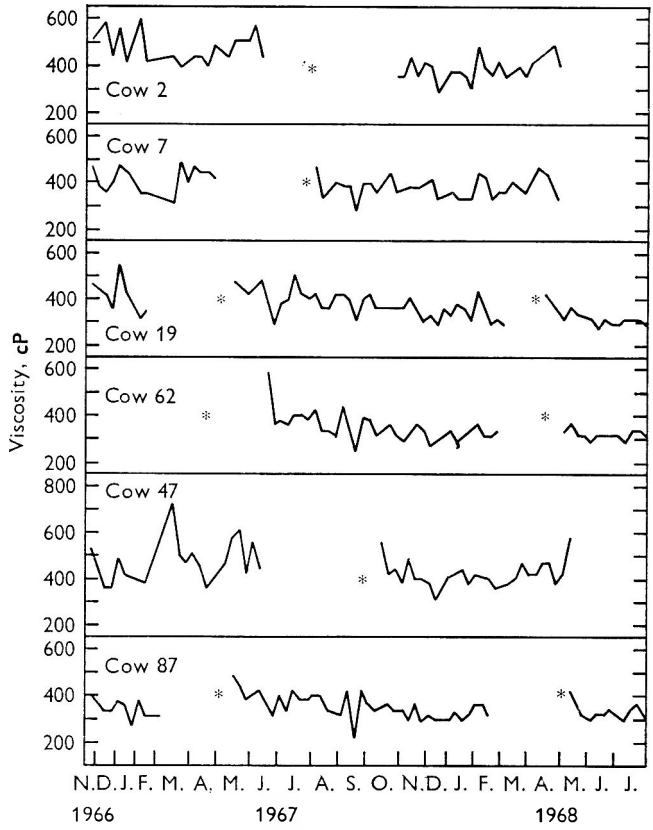


Fig. 2. Variation in the viscosity of paper-coating mixes containing clay and caseins from individual cows during a lactation. Values in centipoises are for a 45% (w/w) dispersion of clay and casein in ammonia (13·5% casein on clay), measured at a shear rate of 50 $s^{-1}$ at 25°C. * Calving date.

## RESULTS AND DISCUSSION

*Casein viscosity*

The viscosity of casein samples from 5 of the 6 cows showed a general decline throughout their lactation (Fig. 1), although casein from cow 47 showed a rather unusual pattern, particularly at the end of lactation. (This pattern was also apparent in the coating viscosity and adhesive strength of the casein from cow 47.) Unfortunately, samples of milk were not received from cow 2 for some 2 months after

calving, and early lactation data on viscosity were therefore not collected. There was no obvious trend in viscosity of casein from this cow.

From an examination of the casein viscosity patterns of all cows except cow 2, it was apparent that the principal cause of the decline was lactational rather than seasonal.

*Coating viscosity*

While not as pronounced as the trend from the casein viscosity data, there was some evidence (from cows 19, 62, 47 and 87) to suggest that coating viscosity follows a similar pattern to casein viscosity (Fig. 2).

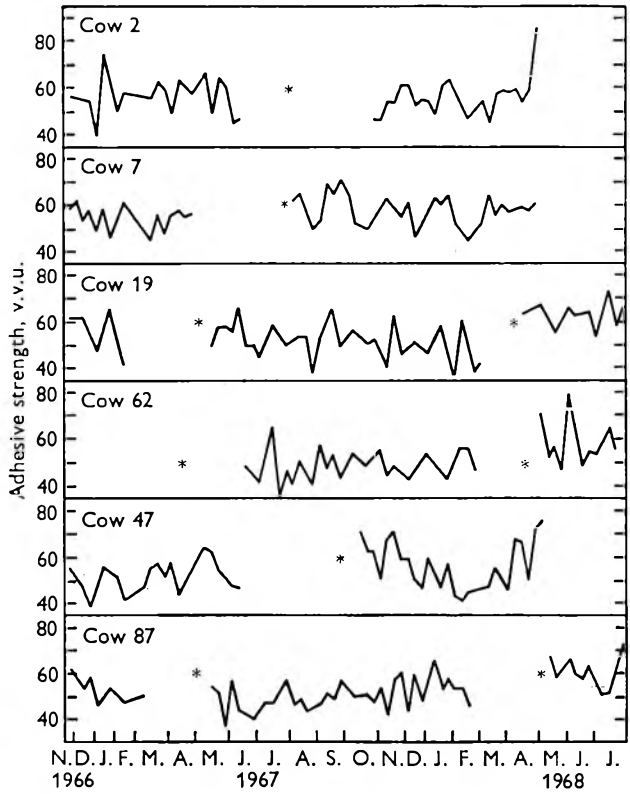


Fig. 3. Variation in the adhesive strength of caseins from individual cows during a lactation. Values (in v.v.u.: kilopoise, cm/s) are expressed in terms of the product of the viscosity of the pick test oil and the pick velocity of paper (coated with a mixture of clay and casein) at 20·5°C and 55% R.H. * Calving date.

*Adhesive strength*

In contrast to results obtained earlier (Southward & Dolby, 1968), no overall pattern can be distinguished in the data from the adhesive strength determination on the caseins from these cows.

The adhesive strength was markedly lower overall than in the previous set of experiments and may be attributable to either (i) a change in coating clay to one of a similar type to, but of different origin from, that used previously (Southward & Dolby, 1968) or (ii) a change in pick test oil, nominally of the same type and viscosity, but different in colour and possibly in composition from that used in the earlier trial.

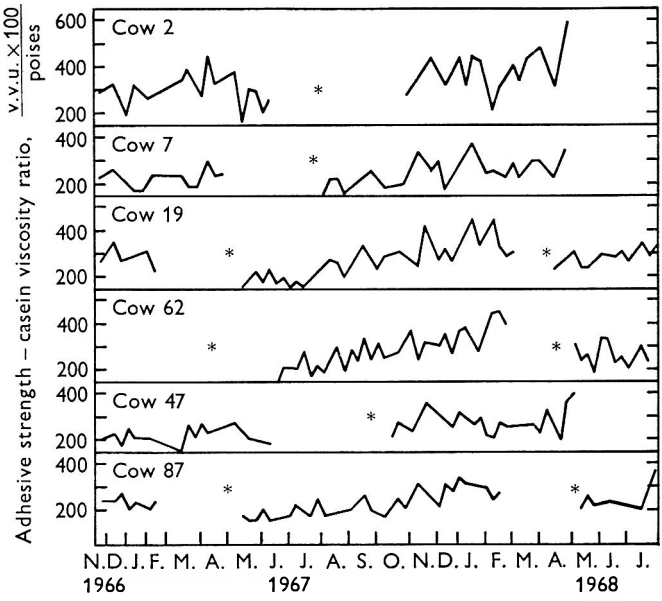


Fig. 4. Variation in the ratio of adhesive strength to viscosity of caseins from individual cows during a lactation. Values are expressed as (v.v.u. × 100)/poises. * Calving date.

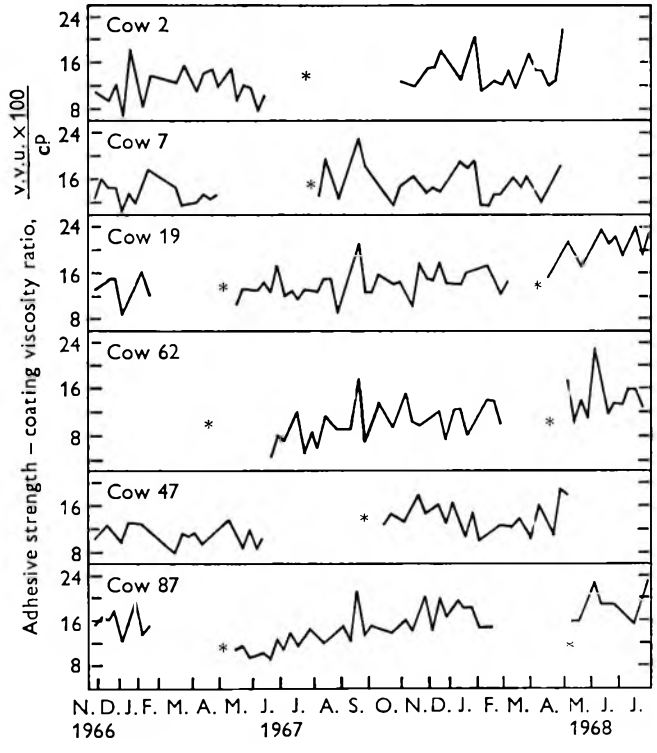


Fig. 5. Variation in the ratio of adhesive strength (of caseins) to viscosity of paper coating mixes of clay and caseins from individual cows during a lactation. Values are expressed as (v.v.u. × 100)/cP. * Calving date.

It is of interest to note that the adhesive strength: casein viscosity and adhesive strength: coating viscosity ratios for casein from the autumn-calving cows (nos 19, 62 and 87), all showed clearly increasing lactational trends, whereas the pattern was much less clear for the spring-calving cows. This was contrary to the previous finding (Southward & Dolby, 1968) where there was a definite upward trend with spring-calving cows as with the autumn-calving cows in the present trial (Figs 4, 5).

While the main trend is lactational, there appear to be some other influences having a short-time effect. Some of these may arise from experimental error, while some are likely to be real changes in casein properties. It appears that weather has little effect on these properties as changes rarely occurred simultaneously with a number of cows.

## CONCLUSIONS

A decline from beginning to end of lactation in casein viscosity and a smaller decline in coating viscosity were found for autumn-calving cows as was previously reported for spring-calving cows. In the present trial this trend was less obvious with spring-calving cows but it is evident that lactational changes are the principal cause of changes in casein viscosity during the season. Little change in adhesive strength through the lactation was noted, but the ratio of adhesive strength to viscosity tended to show a lactational increase.

The authors are grateful to Mr G. F. Wilson for providing samples of milk, to Dr L. K. Creamer for many helpful discussions and to Misses E. A. Pinfold and R. Doyle and Mr M. W. Bysouth for technical assistance.

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# The effects of nutritional level on milk yield and milk composition in cows and heifers*

By S. GORDIN, R. VOLCANI and YEHUDITH BIRK

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Summary. The effects of diets high in concentrates and low in roughage on the yield of milk and milk constituents were studied in 32 cows and heifers throughout lactation. A high level of concentrates in the ration caused an increase in milk yield, a decrease in fat percentage but not in total fat yield, and an increase in protein production.

Milk produced under such feeding conditions is capable of yielding greater quantities of curd than would be expected from its fat content, and should be evaluated accordingly. Feed requirements of the cows should be assessed not solely on the basis of the fat content of their milk but also in accordance with the protein content.

For many years it has been customary to evaluate milk in relation to its fat content. Great efforts have been made to breed animals for high milk fat percentage and for high total fat production, not only because of the importance of milk fat and its products in the human diet, but also because of the belief that a positive correlation exists between the content of milk fat and that of total solids, and in particular that of milk protein (Jenness & Patton, 1959; Lush, 1960; Corbin & Whittier, 1965).

In the U.S.A. increased competition from vegetable fats and oils caused a drastic fall in butter production, and in the commercial value of the milk fat (Coulter, 1966).

In recent years the value of high quality protein in the human diet has become more apparent, as has in particular the importance of milk and some of its products as the cheapest source of essential amino acids (Porter & Garton, 1964).

Change in the pattern of feeding of the Israeli–Friesian dairy herd, involving increase in both the absolute and relative amounts of concentrates, led to a pronounced decline in the milk fat percentage. However, the published evidence concerning the effects on other milk constituents is contradictory (Rook & Line, 1961; Nordfelt & Ruudvere, 1963; Van Soest, 1963; Olson, Reed, Benson, Stewart & Dahncke, 1965, Huber & Boman, 1966).

The development of the dye binding methods for protein estimation has enabled protein determination to be carried out on large numbers of milk samples, in an ordinary laboratory, and has made possible the examination of this question (Kiddy, 1963; Lolkema & de Graaf, 1966).

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Production in the Israeli dairy herd is highly intensive. Natural pasture is non-existent and only very limited areas of cultivated pasture or forage are available. The quantity of water and the area of land available per head of cattle are limited. Concentrates constitute the major part of the ration and in many herds supply over 80 % of the energy intake at certain periods of the lactation. The Israeli–Friesian strain that has been developed is capable of producing a high milk yield with a low fat percentage on a high-concentrate diet (Volcani, 1963).

Table 1. *Average milk production, and fat percentage and production, per cow in the Israeli registered herd*

| Production | 1960 | 1962 | 1964 | 1966 | 1968 | 1969 |
|---|---|---|---|---|---|---|
| Milk, kg | 5637 | 5709 | 5694 | 5939 | 6165 | 6271 |
| Fat, % | 3·42 | 3·37 | 3·28 | 3·26 | 3·25 | 3·26 |
| Fat, kg | 192·8 | 192·4 | 186·8 | 193·6 | 200·4 | 204·4 |

Less than 50 % of local milk production is utilized for the liquid market. The remainder is used almost exclusively in the manufacture of soft and hard cheese.

Powell (1939), Loosli, Lucas & Maynard (1945), Balch, Balch, Bartlett, Cox & Rowland (1952) and Ronning (1960) found that high-energy diets low in roughage and high in concentrates caused a fall in the fat percentage of milk. Holmes, Waite, MacLusky & Watson (1956), Storry & Rook (1966) and McCullough (1966) noted differences in solids-not-fat (SNF) percentages on feeding different levels of concentrates in the ration.

Milk with a low fat percentage is generally expected to have a low protein content, and this, if established, would clearly influence the nutritional and economic value of the milk. The purpose of the present work was to examine the effects of high-energy diets rich in concentrates, on the milk yield and on the fat and protein contents and the relationship between them.

## MATERIALS AND METHODS

Thirty-two cows and heifers of the Israeli–Friesian strain were divided into 2 feeding groups of 16 and housed in open sheds, each individual cow having a fixed feeding stand. The cows were allocated to the groups after consideration of their age, milk yield and body weight (average: 580 kg for cows and 490 kg for heifers); the heifers were also grouped on the basis of their dams records. The experiment was continued over a period of 3 years.

The diet consisted of pelleted concentrates containing 16 % crude protein and 90 % dry matter, and of roughage including fodder, silage and hay, and was supplied individually only when each cow was tied in its station.

Equal amounts of roughage (4 FU*) were eaten daily by each cow in all groups throughout the experiment. Two levels of concentrates were fed: (1) 'Normal' level, calculated weekly from maintenance requirements plus an additional 0·4 FU/kg milk, according to the Breirem (1955) standard; (2) 'High' level: concentrates were

* FU = Scandinavian Feed Unit, 1 FU = 1·65 Mcal net energy for fattening or 2·1 Mcal for milk production.

offered to each cow ad libitum several times daily. The cows of this group were weighed twice weekly. On exhibiting a progressive increase in body weight these cows were transferred gradually to the Normal diet.

Cows found to be infected with mastitis were withdrawn from the experiment and replaced by other animals.

Cows were milked thrice daily and the yields of milk recorded. Samples were collected at 3 consecutive milkings, weekly or fortnightly, and analysed for total solids and for fat as described by American Public Health Association (1960), and for protein as follows: a solution was prepared containing 0·06% Amido Black 10 (Merck AG, Darmstadt), 0·16% disodium hydrogen phosphate and 1·6% citric acid, in distilled water. This solution, of pH 2·5, when diluted 1:100 with distilled water gave an absorbance of 0·28 at 550 nm, as measured with a 'Spectronic 20' spectrophotometer using a cell of 12 mm light path.

To a test tube containing 10 ml of the Amido Black solution at room temperature, a 0·5-ml sample of milk was added. The tube was stoppered and shaken for at least 10 min and centrifuged at 820 *g* for 10 min. One ml of the supernatant was diluted with 10 ml of distilled water and its absorbance measured. A standard absorbancy curve was prepared for each batch of Amido Black solution by nitrogen determination (Kjeldahl method) on the same samples.

SNF was obtained by subtracting the fat percentage from the percentage of total solids.

Statistical treatment of the results was carried out according to Snedecor (1956).

## RESULTS AND DISCUSSION

Data on the feed intake of the animals in the 2 groups are given in Table 2. The data are analysed and reported for 3 periods. (1) The first period was the first 12 weeks of lactation, during which concentrates were given to the High groups ad libitum and

Table 2. *Average feed units (FU*) consumed daily per cow, and percentage of roughage in the diet during the experimental periods*

| Feeding group | | Weeks of lactation 1–12 | | 13–28 | | 29–44 | |
|---|---|---|---|---|---|---|---|
| | | FU | Roughage, % | FU | Roughage, % | FU | Roughage, % |
| Heifers | High | 18·5 | 22 | 13·8 | 29 | 11·2 | 36 |
| | Normal | 11·9 | 34 | 10·9 | 37 | 9·7 | 41 |
| Cows | High | 20·2 | 20 | 15·0 | 27 | — | — |
| | Normal | 13·7 | 29 | 11·0 | 36 | — | — |

* See p. 288.
—, Not determined because of many cases of mastitis.

to the Normal groups in accordance with milk yield and the maintenance requirements. (2) The second period was from the 13th to the 28th week, during which the effects of pregnancy are normally not yet apparent (Lampo, Willems & Vanschoubroek, 1966). Cows of the High group which exhibited gains in body weight accom-

panied by a drop in milk production were gradually transferred to the Normal regime during this period. (3) The third period was from the 29th to the 44th week, during which most of the animals were pregnant. The records of the heifers only are presented, as a large number of the cows were withdrawn from the experiment due to mastitis.

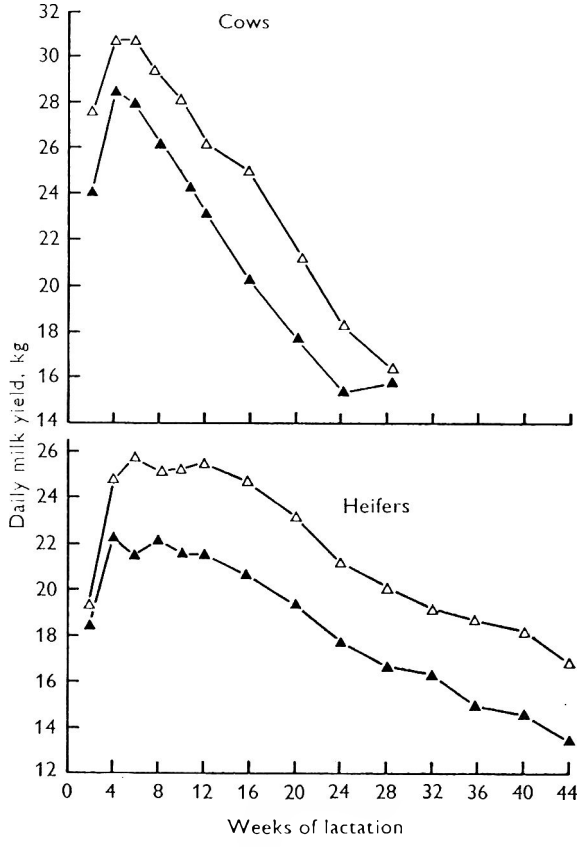


Fig. 1. Average daily milk yield of cows and heifers on Normal (▲) and High energy (△) diets.

During all 3 periods the High group consumed greater amounts of concentrates than did the Normal group and thus the percentage of roughage in the diet remained lower in the High group.

*Milk yield*

Peak milk yield was obtained within 3–6 weeks of the beginning of lactation, the High groups exhibiting significantly higher yields throughout the lactation period.

*Milk composition*

*Fat.* The High groups showed consistently a significantly lower fat percentage than did the Normal group, characterized by a minimum between the eighth and tenth week below the recorded level of the Israeli herd. This minimum was followed by a gradual increase which was maintained until the end of the milking period. Because

of the difference in milk yield between the 2 groups there was no difference in total fat production.

*Protein.* No differences were found in the protein percentage but total protein production was significantly higher both for cows and for heifers of the High groups during the entire test period.

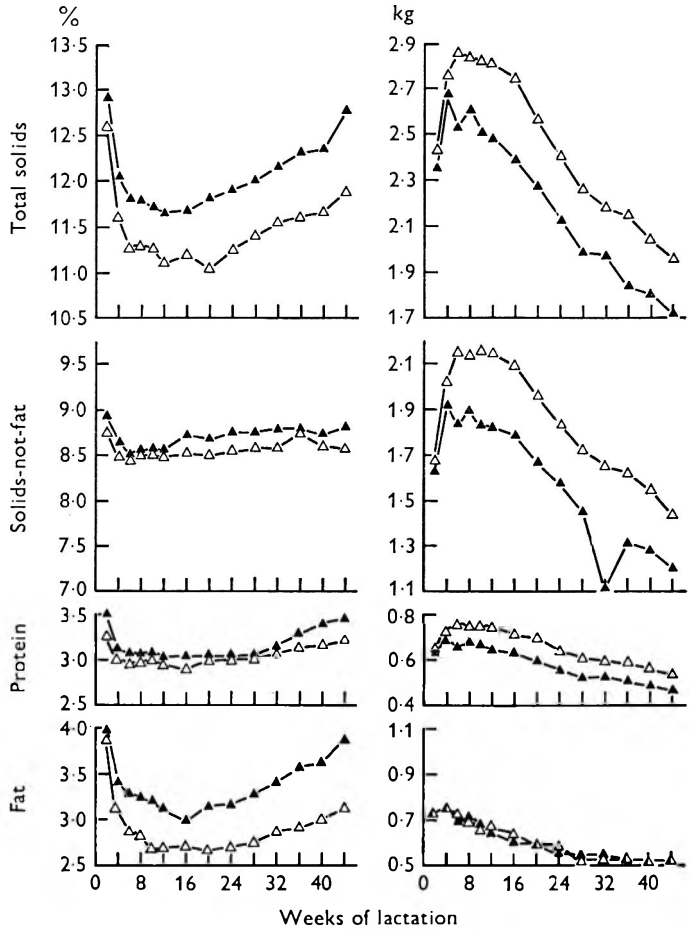


Fig. 2. Average daily yield and percentage of milk constituents of heifers receiving Normal (▲) and High energy (△) diets.

*SNF and total solids.* The pattern for SNF content resembled that for protein in both groups. The pattern for total solids content resembled that for fat, although total production was somewhat higher in the High groups.

Data presented in Table 3 shows a rise in overall milk production in the High group. There was no difference in fat production, but a rise in protein production was evident. These trends were maintained not only in the first 12-week period, during which concentrates were fed ad libitum, but also during the later periods when the feeding regime was the same for both groups. However, the percentage of roughage in the diet remained lower in the High groups.

It has been found that in general a rise in milk production lowers the overall fat and protein percentage, but is accompanied by a rise in fat and protein production (Huber, Graf & Engel, 1964; Swanson, Hinton & Miles, 1967).

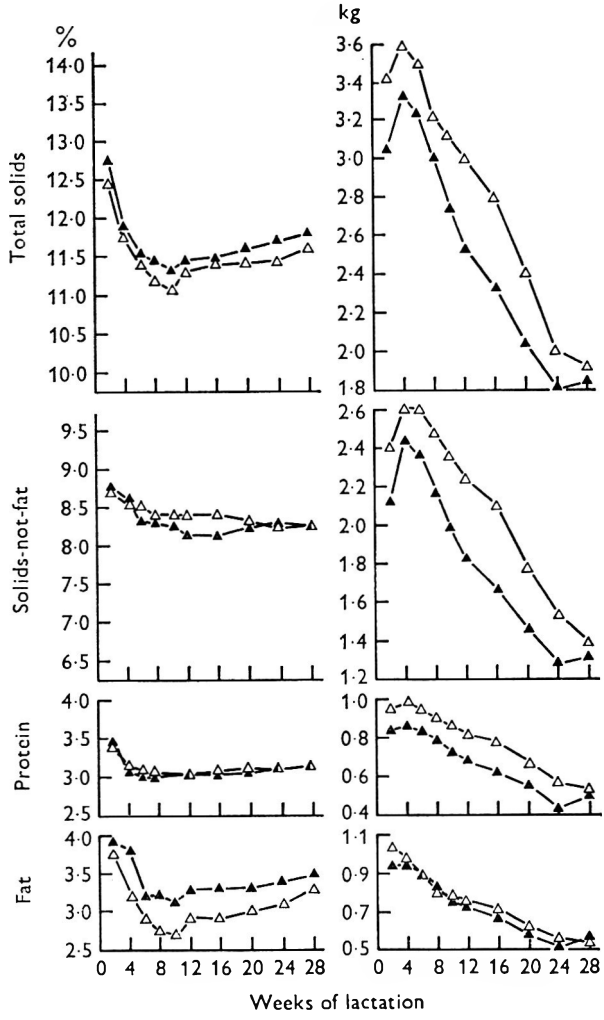


Fig. 3. Average daily yield and percentage of the milk constituents of cows receiving Normal (▲) and High energy (△) diets.

In the present experiment, however, the milk fat percentage was lowered in the High groups, in some cases to less than 2%, which represented a fall in total fat production. At the same time the protein percentage and production were not lowered, and in some cases were even higher. Furthermore, the variations in SNF may reflect rises greater than those due solely to the protein.

Kay & Hobson (1963) reviewed the effect of high-energy diets on milk fat and connected the fall in milk fat percentage on a high-concentrate diet with changes in the production of VFA and in the ratio of acetic to propionic acid in the rumen. High-energy diets lead to enhanced rumen activity and their buffering capacity is insuffi-

cient to prevent changes in the pH. A fall in pH inhibits the acetic producing microflora relative to the propionic acid producing population. Since acetic acid is a major precursor of milk fat, lower levels of acetic acid result in lower milk fat production. Further metabolism of the propionic acid may result in the formation of amino acids which may be used in the synthesis of milk proteins (Van Soest & Allen, 1959; McCullough, 1966; Storry & Rook, 1966).

Table 3. *Production of milk and its constituents during the 3 experimental periods*

| | | Yield, kg | | | |
|---|---|---|---|---|---|
| | | Heifers | | Cows | |
| Weeks of lactation | Component | High | Normal | High | Normal |
| 1–12 | Milk | 2029 | 1778 | 2419 | 2149 |
| | Fat | 59·5 | 59·2 | 73·2 | 71·4 |
| | Protein | 61·5 | 55·7 | 76·6 | 66·6 |
| | SNF | 172 | 153 | 206 | 181 |
| | Total Solids | 232 | 212 | 279 | 252 |
| 13–28 | Milk | 2489 | 2090 | 2227 | 1940 |
| | Fat | 65·5 | 64·0 | 68·6 | 65·0 |
| | Protein | 74·3 | 63·8 | 71·5 | 61·7 |
| | SNF | 272 | 181 | 190 | 160 |
| | Total Solids | 278 | 246 | 259 | 225 |
| 29–44 | Milk | 2035 | 1659 | — | — |
| | Fat | 58·9 | 58·8 | — | — |
| | Protein | 63·4 | 55·0 | — | — |
| | SNF | 175 | 145 | — | — |
| | Total Solids | 234 | 204 | — | — |
| Total | Milk | 6553 | 5557 | 4696 | 4089 |
| | Fat | 183·5 | 182·5 | 141·8 | 136·4 |
| | Protein | 198·9 | 174·5 | 148·1 | 127·3 |
| | SNF | 560 | 479 | 396 | 341 |
| | Total Solids | 744 | 622 | 538 | 477 |

All differences between High and Normal with the exception of fat were significant ($P < 0{\cdot}01$).

Under our experimental conditions the groups receiving the High level of concentrates showed a fall in fat percentage which supports this assumption, but no corresponding fall in protein percentage was found. The theory of a constant relationship between fat and protein percentages in the milk produced under the High feeding regime is thus not correct. Under these conditions estimation of feed requirements based on Fat Corrected Milk (Gaines, 1928) was inadequate, in that the decrease in milk fat percentage was not accompanied by a corresponding fall in the milk protein percentage, and the estimates therefore need to be revised.

The fat content of milk produced under such conditions of feeding is clearly not a suitable index of its dietetic and commercial value, especially if the milk is destined for cheese production.

We thank Dr J. Kali and Dr S. Amir of the Volcani Institute for their cooperation and helpful suggestions.

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# The effect of varying ratios of roughage to concentrates on composition and yield of cow's milk*

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(*Received* 16 *March* 1971)

Summary. Two levels of roughage, 3 FU (feed units) and 5 FU daily, were fed to Israeli–Friesian cows and heifers receiving either a normal (Normal) or a high-energy (High) diet.

Milk yield and composition were examined for 12 weeks following parturition. The animals receiving 5 FU of roughage daily showed a higher milk yield during the first 8 weeks. With the Normal diet the amount of roughage had no effect on milk composition. With the High diet, milk yield was higher than with the Normal, and a depression in fat percentage was observed in the milk of cows and heifers receiving 3 FU daily. No corresponding fall in milk protein percentage was observed – the cows of this group (High-3 FU roughage) showing, in fact, a rise in protein percentage. No differences in milk total solids were found. With these high-energy low-roughage diets no correlation was apparent between milk fat and protein percentages.

In Israel only limited amounts of water are available, and the decision to produce a specific crop is influenced not only by the price of water required to produce it, but also by the alternative income that would be obtainable from the use of the water in the production of other crops. The profitability of irrigated fodder is not high and farmers prefer to utilize their limited water for other more profitable crops. Hence, the amount of roughage fed to the dairy herd is the minimum estimated to be required for normal activity of the digestive system. The relative cheapness of grain, and the savings in labour and storage space that result from its use, have also promoted the wide use of nutritionally balanced concentrates mixtures (Hoglund, 1963). The quantity of roughage allotted per cow daily is almost constant in all seasons regardless of milk yield, rises in yield being accommodated by increased allowance of concentrates. In the period immediately following parturition when milk yield is at its highest, concentrates are often presented ad libitum and may constitute 85–90 % of the ration. Under this feeding regime, in which the amount of roughage consumed is almost constant, the percentage of roughage in the diet is especially low in the high production periods.

The purpose of the present work was to examine the effects of feeding 2 fixed proportions of roughage in both High and Normal diets on the milk yield and composition in cows during the first 12 weeks of lactation.

* Contribution from Volcani Institute of Agricultural Research, Bet Dagan, Israel; 1971 series, no. 1841 E.

MATERIALS AND METHODS

Thirty-two cows and heifers of the Israeli–Friesian strain were divided into 2 groups at 2 nutritional levels immediately after parturition. The Normal level was calculated according to maintenance requirements with an additional 0·4 FU*/kg milk yield (Breirem, 1955). At the High level the animals received concentrate mixtures ad libitum several times daily. Each level was subdivided into 2 groups according to the amount of roughage allotted daily, one receiving 3 FU roughage (Normal-3, High-3), and the other 5 FU roughage (Normal-5, High-5) per animal.

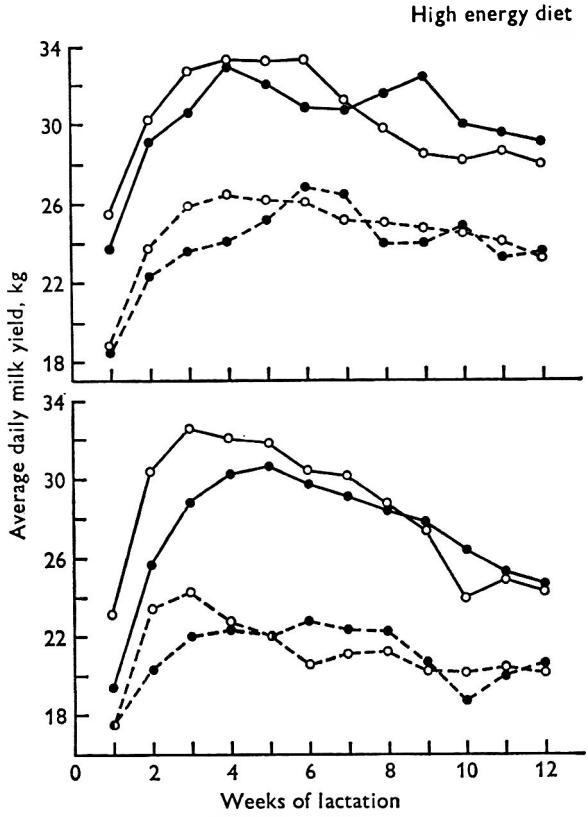


Fig. 1. Average daily milk yield of heifers (– – –) and cows (——) receiving 3 FU (●) or 5 FU (○) of roughage on High energy and Normal diets.

The animals were fed individually and milked thrice daily, and the yields of milk recorded. Samples were collected from consecutive milkings in equivalent amounts and pooled once weekly during the first 12 weeks of lactation. The samples were analysed for total solids, fat, protein and solids-not-fat (SNF) as described in the preceding paper (Gordin, Volcani & Birk, 1971, p. 289). Statistical treatment was according to Snedecor (1956).

* FU = Scandinavian Feed Unit, 1 FU = 1·65 Mcal net energy for fattening or 2·1 Mcal for milk production.

## RESULTS

### *Feed intake*

Roughage comprised some 25–30 % of the intake in group Normal-3 and 30–40 % in group Normal-5. Group High-3 consumed more concentrates and total FU than did group High-5, the difference being not more than 10 %. Since the diet was low in roughage this difference in concentrate intake led to roughage comprising 20 % of the diet in group High-3 and 25–32 % of the diet in group High-5.

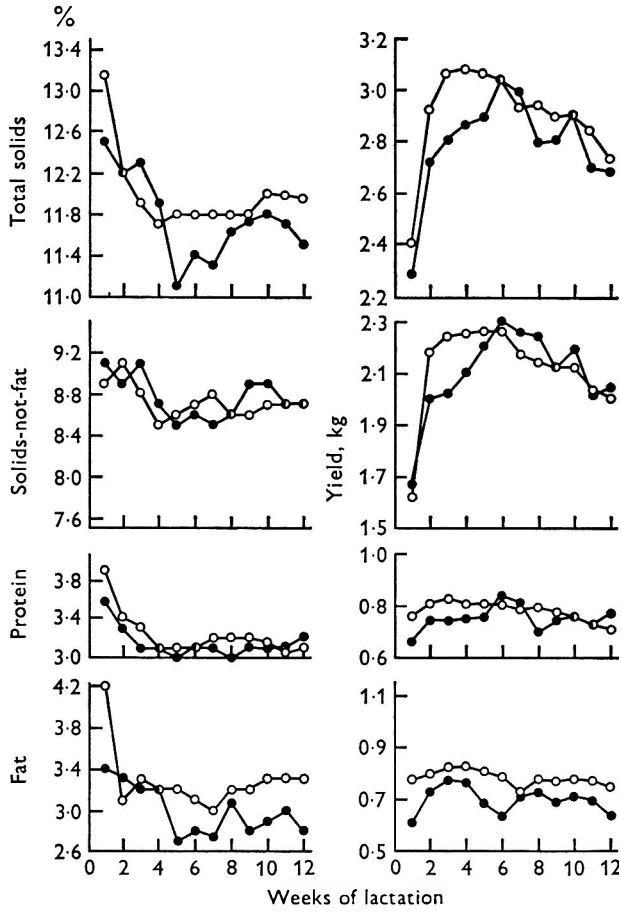


Fig. 2. Average daily yields and percentages of milk constituents of heifers receiving High energy diets containing 3 FU (●) or 5 FU (○) of roughage.

### *Milk yield*

Groups receiving 5 FU of roughage daily produced more milk from the beginning of the experiment until the eighth week than did those receiving 3 FU roughage, at both High and Normal energy levels. After the eighth week production became similar in parallel groups.

*Milk constituents*

*Fat.* At the Normal energy level no differences in milk fat percentage or yield were observed. However, cows of group High-5 produced a higher percentage and total yield of fat throughout the entire period than did group High-3.

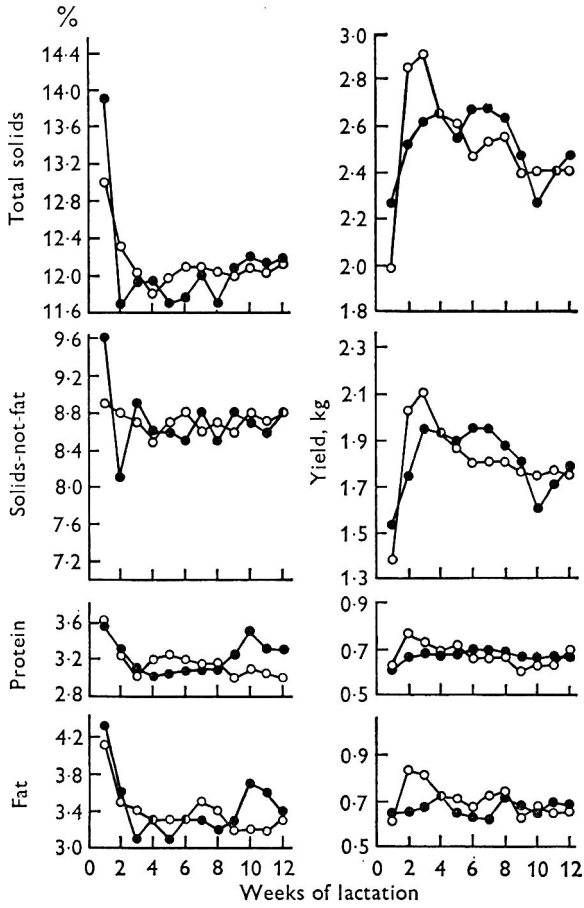


Fig. 3. Average daily yields and percentages of milk constituents of heifers receiving Normal diets containing 3 FU (●) or 5 FU (○) of roughage.

*Protein.* At the Normal energy level no differences were found. However, the cows of group High-3 exhibited a higher percentage and yield of protein, especially after the fourth week of lactation.

*SNF.* The general pattern was similar to that for protein, but the differences resulting from the different dietary treatments were more pronounced.

*Total solids.* No significant differences were apparent.

Table 1 presents a summary of the data for the 12 weeks of the experiment. For heifers and cows of group High-3 the milk fat percentage was lower than for High-5. Cows of group High-3 showed a rise in milk protein percentage over that for High-5.

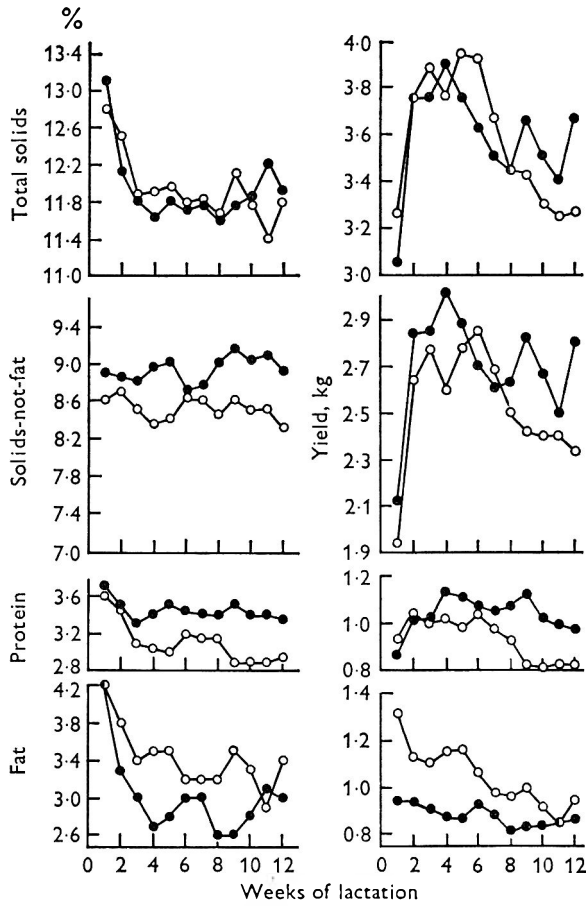


Fig. 4. Average daily yields and percentages of milk constituents of cows receiving High energy diets containing 3 FU (●) or 5 FU (○) of roughage.

At the Normal level the amount of roughage in the diet did not affect the content of milk constituents.

## DISCUSSION

The differences in milk production between the 2 groups fed the High energy level were of the order of 0·3–1·2 kg/day, with group High-5 producing the higher milk yields. The differences between the High and Normal energy levels were approximately 3 kg/day, and similar to those obtained in previous work (Gordin *et al.* 1971).

The observation that on the higher roughage diets milk production was significantly higher in the first 3–5 weeks of lactation was unexpected (cf. Huffman, 1961; Kesler & Spahr, 1964), especially since the feed intake was higher with group High-3. The finding might be attributable to a slower adaptation of the rumen microflora – accustomed to a high roughage diet before parturition – to the high concentrate diet that was given after parturition. This drastic change in diet could well have caused a 'lag period' which lasted for some 8 weeks, while the rumen flora adapted to the new diet. Since a third of the total milk yield is produced during the first 12 weeks of

lactation, it might be useful to begin the dietary change earlier. Alternatively, it is possible that the lower net energy value attributed to roughage in the feed unit calculation may not represent its true net productive value under these conditions of feeding.

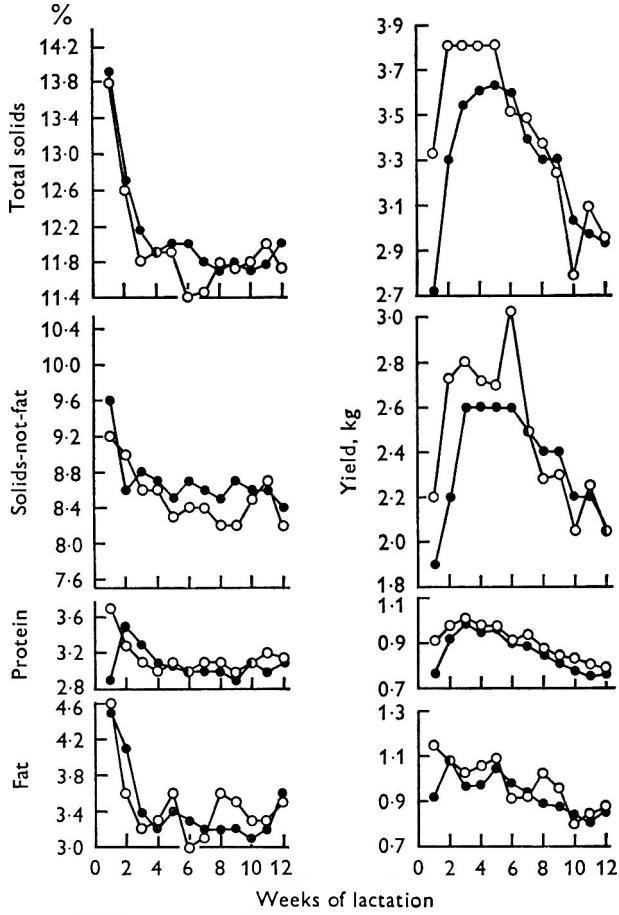


Fig. 5. Average daily yields and percentages of milk constituents of cows receiving Normal diets containing 3 FU (●) or 5 FU (○) of roughage.

At the Normal level the amount of roughage eaten sufficed to maintain normal activity in the rumen and hence no noticeable differences were observed. At the High level however, a depression in fat percentage resulted, as in our previous study (Gordin *et al.* 1971).

Under our experimental conditions the depression in fat percentage was not accompanied by the expected rise in milk yield (Van Soest, 1963; Huber, Graf & Engel, 1964) or by a fall in protein percentage (Jenness & Patton, 1959; Corbin & Whittier, 1965; Gardner, 1969). In fact, cows of Group High-3 showed a rise in protein percentage which rose to above the fat percentage. Heifers, however, did not demonstrate this rise.

No correlation between milk fat and protein percentage was apparent under high energy–low roughage feeding régimes.

Table 1. *The milk yield and composition of the heifers and cows on the experimental diets (12 weeks of lactation)*

| | | | Milk yield | | Fat | | Protein | | Solids-not-fat | | Total solids | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Group | Nutritional level | Roughage, FU* daily | Average daily, kg | Total, kg | Total yield, kg | % | Total yield, kg | % | Total yield, kg | % | Total yield, kg | % |
| Heifers | High | 3 | 23·8 | 2000 | 59·2 | 2·96 | 62·9 | 3·15 | 175·6 | 8·78 | 234·1 | 11·71 |
| | High | 5 | 24·3 | 2040 | 65·3 | 3·20 | 64·9 | 3·18 | 179·5 | 8·80 | 244·2 | 11·98 |
| | Normal | 3 | 20·8 | 1753 | 58·6 | 3·36 | 55·8 | 3·19 | 151·9 | 8·69 | 210·4 | 12·04 |
| | Normal | 5 | 21·1 | 1770 | 58·7 | 3·31 | 55·5 | 3·13 | 152·2 | 8·59 | 210·9 | 11·90 |
| Cows | High | 3 | 30·1 | 2529 | 73·4 | 2·90 | 86·7 | 3·43 | 226·8 | 8·97 | 300·2 | 11·89 |
| | High | 5 | 30·5 | 2562 | 87·6 | 3·42 | 78·2 | 3·05 | 211·9 | 8·27 | 299·0 | 11·67 |
| | Normal | 3 | 27·1 | 2277 | 78·3 | 3·43 | 72·4 | 3·18 | 197·8 | 8·69 | 276·1 | 12·12 |
| | Normal | 5 | 28·3 | 2380 | 81·9 | 3·44 | 75·4 | 3·17 | 207·0 | 8·64 | 289·0 | 12·08 |

* See p. 298.

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# Effects of drenching with a 'pluronic' on bloat and milk production

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Summary. Bloat in identical twin lactating cattle grazing clover-rich pastures was controlled by twice-daily drenching with 7 ml 'Pluronic' L 64 in water. The drench did not alter milk composition and there was no significant difference in milk production between control and treated groups. A significant ($P < 0{\cdot}05$) linear relationship between bloat protection and butterfat production was found between twin sets. Drenching increased milk production in high-bloating cows but slightly depressed production in low-bloating animals.

The use of polyoxypropylene-polyoxyethylene polymers as surfactants in controlling and treating bloat has become widespread in New Zealand and overseas. These anti-foams are most effective when given in water solution as a drench although the addition of 'Pluronics' to drinking water is known to decrease the incidence of serious bloat (Phillips, 1968).

Johns & McDowall (1962), in a 3-week trial using 3 pairs of identical twin cows, found that a single daily dose of 50 ml of Pluronic L 62 had little effect on milk yield or composition. In the U.S.A. Helmer, Bartley & Meyer (1965) showed that daily doses of 10–40 g of another polyoxypropylene-polyoxyethylene block polymer, 'Poloxalene', had no deleterious effect on milk production, milk fat, body weight, feed consumption, conception rate or animal health.

The onset of bloat is usually considered to lower production by lowering food intake and estimates of the economic consequences of bloat include not only deaths of animals but also reduced milk production. When bloat is controlled by the use of an anti-foam, maintenance of production is to be expected.

Attempts to demonstrate this effect in bloating cattle have not been successful (Scott, 1965). The present paper reports the results of a trial testing the effect of routine drenching with the anti-foam Pluronic L 64 on bloat incidence, milk composition and production in identical twin cows grazing clover-rich pastures.

## EXPERIMENTAL

Fifteen sets of identical twin Jersey cows aged from 2 to 8 years were used in the trial. Their ages and calving dates are shown in Table 1. They were divided into control and treated groups, each containing a similar distribution of calving dates. The cows entered the respective groups 4 days after calving. From 24 August 1969

the cows which had calved in the treated group were drenched with 7 ml of Pluronic L 64 as below.

The animals were grazed on a 40-acre area at No. 4 Dairy, Ruakura. The pasture was predominantly white clover, and was rotationally grazed, with each paddock providing enough pasture for three 12-h grazing periods. Throughout the trial, bloat readings were taken 1 and 2 h after the herd had entered the paddock following each milking. Bloat scores were recorded by visual assessment on a 6-point scale:

0 No visible bloat
1 Left flank distended } mild bloat
2 Left flank round }
3 Left flank round and right flank distended – moderate bloat
4 Both flanks round and animal obviously distressed and given treatment } serious bloat
5 Animal seriously distressed and given treatment }

Total bloat scores are the sums of the maximum readings after each milking. To reduce stock losses, any animals reaching a score of 4 were immediately drenched with 7 ml of Pluronic L 64 diluted with 21 ml of water. The Pluronic L 64, a non-ionic polymer of ethylene and propylene glycols, is a product of Wyandotte Chemicals Corporation, U.S.A. An automatic drenching gun was used while the animals were confined in a race. Daily milk yields were recorded and the milk was analysed for fat, lactose and protein at 7-day intervals.

## RESULTS

The effectiveness of preventing bloat in cows by oral dosing with 7 ml Pluronic L 64 twice daily after milking is shown in Fig. 1. The trial continued until 31 January (23 weeks), when drought conditions forced premature drying-off in the herd. Bloat was found throughout most of the trial, the control group consistently having total weekly bloat scores greater than 60 for the first 18 weeks with maximum values of 300–350 during the eighth and ninth weeks. No bloat was found in the last 2 weeks. The readings in the control group have been depressed by the occasional need to drench animals to prevent any deaths. The number of emergency drenches required during each week is shown in Fig. 1. Eleven of the control group cows were drenched on a total of 32 occasions. One cow, number 919, was treated 8 times between 25 August and 24 October and 2 others (numbers 86 and 904) were drenched 4 times. Milk composition values for the 2 groups were:

*Control:* lactose, 4·7 %; protein, 3·5 %; fat, 4·8 %
*Treated:* lactose, 4·8 %; protein, 3·6 %; fat, 4·8 %.

The effect of drenching on milk production was complicated by differences in calving dates of related twins. This effect was reduced by comparing the production of control and treated groups over the 100-day period from 4 October 1969 to 10 January 1970. Over this period the total bloat scores were 1818 and 218 in the control and drenched groups respectively. Only a small difference in total production was found,

Table 1. *Fat production and bloat incidence**

| Cow no.† | Age | Accumulated § bloat score‡ | Butterfat C − D, lb§ |
|---|---|---|---|
| 919 | 2 | 246 | |
| 920 | 2 | 29 | −22·7 |
| 937 | 2 | 315 | |
| 938 | 2 | 50 | −17·9 |
| 936 | 2 | 135 | |
| 935 | 2 | 14 | −12·1 |
| 918 | 2 | 83 | |
| 917 | 2 | 10 | −13·5 |
| 929 | 2 | 98 | |
| 930 | 2 | 19 | −33·4 |
| 904 | 2 | 188 | |
| 903 | 2 | 4 | + 0·3 |
| 931 | 2 | 68 | |
| 932 | 2 | 4 | − 8·1 |
| 173 | 3 | 27 | |
| 174 | 3 | 5 | +26·9 |
| 6 | 4 | 143 | |
| 5 | 4 | 4 | −14·9 |
| 23 | 4 | 59 | |
| 24 | 4 | 9 | +20·0 |
| 62 | 5 | 139 | |
| 63 | 5 | 2 | −57·8 |
| 74 | 6 | 92 | |
| 75 | 6 | 12 | +22·2 |
| 80 | 6 | 28 | |
| 81 | 6 | 8 | +46·4 |
| 86 | 6 | 135 | |
| 87 | 6 | 42 | + 3·0 |
| 99 | 8 | 62 | |
| 98 | 8 | 6 | +25·1 |

* These figures relate to the period 4 October 1969 to 10 January 1970, a total of 100 days.

† The first animal in each twin pair was undosed as a control for the second cow, which was drenched twice daily with 7 ml of Pluronic L 64 diluted with 21 ml of water.

‡ The accumulated bloat score is the sum of the maximum bloat readings after each milking.

§ C, control animal; D, drenched animal.

the drenched group producing 36·51 lb of butterfat more than the control group. This amounted to an extra 0·37 lb/day for 15 cows.

Considerable differences were noted between twin sets. In 7 pairs the control cows produced more than the drenched cows and in the other 8 pairs the results were reversed. A significant ($P < 0·05$) relationship between the differences in bloat scores and fat production between twins was established (Fig. 2). Drenching tended to increase production in drenched cows when the control twin was prone to bloat, but lowered production in twins with low accumulated bloat scores.

## DISCUSSION

This trial confirms the effectiveness of drenching with the polyoxyethylene-polyoxypropylene block polymer Pluronic L 64. It is likely that other members of this series which differ slightly in their ratio of hydrophobic to hydrophilic groups

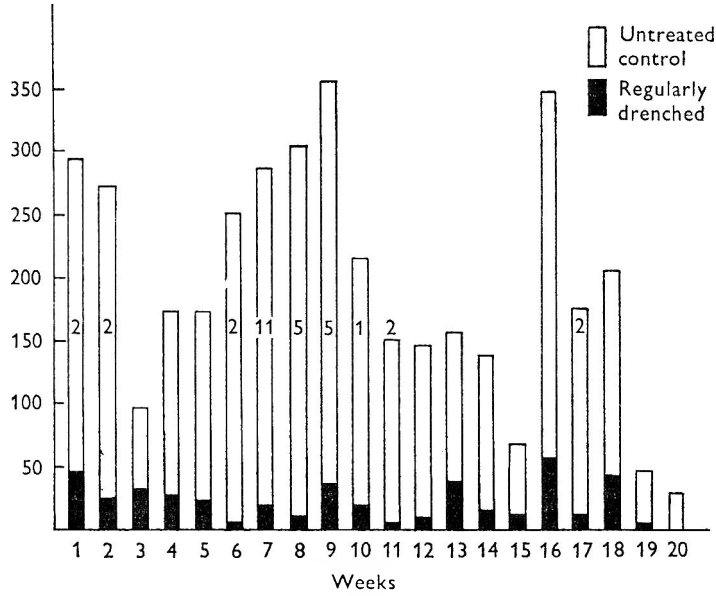


Fig. 1. The effect of twice-daily drenching of 7 ml of Pluronic L 64 on bloat incidence in a herd of identical twin lactating cows. The weekly bloat scores are calculated from the sums of the maximum readings after each milking.

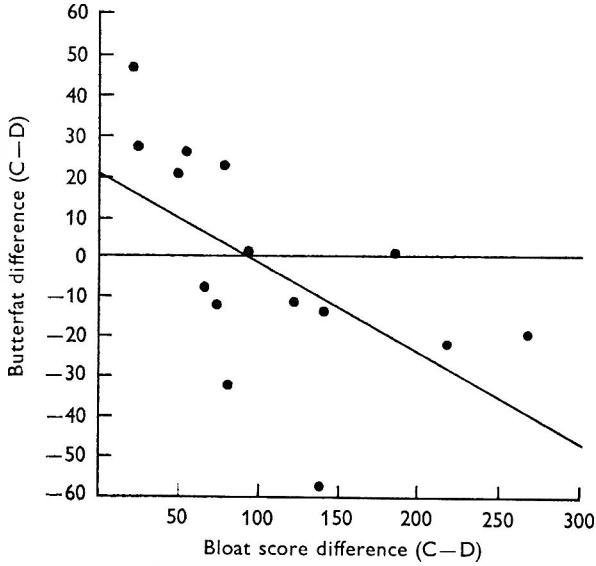


Fig. 2. Relationship between differences between control and Pluronic L 64-drenched cattle for butterfat production ($Y$) and for bloat score ($X$) [$Y = -0{\cdot}22\,(\pm 0{\cdot}09)\,X + 20{\cdot}8\,(\pm 7{\cdot}3)$].

would give similar results, as they are known to possess similar anti-foaming ability both in the laboratory (Wright, 1969) and in the field (Phillips, 1968).

While no deaths occurred in the control animals, the need to drench severely bloated cows on 32 occasions during 9 weeks of the trial emphasizes the need to watch unprotected animals on bloat-inducing pasture.

This trial provides experimental evidence of a relationship between bloat and milk production. Scott (1965) has described a trial in 1963 in which unrelated cows were grazed on sprayed and unsprayed pastures. No differences in production were found in the 2 herds despite differences in the degree of bloat. Scott speculated that the low butterfat-producing cattle used in his trial may have been a factor contributing to the lack of effect. Bartley *et al.* (1965), using 36 dairy steers, 18 of which were given 10 g 'Poloxalene' daily, reported excellent control of bloat but no effects on weight gain, suggesting that bloat in the untreated group did not reduce food intake.

In the present trial a similar conclusion, that bloat and bloat prevention have little effect on milk production, can be drawn from a comparison of the averages of the control and treated groups. However, this apparent lack of effect appears to be due to 2 opposing factors – an increase in milk production caused by drenching those animals which are prone to bloat, and a depression in milk production in animals which are not. In herds containing both types of animals, drenching will not increase total production significantly.

The effect of drenching in maintaining production in high-bloating animals supports the view that the onset of bloat reduces food consumption, but the depressed production in low-bloating cows was unexpected. The Pluronics are known to burst holotrich protozoa (Oxford, 1959), but no other effects of these detergents on rumen micro-organisms have been reported. Further studies are being made of this effect by measuring the production from drenched cattle and the rates of metabolism of micro-organisms in rumen contents.

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# The effects of dietary urea and ammonium sulphate on the yield and composition of sheep milk

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SUMMARY. An experiment lasting 4 years has been carried out with 4 groups (3 test and a control) of 10 sheep. Non-protein nitrogen (NPN) (as urea, ammonium sulphate or a mixture of the two) replaced 25 % of the dietary nitrogen in the first 2 years and 50 % in the second 2 years. The yields of milk, protein and fat were generally depressed in the test groups but not all of the differences reached significance. In the group receiving ammonium sulphate, the milk fat percentage was higher than in the control group in all years and the protein percentage was higher in the last 2 years. Contrary to results elsewhere, the yield of lactose was depressed in all test groups. In the urea group, the content of minerals and of calcium in the milk was significantly less than in the control groups. The total amount of NPN and its components (ammonia, urea and creatine) in milk were not affected by the substitution of NPN in the rations.

Synthetic nitrogen compounds are increasingly used in the diet of ruminants and the effect on productivity of the non-protein nitrogen (NPN) thus provided has been the subject of much research, most of which has been with cows.

Virtanen (1966) has studied the problem in detail. He reported that satisfactory yields of milk were obtained from test cows given purified rations, in which urea and ammonium salts were the only source of N. The same author (Virtanen, 1967) established that the composition of the milk from the test cows was almost the same as that of the control animals, the amount of milk fat being greater with the test animals.

Among the few studies on the effect of NPN, as the only N source in the rations, on the composition and the properties of sheep milk is that of Todorov, Angelova, Mineva, Koumanov, & Ivanov (1969). They found that the difference in the milk yield from the test and the control sheep was not significant, but the percentages of fat and protein were significantly lower with the test animals. The decrease in milk protein was associated with both casein and whey proteins. The amounts of minerals and lactose were not significantly different.

The studies of Briggs & Hogg (1964) showed that the inclusion of urea in rations of cows did not affect the content of urea in the milk. We are not aware of any reports on the amount of creatinine, ammonia or urea in sheep milk but, according to Shahani & Sommer (1951), the concentration of creatinine in cow's milk varies from 0·19 to 0·65 mg/100 ml, that of ammonia-N from 0·17 to 1·19 mg/100 ml and that of urea-N from 6·54 to 10·85 mg/100 ml.

The purpose of the present research was to establish the changes occurring in the composition of milk from sheep fed with rations in which part of the N needed was supplied as urea and ammonium sulphate.

## MATERIALS AND METHODS

The study was carried out with 4 groups of 10 Zlatusha sheep. The animals in each group were balanced for milk yield and percentage of fat and protein. The experiment lasted for 13 weeks, from May 1 to July 31, in 4 successive years.

Table 1. *Net energy and digestible protein in ration ingredients*

| | Net energy, kcal/g* | Digestible protein, g/100 g* |
|---|---|---|
| Barley | 1·697 | 8·0 |
| Corn meal | 1·838 | 8·0 |
| Sunflower meal | 1·414 | 36·0 |
| Corn cobs | 0·707 | 1·6 |
| Grass (mountain pasture) | 0·283 | 1·1 |
| Grass (seeded pasture) | 0·352 | 2·4 |
| Grass (alfalfa pasture) | 0·255 | 3·6 |

* Fresh weight.

Table 2. *Composition of rations, g fresh food/day*

| | Years 1 and 2 | | Years 3 and 4 | |
|---|---|---|---|---|
| | Control (IV) | Test (I, II, III)* | Control (IV) | Test (I, II, III)† |
| Barley | 100 | 75 | 100 | — |
| Corn meal | 200 | 150 | 100 | 50 |
| Sunflower meal | 125 | — | 250 | — |
| Corn cobs | — | 440 | 250 | 966 |
| Grass (mountain pasture) | — | — | 4500 | 5000 |
| Grass (seeded pasture) | 4500 | 4500 | — | — |
| Grass (alfalfa pasture) | — | — | 500 | 400 |
| Chalk | 20 | 20 | 20 | 20 |
| Salt | 10 | 10 | 10 | 10 |

* In years 1 and 2 the test rations I, II and III included respectively 15 g urea, 33 g ammonium sulphate, and 7·5 g urea plus 16·5 g ammonium sulphate.

† In years 3 and 4, the test rations I, II and III included respectively 30 g urea, 66 g ammonium sulphate, and 15 g urea plus 33 g ammonium sulphate.

The rations were designed to provide the daily requirements of 2192–2535 kcal net energy and 160–180 g digestible crude protein as determined for lactating sheep by Platikanov, Tanev, Hincovski & Solomonov (1961). The net energy and digestible protein contents of the foods is shown in Table 1, and the composition of the rations in Table 2. In years 1 and 2 the rations provided 2291 kcal net energy and 177 g digestible protein daily. In the control ration, sunflower meal provided 25 % of the digestible nitrogen. In the test rations the sunflower meal was replaced by urea (I), ammonium sulphate (II) or a mixture of the two (III) providing equivalent amounts of nitrogen (44 g protein equivalent daily).

In years 3 and 4, the rations provided 2277 kcal net energy and 177 g digestible

protein daily. In the control ration sunflower meal provided 50 % of the digestible nitrogen and this was all replaced by the same NPN compounds as were used in the first 2 years; they provided 88 g digestible protein equivalent daily.

In all the years the net energy content of the control and test rations was equalized by adjustments in the amounts of other components, in particular by the inclusion in the test rations of large amounts of corn cobs which have been reported by McLaren, Smith & Peters (1968) to increase the synthesis of protein from urea in the rumen.

Milk was analysed for protein by the Kjeldahl procedure and for fat by the Gerber method. Phosphorous, lactose and calcium were determined by the methods respectively of Gericke & Kurmis (1952), Carrez (1908, 1909) and Schwarz & Hagemann (1950). In addition, ammonia, urea and creatinine were measured by the procedures of Inihov & Brio (1949), Nikolchev (1966) and Jaffe (1886), in order to determine whether the dietary NPN affected the NPN composition of the milk.

## RESULTS

The yield and composition of milk for the first 2 years are shown in Table 3 and for the second 2 years in Table 4. Throughout the experiment the yield of milk by the test groups was less than that by the control groups. The depression in yield was

Table 3. *The average yield and composition of milk from sheep given a control ration or a similar ration in which 25 % of the nitrogen was in the form of NPN (yields are for 3-month periods)*

| | Urea (I) | Ammonium sulphate (II) | Urea plus ammonium sulphate (III) | Control (IV) |
|---|---|---|---|---|
| Milk, l | 726·06 | 689·98 | 719·30 | 734·21 |
| Fat, % | 8·35 | 8·62 | 8·24 | 8·24 |
| Fat, kg | 60·63 | 59·48 | 59·27 | 60·50 |
| Total protein, % | 6·42*** | 6·60 | 6·52** | 6·69 |
| Protein, % | 46·58*** | 45·53*** | 46·86*** | 49·13 |
| Casein, % | 5·15*** | 5·32 | 5·33 | 5·34 |
| Albumin and globulin, % | 0·98 | 0·95 | 0·94 | 0·97 |
| NPN, % | 0·061 | 0·061 | 0·060 | 0·064 |
| Lactose, % | 4·38 | 4·28 | 4·37 | 4·34 |
| Lactose, kg | 31·81 | 29·52*** | 31·43*** | 31·89 |
| Minerals, % | 0·896* | 0·909 | 0·923 | 0·916 |
| Ca, % | 0·198* | 0·200 | 0·206 | 0·203 |
| P, % | 0·132 | 0·134 | 0·134 | 0·138 |

The significance of differences between the test groups (I, II and III) and the control group (IV) is shown as: * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$ or *** $P < 0{\cdot}001$.

particularly large with the ammonium sulphate group and was highly significant ($P < 0{\cdot}001$) for the first 2 years. The results show that substitution of NPN for protein in the diet caused a small depression in the milk yield of sheep under the conditions of our experiment in agreement with similar observations with cows by Scott, de Paula Assis, Gambini & de Sousa Lucci (1965–6) and Flatt, Moe, Oltjen, Putnam & Hooven (1969). Our results also show that the form of NPN was important in this connexion.

The percentage of fat in the milk was significantly increased by ammonium sulphate but not by urea or a mixture of the two. However, because of the depression in milk yield, the amount of fat secreted did not differ significantly from that of the control groups. Our results are not in agreement with those of Todorov *et al.* (1969), or with the conclusion of Virtanen (1966) that the increase in the percentage of milk fat from test cows is due to the influence of NPN in the rations.

Table 4. *The average yield and composition of milk from sheep given a control ration or a similar ration in which 50 % of the nitrogen was in the form of NPN (yields are for 3-month periods)*

| | Urea (I) | Ammonium sulphate (II) | Urea plus ammonium sulphate (III) | Control (IV) |
|---|---|---|---|---|
| Milk, l | 608·55 | 593·05 | 622·27 | 639·02 |
| Fat, % | 8·51 | 9·04* | 8·58 | 8·53 |
| Fat, kg | 51·79 | 53·61 | 53·08 | 54·51 |
| Total protein, % | 6·56 | 6·93 | 6·60 | 6·71 |
| Protein, kg | 39·92 | 41·10 | 41·04 | 42·90 |
| Casein, % | 5·33 | 5·58 | 5·36 | 5·43 |
| Albumin and globulin, % | 1·06* | 1·12 | 1·09 | 1·13 |
| NPN, % | 0·062 | 0·062 | 0·063 | 0·066 |
| Lactose, % | 4·40 | 4·36 | 4·43 | 4·49 |
| Lactose, kg | 26·75*** | 25·83*** | 27·59*** | 28·71 |
| Minerals, % | 0·922*** | 0·958 | 0·964 | 0·949 |
| Ca, % | 0·184** | 0·194 | 0·195 | 0·194 |
| P, % | 0·155 | 0·161 | 0·162 | 0·165 |

The significance of differences between the test groups (I, II and III) and the control group (IV) is shown as: * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$ or *** $P < 0{\cdot}001$.

Sheep milk is used mainly for the production of cheese and kashkaval (yellow cheese) and thus the milk proteins merit special attention. In the first 2 years of the experiment (Table 3) the protein content in the milk was depressed in the 3 test groups, the decrease being significant for group I (urea) ($P < 0{\cdot}001$) and group 3 (mixture) ($P < 0{\cdot}01$) but not for group 2 (ammonium sulphate). During the second period of 2 years (Table 4) the greater rate of substitution of NPN confirmed the pattern of change among the 3 test groups but the depression in groups I and III was smaller and in group II there was a significant increase ($P < 0{\cdot}05$) in protein content compared with the control group. The total yield of protein was less in all test groups throughout, this being highly significant ($P < 0{\cdot}001$) in the first 2 years.

In the first half of the experiment the casein content of the milk in the test groups tended to be less than in the control groups, but the decrease was significant ($P < 0{\cdot}001$) for group I only. In the second period there were no significant differences though the casein content in group II was greater than in the control group.

During the first 2 years of the experiment the content of whey proteins (albumin and globulin) in the milk of the test groups differed little from that of the control group, indicating that these constituents were not markedly affected by the treatments. During the second period all test groups showed a decrease, though this was significant ($P < 0{\cdot}05$) for group I only.

According to Halverson, Williams & Paulson (1968), inclusion of sulphate in the

rations stimulates sulphide production in the rumen of sheep in the same way as does the inclusion of cystine or methionine, and it has been proved that sulphide is an intermediate in protein synthesis. The increased amount of sulphide in the rumen causes an increase in the numbers of those micro-organisms which can utilize it and which are present in only small numbers when normal rations or rations containing urea are given. It is possible that this increase may explain the increased content of total protein and of casein in milk from sheep given rations in which 50% of the nitrogen was in the form of ammonium sulphate.

Throughout the 4 years of the experiments no significant differences were found in the content of NPN (Tables 3, 4), ammonia (0·39–0·62 mg/100 ml), urea (44·09–55·92 mg/100 ml) or creatinine (3·54–4·28 mg/100 ml) in the milks of individual groups.

The lactose content of the milk varied but in only one instance did the values differ significantly from that for the control groups. However, the yield of lactose was less than that of the control groups throughout, the depression being highly significant ($P < 0·001$) with all treatments except the higher level of substitution of urea.

The phosphorus content of the milk in the test groups was lower than that of the control groups throughout the experiment but not significantly so. The calcium content in the urea group was significantly depressed in all years.

## DISCUSSION

The present results show that the NPN given in the conditions of our experiment caused a decrease in the milk yield of sheep, and are in agreement with the results of Scott *et al.* (1965–6) and Flatt *et al.* (1969) with cows. There was also a tendency for a decrease in the content of total protein and of casein in the milk from the sheep receiving urea in their rations, as was reported by Todorov *et al.* (1969). The higher percentage of protein in group II in the second part of the experiment supports the conclusions of Khirwar, Pandit & Sengar (1965) that ammonium sulphate enhances the utilization of the protein in the rations.

The significant depression in the yield of lactose with all groups except the urea group in years 1 and 2 is not in agreement with published results and is difficult to explain.

In considering the results of the experiment as a whole it is important to bear in mind that, in order to keep the content of net energy and digestible protein similar in all the rations, it was deemed necessary to make appreciable changes in the composition of the test rations as compared with the control rations. It is possible that at least some of the changes in yield and composition of milk may have been due in part to ration changes other than the substitution of NPN for protein.

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# The metabolism of [U-$^{14}$C]glucose, [1-$^{14}$C]palmitic acid and [1-$^{14}$C]stearic acid by the lactating mammary gland of the sow

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Summary. (1) Lactating Large White sows were given an intravenous infusion of [U-$^{14}$C]glucose (3 sows), or of triglycerides containing [1-$^{14}$C]palmitic acid (1 sow) or [1-$^{14}$C]stearic acid (1 sow).

(2) The contribution of labelled plasma constituents to the synthesis of milk citrate, lactose, triglyceride glycerol, individual amino acids of the milk proteins and individual fatty acids of the triglyceride fraction was estimated by the 'transfer quotient' method. For the glucose infusions the mean values for the transfer quotients were: lactose, 70 %; citrate, 42 %; glyceride glycerol, 38 %; protein, 3 %; laurate, 6 %; myristate, 12 %; palmitate, 6 %; palmitoleate, 4 % and stearate, 2 %. Palmitic acid of the plasma triglycerides made similar contributions of about 60 % to the palmitic and palmitoleic acids of milk fat, whereas stearic acid made a major contribution (71 %) to the stearic acid of milk fat but a smaller contribution (42 %) to oleic acid.

(3) The results are compared with corresponding values for the goat and the cow.

Arteriovenous-difference studies in the conscious lactating sow (Linzell, Mepham, Annison & West, 1969; Spincer, Rook & Towers, 1969) have shown that plasma glucose and fatty acids of the plasma triglycerides are taken up in considerable amounts by the lactating mammary gland, glucose accounting for about 60 % of the total uptake of materials. An assessment has now been made of the quantitative contributions of plasma glucose and of palmitic and stearic acids of the plasma triglycerides to the synthesis of individual milk constituents, using a radioisotopic tracer technique.

## MATERIALS AND METHODS

*Animals and their management.* Lactating Large White sows in their fifth to sixth week of lactation were used. Piglets were removed immediately before the start of an experiment and the sows were milked out by hand at regular intervals after the intravenous injection of oxytocin (2 i.u.). The normal feeding routine was adopted.

*Experimental procedure.* Before the start of an experiment, catheters were inserted

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under anaesthesia into a jugular and a mammary vein. Infusions of either [U-$^{14}$C]-glucose (3 sows) or triglyceride containing [1-$^{14}$C]palmitic acid (1 sow) or [1-$^{14}$C]stearic acid (1 sow) were made into a jugular vein over a period of 5 h at a constant rate of 0·7 ml/min: with glucose an initial priming dose was given before the start of the infusion (Annison & White, 1961). [U-$^{14}$C]glucose was infused with carrier in 0·9 % saline solution and the triglycerides were infused as an emulsion, prepared by the method of Zeringue, Brown & Singleton (1964). Details are given in Table 1.

Table 1. *Details of the infusions of radioactive materials*

| Sow | Infused material | Priming dose | Rate of infusion |
|---|---|---|---|
| 1 | [U-$^{14}$C]Glucose | 100 $\mu$Ci | 1·3 mg glucose, 1·12 $\mu$Ci/min |
| 2 | [U-$^{14}$C]Glucose | 100 $\mu$Ci | 1·3 mg glucose, 1·12 $\mu$Ci/min |
| 3 | [U-$^{14}$C]Glucose | 200 $\mu$Ci | 1·3 mg glucose, 2·24 $\mu$Ci/min |
| 4 | [1-$^{14}$C]Tripalmitin | — | 32 mg triglyceride, 0·98 $\mu$Ci/min |
| 5 | [1-$^{14}$C]Tristearin | — | 32 mg triglyceride, 0·98 $\mu$Ci/min |

Samples of coccygeal blood and of milk were taken initially at hourly intervals and later at intervals of about 3 h and sampling was continued for a period of at least 24 h. A sample of mammary-venous blood was taken 4 h after the start of an infusion.

*Radioactive materials.* [U-$^{14}$C]glucose, [1-$^{14}$C]palmitic and [1-$^{14}$C]stearic acids were obtained from the Radiochemical Centre, Amersham, Bucks. After dilution with carrier acid, the fatty acids were incorporated with oleic acid into mixed triglycerides in the proportions of palmitic acid: oleic acid, 1:3 (w/w) and stearic acid:oleic acid, 1:3 (w/w). The triglycerides were synthesized from the free acids via the appropriate acid chlorides. In the final stage of the preparation the triglycerides in chloroform-pyridine-diethyl ether-petroleum ether (40–60°) (8:1:5:5, v/v) were washed twice with water, twice with 1 N-HCl, twice with water, twice with 10 % (w/v) $Na_2CO_3$ and then finally with water until the washings were neutral. The solution was dried with anhydrous sodium sulphate and the solvent removed under reduced pressure. The triglycerides were checked for purity by thin-layer chromatography. Only trace amounts of diglyceride and free fatty acid were present and more than 95 % of the radioactivity was in the triglyceride fraction.

*Measurement of radioactivity.* Materials that are soluble in organic solvents were dissolved in toluene (containing 0·5 % (w/v) 2,5-diphenyl oxazole and 0·05 % (w/v) 1,4-bis-2-(5-phenyl oxazolyl benzene)). Water-soluble materials were first dissolved in 0·2 ml of water and then taken up in a dioxane-base scintillator solution (NE 213 Nuclear Enterprises Ltd, Sighthill, Edinburgh, Scotland). A liquid-scintillation spectrometer (Tracerlab Corumatic 25) was used for the measurement of sample radio-activity. Quench correction was carried out using the channels-ratio method of Baillie (1960).

*Analytical methods.* Analyses of lipids of blood plasma and milk were carried out by methods reported previously (Spincer *et al.* 1969). Other methods of milk analysis were as follows: lactose (Ling, 1956), citrate (Marier & Boulet, 1958), protein (Metson, 1956).

*Isolation and degradation of milk and plasma constituents for measurement of radio-activity.* Individual fatty acids were separated as their methyl esters by gas–liquid chromatography (Spincer *et al.* 1969) and collected from the effluent in U-tubes filled

with glass wool and cooled to − 79 °C by a mixture of acetone and solid $CO_2$. Saturated and unsaturated methyl esters were separated prior to gas–liquid chromatography by argentation chromatography (Morris, 1966). The amount of methyl esters collected was determined by the method of Morgan & Kingsbury (1959).

Individual fatty acids were decarboxylated by the method of Brady, Bradley & Trams (1960), who adapted the method of Phares (1951). For the measurement of the specific radioactivity of triglyceride glycerol, the triglycerides were hydrolysed by the method of Hardwick, Linzell & Mepham (1963) and the glycerol was separated as the tribenzoate (Black, Kleiber, Butterworth, Brubacher & Kaneko, 1957).

Milk citrate and lactose were isolated from skim-milk by the method of Lucas, Kaneko, Hirohara & Kleiber (1959) as modified by Hardwick *et al.* (1963). After hydrolysis of lactose (Schambye, Wood & Kleiber, 1957), the individual hexose molecules were separated by the method of Hough, Jones & Wadman (1949). Milk proteins were precipitated by adjusting the pH to 5·5 with 3 N-HCl. Individual amino acids of milk protein were separated, after hydrolysis of the protein (Mepham & Linzell, 1966), by column chromatography using Amberlite IR-120 resin as described previously (Spincer *et al.* 1969). The individual amino acids were collected and the $\alpha$-amino nitrogen content was determined (Henry, 1964).

Plasma glucose was separated as the penta-acetate. One ml plasma was diluted with 4 ml distilled water and 2 ml 10 % (w/v) glucose carrier was added. Plasma proteins were precipitated by the addition of 2 ml 0·3 N-$Ba(OH)_2$ and 2 ml 5 % (w/v) $ZnSO_4$, and the solution was centrifuged. Two drops glacial acetic acid and 2 drops octan-2-ol were added to the supernatant which was evaporated to dryness under reduced pressure. Sixty mg anhydrous sodium acetate and 1 ml acetic anhydride were added to the residue and the mixture was heated at 100 °C for 90 min. The crystals of glucose penta-acetate were recrystallized 3 times from water and dried to constant weight.

Table 2. *The concentration and radioactivity of glucose and triglycerides of the coccygeal and venous blood plasma and the coccygeal-venous differences across the mammary gland in lactating sows*

| | | Plasma concentration, mg/100 ml | | | Plasma radioactivity, $\mu$Ci/100 ml | | |
|---|---|---|---|---|---|---|---|
| Sow | Infused material | Coccygeal | Venous | Coccygeal-venous difference, % | Coccygeal | Venous | Coccygeal-venous difference, % |
| | | | | Glucose | | | |
| 1 | [U-$^{14}$C]Glucose | 100·2 | 70·8 | 29 | 0·15 | 0·11 | 27 |
| 2 | [U-$^{14}$C]Glucose | 96·8 | 69·2 | 29 | 0·19 | 0·12 | 37 |
| 3 | [U-$^{14}$C]Glucose | 94·1 | 64·9 | 31 | 0·44 | 0·28 | 36 |
| | | | | Triglycerides | | | |
| 4 | Triglyceride containing [1-$^{14}$C]palmitic acid | 40·1 | 30·0 | 25 | 0·055 | 0·040 | 27 |
| 5 | Triglyceride containing [1-$^{14}$C]stearic acid | 42·5 | 32·3 | 24 | 0·063 | 0·044 | 30 |

## RESULTS

### *Coccygeal-venous difference measurements*

Coccygeal-venous differences (Spincer *et al.* 1969) in plasma glucose and triglycerides 4 h after the start of each infusion are given in Table 2. In the experiments in which there was an intravenous infusion of [U-$^{14}$C]glucose, the uptake of glucose across the mammary gland was on average 30 % of the coccygeal concentration and the average reduction in total radioactivity was 33 %. Corresponding values for plasma triglycerides in experiments in which there was an intravenous infusion of triglyceride were 25 and 29 %. The close relationship between the uptake of plasma constituents and the reduction in total radioactivity suggests that the infused materials were physiologically acceptable.

### *The contribution of plasma glucose and triglycerides to the synthesis of individual milk constituents*

Towards the end of the 5-h period of infusion of radioactive materials the specific activities of plasma constituents were roughly constant, but steady-state values for the specific activities of milk constituents were not achieved. The calculation of the percentage of a product coming from a precursor as the ratio of the peak specific

Table 3. *The contribution of plasma glucose to the synthesis of lactose, citrate, glyceride glycerol and protein of milk and of individual fatty acids of milk triglycerides in the sow*

(Values are transfer quotients calculated by the method of Kleiber (1954) and expressed as a percentage.)

| | Sow | | |
|---|---|---|---|
| Milk constituent | 1 | 2 | 3 |
| Lactose | 66·6 | 70·3 | 72·0 |
| Citrate | 35·0 | 42·2 | 48·0 |
| Glycerol | 36·1 | 39·3 | 40·4 |
| Protein | 3·0 | 2·8 | 3·8 |
| Laurate | — | — | 5·9 |
| Myristate | — | — | 12·4 |
| Palmitate | — | — | 6·1 |
| Palmitoleate | — | — | 4·0 |
| Stearate | — | — | 1·8 |

activities for product and precursor, the 'product quotient' (Hardwick *et al.* 1963), was not, therefore, possible and the alternative calculation of 'transfer quotient' proposed by Kleiber (1954) was used. In that, a comparison is made of the areas under curves of specific activity against time for the product and precursor respectively. In all sows after the removal of piglets there was a slow decline in milk yield over a period of 22–30 h to about 30 % of the initial level. There was then a sharper decline to a low level and transfer quotients were calculated from measurements made over the first 22 h.

In all experiments, in the milk obtained 22 h after the start of an infusion, the specific radioactivity of lactose was negligible and that of citrate and the triglyceride glycerol of fat was invariably less than 20 % of the peak value. However, the specific

radioactivity of fatty acids of triglycerides and, more particularly, of milk protein did not reach a maximum until about 16 h after the start of an infusion and at 22 h the specific radioactivity was on occasion up to 40 % of the peak value.

[*U*-$^{14}$*C*]*glucose infusions*. There were no significant variations in the concentration of coccygeal plasma glucose in any of the experiments and the transfer of radioactivity to other plasma constituents was negligible. Plasma triglycerides showed the highest level of activity, but in all instances this was less than 1 % of the value for plasma glucose.

Table 4. *The contribution of palmitic or stearic acids of the plasma triglycerides to the synthesis of individual fatty acids of milk triglycerides in the sow*

(Values are transfer quotients calculated by the method of Kleiber (1954) and expressed as a percentage.)

| | | Fatty acid* of milk triglycerides | | | |
|---|---|---|---|---|---|
| Sow | Infused triglyceride | $C_{16:0}$ | $C_{16:1}$ | $C_{18:0}$ | $C_{18:1}$ |
| 4 | [1-$^{14}$C]Palmitic acid | 62 | 51 | 12 | 1 |
| 5 | [1-$^{14}$C]Stearic acid | 1 | 1 | 71 | 42 |

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

Table 5. *Distribution of radioactivity in individual fatty acids of milk triglycerides after the intravenous infusion of triglycerides containing either* [*1*-$^{14}$*C*]*palmitic acid or* [*1*-$^{14}$*C*]*stearic acid into lactating sows*

(Experimental details are described in the Materials and Methods section. Results are given for the total radioactivity in individual fatty acids and for the radioactivity located in the terminal (carboxyl) carbon and are expressed in mnCi.)

| Time after the start of the infusion, h | Infused triglyceride | Fatty acid* | Radioactivity, mnCi: Complete acid | Radioactivity, mnCi: Terminal carbon atom |
|---|---|---|---|---|
| 13 | [1-$^{14}$C]Palmitic acid | $C_{16:0}$ | 1·40 | 1·26 |
| | | $C_{16:1}$ | 1·02 | 0·94 |
| | | $C_{18:0}$ | 0·12 | 0·03 |
| 20 | [1-$^{14}$C]Palmitic acid | $C_{16:0}$ | 1·30 | 1·15 |
| | | $C_{16:1}$ | 1·21 | 1·10 |
| | | $C_{18:0}$ | 0·42 | 0·11 |
| 13 | [1-$^{14}$C]Stearic acid | $C_{18:0}$ | 1·35 | 1·21 |
| | | $C_{18:1}$ | 0·79 | 0·72 |
| 20 | [1-$^{14}$C]Stearic acid | $C_{18:0}$ | 1·98 | 1·79 |
| | | $C_{18:1}$ | 1·20 | 1·09 |

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

The transfer quotients for individual milk constituents are given in Table 3. The largest contribution of plasma glucose was to the synthesis of milk lactose for which the transfer quotient was 67–72 %. Fifty-four per cent of the total radioactivity of lactose was in glucose, and 46 % in the galactose moiety. The contributions to citrate

and triglyceride glycerol were similar, of the order of 40 %. The transfer of radioactivity to milk proteins was low and the small amount of activity was in the non-essential amino acids, especially alanine and glutamate, amino acids closely related metabolically to glucose.

In the first 2 experiments the level of radioactivity in the individual fatty acids of the plasma triglycerides was too low for accurate measurement. In the third experiment, measurements were made on the whole range of fatty acids but radioactivity was detected only in lauric, myristic, palmitic, palmitoleic and stearic acids. The largest contribution was to myristic acid, for which the transfer quotient was 12 %.

*Triglyceride infusions.* There were no significant changes in fatty-acid composition of coccygeal plasma or milk triglycerides or in the concentration of coccygeal plasma triglycerides in either of the experiments. Analysis of individual fatty acids of the plasma triglycerides revealed a small transfer of radioactivity to oleic acid during the infusion of triglyceride containing [1-$^{14}$C]stearic acid. The specific activity of oleic acid rose to a maximum at the end of the infusion period of 10 % of that of the stearic acid. In both experiments small amounts of radioactivity were detected in plasma diglyceride, free fatty acid, cholesterol ester and phospholipid fractions. The highest activity was in the free fatty acids, the value rising to a maximum at the end of the period of infusion of 7 % of the radioactivity of the triglyceride fraction.

The transfer quotients for individual fatty acids of the milk triglycerides are given in Table 4. In these calculations no account was taken of the radioactivity of plasma constituents other than the major infused material. The contribution of palmitic acid of plasma triglycerides was mainly to the palmitic and palmitoleic acids of milk fat, for which the transfer quotients were 62 and 51 % respectively, but there was a more limited contribution (12 %) to stearic acid. Stearic acid of the plasma triglycerides accounted for a high proportion (transfer quotient, 71 %) of the stearic acid of milk fat and a smaller proportion (transfer quotient, 42 %) of the oleic acid. The contribution to other fatty acids was negligible.

Decarboxylation of triglyceride fatty acids of milk obtained after the infusion of triglycerides containing [1-$^{14}$C]palmitic acid indicated that 90 % of the radioactivity in palmitic and palmitoleic acids of milk fat was located in the terminal (carboxyl) carbon whereas for stearic acid the value was only 25 % (Table 5). After the infusion of triglyceride containing [1-$^{14}$C]stearic acid, 90 % of the radioactivity in the stearic and oleic acids of milk fat was located in the terminal carbon.

## DISCUSSION

The loss of milk yield arising from the necessary withdrawal of piglets at the beginning of an experiment restricted the period over which measurements of radioactivity of milk constituents could be made, and the specific activity of the fatty acids of milk triglycerides and of milk protein was still comparatively high at the end of the sampling period. The calculation of transfer quotients is only truly valid when sampling has continued until the specific radioactivities of both the precursor and the product have reached low values. For milk protein this would be of little consequence as the transfer of activity was small, but the transfer of activity to the fatty acids of milk triglycerides was considerable, especially after the intravenous infusion of radioactive triglycerides, and the calculated transfer quotients may be unduly low.

The results of the experiments in which uniformly labelled glucose was infused are in agreement with those of Gütte, Kleiber, Raggi & Black (1961) and Linzell *et al.* (1969) and suggest that glucose is the main source of milk lactose. The reported mean contribution of 70 % is close to that of 77 % established for the goat by Hardwick *et al.* (1963). Some dilution within the gland of the radioactive carbon of the infused glucose with the carbon of unlabelled glucogenic materials would be expected.

Glucose also made a major contribution to the synthesis of milk citrate and triglyceride glycerol. The contribution to citrate in the sow (mean value, 42 %) was similar to that reported by Hardwick *et al.* (1963) for the goat but the contribution to triglyceride glycerol (mean value, 38 %) was about twice that in the goat. This difference is consistent with the smaller uptake of plasma triglycerides in the sow than in the goat demonstrated by arteriovenous-difference studies (Linzell *et al.* 1969; Spincer *et al.* 1969) as the glycerol of plasma triglycerides is the other important source of the glycerol of milk triglycerides (West, Annison & Linzell, 1967).

The transfer of radioactivity from glucose to fatty acids of milk triglycerides indicated only a limited synthesis *de novo* of fatty acids, as did the earlier work of Gütte *et al.* (1961), in contrast to the considerable synthesis suggested by arteriovenous-difference studies (Spincer *et al.* 1969). Linzell *et al.* (1969) have concluded from experiments in which isolated mammary units were perfused with [U-$^{14}$C]-glucose that in mammary tissue of the sow, as in that of the goat (Hardwick *et al.* 1963), there is a large pool of lipid with a slow turnover rate. Measurements based on a short period of infusion of labelled glucose may, therefore, underestimate the contribution of glucose to fatty acid synthesis. However, the present results and those of Linzell *et al.* (1969) show that there is no similar delay in the transfer of activity from plasma glucose to the glycerol of milk triglycerides, and the synthesis *de novo* of fatty acids from glucose may not be closely linked with their subsequent incorporation into milk triglycerides.

The 2 experiments in the present investigation in which labelled palmitic or stearic acids were infused in mixed triglycerides identified the plasma triglycerides as major precursors of the longer chain fatty acids of milk fat. More than 60 % of palmitic acid and 70 % of stearic acid of milk fat were accounted for by uptake from plasma triglycerides. There was also an extensive transfer of activity from palmitic and stearic acids of the plasma triglycerides to the corresponding monounsaturated fatty acids of milk fat, presumably through a desaturation of the acids in the mammary tissue (Bickerstaffe & Annison, 1968). The contribution of palmitic acid of plasma triglycerides to the palmitic and palmitoleic acids of milk fat was similar, but the contribution of stearic acid of plasma triglycerides to oleic acid of milk fat was about half of that to stearic acid, which is consistent with the large arteriovenous-difference in oleic acid (Spincer *et al.* 1969). These observations suggest that in the sow the desaturation mechanism is as active towards palmitic acid as stearic acid, a feature that would account for the characteristically high levels of palmitoleic acid in the milk fat of the sow.

The slight transfer of radioactivity to the stearic acid of milk fat after the infusion of radioactive glucose or of triglycerides labelled with radioactive palmitic acid, and the distribution of radioactivity in the stearic acid, suggest that there was a small amount of elongation within the gland (see also Linzell *et al.* 1969).

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# Changes on storage in milk processed by ultra-high-temperature sterilization

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Summary. Samples of commercially processed ultra-high-temperature (UHT) milk were stored at 4, 20, 30 and 37 °C for up to 2 years or until gelation occurred.

The stability of the proteins to ethanol, calcium ions and rennet decreased with time of storage. However, preliminary autoclaving of the UHT milk induced a high degree of stability.

The extent of protein decomposition that had occurred in the UHT milk samples depended on the time and temperature of storage. There was negligible decomposition when the samples had been autoclaved before storage at 4 and 20 °C. Storage at 37 °C led to significant decomposition.

UHT milk samples gelled after being stored for 13 months at 4, 20 and 30 °C, but not at 37 °C. The autoclaved milk was still fluid after being stored for 2 years at these temperatures.

The time of onset of gelation did not depend on the degree of protein breakdown, and it seemed therefore that proteolysis was not the primary cause of gelation on storage.

It was concluded that the proteins in UHT milk underwent several changes on storage that were apparently independent of each other and led ultimately to coagulation of the milk. These changes included proteolysis, and a progressive loss of stability under conditions that favoured aggregation of the casein micelles.

Milk that has been sterilized by conventional autoclaving in the bottle remains unchanged in appearance during storage for long periods of time. On the other hand, milk sterilized by the use of ultra-high-temperature (UHT) processes often forms a gel after only a few months of storage, the time before gelation occurs being inversely related to the severity of the heat treatment applied (Alfa-Laval, 1967).

The problem of gelation of UHT milk has been investigated by Hostettler, Stein & Bruderer (1957), who observed that all samples of UHT milk examined showed residual phosphatase activity and that those which had gelled had undergone some casein decomposition. These observations led them to propose that protein decomposition and coagulation might be caused by proteolytic enzymes which had regained activity after being temporarily inactivated by the UHT process. Samuelsson & Holm (1966) observed that there was an inverse relationship between the degree of protein decomposition and the time of onset of gelation, and attributed gelation to reactivation of proteolytic enzymes.

Against this, Nakai, Wilson & Herreid (1964) found no evidence for the presence of proteolytic enzymes in UHT-sterilized concentrated milk, although the samples gelled after only a few weeks of storage.

Investigations in our laboratory over the past 2 years have demonstrated that changes occur on storage of commercially produced UHT-sterilized milk which affect its stability to heat and to low pH values, and precede the onset of gelation. The casein micelles in milk remained in colloidal suspension after heat-sterilization but showed a tendency, which increased with age, to aggregate to form a coagulum on exposure to heat and low pH values. The increase in the tendency to coagulation might possibly be the result of changes in the protein molecules themselves or of changes in their environment (e.g. in the calcium ion concentration). Such changes are known to affect the colloidal stability of casein micelles (Jenness & Patton, 1959).

The present report is concerned with changes in the properties of the proteins in UHT-sterilized milk during storage which precede or occur simultaneously with visible manifestations of protein instability. Interest has been centred particularly on changes in the structure of casein micelles which result in decomposition and the release of peptides and glycopeptides. Such changes resemble those that occur during the action of rennet on milk, where hydrolysis of the $\kappa$-casein molecules precedes coagulation.

## EXPERIMENTAL

### *Materials*

Samples of UHT-sterilized milk were obtained from a commercial plant. The milk was heated by an indirect heating method, the milk temperature being raised to 138 °C and held at that temperature for 2–5 s. Some samples of UHT milk were further heat-treated by autoclaving to give 'autoclaved UHT milk'. Autoclaving was carried out by transferring samples of UHT milk into cans and heating the cans in a rotary batch sterilizer for 15 min at 116 °C.

The milks were stored at 4, 20, 30 and 37 °C for up to 2 years or until gelation occurred and were examined at intervals of 2–3 months.

### *Methods*

*Alcohol test.* One ml portions of aqueous ethanol (96, 83, 72 and 62 %, v/v) were added to a series of test tubes, each containing 1 ml milk.

The tubes were examined for evidence of coagulation both immediately after mixing the contents and after standing for 5 min. The minimum concentration of alcohol which produced coagulation after 5 min was noted.

*Calcium sensitivity.* Volumes of 0·1 M-$CaCl_2$ solution ranging from 0 to 4·0 ml were added to 5 ml portions of milk in 15 ml graduated centrifuge tubes. The final volume was made up to 9 ml with distilled water. The tubes were then immersed for 15 min in a water bath at 50 °C, cooled and centrifuged at 900 $g$ for 10 min. After removal of the supernatant the sediment was washed with 9 ml distilled water, re-centrifuged, and its volume noted. A sediment volume of 0·5 ml or more was regarded as a positive indication of precipitation.

*Coagulation by rennet.* Three ml of a solution of commercial rennet (Standard calf rennet, Chrs. Hansen's Laboratory Ltd, London) were diluted to 100 ml with distilled

water. Of this solution 1 ml was added to 50 ml of milk at 37 °C in a conical flask and the mixture was incubated at 37 °C. The film of milk produced on the walls of the flask by swirling was examined at intervals. The time when the film showed particles of precipitate was noted. This was taken as the rennet coagulation time.

*Distribution of nitrogen-containing fractions.* Total N, non-casein N and non-protein N were determined as described by Rowland (1938*a*). Protein was estimated by multiplying N by 6·38.

*Glycopeptide.* Glycopeptide was estimated by determining the *N*-acetyl-neuraminic acid (sialic acid) content of samples of milk from which proteins had been removed by precipitation with trichloracetic acid (TCA), the filtrate being subsequently dialysed.

The protein precipitation was carried out by adding TCA to give a final concentration of 12 % (w/v) in the milk sample. The filtrate obtained was dialysed for 12 h against running tap water using 1 cm Visking dialysis tubing, which had been immersed in water at 95 °C for 5 min to reduce its porosity. A sample of the dialysed solution was heated in 0·1 N-$H_2SO_4$ for 1 h at 80 °C to release bound sialic acid. The free sialic acid was then estimated by a thiobarbituric acid method as described by Aminoff (1961).

## RESULTS

Gelation occurred in all samples of UHT milk examined after about 13 months storage at temperatures of 4, 20 and 30 °C. No gelation took place when they had been stored at 37 °C for 13 months and longer.

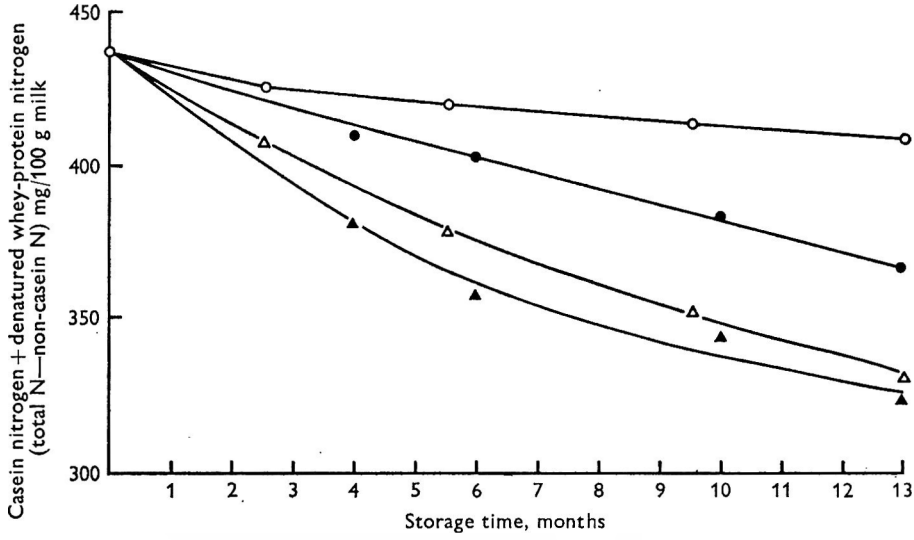


Fig. 1. Storage of UHT milk. Changes in nitrogen-containing constituents at different temperatures. ○, 4 °C; ●, 20 °C; △, 30 °C; ▲, 37 °C.

The depletion of protein in the bulk of the milk resulting from sediment formation and from separation of some protein into the cream layer was determined in a sample of bottled UHT milk that had been stored for 13 months at 37 °C. The casein N content of the middle portion of this milk was compared before and after the sediment and

cream layer had been re-incorporated into the bulk of the milk by mixing in a household liquidizer. The casein N content of the mixed milk was 390 mg/100 g milk and that of the middle portion of the unmixed milk was 370 mg/100 g. Thus, the casein N of the middle portion had decreased during storage by 5 %. This decrease was considered unlikely to account for the failure of the milk to gel, since a sample of UHT-processed skim-milk in which there was a 9 % reduction in protein due to sedimentation was shown to gel on storage at 20 °C after 13 months.

Table 1. *Changes in the stability to ethanol during storage at 20 °C*

| Storage time, months | Minimum concentration of ethanol in water to produce coagulation, % | |
|---|---|---|
| | UHT milk | Autoclaved UHT milk |
| 0 | 96 | > 96 |
| 2 | 96 | > 96 |
| 5 | 83 | > 96 |
| 9 | 62 | > 96 |
| 13 | Gelled | > 96 |

Table 2. *Changes in calcium sensitivity during storage at 4 and 20 °C*

| Storage time, months | ml 0·1 M-$CaCl_2$ required to give positive test (see text) | | | |
|---|---|---|---|---|
| | UHT milk | | Autoclaved UHT milk | |
| | 4 °C | 20 °C | 4 °C | 20 °C |
| 0 | 2·8 | 2·8 | 2·1 | 2·1 |
| 2 | 1·6 | 1·4 | 1·5 | 1·8 |
| 5 | 1·2 | 0·8 | 1·0 | 2·0 |
| 10 | 0·6 | 0·2 | 1·0 | 1·8 |
| 13 | Gelled | Gelled | 0·6 | 2·0 |

Calcium sensitivity of raw milk > 4·0 ml M-$CaCl_2$.

It was decided therefore to examine the relative rates of decomposition of the proteins in UHT milk after storage at 30 and 37 °C. The rates observed at 30 and 37 °C were very similar (Fig. 1) so that the extent of decomposition as judged by the decrease in the concentration of casein plus denatured whey protein (total N – non-casein N) was similar in milks stored for 48 weeks at 37 °C and for 56 weeks at 30 °C. However, samples stored at 30 °C gelled whereas those stored at 37 °C did not.

There was no detectable gelation in autoclaved UHT milk which had been stored at 4, 20 or 37 °C for up to 13 months.

*Stability to ethanol.* The stability to ethanol of the proteins in UHT milk decreased with time, slowly at first and more rapidly later (Table 1). Autoclaved UHT milk, on the other hand, retained its original stability throughout the storage period.

*Calcium sensitivity.* On storage of UHT milk at 4 and 20 °C the stability of casein to precipitation by added calcium decreased progressively until it reached a very low value shortly before gelation (Table 2). Autoclaved UHT milk stored at 20 °C showed no change in its calcium sensitivity over the test period of 13 months and showed no signs of gelation at 24 months. When autoclaved UHT milk was stored at 4 °C,

its calcium sensitivity increased progressively with the time of storage but at a lower rate than that of UHT milk which had not been autoclaved.

*Coagulation by rennet.* Raw milk coagulated in about 7 min. After UHT treatment the rennet coagulation time increased to about 50 min, but during storage at 20 °C the rennet coagulation time progressively decreased. Thus, after storage for 6 months, UHT milk coagulated as quickly as milk that had not been heat-treated (Table 3). There were, however, differences in the appearance of the coagulum – that from raw milk was a firm gel whereas stored UHT milk gave a soft coagulum. On storage at 37 °C rennet coagulation time increased.

Table 3. *Changes in renneting time during storage at 20 and 37 °C*

| Storage time, months | Coagulation time, min: UHT milk, 20 °C | UHT milk, 37 °C | Autoclaved UHT milk, 20 °C |
|---|---|---|---|
| 0 | 50 | 50 | > 120 |
| 2 | 50 | 120 | — |
| 4 | 12 | > 120 | — |
| 6 | 4 | > 120 | — |
| 11 | 4 | — | > 120 |
| 13 | Gelled | > 120 | > 120 |

Coagulation time of raw milk: 7 min.

Table 4. *The effects of ultra-high-temperature (UHT) processing and of autoclaving of milk on the distribution of N among protein and peptide fractions and on the amount of non-dialysable sialic acid soluble in 12% trichloracetic acid (TCA)*

| Heat treatment | None | UHT | Autoclaving |
|---|---|---|---|
| Total N, mg/100 g | 522 | 522 | 522 |
| Non-casein N, mg/100 g | 122 | 65 | 62 |
| Non-protein N, mg/100 g | 29 | 34 | 34 |
| Casein N, mg/100 g | 400 | 457 | 460 |
| Sialic acid soluble in 12% TCA, mg/100 g | 1·1 | 1·5 | 1·2 |
| Whey protein N, mg/100 g | 93 | 31 | 28 |
| Denatured whey-protein N, mg/100 g | — | 62 | 65 |
| Denatured whey-protein N, % of original value | — | 66·5 | 70 |

Autoclaving of UHT milk increased the coagulation time even further, so that no coagulum was observable 2 h after renneting. The rennet coagulation time observed for autoclaved UHT milk did not decrease with increasing time of storage at 20 °C.

*Effect of UHT treatment and autoclaving on the distribution of nitrogen between different fractions and on the release of sialic acid*

Casein N was calculated from the difference between total N and non-casein N (Table 4). The whey protein N fraction was calculated as the difference between non-casein N and non-protein N. The fraction consisted of the milk serum proteins $\beta$-lactoglobulin, $\alpha$-lactalbumin, serum albumin, the various immune globulins and the proteose-peptone fraction.

The non-dialysable, 12% TCA-soluble fraction obtained from unheated milk samples contained about 7% of the total sialic acid measured in those samples. The sialic acid in this fraction is probably present mainly in glycopeptides and in oligosaccharides which are minor constituents of milk (Barker & Stacey, 1963).

After UHT treatment there was an increase in the fraction of the total N measured as casein N and a decrease in whey protein N. These changes were attributable to the precipitation at pH 4·6 of both casein and whey proteins, the latter having been denatured as the result of heating and also forming complexes with casein. The increase in the N content measured as 'casein N' was not precisely equal to the reduction in that measured as 'whey-protein N' since heating caused decomposition of proteins leading to an overall increase in the amount of N-containing compounds soluble in 12% TCA.

The particular UHT treatment used denatured a surprisingly high proportion of the whey proteins and the subsequent autoclaving had only a small additional effect. Taking the content of the heat-stable proteose-peptone fraction as 25% of the serum proteins (Rowland, 1938*b*) the proportions of native whey proteins remaining in UHT milk and in autoclaved UHT milk were calculated as 8 and 5% respectively.

UHT treatment resulted in a slight increase in the sialic acid content of the non-dialysable, 12% TCA-soluble fraction. This suggested that some protein breakdown had occurred. Unexpectedly, autoclaving reduced the amount of this fraction. This reduction may be attributed to subsequent decomposition of some of the glycopeptides formed during UHT treatment due to autoclaving. This would result in the liberation of free and therefore dialysable sialic acid, which would not be measured by the method of analysis used, being specifically a measure of the bound sialic acid content.

*Effect of storage of UHT milk on the distribution of nitrogen between fractions, and on the release of sialic acid*

Some of the proteins in UHT milk have been found to break down into smaller molecules when the milk was stored at various temperatures. Small peptides were formed which contribute to the non-protein N fraction (Fig. 2). There was a linear increase in the non-protein N fraction with time when samples were stored at all 4 temperatures during the test period of 13 months.

On storage there was a decrease in the casein N fraction, consisting of casein together with whey proteins changed by heating, and an increase in the fraction soluble at pH 4·6 but insoluble in 12% TCA, and thought to consist of native whey proteins and proteose-peptone (Figs 1, 3). The increase in the whey protein concentration was thought to be due to products of casein breakdown. It was considered unlikely that there was an increase in the whey protein content due to the restoration of denatured whey proteins to the native state, making them soluble at pH 4·6.

On storage of UHT milk there was also an increase in the proportion of sialic acid present in the non-dialysable, 12% TCA-soluble fraction (Fig. 4). This was attributed to the release from casein of glycopeptides containing sialic acid. It was considered unlikely that these glycopeptides were derived from the proteose-peptone fraction (Marier, Tessier & Rose, 1963) because measurements of the distribution of sialic acid between fractions from freshly prepared and from stored UHT milk indicated that

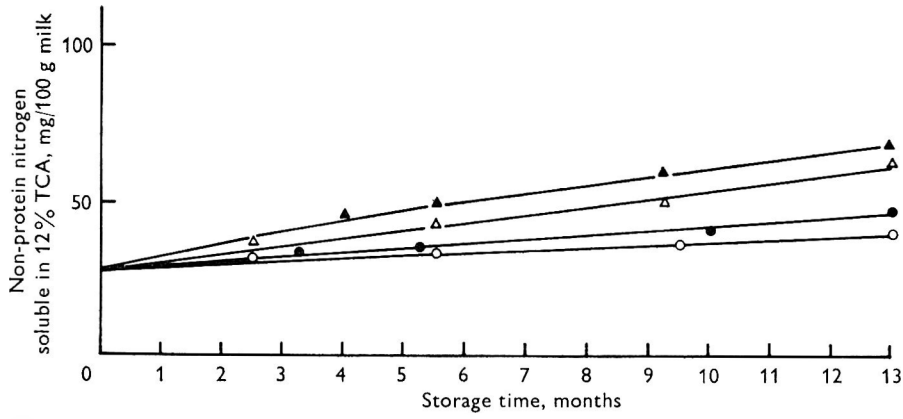


Fig. 2. Storage of UHT milk. Changes in nitrogen-containing constituents at different temperatures. ○, 4 °C; ●, 20 °C; △, 30 °C; ▲, 37 °C.

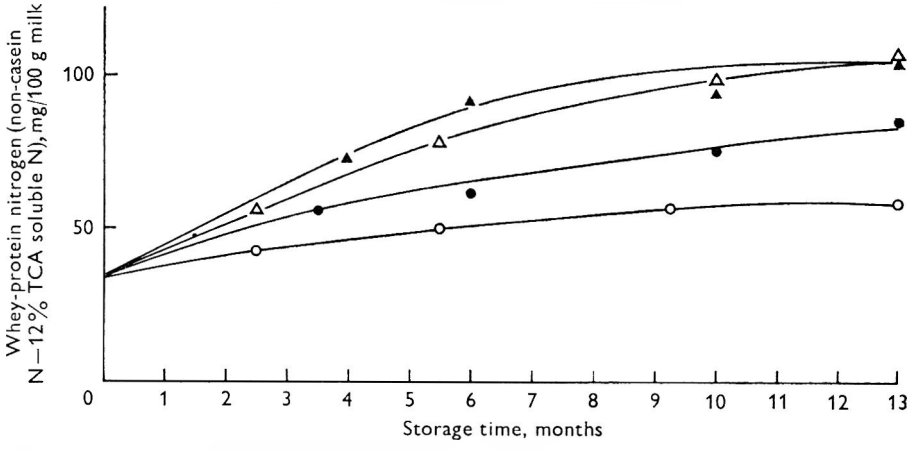


Fig. 3. Storage of UHT milk. Changes in nitrogen-containing constituents at different temperatures. ○, 4 °C; ●, 20 °C; △, 30 °C; ▲, 37 °C.

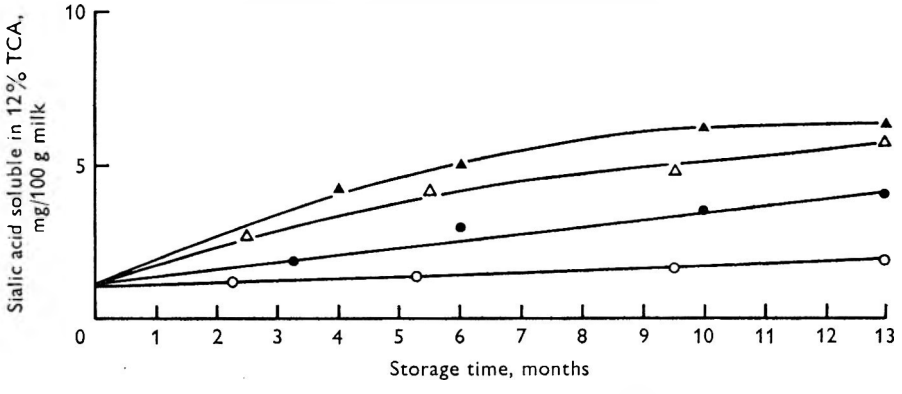


Fig. 4. Storage of UHT milk. Changes in nitrogen-containing constituents at different temperatures. ○, 4 °C; ●, 20 °C; △, 30 °C; ▲, 37 °C.

most of the glycopeptides were derived from the casein fraction rather than from the whey protein fraction (Table 5).

For present purposes it was assumed that the sialic acid attached to non-dialysable peptide material, soluble in 12 % TCA, was released by decomposition of $\kappa$-casein and that the linear increase in the sialic acid content of this fraction with length of storage at 4 and 20 °C (Fig. 4), represented a progressive breakdown of $\kappa$-casein. When UHT milk was stored at 30 and 37 °C the amount of sialic acid in the undialysable portion of the 12 % TCA filtrate appeared to reach a limiting value after about 10 months. After this period at 37 °C the increase in non-dialysable sialic acid was 5 mg/100 g milk, representing about one-third of the observed 14·4 mg/100 g total sialic acid content of the casein fraction.

Table 5. *Sialic acid distribution (mg/100 g) between protein fractions in freshly prepared and stored ultra-high-temperature (UHT) milks*

| | Freshly prepared UHT milk | UHT milk stored for 7 months at 37 °C |
|---|---|---|
| Total sialic acid | 18·2 | 18·2 |
| Whey protein fraction | 2·6 | 2·4 |
| Non-protein N fraction | 1·2 | 5·4 |
| Casein fraction by difference | 14·4 | 10·4 |

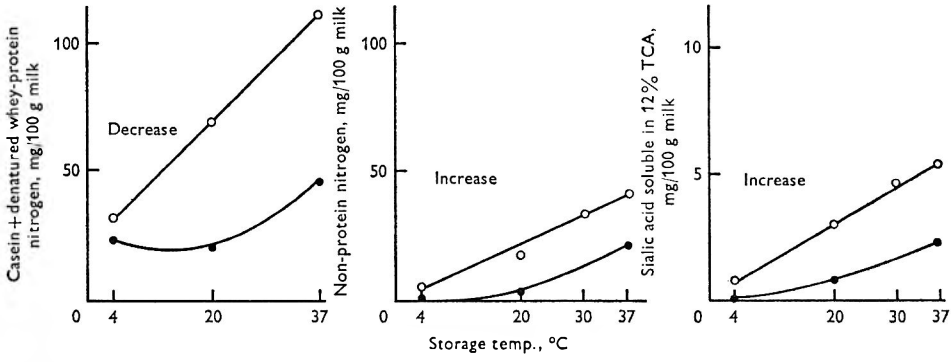


Fig. 5. Changes in nitrogen distribution in UHT milk and autoclaved UHT milk after storage for 13 months at different temperatures (expressed as differences between original distribution and distribution after 13 months' storage). ○, UHT; ●, autoclaved.

*Differences brought about by autoclaving UHT milk*

Fig. 5 shows the marked effect that autoclaving UHT milk had on the changes in nitrogen distribution that took place on storage.

The increase in the non-protein N content and the decrease in the casein + denatured whey protein N content of UHT milk were apparently linear with increasing temperature of storage for the storage period of 13 months. With milk that had been autoclaved before storage there was negligible change in the non-protein N content and casein + whey protein N content after storage for 13 months at 4 or 20 °C. Storage at 37 °C resulted in some protein decomposition.

## DISCUSSION

Gelation in UHT milk followed a gradual decrease with length of time in the stability of the casein micelles to ethanol, calcium ions and rennet. Gelation was probably a further stage in a slow process as a result of which the system became more sensitive to agents that induce protein–protein interactions.

Decomposition of the protein molecules during storage may provide an explanation for the observed changes in the stability of UHT milk. Changes in the distribution of nitrogen between the various fractions of UHT milk during storage (Figs 1–5) suggested that protein decomposition preceded gelation and might therefore be a cause of gelation. Thus, the smaller extent of protein decomposition in autoclaved UHT milk appeared to correlate with a greater resistance to gelation (Fig. 5). However, if gelation were the result of protein decomposition alone then it would be expected to occur when a critical degree of decomposition had occurred. This seems to be true for rennet coagulation, in which the hydrolysis of $\kappa$-casein precedes coagulation (Wake, 1959), but it is not true for the coagulation of UHT milk on storage.

The amount of sialic acid found in the fraction soluble in 12 % TCA, probably from glycopeptides, following storage of UHT milk for 13 months at 4° C, was similar to that found after storage for $3\frac{1}{2}$ months at 20 °C and for $1\frac{1}{2}$ months at 30 °C (Fig. 4). However, gelation occurred after approximately the same period of storage at all 3 temperatures, namely 13 months.

These results clearly indicated that there was little or no correlation between the extent of protein decomposition on storage and the tendency of the milk to gel, which appeared to be almost independent of temperature. Moreover, the observation that protein decomposition still occurred on storage of UHT milk at 37 °C, even after it had been autoclaved (Fig. 5), argued against an involvement of proteolytic enzymes in the decomposition process. Thus, the search for surviving or reactivated protease molecules may be irrelevant to the problem of gelation.

The basis of the long-term stability conferred on milk proteins by the autoclaving process, which involves high temperatures and long times of heating, is clearly of considerable theoretical and practical interest. It is highly significant that storage of UHT milk at 37 °C prevented gelation, an observation already made and discussed by Hostettler *et al.* (1957). Both autoclaving and storage at 37 °C are associated with considerable browning of the milk. Browning of milk results from the reaction of lactose with the lysine residues of proteins. In recent experiments in which the lysine and arginine residues of $\kappa$-casein molecules were blocked there was a resultant loss of sensitivity to rennet coagulation (Hill & Craker, 1968; Hill, 1970). Thus, it should be expected that browning would lead to an inhibition of rennet coagulation. This was in fact observed in autoclaved UHT milk and in UHT milk stored at 37 °C (Table 3).

Hill (1970) concluded that in addition to the previously recognized mechanism of rennet coagulation involving calcium bridging and hydrophobic bonding there may also be an interaction between the separate casein molecules through oppositely charged regions of suitable configuration.

These regions on the molecules owe their configuration and charge to specific groups. Lysine residues may be among those which contribute to the special

configuration of the regions on the micelles which interact (Hill, 1970). These arguments ascribing a significant role to lysine residues in the rennet coagulation of milk may apply also to the storage coagulation of sterile milk.

Therefore, it may be postulated that coagulation occurs partly because slow conformational changes in the molecules on the surface of casein micelles expose regions which subsequently interact to form casein aggregates.

The casein–lactose combination involving lysine residues which precedes browning in UHT milk stored at temperatures above 30 °C and during autoclaving may either prevent these changes or block existing regions that could otherwise take part in protein–protein interaction.

Further studies of the mechanisms involved in the interaction of casein micelles are needed to test this hypothesis.

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# The concentrations of some water-soluble constituents in the milks of cows, sows, ewes and goats

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Summary. The concentrations of lactose, potassium, sodium, chloride and citric acid were determined in milks of cows, sows, ewes and goats. For cows, sows and ewes trends with lactation are reported and for all species correlations between constituents in milks secreted in mid-lactation are given. The results are discussed in relation to the secretion of water-soluble constituents in milk.

Numerous studies have been made of the variations in concentration of the water-soluble constituents in milks from the Friesian cow (see review by Rook & Wheelock, 1967) but little information is available for other breeds or for other species. In the work reported below the concentrations of water-soluble constituents were determined in milks from cows of the Jersey breed and from ewes, sows and goats. To minimize effects on composition due to mammary infection or damage, samples were taken mainly from animals in their first lactation and samples of milk were obtained from individual units of the gland so that abnormal milks could be identified and excluded.

## EXPERIMENTAL

### *Animals*

Milk samples were obtained from 10 Jersey cows, 5 Masham ewes, 7 Large White × Wessex sows and 3 Saanen and 3 British Toggenburg goats. All the cows, ewes and sows and 3 of the goats were in their first lactation. The remaining goats were in their second lactation. Two of the cows, 3 of the sows and 4 of the ewes were sampled at intervals throughout a complete lactation and other animals were sampled over a period of 3–4 weeks in the middle of lactation. Cows were kept in lactation for about 11 months but ewes ceased to lactate when the lambs were weaned at 13–14 weeks of age and sows after about 8 weeks, following the enforced weaning of piglets. In no instance was there any clinical evidence of mammary infection.

### *Collection of samples*

Cows were milked at a morning and evening milking using a machine designed for the collection of milk from separate quarters of the udder and samples of milk were taken from each quarter for analysis. Goats were treated similarly but were milked by

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hand. Ewes and sows were hand-milked once on the day of sampling after an injection of 1 i.u. oxytocin (Syntocinon) into the jugular vein of the ewe and the ear vein of the sow. Samples of milk were taken from each half of the udder of the ewe and from each of 2 left and each of 2 right anterior teats of the sow.

After collection milks were stored at −20 °C until required for analysis. For the cow, ewe, sow and goat, samples were excluded if the standard error of the mean value for sodium in milks from separate gland units was greater than 10 % of the mean.

*Methods of analysis*

Lactose was determined by a modification of the chloramine-T method of Hinton & Macara (1927). Sodium and potassium were determined on protein-free filtrates of milk using a Unicam S.P. 90 flame-photometer. Chloride was estimated by an adaptation of the Volhard method (Davies, 1932) and citric acid by the method of Marier & Boulet (1958), as modified by White & Davies (1963). Total solids were determined gravimetrically (British Standards Institution, 1951).

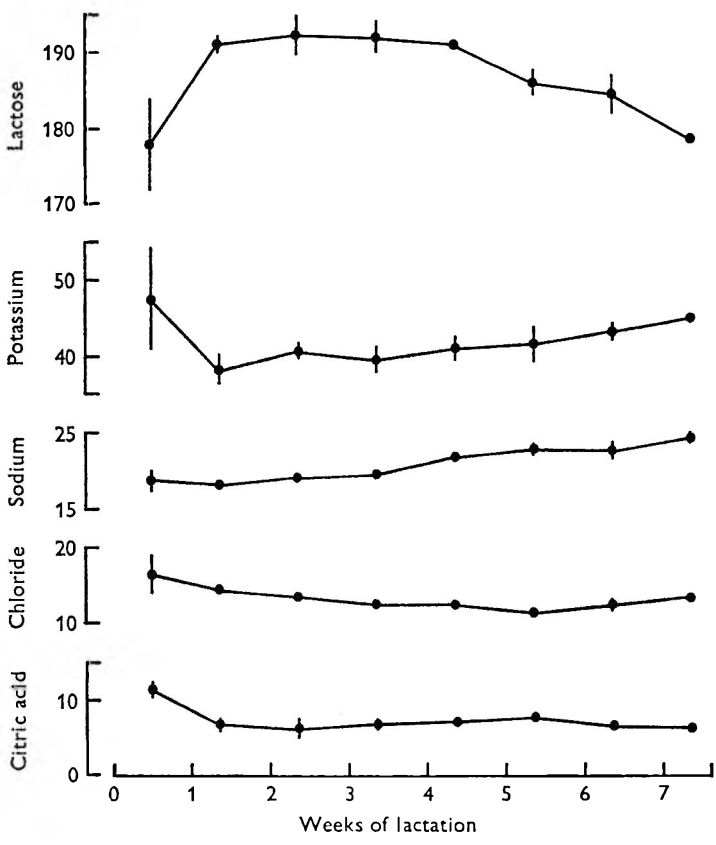


Fig. 1. Variations throughout the first lactation in the concentrations (mmoles/kg of milk water) of lactose, potassium, sodium, chloride and citric acid in ewe's milk. (Values are means with standard errors, for 4 animals.)

RESULTS

*Variations in the composition of the milk associated with stage of lactation*

*Cow*

The trends with lactation were similar to those reported by Rook & Campling (1965). Lactose content increased during the first 2 weeks, the level was maintained until about 15 weeks *post partum* and then decreased until the end of lactation. The trends in potassium were similar, but less pronounced, whereas sodium and chloride contents declined from the beginning of lactation to a minimum at about 8 weeks and then increased to the end of lactation. Citric acid content increased during the first week and then fell slowly throughout lactation.

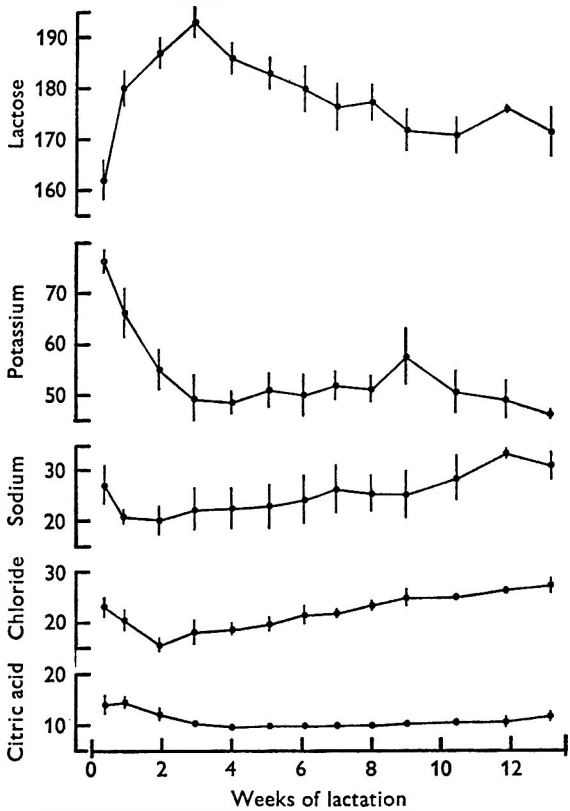


Fig. 2. Variations throughout the first lactation in the concentrations (mmoles/kg of milk water) of lactose, potassium, sodium, chloride and citric acid in sow's milk. (Values are means with standard errors, for 3 animals.)

*Ewe* (*Fig. 1*)

The trends in lactose content were much more pronounced in the ewe than in the cow, values rising sharply to a distinct peak at about the third week of lactation. There was a complementary fall in potassium content over the same period but then there was little change throughout the rest of lactation. Trends in sodium and chloride contents were similar to those in the cow, whereas citric acid content decreased over the first 4 weeks and then increased slowly to the end of lactation.

*Sow* (*Fig. 2*)

Lactose content increased sharply throughout the first week of lactation, remained steady until the fourth week but then fell to the end of lactation. There were opposite trends in potassium content, with a pronounced minimum at the end of the first week. Sodium content increased throughout lactation, whereas chloride content decreased, except over the last 2 weeks. The concentration of citric acid fell during the first week but thereafter showed no clear trend.

Table 1. *Mean values, with standard error, for the concentrations (mmoles/kg of milk water) of lactose, potassium, sodium, chloride and citric acid in the milks of Jersey cows, sows, ewes and goats in mid-lactation*

(Values were calculated for weeks 19–22 of lactation for cows and goats, weeks 3–5 for sows, and weeks 5–7 for ewes.)

| Species | Animal | No. of samples on which values are based | Lactose | Potassium | Sodium | Chloride | Citric Acid |
|---|---|---|---|---|---|---|---|
| Cow | K 60 | 5 | 161·5±0·9 | 63·7±0·3 | 15·0±3·6 | 22·0±0·5 | 11·0±0·2 |
| | K 63 | 4 | 159·0±0·8 | 61·9±3·4 | 17·0±0·9 | 22·2±0·8 | 12·6±0·4 |
| | L 2 | 6 | 162·9±3·8 | 63·3±0·2 | 17·4±0·9 | 22·4±0·7 | 12·3±0·3 |
| | L 9 | 6 | 157·5±0·5 | 61·1±0·7 | 19·3±0·4 | 28·8±0·4 | 9·7±0·7 |
| | L 10 | 4 | 167·0±0·7 | 58·8±0·8 | 15·5±4·3 | 21·0±0·7 | 12·0±0·7 |
| | L 11 | 5 | 158·1±0·6 | 64·2±0·6 | 16·7±3·8 | 25·4±0·7 | 10·1±0·3 |
| | L 20 | 5 | 163·4±0·8 | 61·3±0·9 | 17·4±0·8 | 24·2±0·8 | 11·5±0·2 |
| | L 29 | 6 | 156·3±1·3 | 58·9±0·9 | 22·9±0·7 | 24·6±0·7 | 12·6±0·4 |
| | L 37 | 6 | 164·2±2·5 | 56·6±0·6 | 19·2±0·7 | 20·4±0·2 | 12·3±0·5 |
| | L 62 | 5 | 155·3±4·0 | 58·8±1·0 | 18·0±4·2 | 35·9±0·7 | 9·7±0·6 |
| Mean with S.E. | | | 160·5±1·2 | 60·9±0·8 | 17·8±0·7 | 24·7±1·5 | 11·4±0·4 |
| Sow | 876 | 1 | 201·0 | 37·1 | 17·7 | — | 4·0 |
| | 878 | 1 | 191·5 | 41·5 | 18·2 | — | 6·0 |
| | 1022 | 3 | 188·6±0·8 | 39·4±0·3 | 20·5±0·8 | 13·3±0·5 | 5·6±3·5 |
| | 1229 | 4 | 190·7±1·7 | 43·6±3·2 | 19·9±0·6 | 12·4±0·2 | 7·8±0·2 |
| | 1230 | 3 | 191·9±1·9 | 39·5±4·7 | 22·3±0·8 | 12·1±0·9 | 6·7±0·2 |
| | 1231 | 2 | 184·9±5·1 | 36·6±0·4 | 19·2±0·7 | 13·9±5·3 | 9·0±0·1 |
| | 8839 | 1 | 193·4 | 43·0 | 18·8 | — | 5·0 |
| Mean with S.E. | | | 191·7±1·9 | 40·1±1·0 | 19·5±0·6 | 12·9±0·4 | 6·3±0·6 |
| Ewe | 6 | 3 | 188·0±5·7 | 54·0±5·9 | 18·7±4·9 | 16·9±5·5 | 16·1±0·4 |
| | 9 | 2 | 178·1±3·8 | 52·6±1·0 | 18·3±0·3 | 19·7±5·7 | 7·8±0·5 |
| | 11 | 3 | 191·5±3·6 | 43·0±2·9 | 34·8±0·7 | 22·4±4·8 | 9·9±0·3 |
| | 12 | 3 | 187·1±2·6 | 48·5±5·2 | 20·3±3·8 | 19·9±3·4 | 9·4±0·2 |
| | 14 | 3 | 183·6±7·5 | 52·9±3·9 | 16·1±4·1 | 16·2±0·7 | 10·3±1·0 |
| Mean with S.E. | | | 185·7±2·3 | 50·2±2·0 | 21·6±3·4 | 19·0±1·1 | 10·7±1·4 |
| Goat | Daphne | 16 | 152·4±0·9 | 75·1±0·7 | 19·8±0·2 | 45·7±0·5 | 2·8±0·1 |
| | Gail | 2 | 147·9±0·6 | 58·1±3·8 | 25·0±0·4 | 46·6±0·4 | 3·5±0·0 |
| | Gill | 5 | 135·7±2·0 | 80·2±0·5 | 25·2±0·4 | 36·6±0·5 | — |
| | Moorchick | 4 | 137·3±3·4 | 82·9±3·1 | 23·6±0·1 | 45·7±0·5 | — |
| | Nanette | 2 | 148·8±0·9 | 67·7±0·7 | 14·2±0·0 | 43·1±0·2 | 5·7±0·1 |
| | Pamela | 2 | 145·7±3·9 | 62·5±2·5 | 14·0±0·0 | 48·4±7·0 | 4·9±0·4 |
| Mean with S.E. | | | 144·6±2·7 | 71·0±4·1 | 20·3±2·1 | 44·3±1·7 | 4·2±0·7 |

*Between-species differences in mid-lactation values for the concentrations of water-soluble constituents of milk*

Mid-lactation values were calculated from the values for samples taken in weeks 19–22 inclusive for cows and goats, weeks 5–7 for ewes and weeks 3–5 for sows (Table 1). Within any one species between-animal variations in composition were small, but there were larger and in some instances significant differences between species. Lactose content was highest in the sow and the ewe, with mean values of 191·7 mmoles/kg and 185·7 mmoles/kg respectively. Values for the goat (mean value, 144·6 mmoles/kg) were slightly lower than those for the cow. Values for potassium content were in the reverse order. Between-species differences in sodium content were small but there was a 3-fold variation in chloride content, with the highest values in the goat and the lowest values in the sow. Citrate contents were highest in the cow, with the exception of a very high value for ewe 6, and lowest in the sow and the goat.

*The relationship between water-soluble constituents in milks obtained in mid-lactation*

The correlations between lactose, potassium, sodium and chloride in milks obtained in mid-lactation for cows, sows, ewes and goats and for the pooled values for all species are shown in Table 2. In individual species, for most constituents correlation was poor and did not have statistical significance ($P < 0{\cdot}05$). Exceptions were the negative correlations between lactose and chloride in the cow and sow, and sodium and potassium, and potassium and chloride in the ewe. However, for pooled values for all species there were significant ($P < 0{\cdot}05$) positive correlations between potassium and chloride, and significant ($P < 0{\cdot}05$) negative correlations between potassium and lactose and between chloride and lactose, but there was only a poor correlation between sodium and chloride, sodium and lactose, and sodium and potassium.

## DISCUSSION

The trends in the concentration of water-soluble constituents in cow's milk with stage of lactation are well established (see Rook & Campling, 1965) but information for other species is more limited. The results reported here for the Jersey cow are consistent with earlier observations for the Friesian (Rook & Campling, 1965) but lactational trends for the sow and ewe differed from those of the cow in several important respects. In all species there were corresponding trends in milk lactose content, values rising to a peak in the first weeks of lactation and then declining to the end of lactation. For the cow there was little variation in potassium content from about the third day of lactation onwards to the middle of lactation, then values declined to the end of lactation. However, for the sow and ewe potassium values decreased early in lactation and towards the end of lactation increased in the sow and decreased only slowly in the ewe. In sow's milk the sodium content increased progressively throughout lactation but there was an opposite trend in the chloride levels.

The management of cows involves removal of the calf at birth and milking-out of the udder at frequent intervals. Accumulation of milk within the mammae of this species is thus prevented but in the sow and ewe the volume of milk removed is

Table 2. *The relationships between sodium, potassium, lactose and chloride in milk obtained in mid-lactation from Jersey cows, sows, ewes and goats*

(The concentrations of sodium, potassium and chloride are expressed in mmoles/kg of milk water.)

| Species | Correlation between† | Regression equations | S.E. of regression coefficients | Correlation coefficient |
|---|---|---|---|---|
| Cow | Na and Cl | Na = 0·104 Cl + 15·3<br>Cl = 0·445 Na + 16·7 | $1·67 \times 10^{-1}$<br>$7·12 \times 10^{-1}$ | 0·22 |
| | Na and K | Na = −0·436 K + 44<br>K = −0·546 Na + 69 | $2·76 \times 10^{-1}$<br>$3·45 \times 10^{-1}$ | −0·49 |
| | Na and L | Na = −0·283 L + 64<br>L = −0·830 K + 175 | $1·81 \times 10^{-1}$<br>$5·27 \times 10^{-1}$ | −0·49 |
| | K and Cl | K = 0·050 Cl + 62·1<br>Cl = −0·170 K + 35·0 | $1·91 \times 10^{-1}$<br>$6·49 \times 10^{-1}$ | −0·09 |
| | K and L | K = −0·079 L + 74<br>L = −0·184 K + 172 | $2·29 \times 10^{-1}$<br>$7·90 \times 10^{-2}$ | −0·12 |
| | Cl and L | Cl = −0·896 L + 167<br>L = −0·606 Cl + 176 | $2·90 \times 10^{-1}$<br>$1·99 \times 10^{-1}$ | −0·73* |
| Sow | Na and Cl | Na = 1·18 Cl + 36<br>Cl = 0·461 Na + 22·4 | $7·64 \times 10^{-1}$<br>$2·98 \times 10^{-1}$ | −0·74 |
| | Na and K | Na = 0·536 K + 18·4<br>K = 0·253 Na + 34 | $3·23 \times 10^{-1}$<br>1·49 | 0·11 |
| | Na and L | Na = −0·127 L + 44<br>L = −1·28 Na + 216 | $1·29 \times 10^{-1}$<br>1·30 | −0·40 |
| | K and Cl | K = −2·44 Cl + 71<br>Cl = −0·201 K + 21 | 1·75<br>$1·44 \times 10^{-1}$ | −0·70 |
| | K and L | K = −0·006 L + 41<br>L = −0·020 K + 193 | $2·47 \times 10^{-1}$<br>$8·0 \times 10^{-1}$ | −0·01 |
| | Cl and L | Cl = −0·263 L + 63<br>L = −3·63 Cl + 236 | $4·24 \times 10^{-2}$<br>$5·61 \times 10^{-1}$ | −0·97* |
| Ewe | Na and Cl | Na = 2·5 Cl − 26<br>Cl = 0·28 Na + 13·0 | $9·49 \times 10^{-1}$<br>$1·05 \times 10^{-1}$ | 0·83 |
| | Na and K | Na = −1·54 K + 99<br>K = −0·56 Na + 68 | $3·51 \times 10^{-1}$<br>$1·28 \times 10^{-1}$ | −0·93* |
| | Na and L | Na = 1·0 L − 167<br>L = 0·46 Na + 176 | $6·20 \times 10^{-1}$<br>$2·83 \times 10^{-1}$ | 0·69 |
| | K and Cl | K = −1·59 Cl + 80<br>Cl = −0·49 K + 43 | $5·00 \times 10^{-1}$<br>$1·53 \times 10^{-1}$ | −0·88* |
| | K and L | K = −0·59 L + 151<br>L = −0·72 K + 222 | $3·94 \times 10^{-1}$<br>$4·94 \times 10^{-1}$ | −0·64 |
| | Cl and L | Cl = 0·162 L − 11·1<br>L = 0·67 Cl + 198 | $2·69 \times 10^{-1}$<br>1·10 | 0·33 |
| Goat | Na and Cl | Na = −0·48 Cl + 42<br>Cl = −0·309 Na + 51 | $5·74 \times 10^{-1}$<br>$3·71 \times 10^{-1}$ | −0·38 |
| | Na and K | Na = 0·20 K + 6·6<br>K = 0·71 Na + 57 | $2·42 \times 10^{-1}$<br>$8·89 \times 10^{-1}$ | 0·37 |
| | Na and L | Na = −0·397 L + 78<br>L = −0·66 Na + 158 | $3·32 \times 10^{-1}$<br>$5·54 \times 10^{-1}$ | −0·51 |
| | K and Cl | K = −1·26 Cl + 127<br>Cl = −0·22 K + 60 | 1·01<br>$1·78 \times 10^{-1}$ | −0·53 |
| | K and L | K = −0·91 L + 202<br>L = −0·41 K + 174 | $5·88 \times 10^{-1}$<br>$2·66 \times 10^{-1}$ | −0·61 |
| | Cl and L | Cl = 0·34 L − 5·1<br>L = 0·67 Cl + 115 | $2·60 \times 10^{-1}$<br>$6·70 \times 10^{-1}$ | 0·54 |

Table 2. (*cont.*)

| Species | Correlation between† | Regression equations | S.E. of regression coefficients | Correlation coefficient |
|---|---|---|---|---|
| All species | Na and Cl | Na = 0·006 Cl + 19·4<br>Cl = 0·048 Na + 25 | $7{\cdot}87 \times 10^{-2}$<br>$5{\cdot}51 \times 10^{-1}$ | 0·02 |
| | Na and K | Na = −0·030 K + 21·1<br>K = −0·27 Na + 61 | $6{\cdot}48 \times 10^{-2}$<br>$5{\cdot}82 \times 10^{-1}$ | −0·09 |
| | Na and L | Na = 0·017 L + 16·6<br>L = 0·035 Na + 163 | $4{\cdot}24 \times 10^{-2}$<br>$8{\cdot}93 \times 10^{-1}$ | 0·076 |
| | K and Cl | K = 0·75 Cl + 38<br>Cl = −0·73 K − 15·6 | $1{\cdot}43 \times 10^{-1}$<br>$1{\cdot}37 \times 10^{-1}$ | 0·74*** |
| | K and L | K = −0·60 L + 66<br>L = −1·40 K + 248 | $5{\cdot}09 \times 10^{-2}$<br>$1{\cdot}21 \times 10^{-1}$ | −0·91*** |
| | Cl and L | Cl = −0·55 L + 119<br>L = −1·29 Cl + 200 | $7{\cdot}28 \times 10^{-2}$<br>$1{\cdot}69 \times 10^{-1}$ | −0·84*** |

* $P < 0{\cdot}05$, *** $P < 0{\cdot}001$.

† L, Lactose; Na, sodium; K, potassium; Cl, chloride.

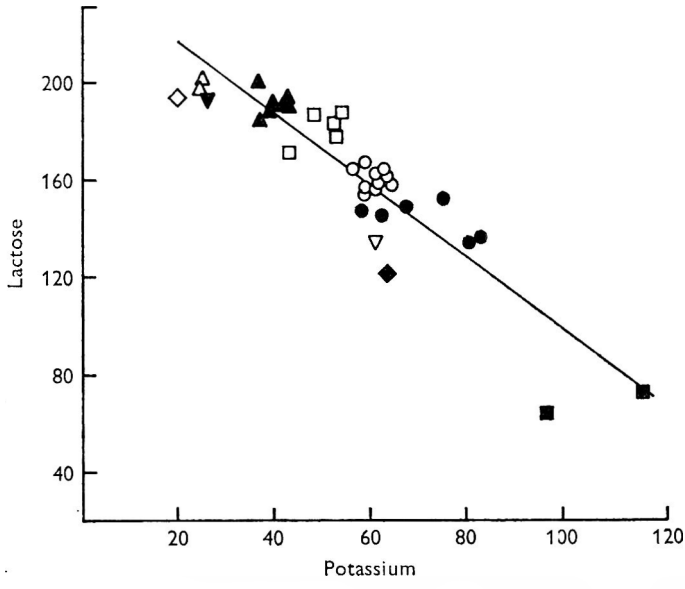


Fig. 3. The relationship between lactose and potassium concentrations (mmoles/kg of milk water) in milks of the cow, ○; goat, ●; ewe, □; sow, ▲ (present experiment); human, △; rabbit, ■ (Konar, Thomas & Rook, unpublished data); elephant, ▽ (McCullagh & Widdowson, 1970); rhinoceros, ▼ (Gregory, Rowland, Thompson & Kon, 1965); mare, ◇ (Ling, Kon & Porter, 1961); rat, ◆ (Cox & Mueller, 1937). The solid line represents the regression: Lactose = 246·5 − 1·47 ± 0·11 potassium ($r = -0{\cdot}90$, $P < 0{\cdot}001$).

determined by the appetite of the piglets or lambs and may be less than that secreted. Consequent engorgement of the udder may result in a partial resorption of lactose which, if unaccompanied by a loss of potassium, could cause the observed high values for potassium in milk removed from the udder. Also, in the sow and ewe the arbitrary termination of lactation when the young are weaned may not allow the development of changes in milk composition characteristic of advanced lactation in the cow.

Differences between species in mid-lactation values for lactose content are well

recognized, but the close inverse relationship with potassium content (Table 2) has not previously been reported, although a similar relationship has been observed in milks of Friesian heifers in mid-lactation (Rook & Wood, 1959). On the basis of values available from the literature and our own observations it seems likely that the relationship between lactose and potassium may extend to the milks of many species (Fig. 3). To account for the relationship in milks from Friesian heifers Rook & Wood (1959) postulated that milk fluid arises by a continuous synthesis of lactose and other constituents within the cell and a movement of water into the cell to maintain osmotic equilibrium until a fairly constant ratio of secretory to intracellular fluid is obtained, when the cellular contents are expelled into the alveolar lumen. The ratio of the secretory to intracellular fluid at the moment of expulsion would vary from animal to animal. The physiological basis of such a process was obscure but recently Coffey & Reithel (1968) have demonstrated that lactose synthetase activity is associated with particulate material derived from the Golgi body of the secretory cell and Brew (1969) has proposed that lactose may be secreted in these 'particles'. If the 'particles' are vesicular in nature (see reviews by Brew, 1970; Keenan, Saacke & Patton, 1970) then the relationship observed in the milk between lactose and potassium may reflect their association in the vesicular contents.

The between-species relationships between lactose and other water-soluble constituents and between potassium and chloride may have a similar origin as some sodium and chloride would be present with potassium in the cell contents. The relatively poor correlation between sodium and chloride and sodium and potassium (Table 2) suggests, however, that there may be important differences between species in the concentration or distribution of sodium within the cell. The close positive correlation between sodium and chloride reported in many previous studies with the cow has probably arisen mainly because of effects of stage of lactation or inter-quarter differences which appear to reflect changes in the extent to which the primary secretion of the alveolar cell is diluted with a transudate of blood plasma (see Rook & Wheelock, 1967).

The results of the present studies indicate that in most species the contribution of citric acid to the osmotic activity of milk is small. On the basis of values for citric acid content in milk reported in the literature for the horse, human and pig, Hardwick (1966) generalized that levels in non-ruminant milks are substantially less than in ruminant milks, a difference that he suggested might be linked to the absence of ATP-citrate lyase (EC 4.1.3.8) from ruminant mammary tissue. The values obtained for the goat in the present investigations are not consistent with such a conclusion although they are somewhat lower than reported by Bosworth & Van Slyke (1916). However, the lactational variations in milk citric acid content may reflect the involvement of ATP-citrate lyase since the activity of the enzyme increases during early lactation (Baldwin, 1966; Baldwin & Milligan, 1966) and during this period most species show a decline in the citric acid content of their milk.

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*With 2 plates*



# Heat-induced interaction of β-lactoglobulin and κ-casein

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Summary. The interaction of β-lactoglobulin and κ-casein at high temperature was studied polarimetrically. The rate of interaction was related to the genetic variant of β-lactoglobulin present. β-Lactoglobulin B, whose thermodenaturation was faster than that of A variant, interacted more rapidly with κ-casein than did the A variant. Velocity sedimentation studies indicated that characteristics of the complex were determined more by the β-lactoglobulin than by the κ-casein. The presence of κ-casein during the thermodenaturation of β-lactoglobulin appeared to prevent the aggregation of β-lactoglobulin from proceeding to completion, the κ-casein complexing with intermediate species and restricting the aggregation process.

A complex is formed when β-lactoglobulin and κ-casein are heated together in solution at neutral pH. Several workers have presented evidence that the formation of this complex during forewarming treatments of milk increases the heat-stability and the rennin clotting time of the colloidal caseinate system (Trautman & Swanson, 1958; Kannan & Jenness, 1961; Zittle, Thompson, Custer & Cerbulis, 1962). The subject has been reviewed recently (Sawyer, 1969). Although the complex is known to form when buffered solutions of the 2 proteins are heated, there is still considerable uncertainty as to whether a similar type of complex forms in the milk medium (Tessier, Yaguchi & Rose, 1969). Nevertheless, the heat stability of a milk sample may be determined at least in part by the genetic composition of the proteins present, since each of the major milk proteins (α-, β-, and κ-casein, β-lactoglobulin and α-lactalbumin) is known to occur in several genetically determined forms which differ only slightly in amino acid composition (McKenzie, 1967). The rate and extent of interaction of β-lactoglobulin with κ-casein might therefore be governed by the genetic variants of these proteins present. The present paper describes a study of the kinetics of this interaction and examines certain characteristics of the complex formed. Differences in the rate of interaction of 2 β-lactoglobulin variants, β-A and β-B, with κ-casein are related to the susceptibility of the β-lactoglobulin variants to thermodenaturation.

## EXPERIMENTAL

### *Materials*

κ-Casein was prepared from whole casein by the method of Zittle & Custer (1963). The method yielded 2·3 g κ-casein from 60 g whole casein. The preparation was stored as the freeze-dried powder.

β-Lactoglobulins A and B were prepared from the milk of homozygous cows by the method of Armstrong, McKenzie & Sawyer (1967). Pooled β-lactoglobulin (3 times recrystallized) was obtained from Koch–Light Laboratories Ltd, Colnbrook, Bucks.

All reagents were of analytical grade and glass-distilled water was used throughout the study. *N*-ethylmaleimide was obtained from Koch–Light Laboratories and Sephadex G-200 from Pharmacia, Uppsala, Sweden. Guanidine hydrochloride, ultrapure grade (GuHCl) was the product of Mann Research Laboratories, Orangeburg, New York. The buffer employed in the study was of the following composition: 0·05 M-sodium cacodylate, 0·01 M-cacodylic acid, 0·05 M-NaCl (pH 6·6, I = 0·1).

### *Methods*

Concentrations of κ-casein and β-lactoglobulin were measured spectrophotometrically at 278 nm using a Zeiss PMQ II instrument. Absorbancy coefficients used were 0·96 l/g cm for β-lactoglobulin (Sawyer, 1966) and 1·22 l/g cm for κ-casein (Zittle & Custer, 1963).

Sedimentation velocity experiments were carried out in a Spinco Model E analytical ultracentrifuge at 20°C using schlieren optics. Velocity experiments in GuHCl were carried out at 25°C and sedimentation coefficients were corrected for temperature and the density of the solvent using the data of Kawahara, Kirshner & Tanford (1965). Values of the sedimentation coefficient so calculated are approximate as no consideration was given to changes in the partial specific volume that may occur in the denaturing solvent.

All other methods, including polarimetric procedures and the use of the Guggenheim procedure for the analysis of single and consecutive first-order reactions, have been described previously (Guggenheim, 1926; Sawyer, Norton, Nichol & McKenzie, 1971).

## RESULTS

### *Optical rotation*

The interaction of β-lactoglobulin and κ-casein was followed polarimetrically under specified conditions of temperature (60–80°C), protein concentration, pH and ionic strength. These experiments established the time required for the reaction to go to completion and were of assistance in the design of sedimentation velocity experiments. Moreover, the results were amenable to kinetic analysis.

Optical rotation was measured as a function of time for 1:1 (w/w) mixtures of β-lactoglobulin and κ-casein. The total protein concentration of each solution was 0·48 g/100 ml, and 6 temperatures were examined between 60 and 80°C. The results are summarized in Fig. 1(*a*). The initial reaction is rapid and extrapolation of the data to zero time is difficult, especially at the higher temperatures. The final specific rotation is different at each temperature: $[\alpha]_{365nm}$ decreases from −349 at 60°C to −356 at 65°C and then increases to −342 at 80°C.

The data could not be satisfactorily described by the equation for a second-order reaction. Treatment of the data in Fig. 1(*a*) according to the procedure of Guggenheim (1926) for the analysis of first-order reactions is shown in Fig. 1(*b*). Apparent first-order reactions are observed at 65·0, 72·5, 77·0 and 79·5°C, the rate constants being 0·0012, 0·003, 0·0092 and 0·0135 $min^{-1}$ respectively. At 60·0 and 69·5°C the

Guggenheim plots are biphasic in a manner which suggests the occurrence of 2 consecutive first-order reactions (Sawyer *et al.* 1971). At all temperatures, Guggenheim plots become non-linear near the completion of the reaction, suggesting the occurrence of reactions of intermediate or higher order. Fig. 2 shows that the reaction is markedly concentration dependent. The final specific rotation, and therefore the final product of the reaction, is different at each protein concentration.

Heat-treatment did not change the specific rotation of κ-casein (Fig. 3*a*). Thus, the total change in the specific rotation observed for mixtures of κ-casein and β-lactoglobulin may be attributed to (i) changes in the conformation and state of aggregation

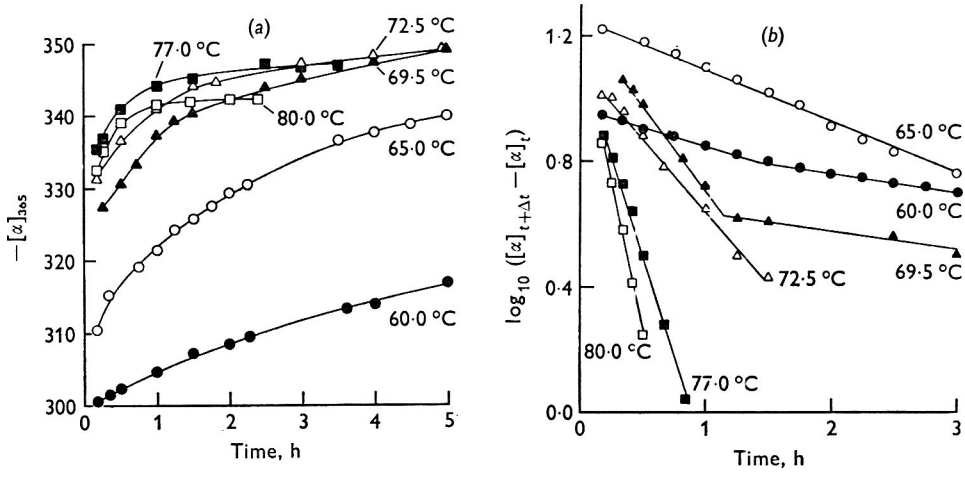


Fig. 1. (a) The dependence of optical rotation on time for solutions of pooled β-lactoglobulin and κ-casein (1:1, w/w), at several temperatures. Total protein concentration, 0·48 g/100 ml. (*b*) Data analysed according to the Guggenheim procedure for the analysis of first-order reactions (Guggenheim, 1926). Values of $\log([\alpha]_{t+\Delta t}-[\alpha]_t)$ were calculated from the smooth curve through the experimental points in Fig. 1(*a*). Values of $\Delta t$ were as follows: 120 min at 60·0°C and 650 °C, 60 min at 69·5°C and 72·5°C, 30 min at 77·0°C and 20 min at 80·0°C.

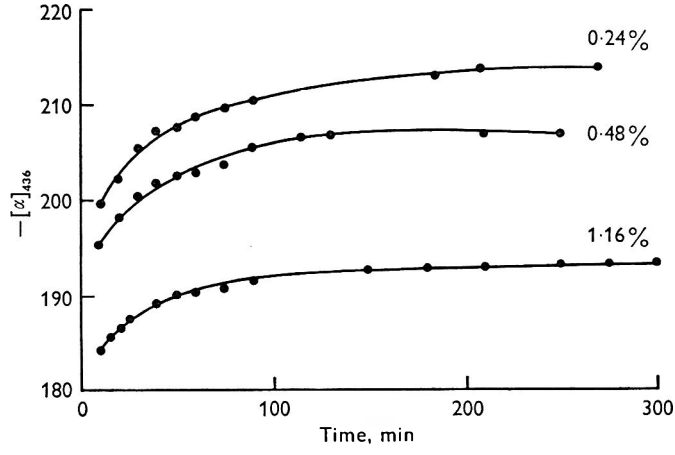


Fig. 2. Time dependence of optical rotation for solutions of pooled β-lactoglobulin and κ-casein (1:1, w/w) at initial total protein concentrations of 0·24, 0·48 and 1·16 g/100 ml. The temperature of heating was 74°C.

of the $\beta$-lactoglobulin and (ii) rotations which may arise at the sites of contact between polypeptide chains of the 2 proteins. It was therefore of interest to relate the observed rotational change to that expected from consideration of the denaturation of $\beta$-lactoglobulin itself. The time course of the denaturation of the A and B variants of $\beta$-lactoglobulin at 74°C is shown in Fig. 3(*a*). The results are in agreement with

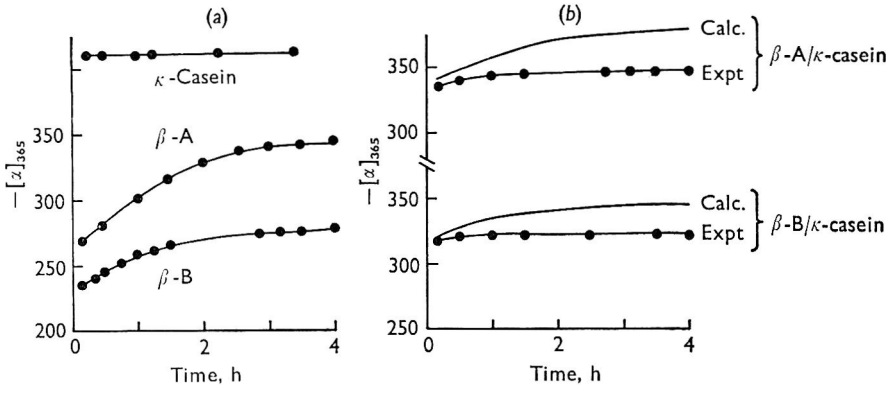


Fig. 3. (*a*) Dependence of optical rotation on time for solutions of $\beta$-lactoglobulin A and B, and $\kappa$-casein heated at 74°C. (*b*) Comparison between the experimental (expt) and the theoretically calculated (calc.) time dependence of optical rotation at 74°C for solutions of $\beta$-A/$\kappa$-casein and $\beta$-B/$\kappa$-casein. The theoretical curve was calculated according to equation (1).

those previously reported (Sawyer *et al.* 1971). If no interaction occurs between the 2 proteins, the expected rotation at any time is determined by the weight average relationship (McKenzie, Sawyer & Smith, 1967):

$$[\alpha]_{\text{calc.}} = \frac{[\alpha]_\beta c_\beta + [\alpha]_\kappa c_\kappa}{c_\beta + c_\kappa}, \tag{1}$$

where $c_\beta$ and $c_\kappa$ are the weight concentrations of $\beta$-lactoglobulin and $\kappa$-casein respectively, and $[\alpha]_\beta$ and $[\alpha]_\kappa$ are the specific rotations of the 2 proteins at any time during the reaction: $[\alpha]_{\text{calc}}$ is the expected specific rotation of the mixture. The results shown in Fig. 3(*b*) show that observed rotations are less than those expected had no interaction occurred. The extent of the rotational change (as reflected by values of the final rotation) as well as the rate of change is less than would be expected in the absence of interaction. Also the final specific rotation is achieved more quickly for the $\beta$-B/$\kappa$-casein solution than for the $\beta$-A/$\kappa$-casein solution, suggesting that $\beta$-B interacts faster with $\kappa$-casein than does $\beta$-A.

### *Sedimentation velocity*

Solutions for sedimentation velocity analysis were prepared in the same buffer as that used in kinetic investigations (cacodylate buffer, pH 6·6, I = 0·1). The concentration dependence of the sedimentation coefficient of $\kappa$-casein is shown in Fig. 4. The dependence is linear and obeys the relationship

$$s_{20,w} = s^\circ_{20,w} - 7{\cdot}1c, \tag{2}$$

where $s^{\circ}_{20,w}$ (20·6S) is the sedimentation coefficient at infinite dilution and $c$ is the protein concentration in g/100 ml. Heat-treatment had no effect on the sedimentation coefficient of κ-casein but a small amount (approx. 3 %) of highly aggregated material was evident during the early stages of the sedimentation velocity experiment.

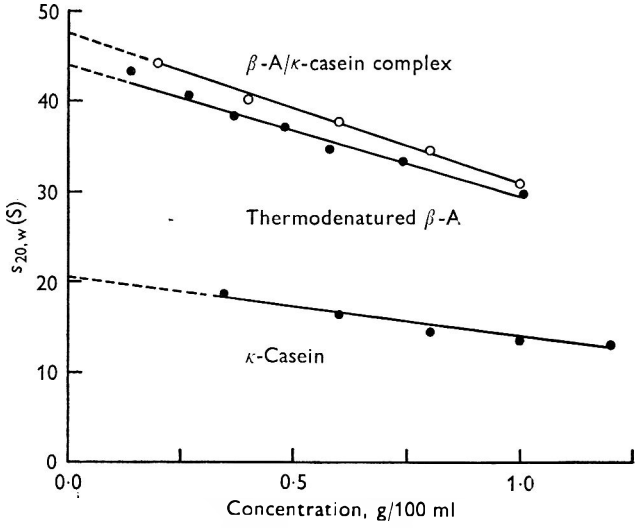


Fig. 4. Concentration dependence of sedimentation coefficients for solutions of κ-casein (unheated) and the β-A/κ-casein complex (74°C for 6 h). The results for thermodenatured β-lactoglobulin A (74°C for 6 h) are those of Sawyer *et al.* (1971) and are included for comparison.

As the sedimentation coefficient of κ-casein is approximately 7 times greater than that of native β-lactoglobulin ($s_{20,w}$ for β-A at pH 6·6 is 2·6S; McKenzie & Sawyer, 1967), a mixture of the 2 proteins can be resolved by velocity sedimentation. However, when the mixture is heated, only one asymmetric peak representing the complex is observed. The sedimentation coefficient of the complex is dependent on the temperature of heating and decreases monotonically from 44 S at 65°C to 32 S at 90°C as shown in Fig. 5. These solutions had been cooled to room temperature only after optical rotation measurements had indicated that the reaction had proceeded to completion. A small amount of low-molecular-weight material remained near the meniscus during sedimentation velocity runs. This material could be removed by passage of the protein through a column of Sephadex G-200. The dependence of the sedimentation coefficient on concentration of this purified complex is shown in Fig. 4. The dependence is linear and obeys the relationship

$$s_{20,w} = s^{\circ}_{20,w} - 16{\cdot}6c, \tag{3}$$

where $s^{\circ}_{20,w}$ = 47·3 S. It should be noted that β-lactoglobulin itself polymerizes during thermodenaturation. The concentration dependence of the sedimentation coefficient of thermodenatured β-A has been reported previously (Sawyer *et al.* 1971) but the results are included in Fig. 4 for comparison.

The results of the polarimetric experiments suggested that the rate of interaction of κ-casein with the B variant of β-lactoglobulin was faster than with the A variant. It was important therefore to determine whether this relationship could be confirmed

by sedimentation velocity studies. Solutions of $\kappa$-casein/$\beta$-A and $\kappa$-casein/$\beta$-B were heated at 74°C for 10 min, 1 h and 6 h. The reactions were terminated by cooling rapidly to room temperature. The schlieren profiles are shown in Plate 1 together with the relevant values of the sedimentation coefficients. The profiles at 6 h are not included as they were similar to those at 1 h. For the $\kappa$-casein/$\beta$-B solution, approximately 95% of the material reacted after 10 min to form a species moving with a velocity of 38 S. On the other hand, 2 partially resolved peaks of similar area were observed for the $\kappa$-casein/$\beta$-A solution after the same time interval. Clearly, the B variant reacts faster with $\kappa$-casein than the A variant. The results therefore agree with those of polarimetric studies.

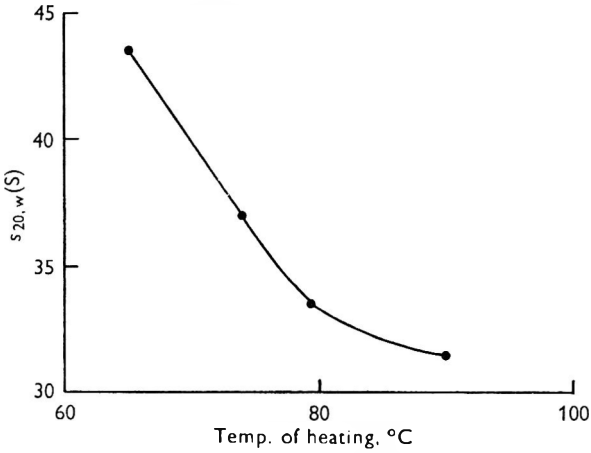


Fig. 5. Variation of the sedimentation coefficient of the $\beta$-A/$\kappa$-casein complex as a function of temperature of heating. Protein concentration, 0·75 g/100 ml. All solutions had been heated until optical rotations remained constant with time.

*Effect of thiol blocking reagent.* The presence of *N*-ethylmaleimide (twice the amount required to bind the single thiol group in $\beta$-A) during heat treatment of the protein mixture prevented complex formation to only a minor degree: 8% of the material sedimented with a velocity characteristic of $\kappa$-casein. The peak representing the complex was hypersharp and sedimented faster (50 S) than the complex formed in the absence of *N*-ethylmaleimide (30 S). Control experiments indicated that the effect of *N*-ethylmaleimide was mainly on the $\beta$-lactoglobulin rather than on the $\kappa$-casein. For example, when $\beta$-A was heated in the presence of a similar amount of *N*-ethylmaleimide, the observed schlieren peak was hypersharp and its sedimentation coefficient (54 S) was greater than that found in the absence of *N*-ethylmaleimide (37 S).

*Interaction with thermodenatured $\beta$-lactoglobulin.* Plate 2(*a*) shows the schlieren profile for a 1:1 (w/w) solution of $\kappa$-casein and heat denatured $\beta$-A. Measurement of the peak areas from enlargements of these photographs indicated that 45% of the material sedimented as the slow component and 55% as the fast component. Thus, there is little tendency for $\kappa$-casein to complex with thermodenatured $\beta$-A when the 2 proteins are mixed at room temperature. On the other hand, when this solution was heated at 75°C for 7·5 h, the proportions of fast and slow components were 76 and 24% respectively (Plate 2*b*), thus indicating that about 50% interaction had

occurred. The presence of *N*-ethylmaleimide (twice the amount required to bind the single thiol group in β-A) during the heat treatment of β-A or heat treatment of the mixture did not affect this result.

*Effect of dissociating agents.* The linear dependence of the sedimentation coefficient of the complex on concentration (Fig. 4) suggested that the complex was extremely stable and did not dissociate at low protein concentrations. It was therefore of interest to determine whether GuHCl could dissociate the complex to any marked extent. A 1:1 (w/w) mixture of κ-casein and β-A (total protein concentration, 0·9 g/100 ml) was heated at 75°C for 8 h and cooled rapidly to room temperature. Two samples of the heated solution were removed and solid GuHCl was added to each to give final concentrations of 3·0 M and 6·0 M after allowing for volume changes. The final protein concentration in each solution was 0·49 g/100 ml. A single schlieren peak was observed for each solution although there was evidence of material remaining close to the meniscus. Sedimentation coefficients were 21·75 S and 14·3 S in 3·0 M and 6·0 M-GuHCl respectively. The decrease in the apparent sedimentation coefficient observed in GuHCl suggests a reduction in the molecular weight of the complex although an increase in the frictional coefficient caused by further denaturation may contribute slightly to this change. In 6·0 M GuHCl the area of the peak was only 70 % of the corresponding area observed in 3·0 M GuHCl. When a solution of the complex was reacted in 6·0 M GuHCl/0·2 M mercaptoethanol for 12 h, complete dissociation of the complex was observed ($s_{25,w}$ = < 2 S) and no peak resolved from the meniscus even after spinning for 60 min at top speed (59780 rev/min).

## DISCUSSION

Kinetic analysis of the optical rotation results indicates that the reaction between β-lactoglobulin and κ-casein does not follow true first- or second-order kinetics. Although linear first-order plots were obtained at several temperatures, the dependence of the rate and extent of the reaction on the initial protein concentration (Fig. 2) shows that true first-order kinetics are not obeyed. The form of the Guggenheim plot for a series of 2 irreversible first-order reactions has been reported previously (Sawyer *et al.* 1971) and the biphasic relationship observed at 60 and 70°C (Fig. 1*b*) supports such a mechanism. However, it should be noted that linearity of the Guggenheim plot does not necessarily indicate the absence of consecutive reactions as a linear plot is observed if the rate constants of the 2 reactions are of a similar magnitude. Although the apparent first-order rate constants are related to the absolute temperature in the manner predicted by the Arrhenius equation, it is inappropriate to attach significance to the value of the activation energy so obtained since the type of complex formed depends on both the time and temperature of heating. For example, the final specific rotation and the sedimentation coefficient of the complex are strongly dependent on the temperature of heating. This behaviour appears to be determined primarily by the β-lactoglobulin component which shows similar complex characteristics on thermodenaturation (Sawyer *et al.* 1971). In contrast, the sedimentation coefficient and optical rotation of κ-casein are unaffected by heat treatment. Thus, the changes in optical rotation observed experimentally for mixtures of the 2 proteins must be due to the thermodenaturation of β-lactoglobulin and to rotational effects caused by

complex formation itself. Although the magnitude of this latter effect is unknown, it is clear from Fig. 3(*a*) and 3(*b*) that the presence of κ-casein limits the final value of the laevorotation which might have been expected in the absence of interaction. When considered in the light of sedimentation velocity experiments discussed below, the result suggests that the presence of κ-casein prevents the denaturation of β-lactoglobulin from proceeding to completion.

Both sedimentation velocity and polarimetric studies show that κ-casein reacts more rapidly with β-B than with β-A. The thermodenaturation of β-lactoglobulin itself proceeds via a complicated series of unfolding and association steps. However, it is significant that the B variant is denatured more rapidly than the A variant. It appears therefore that the rate of denaturation of β-lactoglobulin determines its rate of interaction with κ-casein.

The sedimentation coefficient of the complex is only slightly greater than that for thermodenatured β-lactoglobulin (e.g. 35 S compared to 37 S for thermodenatured β-A at a concentration of 0·6 g/100 ml: Fig. 5). It is clear therefore that κ-casein associates with β-lactoglobulin before the aggregation of β-lactoglobulin itself proceeds to completion. If the association had occurred with fully denatured β-lactoglobulin, the expected sedimentation coefficient of the resulting complex would have been considerably higher than the experimentally observed value although the exact figure cannot be predicted due to uncertainty of changes in the frictional coefficient. In support of this view it should be noted that the interaction of κ-casein with thermodenatured β-A is incomplete, with only 50 % of the κ-casein reacting to form the complex. Our values for the sedimentation coefficient of the complex are in general agreement with those of other workers (Zittle *et al.* 1962; Tessier *et al.* 1969). However, direct comparisons cannot be made due to the use of different conditions of pH, time and temperature of heat treatment.

The linearity of the relationship between the sedimentation coefficient and protein concentration (Fig. 4) indicates that the complex is relatively stable and does not dissociate at low concentrations. Indeed, 6 M-GuHCl is unable to entirely disrupt the complex although a slight degree of dissociation is observed. It is only when mercaptoethanol is included in the solvent that complete dissociation is obtained. This result is in accord with those of previous workers who have shown that intermolecular disulphide bonds are involved in complex formation (Sawyer, Coulter & Jenness, 1963; Purkayastha, Tessier & Rose, 1967). In the presence of *N*-ethylmaleimide, a thiol blocking reagent which prevents disulphide interchange, complex formation is inhibited but not entirely prevented. Thus, although the formation of intermolecular disulphide bonds may be a major factor in stabilizing the complex, some form of less specific association, perhaps involving physical entanglement of polypeptide chains, may also be involved.

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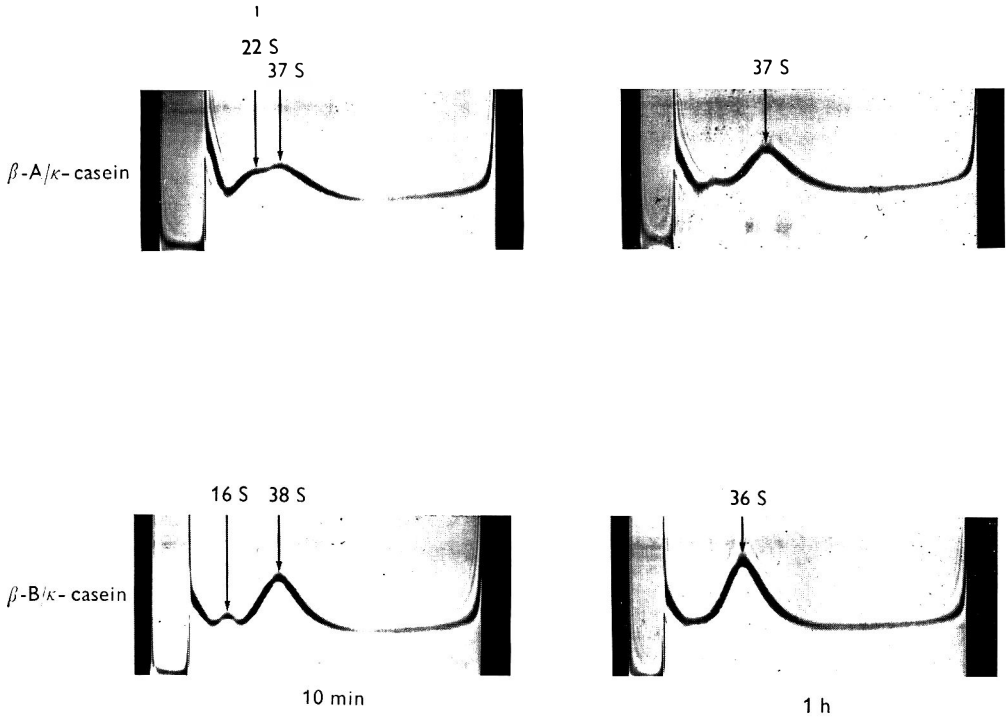
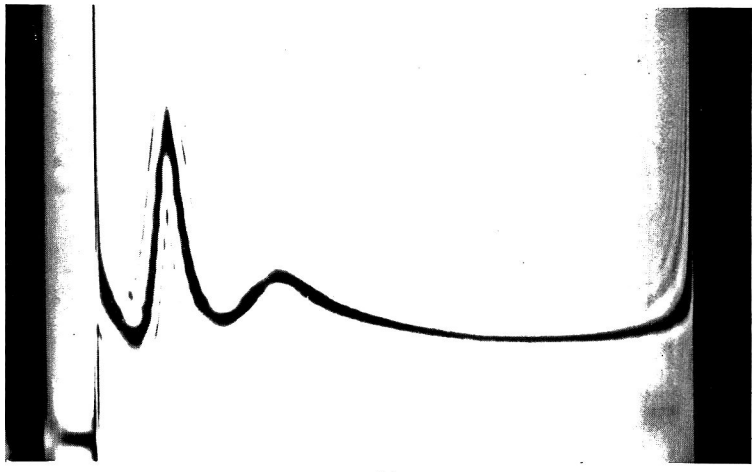
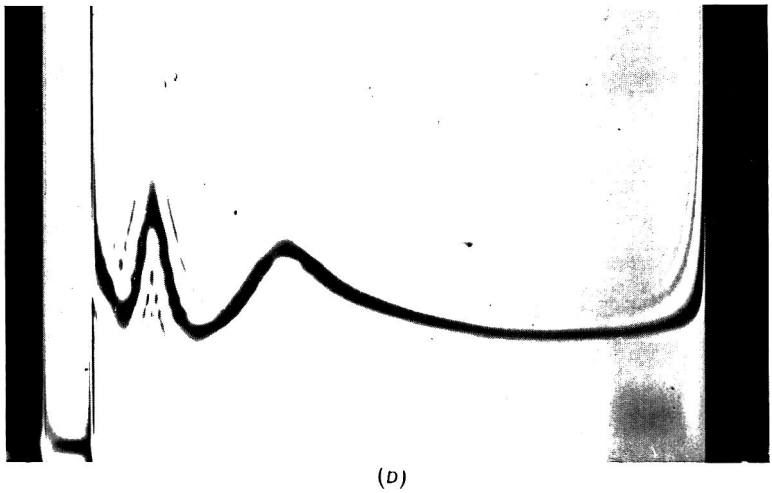

22 S
37 S
37 S
β-A/κ-casein
16 S
38 S
36 S
β-B/κ-casein
10 min
1 h



(a)



(b)

Purkayastha, R., Tessier, H. & Rose, D. (1967). *J. Dairy Sci.* **50**, 764.
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## EXPLANATION OF PLATES

### Plate 1

Sedimentation velocity profiles for solutions of β-A/κ-casein and β-B/κ-casein heated at 74°C for 10 min and 1 h. Sedimentation coefficients ($S_{20w}$) of individual peaks are indicated.

### Plate 2

Sedimentation velocity profiles for (*a*) a solution of κ-casein and thermodenatured β-lactoglobulin A and (*b*) the same solution heated at 75°C for 7·5 h.

*Printed in Great Britain*

# A use of the computer as an aid in diagnosis of metabolic problems of dairy herds

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Summary. The contribution that the computer has made in the interpretation and analysis of a metabolic profile test, which is designed to monitor the metabolic health of a dairy herd and assess the adequacy of dietary intake for production, is described. The test is based on an assessment of blood chemistry for any number of variates, which constitute the metabolic profile, measured for randomly selected cows from 3 groups of a herd: dry cows, cows giving intermediate quantities of milk and cows giving high daily yields. The computer program is designed to give an objective and comprehensive assessment of the test in an easily interpretable form based on a pictorial form of print-out. The profile is displayed as a 'histogram' presented as standard deviations from the normal base-line, and for each variable a scatter diagram is included presenting the individual variates plotted against the particular animal's daily milk yield.

The particular context of this paper is a *metabolic profile* test, as described by Payne, Dew, Manston & Faulks (1970), which is designed to monitor the metabolic health and to help diagnose existing metabolic problems of dairy herds. The test is based on an analysis of blood chemistry on a sample of cows selected at random from separate groups of a herd stratified in accordance to milk yield. At present, the groups adopted are dry cows (or, if there are not enough dry cows, cows giving low yields), cows giving intermediate yields of milk, and cows giving high daily yields. From the blood analysis results for these 3 groups, the adequacy both of maintenance and of production rations can be assessed.

The blood samples are analysed for such variates as packed cell volume, glucose, magnesium, potassium concentrations, and others, and the average values calculated for each group. This set of values is said to constitute the group's *metabolic profile* with respect to the blood measurements made. If this profile is compared with the profile that would be expected for an average normal herd, a large deviation from normal with respect to a particular variable provides evidence of 'abnormality', the larger the deviation the greater being the degree of abnormality. Without the help of the computer this is about as far as one can go for a quick assessment, and there is plenty of scope for error, both numerical and subjective. With the use of the computer, however, it has been possible to examine not only the deviations of mean values from normal, but also the deviations of individual cow values, yet still present the results

in a form that is objective and easily understood. Emphasis has been placed on a pictorial form of print-out, so that assessment of the profile can be made without direct use of statistical tests.

Blood samples taken from the entire dairy herds of 13 farms have been used to compute the normal values and confidence limits. Payne *et al.* (1970) emphasized that these herds might not be representative of the national herd, especially since herd-to-herd variation is likely to be the largest single variable in this type of work, but the herds were selected because they had good records of management. Further, variables such as seasonal trend and age of cows have not been taken into account. Nevertheless, the use of the means and standard deviations calculated from the 13 herds does appear to be giving sensible diagnostic conclusions, so that their selection as a standard is serving a useful purpose. The profile technique has since been applied to a large number of herds and a much larger sample is now available for statistical appraisal of normal values.

The test is thus based on an assessment of an individual herd's metabolic profile compared with the normal. Payne *et al.* (1970) described the blood sampling and analysis techniques, and for a further description of the test the reader is referred to their paper. The present paper demonstrates how the computer has been used as an aid in the interpretation of the test, and it is suggested that this type of presentation might be useful in other health studies, based on a large-scale laboratory analysis, when a rapid assessment of the results is required. The print-out of the program is illustrated by some of the farm tests discussed by Payne *et al.* (1970).

## METHODS

### *Preparation of data*

At the time of the blood sampling, names or identifying codes of cows in the sample together with their current average daily milk yields are recorded on a data sheet. Subsequently the blood analysis results are added. At present this is done manually by copying the figures from a digital paper output produced by the autoanalysers. In the near future it is planned to attach a paper tape punch to one autoanalyser so that this step can be bypassed. When all the results have been assembled, each cow's record consists of its name, milk yield and the blood variables, a typical set being packed cell volume, glucose, urea, inorganic phosphorus, calcium, magnesium, sodium, potassium, total protein, albumin, globulin and haemoglobin. The data are punched on cards and submitted together with parameter values describing the data to the computer.

### *Parameter Cards*

Table 1 illustrates a typical set of input data:

(1) The first line provides a heading for the print-out. Any information describing the herd, such as farm, farmer, veterinary surgeon, date of bleed, range of milk yields in each group, may be included.

(2) The METABOLITES line specifies the number of blood variables measured.

(3) The LABELS line gives the order in which the variables are listed, and the abbreviated labels identify them in the program print-out.

(4) The metabolic profile test relates the particular herd's profile to the average

population values. The MEANS and S.D. lines specify these population values in the form of means and standard deviations, the units being mg/100 ml for glucose, urea, inorganic phosphorus, calcium and magnesium, m-equiv./l for sodium and potassium, and g/100 ml for total protein, albumin, globulin and haemoglobin.

Table 1. *Example of a set of input data*

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HERD | 22 | GROUPS 5–14, 15–24 AND 25+ LBS MILK/DAY | | | | | | | | | | |
| METABOLITES | 12 | | | | | | | | | | | |
| LABELS | 12 | PCV | GLU | UREA | IP | CA | MG | NA | K | TP | ALB | GLOB | HB |
| MEANS | | 29 | 45 | 15·0 | 5·4 | 9·3 | 2·58 | 139·0 | 4·80 | 7·10 | 3·30 | 3·80 | 12·2 |
| S.D. | | 3 | 4·5 | 2·5 | 0·9 | 0·5 | 0·27 | 2·0 | 0·40 | 0·50 | 0·30 | 0·60 | 1·2 |
| LIMITS | 1 | . | . | . | . | . | . | . | . | . | . | . | . |
| | 9 | 5·2 | 9·2 | . | . | . | . | . | . | . | . | . | . |
| GROUPS | 3 | 5 | 15 | 25 | . | . | . | . | . | . | . | . | . |
| SALAD | 14 | 26 | 31 | 15·0 | 6·1 | 8·9 | 2·10 | 137·0 | 4·30 | 7·30 | 3·20 | 4·10 | 11·8 |
| DELPHIUM | 41 | 24 | 33 | 16·0 | 7·0 | 9·1 | 1·90 | 140·5 | 4·65 | 6·40 | 2·75 | 3·65 | 10·0 |
| DOLLY | 8 | 32 | 36 | 14·0 | 5·8 | 9·1 | 2·30 | 139·0 | 4·20 | 7·45 | 3·00 | 4·45 | 13·8 |
| FLORA | 18 | 26 | 36 | 13·0 | 5·8 | 9·3 | 2·30 | 138·0 | 5·40 | 8·45 | 3·00 | 5·45 | 11·0 |
| FLORIN | 18 | 27 | 36 | 13·0 | 6·0 | 9·1 | 2·60 | 140·0 | 5·20 | 7·65 | 2·85 | 4·80 | 11·4 |
| DILIGNCE | 18 | 25 | 42 | 12·5 | 5·7 | 9·1 | 2·10 | 139·0 | 4·30 | 7·65 | 2·85 | 4·80 | 11·0 |
| SALUTE | 36 | 28 | 41 | 11·5 | 5·5 | 8·9 | 2·50 | 137·0 | 4·10 | 7·15 | 3·05 | 4·10 | 11·8 |
| SATIN | 30 | 21 | 35 | 12·0 | 6·4 | 9·1 | 2·20 | 139·5 | 4·40 | 8·00 | 2·80 | 5·20 | 8·8 |
| JACINTH | 31 | 26 | 43 | 13·0 | 5·3 | 8·9 | 2·10 | 138·5 | 3·70 | 7·65 | 2·50 | 5·15 | 10·6 |
| CORVETTE | 16 | 27 | 37 | 9·5 | 4·5 | 8·8 | 1·85 | 139·5 | 4·50 | 8·00 | 2·40 | 5·60 | 11·2 |
| BIANCA | 12 | 22 | 31 | 15·0 | 5·7 | 8·9 | 2·00 | 138·0 | 4·40 | 8·00 | 2·60 | 5·40 | 9·4 |
| CALUMET | 20 | 26 | 45 | 11·0 | 5·8 | 9·4 | 2·20 | 139·5 | 4·40 | 8·30 | 2·65 | 5·65 | 10·6 |
| SKYSCAPE | 10 | 26 | 39 | 14·0 | 6·7 | 9·0 | 1·80 | 137·5 | 4·50 | 8·45 | 2·80 | 5·65 | 11·2 |
| LILAC | 20 | 25 | 36 | 14·0 | 5·4 | 9·6 | 2·10 | 139·0 | 4·35 | 7·65 | 3·45 | 4·20 | 10·8 |
| P. GALNTH | 12 | 31 | 32 | 16·0 | 5·4 | 8·9 | 2·00 | 142·0 | 4·50 | 6·55 | 2·80 | 3·75 | 13·0 |
| KATRINA | 10 | 29 | 33 | 13·0 | 6·7 | 9·9 | 2·10 | 140·0 | 5·00 | 8·80 | 3·20 | 5·60 | 12·8 |
| MAGIC | 12 | 28 | 38 | 12·5 | 6·1 | 9·2 | 2·10 | 135·5 | 4·30 | 8·65 | 2·75 | 4·90 | 11·4 |
| FLOSSY | 24 | 22 | 44 | 14·0 | 6·3 | 9·0 | 1·85 | 139·5 | 4·50 | 7·65 | 2·85 | 4·80 | 9·6 |
| GALINTH | 31 | 29 | 40 | 14·5 | 5·4 | 9·1 | 2·00 | 138·5 | 5·00 | 8·00 | 3·25 | 4·75 | 12·4 |
| FORGETFL | 36 | 26 | 43 | 11·0 | 2·3 | 9·0 | 2·25 | 139·0 | 4·20 | 7·55 | 2·55 | 5·00 | 11·2 |
| ROCHELLE | 28 | 29 | 37 | 15·0 | 6·5 | 9·2 | 1·60 | 139·5 | 4·90 | 9·20 | 3·15 | 5·90 | 11·8 |
| END | | | | | | | | | | | | | |

The first column of figures after the cows' names is the daily milk yield in lb. The blood variables follow in the order described in the LABELS line.

The first 8 columns on a card are available for the cows' name; an abbreviation is necessary if the name contains more than 8 letters.

A list of labels, means and standard deviations is defined in the program. Thus, the LABELS, MEANS and S.D. lines in Table 1 are optional and are required only if the variables measured differ from the standard list.

(5) Ranges of values within which the data are generally expected to lie are defined in the program. These ranges are used to exclude unreasonable values that may arise from errors in punching and from other causes, and also to define suitable scales in the scatter diagrams that are produced. In particular cases ranges can be redefined. The LIMITS line in Table 1 illustrates this, in that the range for the one variable total protein (the ninth variable in the list) is redefined as 5·2–9·2 so that the abnormally high total blood protein concentration of the cow Rochelle can be included.

(6) The GROUPS line specifies the milk yield groups into which the data are to be divided. In this case there are 3 groups, comprising animals yielding 5–14, 15–24 and 25 lbs or more of milk per day.

(7) The individual cows' records follow; cows may be listed in any order. Individual values not recorded, due, for example, to blood clotting or lost samples, may be left blank. These are treated as missing values and are not included in any subsequent calculation.

(8) The END line signifies that all the data have been read. Metabolic profile tests for any number of farms may be run at one time; for all except the last farm the END line is replaced by a CONTINUE line, which signifies that data for another farm follow.

## RESULTS

### *Computer print-out*

Figs 1–3 demonstrate a selection of the computer print-out for 3 tests. As the computer print-out could not be photographed directly, these figures are reproductions of typed copies of the print-out. The resemblance to the actual print-out as produced by the computer, however, is close. Each print-out includes for the main part a *summary table of means*, a '*histogram*' for each group giving a general pictorial presentation of the herd's profile, and *scatter diagrams* in which variables for individual cows are plotted against individual daily milk yields.

### *Summary tables*

The summary tables (Figs 1–3) compare the mean sample values with the population means, which are the normal standards. The population means and standard deviations differ slightly from those published by Payne *et al.*, but only in that they have been rounded off to the number of significant digits that the analytical procedures give. The sample standard deviations are calculated as 'pooled within group' standard deviations, and exclude differences between herds and between groups within a herd. In the tables these are printed alongside the population standard deviation values, calculated on the same basis, so that the herd's variability can be compared with the average.

Missing values and values outside the assigned limits are not included in the calculations of means and standard deviations. The figures printed alongside the group numbers in the summary tables give the total numbers of cows for which blood chemical analyses have been carried out.

### *Histograms*

The 'histograms', produced for each group, represent the mean values given in the summary tables but in pictorial form so that imbalances in the profile can be seen quickly. The horizontal line through the MEAN represents the population mean for each variable, and is used as the base-line. The left-hand vertical axis is divided up into half standard deviation units. The standard deviation, of course, has a different numerical value for each variable, but, by expressing differences between sample and population means in terms of standard deviations, departures from normality can be compared for all variables on the same scale. The horizontal sections of the histograms give the positions of sample means relative to the base-line. A group with as few as 5 cows sampled at random can be considered to be normal with respect to a particular variable if the mean value lies within 1 S.D. of the mean and the herd is no

more variable than average. The further the histogram is above or below 1 S.D. from the base-line the greater the deviation from normality. A mean value falling 2 S.D. below the mean, for example, implies that about half the cows in the group have values below the 95% confidence limit for that variable.

| GROUP | NO. | PCV | GLU | UREA | IP | CA | MG | NA | K | TP | ALB | GLOB | HB |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 7 | 33·57 | 43·29 | 23·93 | 6·64 | 9·37 | 2·39 | 140·50 | 5·71 | 7·42 | 4·81 | 2·61 | 13·67 |
| 2 | 7 | 32·43 | 46·43 | 21·21 | 6·40 | 9·23 | 2·10 | 141·14 | 5·76 | 7·72 | 4·79 | 2·94 | 13·59 |
| 3 | 7 | 30·14 | 45·71 | 19·29 | 6·46 | 9·59 | 2·17 | 140·21 | 6·06 | 7·79 | 4·69 | 3·09 | 12·17 |
| MEAN | 21 | 32·05 | 45·14 | 21·48 | 6·50 | 9·40 | 2·22 | 140·62 | 5·84 | 7·64 | 4·76 | 2·88 | 13·14 |
| POP. MEAN | | 29·00 | 45·00 | 15·00 | 5·40 | 9·30 | 2·58 | 139·00 | 4·80 | 7·10 | 3·30 | 3·80 | 12·20 |
| SAMPLESD | | 2·12 | 4·61 | 2·39 | 0·84 | 0·41 | 0·33 | 1·31 | 0·55 | 0·65 | 0·53 | 0·82 | 0·69 |
| POP.SD | | 3·00 | 4·50 | 2·50 | 0·90 | 0·50 | 0·27 | 2·00 | 0·40 | 0·50 | 0·30 | 0·60 | 1·20 |

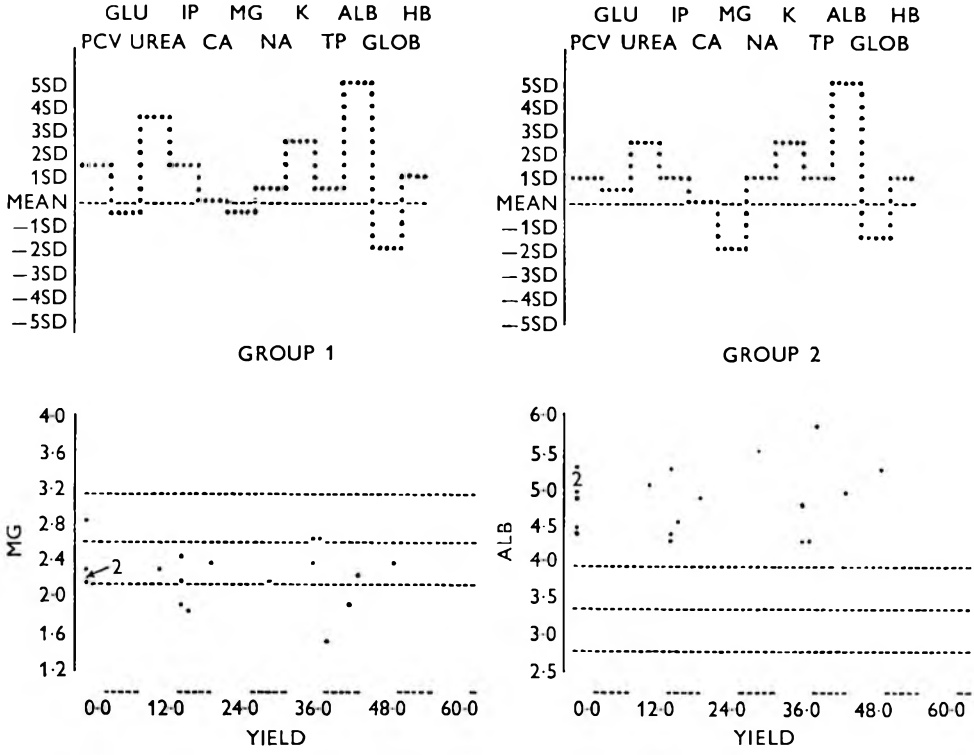


Fig. 1. A selection of the computer print-out for herd 16 – a relatively hypomagnesaemic herd.

*Scatter diagrams*

The histograms are followed by scatter diagrams which demonstrate how the results for individual cows are distributed about their population means, so that it can be seen, for example, how many tend to have extreme concentrations of a particular variable. Each individual variate is plotted against that animal's daily milk yield. A scatter diagram is produced for each variable. Each diagram contains 3 horizontal lines, the central line corresponding to the population mean, the upper and lower to the mean ± 2 population standard deviations. For a 'normal' herd the majority of points should be distributed within this band. By chance an average of about 1 in 20 points will fall outside.

*Examples*

In Figs 1–3 the computer print-out described so far is illustrated for 3 of the herds 16, 22 and 25 discussed by Payne *et al.* Two scatter diagrams and the histograms for groups 1 and 2 are selected in each case for demonstration. The histograms for group 3 (maximum yielding cows) were in each case similar to the histograms for group 2 (intermediate yielding cows).

| GROUP | NO. | PCV | GLU | UREA | IP | CA | MG | NA | K | TP | ALB | GLOB | HB |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 7 | 27·71 | 34·29 | 14·21 | 6·07 | 9·13 | 2·06 | 138·43 | 4·46 | 7·89 | 2·91 | 4·84 | 11·91 |
| 2 | 7 | 25·43 | 39·43 | 12·43 | 5·64 | 9·19 | 2·14 | 139·21 | 4·66 | 7·91 | 2·86 | 5·04 | 10·80 |
| 3 | 7 | 26·14 | 38·86 | 13·29 | 5·49 | 9·04 | 2·08 | 138·64 | 4·42 | 7·71 | 2·89 | 4·82 | 10·94 |
| MEAN | 21 | 26·43 | 37·52 | 13·31 | 5·73 | 9·12 | 2·09 | 138·76 | 4·51 | 7·83 | 2·89 | 4·90 | 11·22 |
| POP. MEAN | | 29·00 | 45·00 | 15·00 | 5·40 | 9·30 | 2·58 | 139·00 | 4·80 | 7·10 | 3·30 | 3·80 | 12·20 |
| SAMPLESD | | 2·85 | 3·91 | 1·66 | 1·02 | 0·27 | 0·25 | 1·50 | 0·41 | 0·72 | 0·29 | 0·71 | 1·19 |
| POP.SD | | 3·00 | 4·50 | 2·50 | 0·90 | 0·50 | 0·27 | 2·00 | 0·40 | 0·50 | 0·30 | 0·60 | 1·20 |

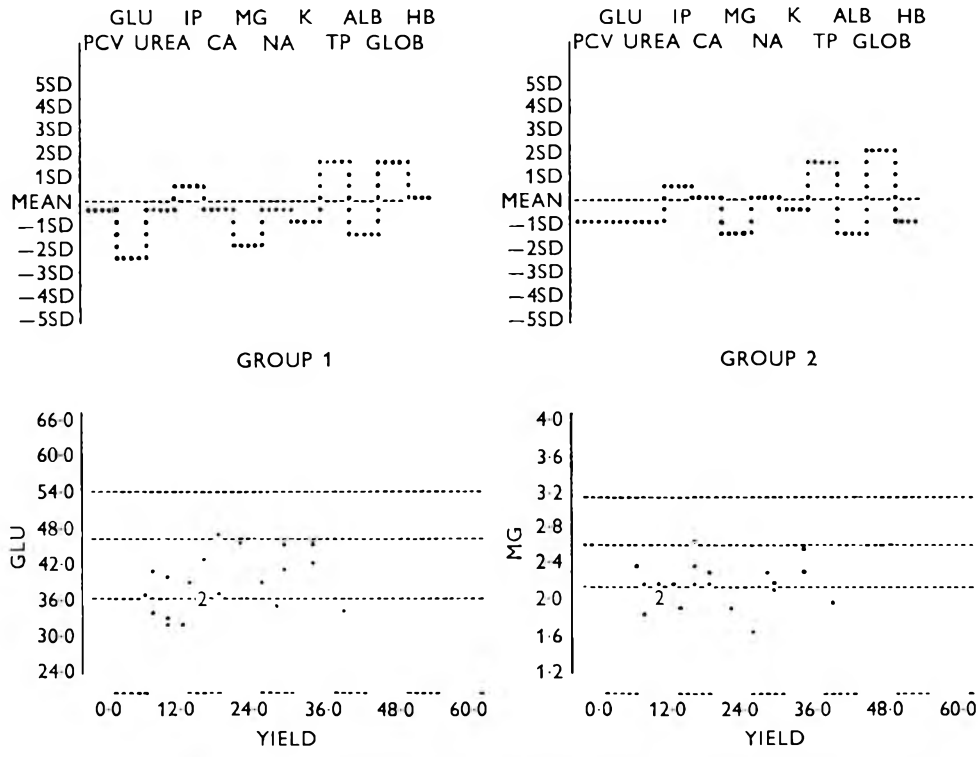


Fig. 2. A selection of the computer print-out for herd 22 – a Jersey herd with generally inadequate diet and high incidence of ketosis and milk fever.

*Herd 16* was reported to be suffering from an unusually large number of cases of milk fever. The histograms (Fig. 1) show this to be a relatively hypomagnesaemic herd, and the departure from normality to be most pronounced for milking cows (groups 2 and 3). Of the magnesium concentrations all but 2 were below average for the milking cows, and half the remainder were outside the lower 2 standard deviation limit. The histograms also show that concentrations of some of the other variables

were abnormally high. The scatter diagram for albumin, for example, shows that in every instance concentrations were above normal. This could in part be explained by the fact that the herd had been grazing on highly fertilized pasture. The computer print-out demonstrates very clearly the abnormality of the profile of this herd.

| GROUP | NO. | PCV | GLU | UREA | IP | CA | MG | NA | K | TP | ALB | GLOB | HB |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 6 | 34·00 | 44·33 | 12·33 | 5·62 | 9·82 | 2·33 | 140·50 | 4·48 | 8·43 | 3·26 | 5·17 | 13·30 |
| 2 | 7 | 32·00 | 50·00 | 11·86 | 5·44 | 9·13 | 2·54 | 139·00 | 4·60 | 7·90 | 3·46 | 4·44 | 12·44 |
| 3 | 7 | 29·14 | 50·29 | 9·31 | 5·87 | 9·80 | 2·59 | 139·21 | 4·87 | 8·24 | 3·39 | 4·84 | 11·47 |
| MEAN | 20 | 31·60 | 48·40 | 11·11 | 5·64 | 9·57 | 2·49 | 139·52 | 4·66 | 8·18 | 3·38 | 4·80 | 12·36 |
| POP. MEAN | | 29·00 | 45·00 | 15·00 | 5·40 | 9·30 | 2·58 | 139·00 | 4·80 | 7·10 | 3·30 | 3·80 | 12·20 |
| SAMPLESD | | 2·04 | 3·59 | 2·19 | 0·67 | 0·57 | 0·26 | 1·52 | 0·40 | 0·48 | 0·35 | 0·75 | 0·73 |
| POP.SD | | 3·00 | 4·50 | 2·50 | 0·90 | 0·50 | 0·27 | 2·00 | 0·40 | 0·50 | 0·30 | 0·60 | 1·20 |

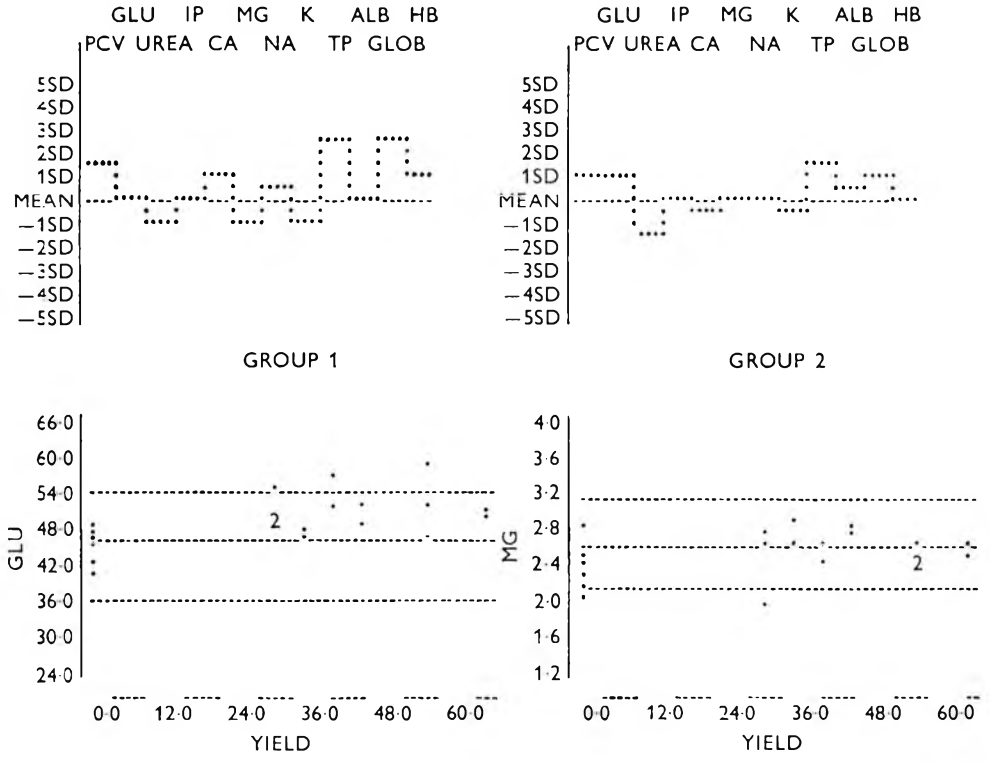


Fig. 3. A selection of the computer print-out for Herd 25 – a high-yielding and exceptionally well-managed Friesian herd.

*Herd 22* (Fig. 2) is chosen to demonstrate a herd that was deficient in energy, magnesium and protein. The histograms show this with the means for blood glucose, magnesium and albumin all 1 S.D. or more below the mean. The scatter diagrams for magnesium and glucose highlight the situation. Deductions from the computer print-out corresponded with nutritional deficiencies subsequently found in the herd.

*Herd 25* (Fig. 3) was one of the more 'normal' herds tested. Apart from urea concentrations which were lower than average, the histograms for the milking cows (groups 2 and 3) demonstrate an almost 'ideal' profile. The profile for dry cows (group 1) was good too, with only the magnesium values tending to be a little low. Examina-

tion of the scatter diagram for magnesium, however, indicates little abnormality. Glucose concentrations for milking cows are above average, and the inorganic phosphorus concentrations are normal. To quote Payne *et al.* (1970): 'Herd 25 illustrates an almost ideal profile. Diets were carefully planned for high production, there were few clinical problems...The few animals falling outside the confidence limits suggest generous feeding as opposed to any imbalance.'

*Abnormal cows*

The herd data are listed in the computer print-out, so that individual cow values can be looked at more closely. This is followed by a list of those cows which have one or more variates further than $\pm 3$ standard deviations from the population means.

Table 2. *Cows in herd 22 with exceptionally low* (−) *and exceptionally high* (+) *levels more than 3 standard deviations from the population means*

| | PCV | GLUC | UREA | IP | CA | MG | NA | K | TP | ALB | GLOB | HB |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CORVETTE | . | . | . | . | . | . | . | . | . | − | + | . |
| BIANCA | . | − | . | . | . | . | . | . | . | . | . | . |
| CALUMET | . | . | . | . | . | . | . | . | . | . | + | . |
| SKYSCAPE | . | . | . | . | . | . | . | . | . | . | + | . |
| KATRINA | . | . | . | . | . | . | . | . | + | . | + | . |
| MAGIC | . | . | . | . | . | . | . | . | + | . | . | . |
| FORGETFUL | . | . | . | − | . | . | . | . | . | . | . | . |
| ROCHELLE | . | . | . | . | . | − | . | . | + | . | + | . |

Table 2 illustrates the form of print-out for herd 22. Whenever a concentration lies outside these limits a '+' (for above) or a '−' (for below) is printed in its appropriate column. When a herd metabolic imbalance is demonstrated by the histograms, this table identifies those cows for which the imbalance is most pronounced. The table also identifies cows that may be out of line with the rest of the herd. The farmer might be able to suggest why such a cow might be exceptional; for example, perhaps it has always been a poor yielder, or was particularly susceptible to ketosis.

In Table 2 cows Bianca and Rochelle are the extreme examples of a general deficiency in blood glucose and magnesium respectively in herd 22. In addition to glucose Bianca had a low packed cell volume and low magnesium, albumin and haemoglobin concentrations (Table 1), all more than 2 standard deviations below the population mean. The extremely low concentration of inorganic phosphorus for Forgetful is completely out of line with the rest of the herd. Otherwise, apart from albumin, this cow's profile was within the 2 standard deviation limits, so that the inorganic phosphorus value could well be due to an error in recording.

*Estimate of percentage number abnormal in herd*

It is quite by chance that any particular cow is selected in the sample, and although extra information on individual animals might be helpful, this is not the aim of the metabolic profile technique. Rather it is a method for assessing the normality of the whole herd in terms of the blood measurements made. Thus, in addition to identifying particular abnormal animals in the sample, the program gives for each variable estimates of the total numbers of cows in the groups likely to deviate from normality

by more than 3 standard deviations. Table 3 demonstrates this, giving estimates for herd 22 of the number of cows with abnormally low concentrations for 3 of the variables – blood glucose, albumin and magnesium. For glucose, 24% of the cows in group 1 were estimated as having abnormally low concentrations, and about 10% of the cows throughout the herd gave abnormally low magnesium concentrations.

Table 3. *Estimated percentages of cows in herd 22 with abnormal concentrations more than 3 standard deviations below population means*

| Group | Glucose | Magnesium | Albumin |
|---|---|---|---|
| 1 | 24 | 12 | 4 |
| 2 | 2 | 7 | 6 |
| 3 | 3 | 11 | 5 |

## DISCUSSION

The purpose of the metabolic profile test is to compare a herd's profile with the national average for clinically normal animals, not with other herds. The confidence limits need to be based on standard deviations that represent variation only among the individual animals in a herd, not among individuals in the population at large. It is emphasized, therefore, that in the calculation of the population standard deviations differences among the 13 herds have been excluded. Thus, in the interpretation of the scatter diagrams, animals in a 'normal' herd will be distributed with about 19 out of 20 within the 95% confidence bands. If the standard deviations had been calculated ignoring differences among herds, the confidence bands would be wider, and it would not be possible to say how many cows might by chance lie outside.

The 2 standard deviation limits (approximately 95% confidence limits) used in the scatter diagrams provide a means of giving advanced warning of possible metabolic disorders that might develop, the more points falling outside these limits, the more abnormal the herd's profile. This will also be reflected in the positions of the mean values in the histograms relative to the base-line. However, it is emphasized that the cows outside the 2 standard deviation limits are not necessarily abnormal cows in the clinical sense. To distinguish between clinically normal and abnormal cows a wider range is needed. This is the aim of the 3 standard deviation limits (approximately 99·9% confidence limits). Future experience will suggest whether on clinical grounds these 3 standard deviation ranges are reasonable.

Fifteen blood samples analyzed from 5 dry cows, 5 cows giving intermediate milk yields and 5 cows giving high milk yields, chosen *at random* from the herd, are adequate, even for large groups, to show statistically significant ($P < 0{\cdot}05$) evidence of departure from normality of groups with mean values 1 standard deviation or more from the population average. Mean values within 1 standard deviation of the base-line imply that the herd is normal with respect to the particular variable. Thus, a sample of this size provides an adequate interpretation of the herd's profile, and there is generally little to be gained by increasing the sample. However, interpretation of the results does depend on the sample of cows being chosen at random and an attempt is being made to ensure that this is done; otherwise the sample mean values could

provide biased estimates of the herd's profile. Payne *et al.* (1970) proposed 7 cows/group. From the experience since gained in the application of the test it appears that 5/group may be adopted as a satisfactory working number.

In conclusion, this work demonstrates the valuable contribution that the computer can make in a study of this nature when a large-scale laboratory analysis is involved. The computer print-out is providing a rapid, objective and comprehensive assessment of a herd's metabolic profile in an easily interpretable form.

The computer program is written in FORTRAN IV and is suitable for a computer such as an IBM 360.

We are grateful to Dr J. M. Payne for helpful advice and encouragement given during the course of this work, and to F. B. Leech and Penelope K. Leech of the Statistics Department, Rothamsted Experimental Station, for the processing of the data which provided estimates of the normal values and confidence limits.

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*Printed in Great Britain*

# Some effects of unsaturated oils given to dairy cows with rations of different roughage content

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Summary. Three experiments were conducted to determine the effect of feeding polyunsaturated fish oils to dairy cows receiving rations of high, medium or low roughage content. In the rumen, the decreases in the proportion of acetic acid and increases in propionic acid induced by the oils became greater as the amount of oil given was increased but the magnitude of the response to any dose depended upon the composition of the basal diet. The effects on volatile fatty acids (VFA) proportions of small amounts of the oils (125–150 ml/day) were greatest with the low-roughage rations. With large doses of oil (375–450 ml/day) the responses were variable but it is concluded that, in general, changes in VFA proportions are least with low-roughage rations.

The fat content of milk was more sensitive to dietary oil supplementation than were the rumen VFA proportions. When the unsaturated oils were given there was a decrease in milk fat percentage and an increase in the proportion of unsaturated fatty acids in the fat; there was also increased incorporation of fatty acids with more than 18 carbon atoms in the milk fat. The metabolism in the rumen tended to become adapted to the feeding of 150 ml/day of oil, the VFA pattern returning during the second and third week of supplementation to that observed before the addition of oil.

The inclusion of cod liver oil (CLO) in the ration of ruminants often causes a marked fall in the ratio of acetic acid to propionic acid in the rumen and a reduction in milk fat secretion. The effects on rumen fermentation are probably due to the high content of polyunsaturated long-chain fatty acids in CLO. However, the magnitude of the responses reported in the literature is not consistent. For instance, when Nicholson, Cunningham & Friend (1963) gave 60 ml CLO daily to steers receiving an all-concentrate ration, they found marked decreases in the molar proportions of acetic and butyric acids and an increase in propionic acid. In contrast, Broster, Sutton, Tuck & Balch (1965) found only small effects on the molar proportions of volatile fatty acids (VFA) when over twice as much oil, 150 ml/day, was given to

dairy heifers receiving a ration consisting of approximately 5 kg hay and 2 kg concentrates daily.

The present experiments were designed to determine whether the varying responses of rumen VFA proportions could be attributed to an interaction between the roughage content of the rations and the amount of unsaturated oil given in the diet. In addition, the effect on milk composition was determined in 2 of the 3 experiments.

## EXPERIMENTAL

*Expt 1, dry cows* (Shinfield)

Six dry Friesian cows fitted with rumen cannula were used in a 3 × 3 Latin-square experiment with 2 replicates. The rations were designed to be approximately equal in metabolizable energy and digestible protein content but to give high, medium or low roughage levels (Table 1). The cows were given their concentrate ration at 9.30 h and their roughage allowance at 12.30 h. Cows given only rolled barley received half their daily allowance at each of the above times. Water and salt licks were always available.

Table 1. *Expt 1: composition of the rations, kg/day*

| | Rations | | |
|---|---|---|---|
| Content | High roughage | Medium roughage | Low roughage |
| Barley straw | 6·0 | 0 | 0 |
| Grass hay | 0 | 3·0 | 0 |
| Rolled barley | 1·0 | 2·5 | 5·0 |
| Groundnut meal | 0·5 | 0·5 | 0 |

The cows were allowed 3 weeks to become adjusted to each ration change. Each 3-week adjustment period was then followed by three 1-week periods in which each cow received successively 125, 250 and 375 ml/day of CLO (British Codliver Oils (Hull and Grimsby Ltd), with an iodine value of 162; the CLO was mixed with the concentrates given at 9.30 h. For statistical analysis the oil levels were considered as split plots within the Latin square design. Rumen fluid samples were taken at 9.00, 12.00 and 17.00 h on the last 2 days of each 3-week adjustment period and on the sixth and seventh days of each week when the CLO supplements were given. The pH was determined immediately and the samples stored at −17°C until required for chemical analysis.

*Expt 2, lactating cows* (Fredericton)

Six Friesian and 3 Ayrshire lactating cows were used in a 3 × 3 Latin-square design with 3 replicates. The procedures used were similar to those in expt 1 wherever possible. However, changes had to be made in the ration to provide the energy, protein and minerals required by the lactating cows. The rations fed to the Friesian cows are shown in Table 2. Allowances for the Ayrshires were 85% of those for the Friesians. The cows were fed twice daily at 7.30 h and 16.30 h, with more than half the daily allowance being given at the evening feed. The unsaturated oil used was a whole body fish oil (WBFO) (National Sea Products Ltd, Halifax, N.S.) which had an iodine

value of about 120. The daily allowances of 150, 300 or 450 ml oil were mixed individually with the evening feed. Rumen fluid was obtained by stomach tube at 10.00 h on the last day of each period. Milk production was recorded on the last day of each period. Milk samples were taken at the final evening and morning milkings for the determination of protein, solids-not-fat (SNF), total fat, and fatty acid composition of the fat. All samples were stored frozen.

Table 2. *Expt 2: composition of the rations, kg/day**

| Content | Rations | | |
|---|---|---|---|
| | High roughage | Medium roughage | Low roughage |
| Timothy hay | ad lib.† | 8·00 | 1·00 |
| Soybean meal | 1·00 | 1·20 | 1·15 |
| Ground barley | 0·43 | 6·68 | 11·30 |
| Dicalcium phosphate | 0·07 | 0·12 | 0 |
| Ground limestone | 0 | 0 | 0·15 |

* The allowances shown are for Friesian cows. Ayrshires were given 85% of the Friesian allowances.

† The average daily consumption of hay by the Friesians was 15·2 kg and by the Ayrshires 13·4 kg.

*Expt 3, adaptation in lactating cows* (Fredericton)

At the conclusion of expt 2 the 3 cows that had been receiving the medium-roughage ration continued on this ration for a further 6 weeks. For the first 3 weeks no oil was given but for the second 3-week period the ration was supplemented with 150 ml WBFO daily to determine whether the progressive changes found in expt 2 could be an effect of time rather than of the increasing levels of oil. Rumen fluid and milk were sampled on the last day before introduction of the WBFO and at the end of each 7-day period as in expt 2.

*Chemical analyses*

Rumen fluid samples from expt 1 were analysed for the concentration and molar proportions of VFA using a Varian Aerograph 600-3 gas chromatograph with a hydrogen-flame ionization detector. The VFA were separated on a stainless-steel column (183 × 0·32 cm) packed with 7·5% (w/w) polyethylene glycol 400 monostearate and 0·75% (w/w) orthophosphoric acid on 60/80 mesh acid-washed chromosorb W (Sutton & Johnson, 1969). In expts 2 and 3 a Varian Aerograph Model 2100 gas chromatograph with a detector and column packing similar to the above was used.

Total milk fat was determined by standard Babcock test procedures. The fatty-acid composition was determined with the Model 2100 gas chromatograph fitted with a stainless-steel column (198 × 0·32 cm) packed with 8% (w/w) 1:4 butanedial succinate polyester on acid-washed H.M.D.S. Chromosorb W, 80/100 mesh. Atpet 80 was added to the stationary phase at the rate of 0·5% (w/w) before coating. The milk fat was extracted with chloroform-methanol (2:1, v/v) and methylated by heating it at 100°C for 1 h in a closed tube with sodium methoxide. The resulting esters were

injected into the gas chromatograph immediately upon opening the tube. The peak areas of the chromatograms for the $C_{10}$ and lower fatty acids were converted to weight percentages by the factors of deMan (1964).

## RESULTS

Results in Tables 3–5 are presented both as the mean values for all levels of oil supplementation for each roughage level (roughage level) and as the mean of all roughage levels for each level of oil supplementation (CLO or WBFO level). Results for individual treatments are presented in Figs 1 and 2 and are discussed in the text when interactions between oil and roughage level are considered.

### *Expt 1, dry cows*

As the roughage level in the ration decreased, the pH and the molar per cent of acetic acid decreased in the rumen fluid (Table 3). The concentration of VFA and the molar percentages of propionic and valeric acids increased as the roughage level decreased. The molar percentage of butyric acid was higher for the medium-roughage ration than for either the low- or the high-roughage rations.

Table 3. *Expt 1: mean pH and the concentrations and molar proportions of VFA in rumen fluid*

| | | | VFA, molar % | | | |
|---|---|---|---|---|---|---|
| Roughage level | pH | Total VFA, mmoles/l | Acetic | Propionic | Iso+n-butyric | Iso+n-valeric |
| High | 6·75$^{a}$ | 70·3$^{a}$ | 66·0$^{a}$ | 23·1$^{a}$ | 9·1$^{a}$ | 1·8$^{a}$ |
| Medium | 6·65$^{a}$ | 74·4$^{a}$ | 57·5$^{b}$ | 27·3$^{b}$ | 11·6$^{b}$ | 3·6$^{b}$ |
| Low | 6·10$^{b}$ | 95·7$^{b}$ | 46·7$^{c}$ | 37·9$^{c}$ | 9·7$^{a}$ | 5·7$^{c}$ |
| CLO level, ml/day | | | | | | |
| 0 | 6·59$^{a}$ | 77·8$^{a}$ | 62·8$^{a}$ | 22·9$^{a}$ | 10·9$^{a}$ | 3·4$^{a}$ |
| 125 | 6·53$^{a}$ | 79·2$^{a}$ | 57·4$^{b}$ | 28·8$^{b}$ | 10·1$^{a}$ | 3·8$^{a}$ |
| 250 | 6·36$^{b}$ | 82·5$^{a}$ | 55·2$^{b}$ | 31·1$^{bc}$ | 10·2$^{a}$ | 3·6$^{a}$ |
| 375 | 6·51$^{ab}$ | 81·0$^{a}$ | 51·6$^{c}$ | 35·1$^{c}$ | 9·4$^{a}$ | 4·0$^{a}$ |

Superscripts indicate the significance of differences within columns for roughage levels or CLO levels. Means followed by different superscripts are significantly different ($P < 0·01$).

Increasing the level of CLO did not have a consistent effect on the pH, VFA concentration or molar proportions of butyric and valeric acids in the rumen fluid (Table 3). It did cause significant decreases in the molar proportion of acetic acid and increases in propionic acid. The ratio of acetic to propionic acid dropped from 3·2 when no CLO was given to 1·5 at the highest (375 ml) level of oil supplementation.

Significant ($P < 0·05$) interactions occurred between roughage and CLO levels for molar proportions of acetic (Fig. 1*a*) and propionic acids (Fig. 1*b*). The first increment of CLO (125 ml) caused a marked drop in the proportion of acetic acid with the low- and medium-roughage rations but only a slight drop with the high-roughage ration (Fig. 1*a*). Successive increments of CLO had little additional effect on acetic acid when the low-roughage ration was given. With the medium-roughage ration, the magnitude of the drop in acetic acid with the second and third increments of CLO was about half of that caused by the first increment. With the high-roughage ration

the second increment of CLO caused a marked fall and a further substantial drop occurred with the third increment. Although the initial increment of CLO to the high-roughage ration caused only a small change in the proportion of acetic acid, the overall difference due to feeding the highest level of oil amounted to 13·5 percentage units compared with 14·0 units for the medium-roughage ration and 6·2 units for the low-roughage ration.

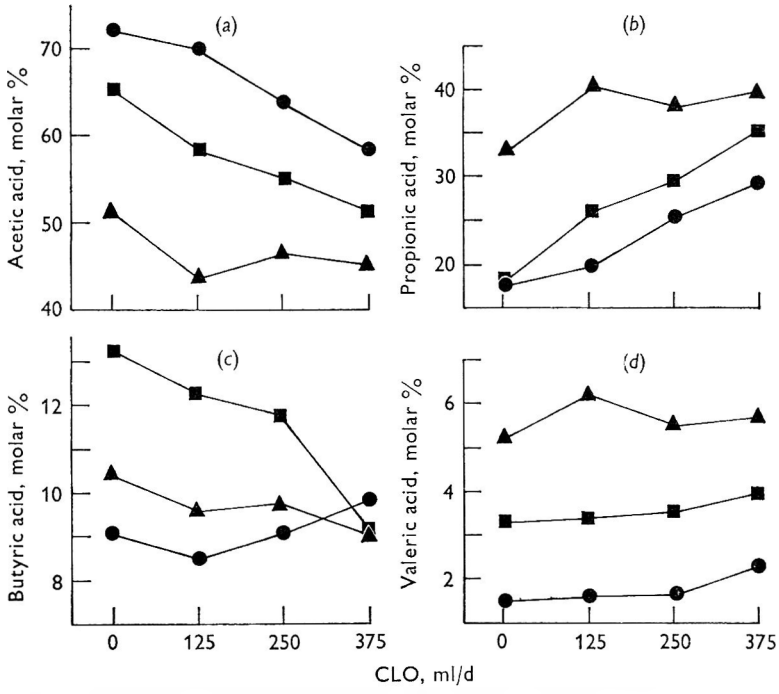


Fig. 1. Expt 1: the interactions between level (ml/day) of CLO administered and molar percentage of acetic acid (*a*), propionic acid (*b*), butyric acid (*c*) and valeric acid (*d*) in rumen fluid of cows receiving rations of high- (●), medium- (■) or low- (▲) roughage levels.

Changes in the molar per cent of propionic acid were generally the reverse of those noted for acetic acid (Fig. 1*b*). The increase in propionic acid due to increasing CLO was greater for the medium-roughage ration (17·5 units) than for the high-roughage ration (12·0 units).

The interaction between roughage and CLO levels failed to reach statistical significance ($P < 0{\cdot}05$) for the change in the proportion of butyric acid although each ration produced a distinctly different pattern of change (Fig. 1*c*). There was little difference between any of the rations in pattern of change for valeric acid (Fig. 1*d*).

*Expt 2, lactating cows*

*Rumen.* Mean values for pH and concentration of VFA in rumen fluid are not given because samples were contaminated with variable amounts of saliva. This contamination does not invalidate the information on relative proportions of the VFA which are presented in Table 4. The changes produced in the proportions of the VFA

by increasing the roughage or WBFO level were all similar in direction to those in expt 1. The proportion of acetic acid tended to be higher and those of propionic and valeric acids lower in expt 2.

Increasing the level of WBFO had a relatively small effect on the proportions of the VFA although the changes were statistically significant ($P < 0{\cdot}01$) for acetic, propionic and butyric acids (Table 4). As in expt 1, proportions of butyric acid were higher ($P < 0{\cdot}01$) on the medium-roughage ration than on the high- or low-roughage rations.

Table 4. *Expt 2: mean proportions of VFA in the rumen fluid and the yield and composition of the milk*

| | VFA, molar % | | | | | Milk Composition, % | | |
|---|---|---|---|---|---|---|---|---|
| Roughage level | Acetic | Propionic | Iso+n-butyric | Iso+n valeric | Milk yield, kg/day | Protein | Fat | SNF |
| High | $70{\cdot}3^{Aa}$ | $19{\cdot}8^{A}$ | $9{\cdot}1^{A}$ | $0{\cdot}9^{A}$ | 15·6 | $3{\cdot}3^{a}$ | $3{\cdot}4^{a}$ | 8·1 |
| Medium | $66{\cdot}4^{Ab}$ | $20{\cdot}0^{A}$ | $12{\cdot}2^{B}$ | $1{\cdot}3^{A}$ | 17·5 | $3{\cdot}1^{b}$ | $3{\cdot}4^{a}$ | 8·1 |
| Low | $54{\cdot}8^{B}$ | $33{\cdot}4^{B}$ | $9{\cdot}0^{A}$ | $2{\cdot}7^{B}$ | 15·2 | $3{\cdot}4^{a}$ | $2{\cdot}7^{b}$ | 8·1 |
| WBFO level ml/day | | | | | | | | |
| 0 | $66{\cdot}1^{A}$ | $20{\cdot}8^{A}$ | $11{\cdot}3^{Aa}$ | 1·6 | 16·6 | $3{\cdot}5^{Aa}$ | $3{\cdot}8^{A}$ | $8{\cdot}2^{Aa}$ |
| 150 | $65{\cdot}6^{A}$ | $22{\cdot}1^{A}$ | $10{\cdot}7^{Aa}$ | 1·6 | 16·6 | $3{\cdot}2^{Bbc}$ | $3{\cdot}4^{B}$ | $8{\cdot}1^{Aab}$ |
| 300 | $62{\cdot}1^{B}$ | $26{\cdot}2^{B}$ | $10{\cdot}0^{Ab}$ | 1·7 | 16·0 | $3{\cdot}1^{Bc}$ | $2{\cdot}9^{C}$ | $8{\cdot}1^{ABb}$ |
| 450 | $61{\cdot}6^{B}$ | $28{\cdot}5^{C}$ | $8{\cdot}3^{B}$ | 1·6 | 15·2 | $3{\cdot}3^{ABb}$ | $2{\cdot}5^{D}$ | $8{\cdot}0^{Bc}$ |

Superscripts indicate the significance of differences within columns for roughage levels or WBFO levels. Means followed by different superscripts are significantly different at $P < 0{\cdot}05$ for upper case ($A$, $B$, $C$) or $P < 0{\cdot}01$ for lower case ($a$, $b$, $c$).

There were significant ($P < 0{\cdot}01$) interactions between rations and WBFO levels for acetic acid and propionic acid as illustrated in Fig. 2(*a*) and (*b*) respectively. The inclusion of WBFO in the high-roughage ration did not change the proportion of acetic acid. The first increment of WBFO had no effect with the medium-roughage ration but the second increment caused an appreciable decline of 3·5 units. With the low-roughage ration, there was an appreciable decline due to each of the first 2 increments of CLO, but no further decline resulted from the third increment. The change in propionic acid was the inverse of that in acetic acid except that the proportion of propionic acid continued to increase with the third increment of WBFO on the low-roughage ration.

*Milk.* The roughage level of the ration had little effect on the protein or SNF contents of the milk. The milk fat percentage was lower ($P < 0{\cdot}05$) for the low-roughage ration than for the other 2 rations (Table 4). The average daily milk yield of all the cows was 16·1 kg. Because of the short time periods between treatment changes and the sequential increase in WBFO levels, little significance can be attached to differences in yield between treatments. The protein content of the milk decreased ($P < 0{\cdot}01$) from 3·5% at the 0 level of WBFO to 3·1% at the 300 ml level but increased ($P < 0{\cdot}05$) to 3·3% at the 450 ml level. The percentage of fat and SNF in the milk decreased with each successive increment of WBFO.

The significant ($P < 0{\cdot}05$) interaction between ration and WBFO level for milk

fat (Fig. 2*c*) was the result of a much sharper drop (0·7 unit) caused by the first increment of WBFO with the low-roughage ration compared with much smaller decreases with the other 2 rations (0·2 unit). The second and third increments of WBFO each caused decreases of 0·5 and 0·6% unit with the high- and medium-roughage rations while with the low-roughage ration the decreases were 0·5 and 0·1 unit.

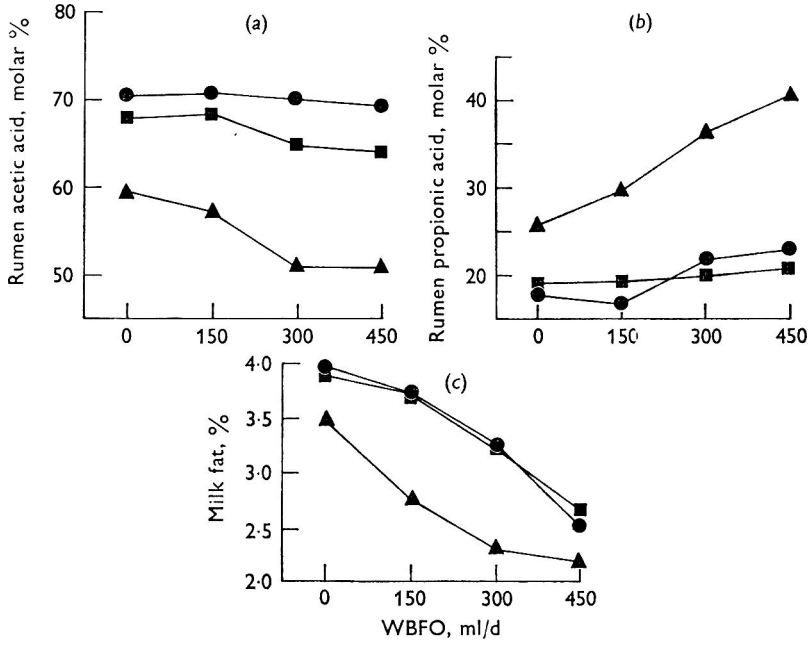


Fig. 2. Expt 2: the interactions between level (ml/day) of WBFO administered and molar percentage of acetic acid (*a*) and propionic acid (*b*) in rumen fluid, and fat content (%) in milk (*c*) of cows receiving rations of high- (●), medium- (■) or low- (▲) roughage levels.

Variation in the roughage level in the ration did not produce any consistent change in the major fatty acid composition of the milk fat (Table 5). Increasing the level of WBFO did cause a consistent decrease in the weight percentages of the saturated acids, except $C_{15}$, and an increase in the unsaturated acids, except $C_{18:2}$. Longer-chain fatty acids, tentatively identified as $C_{20:0}$ and $C_{20:4}$, were observed in increasing amounts as the level of WBFO in the diet increased. At the highest level of WBFO these acids made up as much as 10% of the fatty acids in some milk samples. The results for these longer-chain acids were not included in the statistical analyses because several samples contained them in insufficient amounts for accurate analysis.

*Expt 3, adaptation in lactating cows*

The introduction of 150 ml/day of WBFO into the diet resulted in a depression of acetic acid and an increase in propionic acid at the end of the first week (Table 6). There was also a depression in milk fat percentage and an increase in the weight percentage of unsaturated fatty acids in the milk fat. When the cows were continued on this same level of WBFO for 2 more weeks, the rumen VFA pattern returned to that before the addition of WBFO but the amount of unsaturated fatty acids in the milk

Table 5. *Expt 2: mean weight percentages of the major fatty acids in the milk fat*

| Fatty acid | Roughage level | | | WBFO level, ml/day | | | |
|---|---|---|---|---|---|---|---|
| | High | Medium | Low | 0 | 150 | 300 | 450 |
| 4:0 | 1·8 | 2·2 | 1·6 | 1·9 | 1·8 | 1·9 | 1·8 |
| 6:0 | $2·7^{A}$ | $2·8^{A}$ | $2·2^{B}$ | $2·8^{A}$ | $2·8^{A}$ | $2·5^{B}$ | $2·1^{C}$ |
| 8:0 | 1·5 | 1·7 | 1·4 | $1·7^{A}$ | $1·8^{A}$ | $1·5^{B}$ | $1·2^{C}$ |
| 10:0 | $3·1^{a}$ | $3·8^{b}$ | $3·3^{a}$ | $4·0^{Aa}$ | $3·6^{ABb}$ | $3·3^{Bb}$ | $2·7^{C}$ |
| 12:0 | $4·0^{a}$ | $4·8^{b}$ | $4·9^{b}$ | $5·0^{a}$ | $4·7^{ab}$ | $4·4^{b}$ | $4·2^{b}$ |
| 14:0 | 15·3 | 15·7 | 14·7 | 15·5 | 15·4 | 15·2 | 14·9 |
| 14:1 | $1·4^{a}$ | $1·6^{a}$ | $2·0^{b}$ | $1·4^{Aa}$ | $1·6^{ABbc}$ | $1·7^{BCc}$ | $1·9^{Cd}$ |
| 15:0 | $1·8^{a}$ | $1·3^{b}$ | $2·0^{a}$ | $1·4^{Aa}$ | $1·6^{ABb}$ | $1·8^{BCc}$ | $1·9^{Cc}$ |
| 16:0 | 36·0 | 38·3 | 36·6 | $39·1^{Aa}$ | $37·4^{ABb}$ | $36·6^{BCb}$ | $34·8^{Cc}$ |
| 16:1 | $2·5^{AB}$ | $2·2^{A}$ | $3·0^{B}$ | $1·7^{A}$ | $2·2^{B}$ | $2·9^{C}$ | $3·5^{D}$ |
| 18:0 | 1·2 | 1·0 | 0·8 | $1·4^{A}$ | $1·9^{A}$ | $0·6^{B}$ | $0·2^{B}$ |
| 18:1 | 23·2 | 19·4 | 23·2 | $19·9^{Aa}$ | $20·8^{Aab}$ | $22·7^{ABbc}$ | $24·3^{Bc}$ |
| 18:2 | $0·8^{A}$ | $1·4^{B}$ | $1·7^{C}$ | $1·7^{A}$ | $1·3^{Ba}$ | $1·1^{Bab}$ | $1·0^{Bb}$ |
| 18:3 | $2·0^{a}$ | $1·8^{a}$ | $1·1^{b}$ | $0·2^{Aa}$ | $0·7^{ABa}$ | $1·8^{Bb}$ | $3·8^{C}$ |
| Total unsat. | $29·8^{ab}$ | $26·3^{a}$ | $31·0^{b}$ | $24·8^{A}$ | $26·7^{A}$ | $30·4^{B}$ | $34·4^{C}$ |

Superscripts indicate significance of differences within lines for roughage levels or WBFO levels. Means followed by different superscripts are significantly different at $P < 0·05$ for upper case ($A$, $B$, $C$) or $P < 0·01$ for lower case ($a$, $b$, $c$).

Table 6. *Expt 3: mean proportions of VFA in the rumen fluid and composition of the milk and of the milk fat*

| | Week | | | |
|---|---|---|---|---|
| | 0* | 1 | 2 | 3 |
| | VFA, molar % | | | |
| Acetic | 70·4 | 64·7 | 71·7 | 69·0 |
| Propionic | 16·8 | 20·7 | 16·4 | 17·3 |
| Iso + n-butyric | 11·6 | 12·6 | 11·0 | 12·1 |
| Iso + n-valeric | 1·2 | 2·0 | 1·0 | 1·6 |
| | Milk composition, % | | | |
| Protein | 3·5 | 3·4 | 3·4 | 3·5 |
| Fat | 3·5 | 3·1 | 3·6 | 3·2 |
| SNF | 8·1 | 8·0 | 8·0 | 8·1 |
| | Weight percentages of major fatty acids in milk fat | | | |
| 4:0 | 3·7 | 2·2 | 2·6 | 3·5 |
| 6:0 | 3·0 | 3·5 | 3·4 | 3·6 |
| 8:0 | 1·9 | 2·0 | 2·0 | 2·1 |
| 10:0 | 5·0 | 4·3 | 5·4 | 4·2 |
| 12:0 | 6·2 | 6·6 | 6·9 | 5·9 |
| 14:0 | 16·8 | 18·9 | 18·5 | 17·7 |
| 14:1 | 1·2 | 1·4 | 1·5 | 1·6 |
| 15:0 | 1·3 | 1·3 | 1·3 | 1·3 |
| 16:0 | 42·6 | 40·4 | 37·0 | 36·8 |
| 16:1 | 1·9 | 1·3 | 1·1 | 1·5 |
| 18:0 | 1·7 | 0·7 | 0·7 | 2·7 |
| 18:1 | 11·8 | 15·6 | 15·6 | 17·4 |
| 18:2 | 0·7 | 1·1 | 1·3 | 0·9 |
| Total unsaturated | 15·6 | 19·4 | 19·4 | 21·4 |

* Week 0 refers to the sample taken before introducing WBFO into the ration. Weeks 1, 2 and 3 refer to samples taken after 1, 2 or 3 weeks during which 150 ml/day of WBFO were given.

fat remained elevated. The variation in milk fat percentage in the last 2 weeks probably reflected the small number of cows in the experiment rather than any treatment effect. There was not a progressive change with time in any of the factors studied with the possible exception of the proportion of unsaturated fatty acids in the milk fat.

## DISCUSSION

The results of expts 1 and 2 showed that changes in the proportions of rumen VFA, induced by the inclusion of fish oils in the ration, increased with the amount of oil given but that the size of the response to any given amount of oil depended on the composition of the basal ration.

By analogy with the effect of linseed oil on rumen fermentation (Czerkawski, Blaxter & Wainman, 1966) it seems likely that fish oils alter rumen VFA proportions because of their high content of polyunsaturated long-chain fatty acids, and that the changes induced depend on the maximum concentration of oil in the rumen rather than on the average concentration over the day. Thus, a single dose each day has been found to be more effective than the same amount of oil given continuously over the day (Moore, Hoffman & Berry, 1945).

In expts 1 and 2 the effect of the lowest level of oil supplementation on VFA proportions was greatest with the low-roughage rations. However, further comparison of the effects of the oils on VFA proportions shows considerable disagreement between the results of the 2 experiments. This disagreement probably reflects mainly the differences between the experiments in the amount and type of oil given and in the amount and composition of the basal rations. If one takes into account the lower iodine value of the oil used in expt 2, the amount of unsaturated oil given per unit of food consumed at the highest level of oil supplementation in expt 2 was only a little greater than that achieved with the lowest level of supplementation in expt 1. When the experiments are compared in these terms the changes in VFA proportions are similar. For instance, the reductions in the proportion of acetic acid caused by the addition of 125 ml CLO in expt 1 or 450 ml WBFO in expt 2 were 1·7 and 1·2% units, respectively, for the high-roughage rations, 7·0 and 3·8 for the medium-roughage rations, and 7·6 and 8·7 for the low-roughage rations.

Although with low levels of oil supplementation, of the order of 20 ml CLO/kg air-dry food, the effects on VFA proportions were greatest with the low-roughage rations, with the relatively higher levels of supplementation, up to about 60 ml CLO/kg food, achieved in expt 1 the changes in the proportions of both acetate and propionate were less for the low-roughage ration than for the medium- and high-roughage rations. Thus, the arguments of Demeyer, Van Nevel, Henderickx & Martin (1969) that the effects on rumen fermentation of unsaturated oils would be least with rations containing large amounts of readily fermentable carbohydrate appear to apply only when large amounts of oil are given.

Fat content of milk appears to be more sensitive to change by supplementation of the diet with fish oil than are the rumen VFA proportions. In expt 2 increasing levels of oil had only a small effect on the proportions of acetic or propionic acids with the high-roughage ration, but caused a marked drop in milk fat secretion. Similar results were obtained by Beitz & Davis (1964). A depression of milk fat secretion has also

been induced by intravenous infusion of CLO (Storry, Hall, Tuckley & Millard, 1969). A full explanation of these various observations has yet to be made. There is good reason to believe that fish oils can cause a depression in milk fat secretion by some means in addition to their effect on rumen VFA proportions but Davis & Brown (1970) have recently disputed the conclusions of Hilditch & Thompson (1936) and Storry *et al.* (1969) that the alternative site of action is within the udder.

The changes that occurred in the fatty-acid composition of the milk fat confirmed other observations (Hilditch & Thompson, 1936; Brown, Stull & Stott, 1962; Beitz & Davis, 1964) that, despite the hydrogenation that occurs in the rumen, the inclusion of unsaturated oils in the ration results in an increase in the proportion of unsaturated fatty acids in the milk fat. The results show also that dietary acids of chain-lengths greater than 18-C can be incorporated into milk fat, supporting the early results of Hilditch & Thompson (1936).

Periods of 1 week for studying the effect of each oil level are unusually short compared with the length of periods normally used in lactation experiments, but Shaw & Ensor (1959) showed that the response of rumen fermentation and milk fat secretion to the inclusion of CLO in the ration is very rapid. The results of expt 3 confirm the speed of response, but also show that adaptation may occur after the first week. That such adaptation can occur may well provide a partial explanation for the different responses to CLO supplementation reported in the literature but it clearly does not occur in all experiments. Petersen (1932), for example, observed an increasing depression of milk fat content up to 20 days after starting to feed CLO. More needs to be known about the factors that affect adaptation before a full explanation can be offered.

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# Concentration of milk by ultrafiltration and reverse osmosis

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Summary. Whole milk from cows was concentrated 2-fold by reverse osmosis in a small pilot scale apparatus. All the main constituents of the milk were retained in the concentrate.

The experiment showed that protein was a major cause of blocking of the membrane. Laboratory trials were therefore made on the ultrafiltration of milk to remove the protein with the aim of developing a 2-stage process for the concentration of milk by ultrafiltration followed by reverse osmosis with subsequent recombination of the concentrates from the 2 operations. The rates of both processes and the retentions of the constituents of milk by the membranes were measured.

The processes of ultrafiltration and reverse osmosis offer the attractive prospect of concentrating milk at ambient temperatures, and so of avoiding chemical damage and flavour changes caused by the heating employed in other methods of concentration. So far the application of these methods to dairy products has been focused mainly on whey (Marshall, Dunkley & Lowe, 1968; McDonough & Mattingly, 1970) with the aim of recovering protein and lactose and at the same time of reducing the biological oxygen demand of the whey before disposal of the effluent (Horton, Goldsmith, Hossain & Zall, 1970). Milk has been concentrated by factors of 2·5 (Nielsen, Olsen, Neilsen & Madsen, 1970) and 3·6 (Coton, Clark & Fraser, 1970), but no analysis of the products was given.

Ultrafiltration is distinguished by the use of membranes having relatively open structures, which allow the passage of molecules of all sizes up to the pore size of the membranes. Hence, it is a process for separating solutes of different molecular weights according to the membrane employed. The driving forces are usually low pressures in the region of 0·2–0·5 MN $m^{-2}$ (30–70 lb/$in^2$). The membranes employed in reverse osmosis have a much more closely knit structure which blocks the passage of most solute molecules, whilst solvent is free to pass through under a pressure in excess of the osmotic pressure of the solution being treated. Working pressures are usually in the region of 4 MN $m^{-2}$ (600 lb/in.$^2$).

In the present study separate experiments were carried out on whole milk, and also on skim-milk and whey, using these 2 methods in order to make a preliminary assessment of their potentialities.

## MATERIALS AND METHODS

### *Materials*

The materials were prepared from bulk raw milk from the Institute's British Friesian herd on the day before the ultrafiltration experiments were done and stored frozen in a refrigerator overnight. The milk was not pasteurized. Skim-milk, containing less than 0·05 % fat, was prepared from the raw milk by warming to 32°C and centrifuging at about 5000 *g*. Rennet whey was obtained from the Institute Dairy. It was a by product of the Cheddar cheese-making process and was drawn off after scalding the curd at 39°C.

### *Ultrafiltration*

Ultrafiltration was done on the small laboratory-scale apparatus (Dorr Oliver Co. Ltd, Croydon, Surrey) illustrated in Fig. 1, using 180-ml samples. The membrane was in the form of a flat sheet, designated as type XPA, having a nominal cut-off at mol.

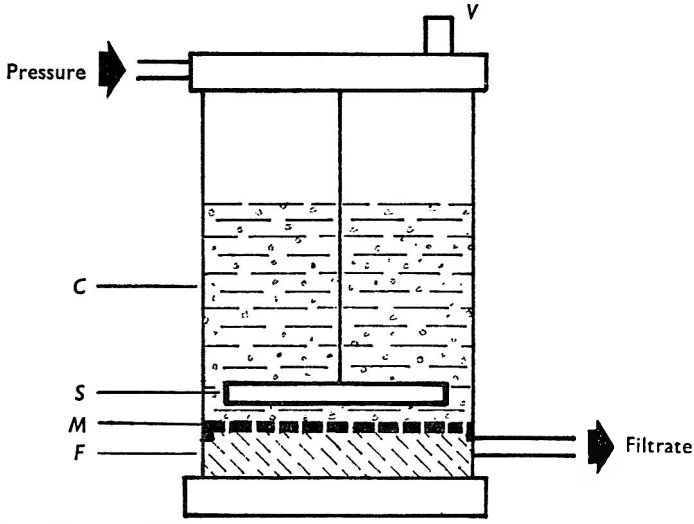


Fig. 1. Ultrafiltration apparatus–laboratory scale. *C*, concentrate; *F*, filtrate; *M*, membrane, mounted on porous plastic supporting disc; *S*, magnetic stirrer; *V*, filling tube and relief valve. Height 15 cm, diam. 7 cm; membrane area, 28 $cm^2$; volume of sample, 180 ml; pressure, 0·2 MN $m^{-2}$, from gas cylinder.

wt. 45000. This value may have been only approximate since it was found that another membrane having a nominal cut-off at mol. wt. 12000 had much the same permeation rate for milk. Pressure was applied from a nitrogen cylinder at 0·2 MN $m^{-2}$ (30 lb/$in.^2$) and the experiments were carried out at temperatures between 25 and 37°C.

### *Reverse osmosis*

The reverse osmosis experiments were conducted on a much larger scale, using a membrane assembly manufactured by Patterson Candy International Ltd, Whitchurch, Hants (Fig. 2). The membrane of cellulose acetate on a fibre support lined the inside of 7 perforated stainless-steel tubes, each 1·3 cm in int. diam. and 250 cm

long, mounted in series inside a plastic tube. As the milk flowed through the tubes under a pressure of about 4·1 MN $m^{-2}$ (600 lb/in.$^2$), water passed through the membrane radially out of the tubes into the outer plastic case. For experimental purposes the concentrate was recirculated so that the sample passed in closed circuit many times over the membrane. Volumes of 45 l were used in each experiment.

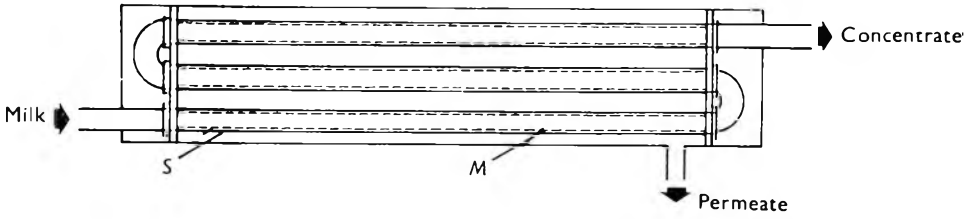


Fig. 2. Reverse osmosis module PCI type B1; pilot plant scale schematic diagram. Seven tubes, 250 cm long and 1·3 cm in int. diam; area of membrane, 0·5 $m^2$; *S*, perforated steel tube; *M*, membrane on fibre liner. Module connected in circuit with pump and control valves. Volume of sample in apparatus, 11 l; flow rate, 9 l/min; pressure, 4·1 MN $m^{-2}$.

In the experiments on the concentration of milk, skim-milk and whey, P.C.I. type 'B' 20/15 membrane was used with a quoted performance of 95 % salt rejection and a flow rate of 0·75 $m^3$ of permeate per $m^2$ of membrane per day from a 0·5 % sodium chloride solution at 25°C under a pressure of 4·1 MN $m^{-2}$ (600 lb/in.$^2$). In the experiment to test for the retention of vitamins, the membrane used was type 'B' (10/15), having a 90 % salt rejection and a permeate flow-rate of 0·75 $m^3$ per $m^2$ per day under the same conditions. With tap water containing about 350 ppm of total dissolved solids, at 25°C and 3·8 MN $m^{-2}$ (550 lb/in.$^2$) pressure, these membranes gave permeate flow rates of 0·9 $m^3$ per $m^2$ per day and 0·7 $m^3$ per $m^2$ per day respectively. Permeate flow rates were measured at intervals throughout the period of each experiment since it was expected that the rate of filtration would decrease with time.

### *Analysis of products*

For the chemical analyses one sample of the whole volume of the milk was taken before the experiment, and one sample from the final concentrate and one sample from the permeate at the end of the experiment. Total solids were determined by the method of Rowland (1957), fat by the Gerber method (British Standards Institution, 1969), nitrogen by the conventional macro Kjeldahl method employing selenium catalyst, and lactose by the infra-red absorption method of Goulden (1964). The methods of vitamin assay were all as described by Ford, Porter, Thompson, Toothill & Edwards-Webb (1969).

## RESULTS

### *Rates*

*Ultrafiltration.* Filtration of milk was started at room temperature, 16°C, but proceeded very slowly at a permeate flux of only 0·13 $m^3$ per $m^2$ per day. The temperature was therefore raised to 38°C, which was considered to be the highest temperature at which the process could be safely operated without causing physical damage to the fat globules. At this temperature the initial permeate flux increased to

0·21 $m^3$ per $m^2$ per day. (J. H. Prentice, personal communication). At 25°C, the permeate fluxes, and therefore rates of concentration, for skim-milk and whey were 1·4 and 3·1 times faster than those for whole milk. Hence, the filtration of milk was hindered to some extent by the fat, but much more by the protein.

For all the materials the permeate flux decreased as the process continued. The flux for milk and for skim-milk increased over the first 40 min and then decreased continuously, whereas for whey the flux decreased from the start of the process, at first rapidly, and then more slowly until, after about 90 min, filtration proceeded at a constant rate. It is unlikely that initial rises in flux were due to temperature changes since the filtration apparatus was placed in a water-bath held at a temperature of 38 ± 0·5°C. Because of these different patterns in change of flux, the rates of decrease given in Table 1 are average values for the whole period of the experiment.

Table 1. *Rates of the ultrafiltration process*

| | Original volume, ml | Final volume, ml | Time, h | Concentration factor | Temp., °C | Permeate flux-average, $m^3$ per $m^2$ per day | Rate of decrease of permeate flux, $m^3$ per $m^2$ per d per h |
|---|---|---|---|---|---|---|---|
| Whole milk | 180 | 90 | 3·7 | ×2·0 | 38 | 0·21 | 0·005 at a level of 0·21, fall continuing at 90 min |
| Skim-milk | 180 | 115 | 2·4 | ×1·6 | 25 | 0·23 | 0·002 at a level of 0·23, fall continuing at 90 min |
| Whey | 180 | 62 | 2·0 | ×2·9 | 26 | 0·51 | 0·023 at a level of 0·51, no further fall after 90 min |

Table 2. *Rates of the reverse osmosis process*

| | Original volume, l | Final volume, l | Time, h | Concentration factor | Permeate flux average, $m^3$ per $m^2$ per d at 25°C | Rate of decrease of permeate flux, $m^3$ per $m^2$ per d per h |
|---|---|---|---|---|---|---|
| Whole milk | 45·5 | 22·8 | 6·5 | ×2·0 | 0·14 | 0·019 |
| Skim-milk | 50·0 | 22·8 | 5·3 | ×2·2 | 0·17 | 0·016 |
| Whey | 50·0 | 18·2 | 3·1 | ×2·7 | 0·34 | 0·048 |

*Reverse osmosis.* For all the materials the permeate flux decreased very rapidly during the first few minutes and thereafter more slowly but continuously over a period of hours as long as the experiment proceeded. Experiments were conducted at temperatures between 15 and 25°C, and all permeate fluxes were corrected to 25°C using tables supplied by the manufacturers of the membrane. Table 4 gives the rates of the process and the rates of decrease in permeate flux for the 3 materials over the main part of the experiment; that is, excluding the large decrease which occurred in the first few minutes.

The permeate fluxes were approximately two-thirds of those obtained in the ultrafiltration experiments at 38°C. From experience of the operation of larger-scale ultrafiltration apparatus other than that used in these experiments, the manufacturers suggest that the relative fluxes in these 2 processes on the same pilot plant scale

would more probably be 1:4. The patterns in rates of fall in flux in both processes were similar: the fall was least for skim-milk and highest for whey. The rate of concentration of whey was 2·5 times that of milk, indicating that with reverse osmosis, as with ultrafiltration, the milk protein was a major cause of hindrance.

### *Composition of Products*

*Ultrafiltration.* The performance of the membrane was assessed from the analyses of the starting materials and the permeates. The figure for nitrogen represents mainly nitrogen from the protein but includes also a small amount from the non-protein

Table 3. *Chemical analyses of fractions and levels of retention of the membrane in ultrafiltration*

(Concentrations as percentage by weight of fluid. Membrane retention stated as amount retained in the concentrate expressed as percentage of the component in the original sample.)

| | Total solids | Fat | Nitrogen | Lactose | Ash |
|---|---|---|---|---|---|
| Whole milk | 11·8 | 2·9 | 0·55 | 4·9 | 0·7 |
| Concentrate | 17·0 | 5·4 | 0·95 | 5·1 | 0·9 |
| Permeate | 5·4 | nil | 0·05 | 4·6 | 0·5 |
| Membrane retention | 78·4 | 100 | 94·6 | 57·0 | 68·4 |
| Skim-milk | 9·4 | — | 0·58 | 4·7 | 0·8 |
| Permeate | 3·3 | — | 0·03 | 3·1 | 0·3 |
| Membrane retention | 87·3 | — | 96·4 | 76·1 | 85·7 |
| Whey | 6·6 | — | 0·16 | 4·5 | 0·6 |
| Permeate | 5·3 | — | 0·08 | 4·2 | 0·5 |
| Membrane retention | 31·1 | — | 67·1 | 38·1 | 69·1 |

nitrogenous compounds which in whole milk account for about 5 % of the total N by weight. Table 2 shows that the milk concentrate retained all the fat and nearly all the nitrogenous material, and that the lactose and other small molecular weight components of the aqueous phase were filtered off. With whey, the retention of nitrogen appeared to be low because most of the nitrogenous material in whey is protein small enough in molecular size to pass through the membrane. The permeates from all 3 materials were clear watery liquids.

Retention rate was calculated as the weight of component retained in the concentrate expressed as a percentage of the weight in the original material. The rates in the experiments depended on a number of factors: the duration of the process, the different compositions of the materials being filtered, and the different components which could collect at the membrane and obstruct filtration. Thus, the retention levels of the solids and nitrogenous materials in whey were much lower than those for milk, partly because the filtrable components formed a higher proportion of the total solids in whey than in the milk, and partly because whey contained less protein to block the membrane. This latter point was illustrated by the behaviour of the lactose which was filtered out of whey at a greater rate than from either whole or skim-milk.

The only vitamins completely retained in the concentrate were folic acid and vitamin $B_{12}$, which are partly or wholly protein-bound (Ford, Salter & Scott, 1969; Gregory, 1954). Vitamin C is very sensitive to light and was largely lost from the skim-milk and whey during their preparation; hence the vitamin was assayed only in milk. No

assays were made of the fat-soluble vitamins A and E and carotene, which were wholly retained in the concentrate. As with lactose, it was observed that more of the filtrable vitamins were lost into the filtrate from the whey than from the milk.

*Reverse osmosis.* Table 5 shows that the membrane retained almost completely all of the components analysed with the exception of the ash which represented the small inorganic molecules, mainly chlorides. Most of the vitamin content in milk was also completely retained in the concentrate. The only losses were of a small part of the nicotinic acid and vitamin $B_6$ (Table 4).

Table 4. *Levels of retention (%) of vitamins by the membrane in ultrafiltration and reverse osmosis*

| | Vitamin C | Panto-thenic acid | Nico-tinic acid | Ribo-flavin | Biotin | Vitamin $B_{12}$ | Thiamin | Vitamin $B_6$ | Folic acid |
|---|---|---|---|---|---|---|---|---|---|
| Mol. wt. | 176 | 219 | 122 | 376 | 244 | 1357 | 301 | 170 | 441 |
| | | | | Ultrafiltration | | | | | |
| Whole milk | 13 | 68 | 59 | 61 | 63 | 98 | 62 | 64 | 95 |
| Skim-milk | — | 74 | 69 | 79 | 84 | 100 | 77 | 80 | 100 |
| Whey | — | 38 | 31 | 50 | 40 | 100 | 33 | 38 | 99 |
| | | | | Reverse osmosis | | | | | |
| Whole milk | — | 100 | 92·1 | 100 | 100 | 100 | 100 | 96·6 | 100 |

Table 5. *Chemical analyses of fractions and levels of retention of the membrane in reverse osmosis*

(Concentrations as percentage by weight of fluid. Membrane retention stated as amount retained in the concentrate expressed as percentage of the component in the original sample.)

| | Total solids | Fat | Nitrogen | Lactose | Ash |
|---|---|---|---|---|---|
| Whole milk | 11·7 | 3·2 | 0·48 | 4·3 | 0·70 |
| Permeate | 0·08 | nil | nil | nil | 0·03 |
| Membrane retention | 96·6 | 100 | 100 | 100 | 97·8 |
| Skim-milk | 8·8 | — | 0·49 | 4·7 | 0·70 |
| Permeate | 0·33 | — | nil | nil | 0·20 |
| Membrane retention | 98·0 | — | 100 | 100 | 84·6 |
| Whey | 6·8 | — | 0·13 | 4·4 | 0·60 |
| Permeate | 0·11 | — | nil | nil | 0·09 |
| Membrane retention | 99·0 | — | 100 | 100 | 90·3 |

## DISCUSSION

The results reported here are from preliminary trials without selection of the membrane specifically for use with milk and without seeking the optimum conditions for the process, with respect to working pressure, flow-rate, and the geometry of the membrane assembly. The degrees of concentration relate only to these arbitrarily chosen conditions and are undoubtedly not the highest attainable.

The greatest problem in the process is the blocking of the membrane. It is recognized (Toh Hoy Lim, Dunkley & Merson, 1971) that protein is a major cause of the blocking, as is illustrated in these present experiments by the large difference in permeate

fluxes for milk and whey. The protein forms a gel which adheres to the membrane and in effect adds another filtering layer. It has been shown (Blatt, Dravid, Michaels & Nelson, 1970) that the permeate flux is dependent on the transport of concentrated solute away from the membrane into the solution, a process which can be assisted by operating at high rates of shear or high Reynolds numbers.

The results show that milk can be effectively concentrated by reverse osmosis alone, but further work is needed to accelerate the rate. It is probable that some increase could be obtained by first concentrating the protein by ultrafiltration (as illustrated by the laboratory-scale experiment) and such a combination of the 2 processes of ultrafiltration and reverse osmosis is now under investigation.

I am happy to acknowledge the contributions to this work made by the Dorr-Oliver Co. Ltd, and by Patterson Candy International Ltd, who provided the facilities for the ultrafiltration and reverse osmosis experiments. I thank my colleagues in the Analytical Chemistry Unit and in the Nutrition Department for the analyses.

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# Effect of fibre level in the diet of the dairy cow on milk yield and composition

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Summary. Thirty-six lactating cows were used in a 3 × 2 factorial design experiment to study the effects of level of energy and fibre intake on milk yield and composition over an 8-week experimental period. Three levels of fibre intake – 1 g of crude fibre per 25, 17·5 and 10 kcal of estimated metabolizable energy (ME) requirements – were given in diets supplying both 80 and 100 % of energy requirements. The level of energy intake significantly affected milk yield, milk energy output and the percentage solids-not-fat (SNF) and protein in the milk. The effects of fibre intake on both milk yield and milk energy output were marked but not significant. Fibre intake had a significant curvilinear effect on both the SNF and protein in the milk with the highest fibre intake resulting in a significant decrease in both fractions. A significant linear decrease was obtained in the lactose fraction as the fibre intake increased.

The total volatile fatty acid (VFA) level in the rumen was significantly affected by both energy and fibre intake. The level of energy intake only significantly affected the proportion of propionic acid. Fibre intake significantly affected the proportion of both acetic and butyric acids resulting in mean proportions of acetic acid of 66, 70 and 72 at the low, medium and high fibre intakes. The correlations between the rumen acids and lactation efficiencies are also presented.

Multiple regression analysis within each fibre level has been used to partition the ME available for production between that used for milk energy output and liveweight change. The results showed efficiencies of utilization of ME for milk output of 66, 65 and 56 on the low, medium and high fibre diets respectively.

Nitrogen balance data are presented.

In 1962 Castle, Drysdale & Watson gave cows equal levels of dry-matter intake from forages at differing stages of maturity and obtained a decline in both milk yield and solids-not-fat (SNF) content with increased forage maturity. The effects of forage quality per se have been examined by Gordon & Forbes (1971) using 2 hays of differing fibre content given at equal net energy intakes. They obtained a lower milk production from the higher fibre roughage but there were no significant effects on the SNF content of the milk. However, there were indications from an earlier trial (Gordon & Forbes, 1970*a*) that high fibre intakes may considerably lower the SNF content of milk when given over a longer period even though adequate energy is provided. It is, therefore, important that the longer-term effects of dietary fibre level are clarified at

both adequate and low levels of energy intake. This main objective was examined in the present investigation with the effect of 3 levels of fibre intake being studied at a normal and low level of energy intake. As reductions in energy intake are usually associated in practice with a reduction in concentrate feeding resulting in little change in total fibre intake, the fibre intakes were equalized between energy levels. As a result the crude fibre content of the dry matter at each fibre level differed considerably between energy levels.

## EXPERIMENTAL

Thirty-six mixed Friesian and Shorthorn cows in their sixth–tenth weeks of lactation were used in a randomized block experiment. The cows were housed in individual stalls and milked twice daily. During the standardization period of 3 weeks they were given sufficient medium-quality hay for maintenance (Blaxter, 1959) and concentrates at the rate of 0·4 kg/kg milk produced. At the end of the standardization period the animals were blocked according to breed and milk yield and allocated at random to the following treatments:

| Treatments | Total fibre intake | Energy intake |
|---|---|---|
| LF:LE | 1 g crude fibre/25 kcal metabolizable energy (ME) requirement (LF) | 80% of requirement* (LE) |
| LF:HE | 1 g crude fibre/25 kcal ME requirement (LF) | 100% of requirement (HE) |
| MF:LE | 1 g crude fibre/17·5 kcal ME requirement (MF) | 80% of requirement (LE) |
| MF:HE | 1 g crude fibre/17·5 kcal ME requirement (MF) | 100% of requirement (HE) |
| HF:LE | 1 g crude fibre/10 kcal ME requirement (HF) | 80% of requirement (LE) |
| HF:HE | 1 g crude fibre/10 kcal ME requirement (HF) | 100% of requirement (HE) |

* Energy requirements were those given by Blaxter (1959) which were converted to ME values using the factor of 1 kg SE = 4·28 Mcal ME given by the Agricultural Research Council (1965).

The animals were maintained on these treatments for 8 weeks and intakes adjusted every second week by the method of equalized feeding (Lucas, 1943). Milk yields were recorded daily and samples taken at the end of the standardization period and during each of weeks 4–8 of the experimental period. In each case sampling was carried out for 3 consecutive days. The cows were weighed on the last 3 consecutive days of the standardization period and on the second, fourth and sixth days of each week during the experimental period. The mean of the weighings taken during the standardization period was used to calculate the metabolic body-weights ($W^{0·73}$) for use throughout the remainder of the trial.

To obtain the required differences in fibre intake it was necessary to use a lower-fibre hay on the 2 low-fibre treatments than on the other treatments. The chemical analysis of both hays are given in Table 1. A percentage of oatfeed (19·5% fibre) was also added to the highest fibre diets to increase fibre intake. As this material is unmilled and consists of a large proportion of oat husks with the fibre in the long form it was felt that it would have similar effects to those associated with the fibre fraction of hay. Within each fibre level the proportion of the animals' theoretical requirements supplied from hay was held constant, the lower-energy treatment being obtained by reducing the level of concentrate feeding. The fibre content of the concentrates on the low energy treatments was also increased slightly by the inclusion of oatfeed to maintain the level of total fibre intake. The composition of the concentrate mixtures used is given in Table 2.

The results were analysed as a 3 × 2 factorial design. The milk yield and composition data was adjusted by covariance analysis. Weight changes were calculated by linear regression using all the data obtained throughout the experimental period. A multiple regression technique was used to relate the milk yield and weight change within each fibre level to the level of energy intake.

Table 1. *Chemical analysis of hays*, %

| | Treatments | |
|---|---|---|
| | LF | MF and HF |
| Dry matter | 85·6 | 84·0 |
| Composition of dry matter | | |
| Crude protein | 10·4 | 10·9 |
| Crude fibre | 23·8 | 31·6 |
| Ether extract | 1·6 | 1·8 |
| Ash | 7·4 | 7·1 |

LF, low fibre; MF, medium fibre; HF, high fibre.

Table 2. *Composition of the concentrate mixtures*, %

| | Treatments | | | | | |
|---|---|---|---|---|---|---|
| | LF:LE | LF:HE | MF:LE | MF:HE | HF:LE | HF:HE |
| Rolled barley | 33 | 40 | 32 | 52 | — | 39 |
| Ground maize | 30 | 44 | 30 | 32 | — | — |
| Oatfeed | 7 | — | 8 | — | 72 | 47 |
| Soybean | 26 | 12 | 26 | 12 | 24 | 10 |
| Minerals* | 4 | 4 | 4 | 4 | 4 | 4 |

LF, low fibre; LE, low energy; HE, high energy; MF, medium fibre; HF, high fibre.
* Declared composition: P, 5·7%; Ca, 21·4%, NaCl, 33·3%; Fe, 0·3%; Mn, 800 ppm; Cu, 300 ppm; Co, 100 ppm; I, 200 ppm.

Digestibility and nitrogen (N) balance trials were carried out during the 18th to 25th and 46th to 53rd days of the experimental period using 3 animals/treatment. The balance procedures were similar to those described previously (Gordon & Forbes, 1970*b*). Gross energy determinations on feed and faeces were carried out by bomb calorimetry. Metabolizable energy was calculated by the formula of digestible energy × 0·82 (Agricultural Research Council, 1965).

Samples of rumen liquor were obtained by a suction strainer and stomach-tube technique similar to that described by Raun & Burroughs (1962). Samples were taken from each animal at 3 and 6 h after it received the evening feed on the 28th and 56th days of the comparison period. Saturated mercuric chloride was added at the level of 2·5% (v/v) to the samples immediately after they were taken and they were then stored at −15°C until analysed. Total volatile fatty acids (VFA) were determined by the steam distillation method reported by Balch & Rowland (1957). The molecular percentages of the individual fatty acids were determined on a composite sample for each animal by gas chromatography. Before separation, the acids were converted to octyl esters by heating the dried sodium salts from rumen contents with a mixture of octan-1-ol and concentrated sulphuric acid.

The milk samples within each 3-consecutive-day-period were bulked according to

yield. The composite sample was analysed for butterfat by the Gerber method, for total solids by the gravimetric method, for protein by the macro-Kjeldahl method (N × 6·38), for lactose by Rowland's modification of the method of Hinton & Macara as given by Ling (1956) and for ash by the method of Ling (1956). Milk energy was calculated from the total output of milk constituents and the energy content of these constituents as given by Kleiber (1961).

## RESULTS

The intakes of digestible energy and digestible crude protein (DCP) were measured directly for 3 replicates/treatment, and were calculated for the remaining 3 replicates using the mean digestibility values for each treatment. These intakes expressed as a

Table 3. *Feeding scales used and mean intakes of metabolizable energy (ME), digestible crude protein and crude fibre*

| | Treatments | | | | | |
|---|---|---|---|---|---|---|
| | LF:LE | LF:HE | MF:LE | MF:HE | HF:LE | HF:HE |
| Hay intake, g/kcal ME requirement | 0·164 | 0·164 | 0·184 | 0·184 | 0·284 | 0·284 |
| Conc. intake, g/kcal ME requirement | 0·185 | 0·291 | 0·191 | 0·272 | 0·162 | 0·268 |
| ME intake, per cent of requirements | 81 | 107 | 83 | 100 | 85 | 105 |
| DCP intake, per cent of requirements | 94 | 104 | 108 | 95 | 94 | 105 |
| Ratio of fibre intake, g: ME requirement, kcal | 24·7 | 24·7 | 18·0 | 17·6 | 10·1 | 10·5 |
| Crude fibre, per cent of dry matter | 13·8 | 10·8 | 18·0 | 14·9 | 25·6 | 21·8 |

LF, low fibre; LE, low energy; HE, high energy; MF, medium fibre; HF, high fibre.

percentage of theoretical requirements (Blaxter, 1959) are given in Table 3. The levels of ME intake were close to those planned but due to the larger than expected degree of variation between treatments in nitrogen digestibility the mean levels of DCP intake ranged from 94 to 108% of requirements. However, the maximum mean difference in DCP intake between either of the main factors, i.e. fibre or energy levels, was only 2·7 percentage units. The levels of fibre intake, expressed as a ratio of fibre intake to theoretical ME requirements are also given in Table 3. These remained relatively constant within each fibre level. The percentage crude fibre in the overall diets are also given in Table 3.

The mean milk yield and milk energy output, adjusted by covariance are given together with the bodyweight changes in Table 4. Milk yield and milk energy were significantly affected by the level of energy ($P < 0·001$) but not by fibre intake. The effect of fibre level on milk energy output almost reached significance, with a significant linear effect ($P < 0·05$) of fibre being obtained in polynomial analysis. There was a mean bodyweight loss on all treatments but this loss was significantly ($P < 0·001$) lower at the high (−0·28 kg/day) than at the low (−0·51 kg/day) level of energy intake. The weight loss was also significantly ($P < 0·05$) greater at the high than the low levels of fibre intake (−0·49 and −0·33 kg/day respectively).

The treatment means for the composition of the main milk constituents on the eighth week of the experimental period are given in Table 5. The butterfat content was not significantly affected by any of the treatments used. The SNF was significantly affected by the level of both energy ($P < 0{\cdot}001$) and fibre intake ($P < 0{\cdot}01$). The effect of fibre on SNF was curvilinear ($P < 0{\cdot}05$) with the content at the highest fibre level being significantly lower than at both other levels ($P < 0{\cdot}01$). The effects on milk protein content showed similar trends to those on SNF with significant effects being produced by the level of both energy ($P < 0{\cdot}001$) and fibre intake ($P < 0{\cdot}01$). Again, the effect of fibre was curvilinear ($P < 0{\cdot}05$) with the protein content at the

Table 4. *Milk yield, milk energy output and body-weight changes at the end of the experimental period*

| | Treatments | | | | | | | Comparison of main effects | |
|---|---|---|---|---|---|---|---|---|---|
| | LF:LE | LF:HE | MF:LE | MF:HE | HF:LE | HF:HE | S.E. of mean | Energy | Fibre |
| Milk yield, kg/day | 15·2 | 17·0 | 14·0 | 17·6 | 13·4 | 16·6 | ±0·82 | LE < HE*** | NS |
| Milk energy, kcals/day | 10035 | 11826 | 9250 | 12000 | 8890 | 10684 | ±554 | LE < HE*** | NS |
| Weight change, kg/day | −0·48 | −0·18 | −0·43 | −0·28 | −0·63 | −0·36 | ±0·07 | LE < HE*** | HF < LF* |

LE, low energy; HE, high energy; LF, low fibre; MF, medium fibre; HF, high fibre.
* $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$.

highest fibre intake being significantly lower than at the medium ($P < 0{\cdot}01$) and lowest ($P < 0{\cdot}05$) fibre levels. Lactose percentage was unaffected by the level of energy intake but showed a significant linear decrease ($P < 0{\cdot}05$) as the level of fibre intake was increased. The ash content of the milk was not affected by either energy or fibre intake, although the individual treatment comparisons showed a significantly lower ash content on treatment 5 than on treatment 2. The total solids content gives a measure of the cumulative effects of the treatments on the individual milk constituents. Although the effect of energy intake failed to reach significance level the total solids content at the highest fibre level was significantly lower ($P < 0{\cdot}05$) than that at the lowest fibre level.

The total VFA concentrations in the rumen liquor at 3 and 6 h after feeding are given in Table 6. The level of energy intake had a significant effect on rumen VFA concentration at both 3 ($P < 0{\cdot}001$) and 6 h ($P < 0{\cdot}05$) after feeding although the effect declined with time after feeding. At 3 h after feeding there was a significant linear ($P < 0{\cdot}05$) increase in total VFA concentration as the level of fibre decreased, but this trend was not found at the 6-h stage. The molar percentages of acetic, propionic and butyric acids in the composite samples (mixture of 3- and 6-h samples) are also given in Table 6. The proportion of acetic acid was unaffected by level of energy intake, but showed a significant linear increase ($P < 0{\cdot}001$) as fibre intake was increased. The proportion of propionic acid was increased significantly ($P < 0{\cdot}01$) by reducing the level of energy intake but was unaffected by changes in fibre intake. In contrast, the proportion of butyric acid was not affected by energy intake, but showed a significant linear decrease ($P < 0{\cdot}001$) as fibre intake was increased. The

Table 5. *Composition of milk at end of experimental period*

| | Treatments | | | | | | s.e. of mean | Comparison of main effects | |
|---|---|---|---|---|---|---|---|---|---|
| | LF:LE | LF:HE | MF:LE | MF:HE | HF:LE | HF:HE | | Energy | Fibre |
| Total solids, % | 11·65 | 12·06 | 11·57 | 11·86 | 11·53 | 11·48 | ±0·13 | NS | HF < LF* |
| Butterfat, % | 3·29 | 3·60 | 3·29 | 3·43 | 3·54 | 3·28 | ±0·12 | NS | NS |
| SNF, % | 8·29 | 8·49 | 8·23 | 8·52 | 7·99 | 8·25 | ±0·07 | LE < HE*** | HF < MF**, HF < LF** |
| Protein, % | 2·94 | 3·12 | 2·98 | 3·14 | 2·80 | 3·01 | ±0·05 | LE < HE*** | HF < MF**, HF < LF* |
| Lactose, % | 4·68 | 4·67 | 4·58 | 4·74 | 4·53 | 4·53 | ±0·06 | NS | HF < MF*, HF < LF* |
| Ash, % | 0·67 | 0·69 | 0·68 | 0·68 | 0·63 | 0·67 | ±0·01 | NS | NS |

LF, low fibre; LE, low energy; HE, high energy; MF, medium fibre; HF, high fibre.

Table 6. *Volatile fatty acid (VFA) composition of rumen samples and lactation efficiency values*

| | Treatments | | | | | | s.e. of mean | Comparison of main effects | |
|---|---|---|---|---|---|---|---|---|---|
| | LF:LE | LF:HE | MF:LE | MF:HE | HF:LE | HF:HE | | Energy | Fibre |
| Total VFA at 3 h m-equiv./100 ml | 8·7 | 10·5 | 8·7 | 9·7 | 7·6 | 9·7 | ±0·41 | LE < HE*** | HF < LF* |
| Total VFA at 6 h m-equiv./100 ml | 9·4 | 10·9 | 8·8 | 9·3 | 8·4 | 9·8 | ±0·55 | LE < HE* | NS |
| Mean total VFA, m-equiv./100 ml | 9·1 | 10·7 | 8·7 | 9·5 | 8·0 | 9·8 | ±0·44 | LE < HE** | HF < LF* |
| Acetic, molar % | 65·5 | 66·8 | 68·6 | 70·8 | 72·4 | 71·5 | ±0·96 | NS | LF < HF**, LF < MF**, MF < HF* |
| Propionic, molar % | 18·9 | 16·6 | 17·6 | 15·1 | 16·4 | 16·6 | ±0·68 | HE < LE** | NS |
| Butyric, molar % | 15·6 | 16·6 | 13·8 | 14·2 | 11·3 | 11·9 | ±0·65 | NS | HF < LF**, HF < MF**, MF < LF** |
| Gross lactation efficiency | 0·42 | 0·36 | 0·36 | 0·40 | 0·34 | 0·35 | ±0·020 | NS | NS |
| Net lactation efficiency | 0·89 | 0·60 | 0·72 | 0·68 | 0·67 | 0·56 | ±0·046 | HE < LE** | HF < LF** |

LF, low fibre; LE, low energy; HE, high energy; MF, medium fibre; HF, high fibre.

Table 7. *Nitrogen balance data*

| | Treatments | | | | | | s.e. of mean | Comparison of main effects | |
|---|---|---|---|---|---|---|---|---|---|
| | LF:LE | LF:HE | MF:LE | MF:HE | HF:LE | HF:HE | | Energy | Fibre |
| N intake, g/day | 222·2 | 255·8 | 205·7 | 261·3 | 240·1 | 284·9 | ±15·1 | — | — |
| App. digestibility of N, % | 72·3 | 66·9 | 73·4 | 65·9 | 70·6 | 66·2 | ± 0·8 | HE < LE*** | NS |
| Retained N, g/day | −10·9 | +7·9 | −7·8 | −0·4 | −16·4 | +8·8 | ± 5·8 | LE < HE** | NS |
| Retained N+milk N, g/day | 62·5 | 97·5 | 47·4 | 85·6 | 47·7 | 92·5 | ± 9·6 | LE < HE*** | NS |

LF, low fibre; LE, low energy; HE, high energy; MF, medium fibre; HF, high fibre.

gross and net lactation efficiencies (kcal of milk/kcal of ME intake and kcal of milk/kcal of ME available for production, respectively) are also given in Table 6. The ME available for production was calculated using the maintenance value of 123·8 kcal/kg $W^{0.73}$ (Van Es & Nijkamp, 1966). Although the gross efficiency was not affected by either the level of energy or fibre intake the net efficiency was affected ($P < 0.01$) by both these factors.

The gross energy digestibilities of the total diets – 78·7, 78·7, 75·5, 74·7, 64·8 and 64·6 % on treatments 1–6 respectively – were significantly affected by the level of fibre ($P < 0.001$) but not by energy intake. The treatment means for the N balance data are given in Table 7. The level of fibre intake had no significant effect on either the apparent digestibility of N, the amount of N retained, or N retained + milk N. However, the digestibility of N was significantly lower ($P < 0.001$) on the high-energy intake compared with the low energy intake. The N retained and N retained + milk N were both significantly greater ($P < 0.01$ and $P < 0.001$ respectively) on the high energy intake.

## DISCUSSION

### *Effect of energy intake on milk yield and composition*

The level of energy intake significantly affected both milk yield and milk energy output. These effects increased as the experimental period progressed. The 20 % lower energy intake resulted in a mean decrease in milk yield of 1·7, 2·3, 2·7 and 2·9 kg/day during the fifth–eighth weeks respectively although the method of equalized feeding was used to determine energy intake. The effect of energy intake on milk yield was greater than that reported earlier (Gordon & Forbes, 1970*b*; Gordon & Forbes, 1971). This could be attributed to the higher mean milk yield in the present investigation (as suggested by Blaxter, 1966). Alternatively the changeover design used in the 2 previous trials, or the maintaining of a constant fibre intake rather than a constant percentage fibre between energy levels may account for the variation in the response to energy. However, the effects reported here are in agreement with those given by Burt (1957). The lower energy intake also reduced the SNF and protein content of the milk by 0·25 and 0·10 percentage units respectively. These effects agree with those reported by Burt (1957), but again are greater than those reported earlier (Gordon & Forbes, 1970*b*; Gordon & Forbes, 1971). The level of energy intake had no significant effect on the content of any of the other major milk constituents.

### *Effect of fibre intake on milk yield and composition*

The mean milk yield at each level of fibre intake (16·1, 15·8 and 15·0 kg of milk on the low, medium and high fibre diets respectively) showed a marked, but non-significant, trend towards a decline in yield as the fibre intake increased. This trend was more marked in the case of milk energy output, with a significant linear decrease being obtained as the fibre intake was increased.

The effect of level of fibre intake on the milk energy output from diets of equal ME content was further examined within each dietary fibre level using a multiple regression technique. Within these relationships both ME and $ME^2$ were significant at the 5 % level.

The best-fit relationships were:

Low fibre: $Ye = 1{\cdot}006\text{ME} - 0{\cdot}000{,}01098(\text{ME})^2 - 7681$, residual standard deviation (RSD) 2265;

Medium fibre: $Ye = 0{\cdot}560\text{ME} - 0{\cdot}000{,}00186(\text{ME})^2 - 2864$, RSD 1229;

High fibre: $Ye = 0{\cdot}556\text{ME} - 0{\cdot}000{,}00323\text{ME})^2 - 2999$, RSD 1264;

where $Ye$ is the milk energy in kcal/day and ME is the ME intake in kcal/day.

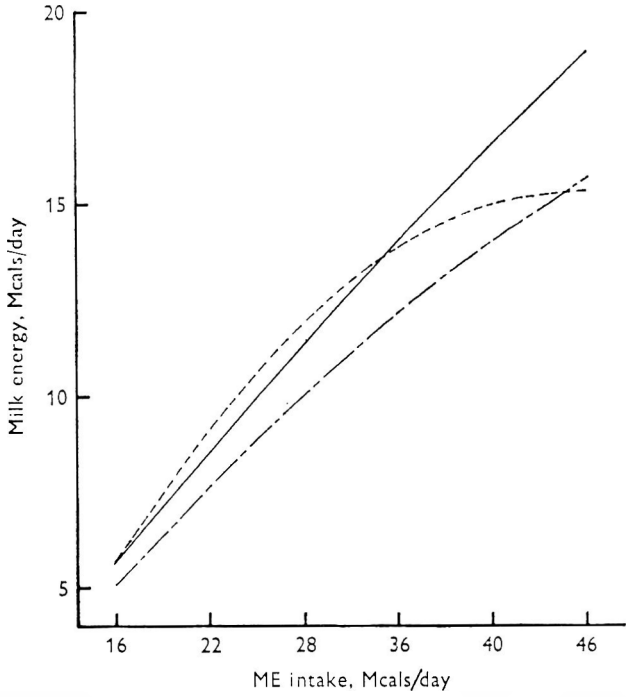


Fig. 1. The relationship between the output of milk energy and the metabolizable energy (ME) intake at each of the 3 levels of fibre: - - - - -, low fibre; ——, medium fibre; – – –, high fibre.

These equations are presented graphically in Fig. 1 and indicate the lower productive value of the highest fibre diet at all levels of energy intake. At the lowest level of fibre intake there was a fall-off in productive value when high levels of energy intake were given. These effects are in agreement with those obtained by Nordfeldt, Iwanaga, Morita, Henke & Tom (1950), who obtained yields of 20·67, 21·75, 23·45 and 23·75 lb/day from rations of equal total TDN content but containing 23, 20, 16 and 12% fibre. This curvilinear response in yield to a decrease in fibre intake from diets of equal digested nutrient content was further demonstrated by Martin, Stoddard & Allen (1954) and Logan & Miles (1963).

At the end of the 8-week experimental period, the SNF content of the milk produced at the highest fibre intake was considerably lower than at each of the other 2 levels. The weekly results showed a difference in SNF between the high and medium

fibre diets of 0·08, 0·12, 0·14, 0·20 and 0·27 percentage units during the fourth to eighth weeks respectively, indicating a marked increase in the response to high fibre intakes as the period on the treatments progressed. This increasing effect of fibre intake on SNF with time is in line with the results obtained in an earlier feeding trial (Gordon & Forbes, 1970*a*) when a marked decline in SNF was obtained after 11 weeks on a high-fibre diet even though adequate energy was supplied. This may also explain why there was no depressing effect of high fibre levels on SNF in an earlier trial using 4-week experimental periods (Gordon & Forbes, 1971).

The decrease in SNF at the highest fibre intake was associated with reductions in both the protein and lactose fractions of the milk. Similar trends in the SNF and protein fractions when high fibre diets were used, even though equal net energy intakes were given, have been obtained by Hinders & Owen (1963) and Nordfeldt & Ruudvere (1963). There is little supporting evidence available on the effect on lactose due to variations in fibre intake. However, a significant reduction was noted by Tsai, Castillo, Hardison & Payne (1967) under humid tropical conditions as fibre intake was increased. This is contrary to the results obtained by Nordfeldt & Ruudvere (1963) who found a significant increase in the lactose fraction as the fibre content was increased from 15 to 25% while maintaining an equal net energy intake.

### *Effect of fibre intake on rumen VFA*

The mean total VFA concentrations obtained showed a significant linear increase as fibre intake decreased. This may not necessarily mean an increased production of VFA at the lower fibre level over the 24-h cycle, as a more uniform production of VFA throughout the day is obtained on high fibre diets (Balch & Rowland, 1957) coupled with the lowering of rumen volume on low fibre diets (Emery, Smith & Lewis, 1958). Hinders & Owen (1963) took rumen samples 0, 4 and 8 h after feeding and obtained a decrease in the extent of diurnal variation and an increase in mean VFA concentration as fibre intake increased. The increase in acetate and decrease in butyrate concentration obtained as fibre intake was increased agrees with the general trends reported in the literature (Elliot & Loosli, 1959; Hinders & Owen, 1963; Coppock, Flatt, Moore & Stewart, 1964). However, no significant overall trend in propionate concentration was obtained, which although agreeing with the results of Hinders & Owen (1963) conflicts with those of Elliot & Loosli (1959), who found an increase in propionate as fibre intake decreased. A similar trend in propionate to that reported by Elliot & Loosli (1959) was obtained within the lower level of energy feeding.

Simple correlation analyses were carried out to study the relationships between the productive efficiency and the relative proportions of the rumen VFA, within each level of energy feeding. Using the data obtained at the lower energy level, the correlations between gross efficiency and per cent acetic, propionic and butyric acids were $-0{\cdot}62$, $0{\cdot}52$ and $0{\cdot}53$ respectively. The correlations with net efficiency were $-0{\cdot}47$, $0{\cdot}42$ and $0{\cdot}37$ respectively. In the correlations with the ratios of the acids only the acetic–propionic and acetic–butyric ratios were significantly correlated with efficiency. The coefficients with the gross efficiency were $-0{\cdot}56$ and $-0{\cdot}53$ respectively and for the net efficiency were $-0{\cdot}46$ and $-0{\cdot}39$ respectively. These relation-

ships obtained are similar to those reported by Elliot & Loosli (1959), Hinders & Owen (1963) and Coppock *et al.* (1964).

No significant simple correlations were obtained using the data from the higher level of energy intake. It would seem that this was due to the decline in efficiency on the high energy level at the lowest fibre intake (Table 6) which was not reflected in the rumen VFA pattern.

*Effect of fibre intake on energy utilization*

As shown by the milk energy output data and both the gross and net efficiency figures given in Table 6, the level of fibre intake had a considerable effect on the overall utilization of ME. As fibre level has little effect on the ME required for maintenance (Blaxter, 1962; Van Es, 1966), the ME available for productive purposes was obtained by deducting the maintenance requirement 123·8 kcal/kg $W^{0.73}$ as given by Van Es & Nijkamp (1966). The production ME, within each fibre level, was related by multiple regression analysis to the milk energy output and body-weight change assuming all the production ME available was used for these purposes. The equations obtained were:

low fibre: production ME = $1{\cdot}51\,Ye + 5278$ weight change (WC), RSD = 4127;
medium fibre: production ME = $1{\cdot}55\,Ye + 3634$ WC, RSD = 2253;
high fibre: production ME = $1{\cdot}79\,Ye + 2544$ WC, RSD = 4127;

where the production ME is in kcal, $Ye$ is the yield of milk energy in kcal/day and WC is the weight change in kg/day.

Using the partial regression coefficients on milk energy output and assuming zero tissue balance, an increase of 1·51, 1·55 and 1·79 kcal ME would be required to produce 1 kcal increase in milk energy output on the low, medium and high fibre diets respectively. The standard errors of these coefficients were 0·08, 0·11 and 0·11 respectively. These correspond to efficiencies of conversion of ME to milk energy of 66, 65 and 56 % on the low, medium and high fibre diets respectively. These efficiencies are in line with those of 69 and 54 obtained in an earlier trial (Gordon & Forbes, 1971) when diets containing 15 and 23 % fibre were used. They also agree with those obtained by Coppock *et al.* (1964), who, from calorimetric trials, obtained efficiencies of 65, 61 and 54 % from diets containing 16, 22 and 28 % fibre. These efficiencies were related to rumen acetate levels of 65, 68 and 71 which are in line with the efficiencies of 66, 65 and 56 from diets giving rumen acetate levels of 66, 70 and 72 obtained in the present investigation.

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# The influence of pH and heat treatment on the colour and stability of ultra-high-temperature sterilized milk

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(*Received* 10 *May* 1971)

Summary. When the pH of milk was varied within the range 7·1 to 6·3 by addition of acid or alkali or through bacterial action, the reflectance of the milk after subsequent ultra-heat-treatment (UHT) was at a maximum of about pH 6·70. Below this value the reflectance dropped rapidly with decrease in pH. The cause of this decrease was the development of increasing amounts of sediment in the product. At pH 6·4–6·5, at least 90 % of the casein and 40 % of the whey proteins had been precipitated. The addition of 0·1 % sodium di-hydrogen phosphate or 0·1 % sodium citrate to the raw milk prevented the formation of the sediment. The role of calcium appeared important as small additions of calcium chloride or EDTA altered the patterns of sediment formation and reflectance with changing pH. Addition of 0·3 % EDTA prevented sediment formation as the pH dropped.

Studies on factors that influence the change of colour which occurs on heating milk have shown that at least 2 separate and apparently unrelated reactions are involved. It has been suggested that the first, a whitening effect that occurs on relatively mild heat treatment, is due to denaturation and subsequent coagulation of some of the serum milk proteins, resulting in an increase in the number of optically opaque particles (Burton, 1955*a*). The second, a browning effect of more importance at higher temperatures, is a result of casein–lactose interactions (Burton, 1954, 1955*a*,*b*; Burton & Rowland, 1955). In the case of ultra-heat-treatment (UHT) of whole milk using direct steam injection, the effect of heat on milk colour is further complicated by homogenization either applied as a separate process or occurring as a result of steam injection as reported by Zadow (1969).

Heat-induced denaturation and coagulation of the whey proteins may lead to the formation of sediment in sterile milk. The importance of using high-quality raw milk to avoid this possibility in the manufacture of UHT products is well recognized.

The present study aimed to examine the influences of pH, treatment temperature, homogenization and of calcium on the colour and degree of sediment formation in UHT milk.

## EXPERIMENTAL

### *Sample preparation*

Fresh raw skim-milk or whole milk was used for all trials except where specified. When necessary the pH of samples was adjusted by dropwise addition of either 2 N-hydrochloric acid or 2 N-sodium hydroxide into the vortex formed in the milk by

an Ultra-Turrax (Janke & Kunkel) mixer. The pH was continuously monitored and mixing was continued for 10 min after the completion of each addition. Distilled water was added to adjust the solids contents of all samples to the same level. Where other additives were used to adjust pH or sequester calcium, a similar technique was used.

*Heat-treatment and cooling*

The Alfa-Laval VTIS/VTS plant used for processing has been described in previous papers by Zadow (1969, 1970). In the work now described steam injection only was used, with the homogenizer by-passed when required. The range of temperatures employed for processing was from 97 to 150 °C with a fixed holding time of 3·0 s. Samples were taken after 5 min steady running at each temperature.

*Analytical*

The reflectance of samples was determined at ambient temperature with an Agtron reflectometer (Magnuson Eng. Inc., California). This instrument determines the reflectance of samples over the visible spectrum.

Sediment depths were determined in mm in a 50-ml conical centrifuge tube after centrifugation of a 40-ml sample at 300 *g* for 5 min. It should be noted that sediment volume is related to the cube of sediment depth.

Non-casein N determinations were made by the method of Aschaffenburg & Drewry (1959).

pH was measured on a Radiometer pH meter Model pH 22 with scale expander. Results were expressed to the nearest 0·005 pH unit.

## RESULTS

Fig. 1 shows the reflectance of unhomogenized skim-milk, sterilized at 150°C for 3 s as a function of pH, together with the sediment depth found for each sample. In this experiment pH modifications were made by addition of hydrochloric acid or sodium hydroxide. The shape of the pH-reflectance curve in Fig. 1 on the acid side of the control value differs from that reported by Burton (1955*a*) in studies in which milk was heated at 90 °C for 4 min and 15 min. He reported a continuous rise in reflectance with decreasing pH. Fig. 1 shows a rapid rise in reflectance to a maximum 0·15 pH unit below the control value and then a sharp decrease in reflectance, corresponding with increasing depth of sediment. The formation of this sediment and the corresponding reduction in the number of light scattering particles was obviously responsible for the downturn of the pH-reflectance curve.

The effect of homogenization of UHT (150 °C, 3 s) skim-milk on the pH-reflectance–sediment curves is shown in Fig. 2. Samples were homogenized downstream in 2 stages at 1500 and 500 lb/in$^2$ respectively using an aseptic Manton Gaulin single-piston homogenizer, Model 15 M-87 A, following pH adjustment with either hydrochloric acid or sodium hydroxide. The shapes of the curves in Fig. 2 are similar to those in Fig. 1, although the peak of the pH-reflectance curve is lower.

Acidification of raw skim-milk by the addition and hydrolysis of glucono-Δ-lactone or by the natural action of chance contaminants yielded, after UHT

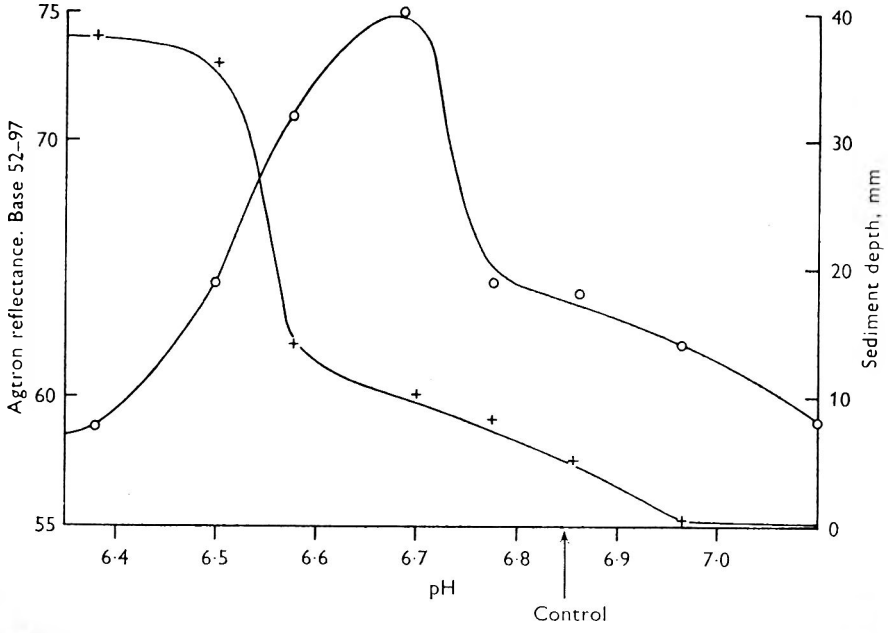


Fig. 1. The reflectance and sediment depth in samples of unhomogenized UHT skim-milk as a function of pH. ○, Agtron reading; +, sediment depth.

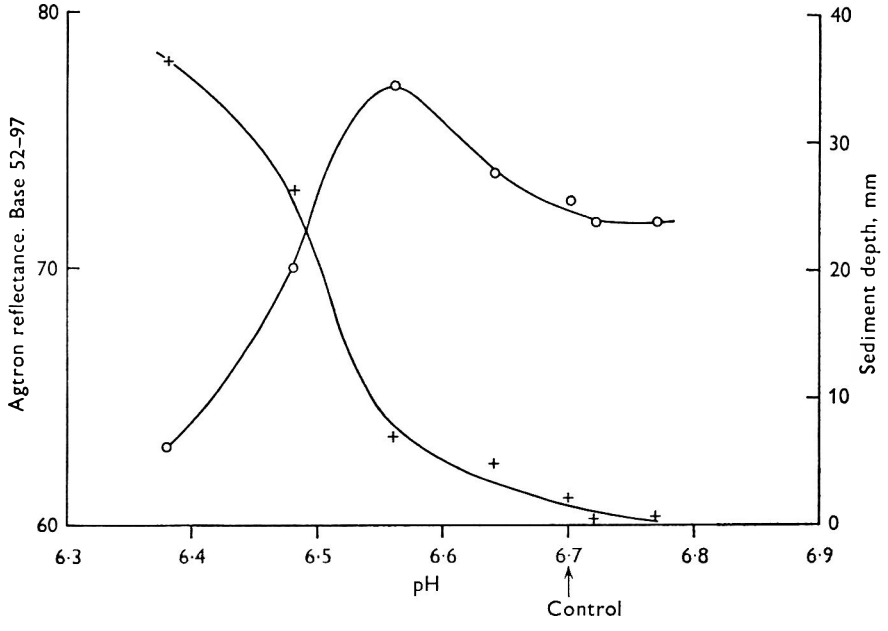


Fig. 2. The reflectance and sediment depth in samples of homogenized (1500 + 500 lb/in²) UHT skim-milk as a function of pH. ○, Agtron reading; +, sediment depth.

treatment (without homogenization), pH-reflectance-sediment curves similar to those in Fig. 1. It may thus be concluded that the method used for adding hydrochloric acid did not cause localized precipitation.

Whole milk processed by UHT with downstream homogenization at 1500 lb/in², followed by 500 lb/in² yielded the pH-reflectance–sediment curves shown in Fig. 3. The change in reflectance was moderate, with only a small peak at a slightly lower pH than that occurring with homogenized skim-milk. The peak of the maximum reflectance of the UHT homogenized whole milk was much higher than that of the skim (91 Agtron units compared with 77 Agtron units) due to the presence of the finely divided fat globules. The pH-sediment curve in Fig. 3 indicates a very large amount of sediment at low pH values.

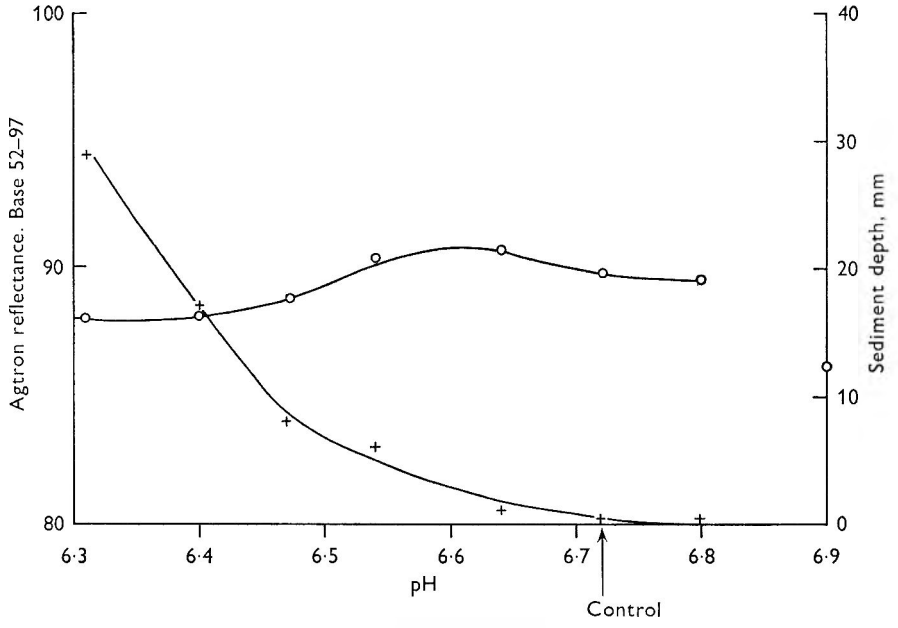


Fig. 3. The reflectance and sediment depth in samples of unhomogenized UHT whole milk as a function of pH. ○, Agtron reading; +, sediment depth.

The rapid increase in the amount of sediment present in UHT unhomogenized skim-milk as a function both of pH and of sterilizing temperature is shown in Fig. 4, which covers the pH range 6·3–6·9 and temperatures from 100 to 142 °C. A very rapid change in the shape of the curves was observed over the pH range, 6·70–6·50, with the volume of sediment present increasing about 64-fold at a sterilization temperature of 142 °C over this pH range. For milk processed above 135 °C, sediment depth increased rapidly with the drop in pH, until at approximately pH 6·4, a maximum value was reached. The sediment at or below this pH represented at least 90 % of the casein and 40 % of the whey proteins of the milk as estimated by nitrogen balance.

It is well known that the denaturation of whey protein during heating of milk is pH-dependent. With the UHT milk in this study only relatively minor changes occurred in the pH range 6·38–7·11 (Table 1).

Washed and dried samples of the sediment exhibited properties similar to those reported by Muller, Hayes & Snow (1967) as characteristic of a rather insoluble medium calcium co-precipitate. When whole milk was processed the sediment contained approximately 8 % fat on a dry basis.

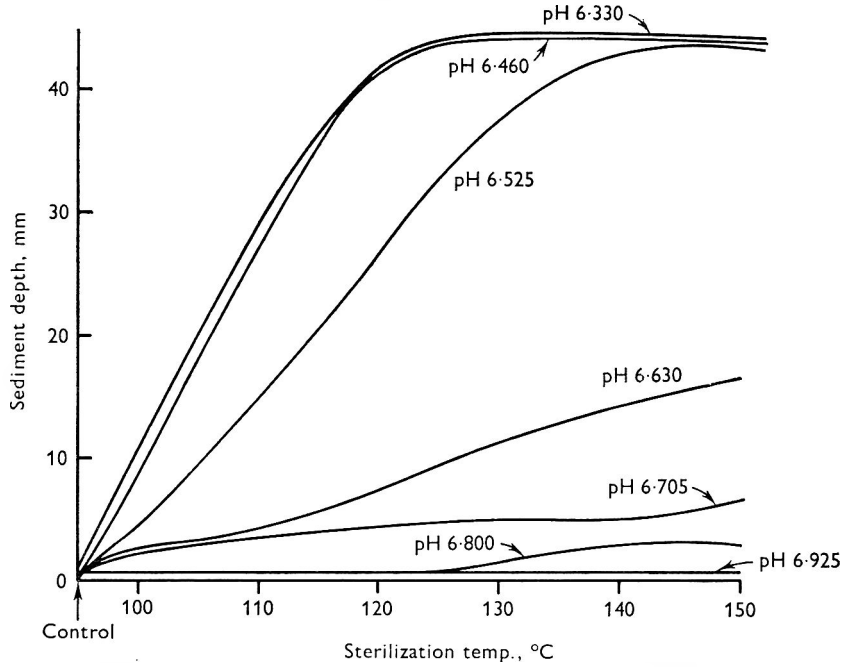


Fig. 4. The sediment depth present in samples of unhomogenized UHT skim-milk as a function of pH and sterilizing temperatures. Tests carried out after sterilization at 100, 110, 120, 129, 140 and 150°C.

Table 1. *The non-casein N contents of directly processed UHT milk (145 °C for 3 s)*

| | pH | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 7·11 | 6·98 | 6·85 | 6·77 | 6·68 | 6·57 | 6·49 | 6·38 |
| Non-casein N, mg/ml | 1·04 | 1·03 | 1·02 | 1·02 | 0·98 | 0·99 | 0·96 | 0·92 |

Separation of the very fine sediment from the milk by gravity was relatively slow. It was observed in gross amounts in the bottom of containers approximately 1 h after filling. Coagulation of this fine sediment into a product that could be more readily handled was achieved by heating the milk at 90 °C for 10 min, or by decreasing the pH to 5·5 or lower. The precipitation of proteins by UHT treatment may provide an alternative method for the manufacture of co-precipitate.

The reflectances of the samples shown in Fig. 4 are seen in Fig. 5 as a function of sterilization temperature and pH. Again, very significant changes in the shape of the curve occurred in the pH range 6·70–6·50. It should be noted that the temperature required for maximum reflectance decreased with fall in pH.

The influence of additives on the reflectance and sediment formation of unhomogenized UHT skim-milk was also examined. The use of sodium di-hydrogen

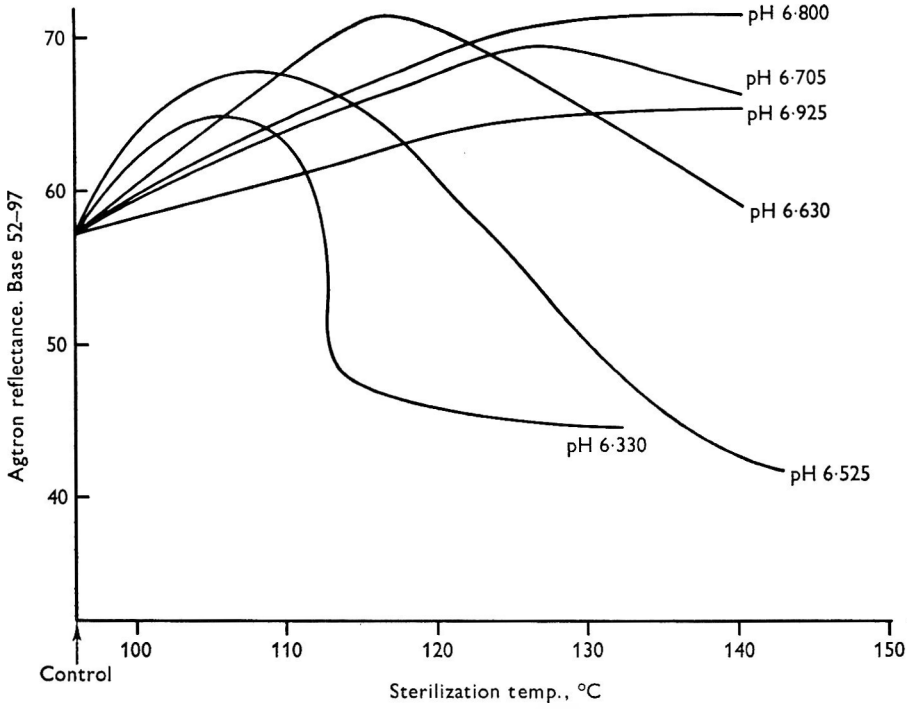


Fig. 5. The reflectance of unhomogenized UHT skim-milk as a function of pH and sterilizing temperatures. Tests carried out after sterilization at 100, 110, 120, 129, 140 and 150°C.

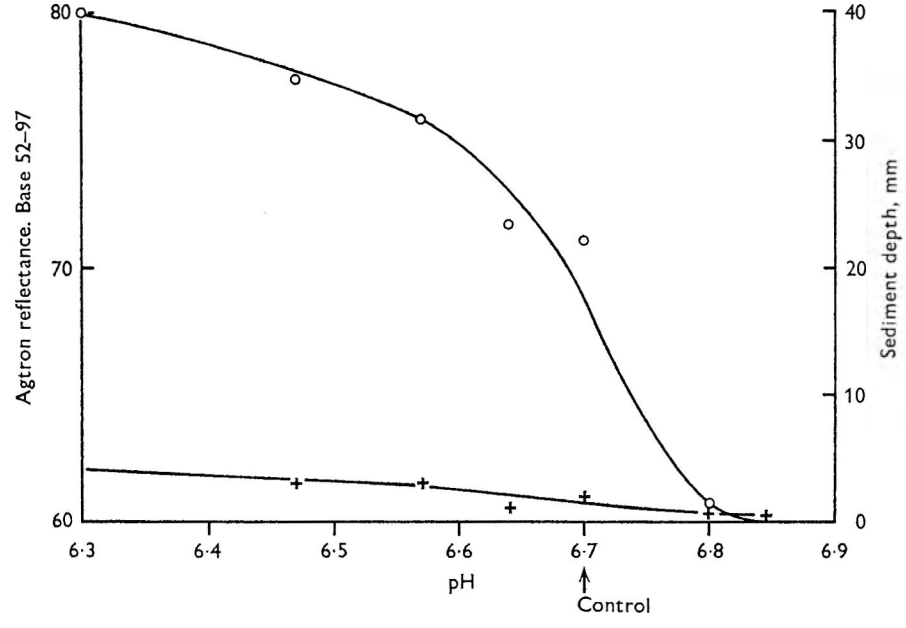


Fig. 6. The effect of pH adjustment by the addition of sodium di-hydrogen phosphate and di-sodium hydrogen phosphate on the reflectance and sediment of unhomogenized UHT skim-milk. ○, Agtron reading; +, sediment depth.

phosphate and di-sodium hydrogen phosphate to adjust the pH of skim-milk yielded the pH-reflectance and pH sediment curves shown in Fig. 6. The reflectance curve was similar to that reported by Burton (1955*a*) and, as would be expected, the degree of sediment formation was very small. Similar curves were observed upon addition of

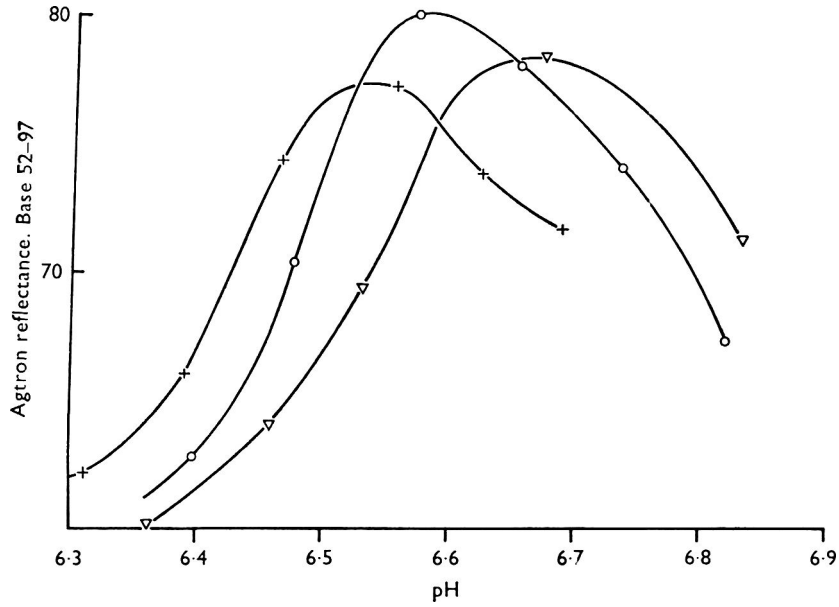


Fig. 7. The effect of the addition of calcium chloride or EDTA on the reflectance of unhomogenized UHT skim-milk. ▽, 0·05% $CaCl_2$ series; +, 0·10% EDTA series; ○, control series.

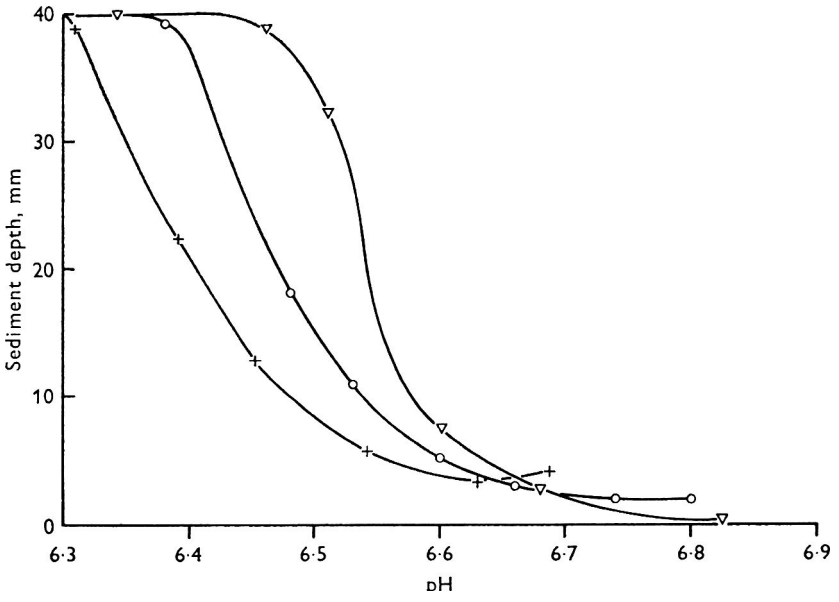


Fig. 8. The effect of the addition of calcium chloride or EDTA on the sediment depth in unhomogenized UHT skim-milk. ▽, 0·05% $CaCl_2$ series; +, 0·10% EDTA series; ○, control series.

0·2 % sodium citrate to the skim-milk followed by pH adjustment with hydrochloric acid. When either acetic acid or orthophosphoric acid was used for pH adjustment of raw skim-milk, the pH-reflectance–sediment curves were similar to those shown in Fig. 1.

The action of phosphate and/or citrate salts points to a specific role of calcium in the sediment formation. To clarify this role, further tests were carried out with the addition of either 0·05 % calcium chloride or 0·1 % ethylenediaminetetraacetic acid di-sodium salt (EDTA) followed by pH adjustment with hydrochloric acid or sodium hydroxide. The reflectance-pH curves from these series (together with a control) are shown in Fig. 7 and the sediment–pH curves in Fig. 8. The use of calcium chloride shifted the peak of the pH-reflectance curve to a higher pH value, whereas the use of 0·1 % EDTA resulted in a more acid-stable milk with a maximum at a lower pH value. The curves obtained at higher levels of EDTA addition (0·3 %) were similar to those in Fig. 6. The addition of enough EDTA (0·3 %) to bind about one-third of the calcium present prevented the sediment formation as the pH decreased.

## DISCUSSION

It has been shown by Zadow (1969) that the effect of UHT treatment on the reflectance of milk at normal pH is controlled mainly by the sterilization temperature and holding time employed in the process. As the sterilization temperature is increased with constant holding time, the reflectance initially increases, reaches a maximum and then decreases. Rowland (1933) suggested that heat-coagulation of the soluble proteins of milk occurred in 2 stages, an initial denaturation being followed, under suitable conditions, by precipitation of the protein. Only protein precipitated in particles large enough to scatter light will influence the reflectance of the milk. The pH of the system will be a major factor influencing the degree of coagulation of the denatured protein. The level of non-casein N remaining in UHT milk is pH-dependent, but in these studies only relatively small changes occurred in this value. This suggests that a change in the degree of whey protein denaturation with pH is not a major factor influencing the pH–reflectance curves.

In studies on the variation of whitening in milk occurring on heating to 90 °C Burton (1955*a*) reported an almost linear rise in reflectance with decreasing pH down to pH 6·15, the lowest level examined. The effect of UHT treatment on reflectance did not follow this pattern; a maximum reflectance was found at approximately pH 6·7. Below this value a rapid drop in reflectance with decreasing pH was observed.

The sediment-reflectance–pH curves in Figs 4 and 5 show that the unexpected decrease in whiteness with decreasing pH results from formation of gross amounts of proteinaceous precipitate. The cause of the formation of this casein–whey protein complex is difficult to determine, particularly as a relatively small change in pH (0·3 units) shifts the quantity of the precipitate from almost zero to about its maximum value on UHT treatment. Yoshiro, Wilson & Herreid (1961), in studies on the size of protein particles in UHT milk, determined sedimentation values on skim-milk acidified with either acetic or phosphoric acid. The rate of change in sedimentation values with pH increased rapidly as the pH was decreased from 6·58, reaching a maximum at approximately pH 6·35. With further decrease in pH, the rate of change

of sedimentation decreased rapidly. However, gross formation of precipitate was not reported to occur in these studies.

For their work, Yoshiro *et al.* (1961) employed a Mallory indirect tubular heater with a sterilization temperature of 145 °C and no intentional holding time. This contrasts with the direct steam injection system operating at 149 °C with a holding time of 3·0 s used in this work.

Homogenization of skim-milk exerts only a minor effect on the pH-reflectance–sediment curves. In comparison, homogenization of whole milk results in a pH-reflectance curve with only minor variations, although heavy sediment formation is observed at low pH values. The absence of major changes in reflectance may be due to the masking effect of the homogenized fat globules or to an influence of fat–protein complex formed during homogenization.

Of major importance is the stabilizing effect of sodium phosphates and sodium citrate. Both of these compounds sequester calcium and it is therefore possible that a change in calcium equilibria is responsible for the formation of the precipitate. The effect of the addition of calcium chloride to the milk supports this view. Stabilization may be simply a matter of calcium distribution, as it was possible to produce sediment-free samples by addition of EDTA. Of further interest is the fact that bacterial action in raw milk before UHT treatment, resulting in a pH decrease of 0·2–0·3 units, yielded a product containing gross amounts of sediment. This emphasizes yet again the need to use only high quality raw milk for the manufacture of UHT products.

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# Comparison of milks processed by the direct and indirect methods of ultra-high-temperature sterilization

## V. Denaturation of the whey proteins

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Summary. The distribution of protein nitrogen was determined in milk treated by an experimental ultra-high-temperature (UHT) plant, operating alternatively as an indirect or as a direct heating plant.

The extent of denaturation of $\beta$-lactoglobulin can be used to assess the relative severity of UHT heat treatments; this criterion was used to compare the indirect and direct arrangements of the plant with each other and with other published results.

The time-temperature profiles of the plant were used to calculate the expected extent of denaturation of $\beta$-lactoglobulin during treatment of the milk. The results of the calculations are in fair agreement with the analytical results.

An experimental ultra-high-temperature (UHT) plant has been described by Burton & Perkin (1970) which is capable of heating milk by either direct steam injection or by indirect heating; for both arrangements, the time-temperature profiles are known. Although the 2 arrangements have almost identical sporicidal effects (Franklin, Underwood, Perkin & Burton, 1970), the extent of denaturation of the whey proteins is not expected to be the same (Burton & Perkin, 1970). Accordingly, the distribution of protein nitrogen in milk treated by the plant was measured, and the results compared with values found by other workers.

### EXPERIMENTAL METHODS

The UHT plant and its operation have been described by Burton & Perkin (1970). After taking a sample of unheated milk for analysis, half of each bulk milk was treated by the plant operating as an indirect heating plant, and the other half by the plant operating as a direct heating plant. The time–temperature profiles were slightly different from those given by Burton & Perkin (1970) and are shown in Figs 1 and 2: maximum temperatures were 141·1 °C for indirect heating and 143·9 °C for direct.

Milk samples were fractionated and analysed as described by Aschaffenburg & Drewry (1959).

## RESULTS AND DISCUSSION

### *The distribution of protein nitrogen*

Any UHT treatment of milk causes at least partial denaturation of some of the whey proteins; on fractionation, this leads to an apparent decrease in the non-casein

Table 1. *Distribution of nitrogen, in mg N/100 g milk, in milk processed by the direct and indirect UHT method*

| | Unheated | Indirect process | Direct process |
|---|---|---|---|
| Total N | (523·4)* | 506·2 | 504·5 |
| Casein N | 384·0 | 448·7 | 436·6 |
| Non-casein N | 121·3 | 57·5 | 67·9 |
| Non-protein N | 26·2 | 26·1 | 25·7 |
| Non-casein protein N | 95·1 | 31·5 | 42·2 |
| Proteose-peptone N | 10·8 | 7·2 | 8·0 |
| Globulin N | 13·0 | 2·2 | 4·0 |
| Total albumin N | 71·3 | 22·1 | 30·2 |
| β-Lactoglobulin N | 40·1 | 8·5 | 14·0 |
| Residual albumin N | 31·2 | 13·6 | 16·2 |

* Value probably in error; 505·3 assumed for calculation of casein N.

Table 2. *Residual native β-lactoglobulin as a percentage of the initial concentration, in milk treated by various UHT plants*

| Type of plant | Native β-lactoglobulin, % | Reference |
|---|---|---|
| Direct heating plants: | | |
| Alfa-Laval (pilot plant) | 65·5 | (1) |
| APV (at N.I.R.D.) | 34·9 | (2) |
| Uperiser (in Switzerland) | 27·1 | (3) |
| VTIS | 26·4 | (4) |
| Uperiser (in Germany) | 19·0 | (4) |
| Indirect heating plants: | | |
| APV (at N.I.R.D.) | 21·2 | (2) |
| Ahlborn | 11·1 | (4) |
| Sterideal | 9·7 | (4) |
| Alfa-Laval (pilot plant) | 8·9 | (1) |
| APV (in Germany) | 6·9 | (4) |
| APV (in Italy) | 0–15·4 | (5) |
| APV (in Russia) | 0 | (6) |

(1) Zadow (1969), from the figures for milk heated to 135°C.
(2) This paper: see Table 1.
(3) Hostettler, Imhof & Stein (1965).
(4) Lembke, Frahm & Wegener (1968).
(5) Corradini & Bottazzi (1966).
(6) Zhdanova & Sergeeva (1968).

N, since denatured whey proteins precipitate with the casein. On further fractionation of the non-casein N, this decrease will be partitioned among the various whey proteins according to their heat stabilities and the severity of the heat treatment. This is illustrated by Table 1, which shows the nitrogen distributions found for one of the milks treated by the UHT plant; 2 other bulk milks which were treated in the same

way gave very similar results. From these results, it is clear that the direct method gives milk with more undenatured whey protein than the indirect method, in spite of the processing temperature being slightly higher in order to achieve the same sporicidal effect. This is as expected; the reasons are discussed by Burton (1969) and Burton & Perkin (1970).

To compare the effect of different UHT plants, it is convenient to select one of the fractions shown in Table 1, such as $\beta$-lactoglobulin, and to calculate the residual native protein as a percentage of the $\beta$-lactoglobulin present in the milk before heating; this is necessary because the amount of $\beta$-lactoglobulin (and the other whey proteins) in raw milk varies considerably. Table 2 shows the result of such calculations applied to the figures in Table 1 and to some other published figures. The results have been grouped according to the method of heating, and it is clear that in general less $\beta$-lactoglobulin is denatured in the direct heating plants than in the indirect. Unfortunately nothing is known of the temperature profiles or sporicidal efficiencies of these plants (except for the APV plant at the N.I.R.D.), and even the maximum temperature is not known in most cases. However, the results in Table 2 suggest that the values of the percentage native $\beta$-lactoglobulin can be taken as an index of the severity of heat-treatment in UHT plants.

### *Calculations of $\beta$-lactoglobulin denaturation*

An empirical equation for the denaturation of $\beta$-lactoglobulin as a function of time of heating was found by Lyster (1970). The equation is:

$$\frac{c}{c_0} = \frac{1}{1 + c_0 kt}, \tag{1}$$

where $c$ is the concentration of $\beta$-lactoglobulin in g $l^{-1}$ at time $t$ in seconds, $c_0$ is the initial concentration and $k$ is the rate constant in $g^{-1}\,l\,s^{-1}$. Other equations expressing $k$ as a function of temperature were also given, valid for different temperature ranges; they are:

$$\log k = 37{\cdot}95 - 14{\cdot}51\ (10^3/T) \tag{2}$$

for the range between 68 and 90 °C, and

$$\log k = 5{\cdot}98 - 2{\cdot}86\ (10^3/T) \tag{3}$$

for the range between 90 and 135 °C, where $T$ is the temperature in K. These equations can be used with the temperature profiles to calculate the value of $k$ at any point in the UHT plant, if it is assumed that the second equation can be extrapolated without error from 135 to 143·9 °C. The results for the 2 arrangements of the plant are shown in Figs 1 and 2 as profiles of rate constant against time, with the temperature profiles also shown in the same figures. The maximum temperatures in these figures are those actually measured on the plant and are 141·1 °C for the indirect heating arrangement and 143·9 °C for the direct. Other differences between the profiles in these figures and those in Burton & Perkin (1970) arise from minor alterations in connecting pipes.

These profiles of rate constant versus time can be used to calculate the extent of denaturation of $\beta$-lactoglobulin during passage through the plant. There are 2 parts to

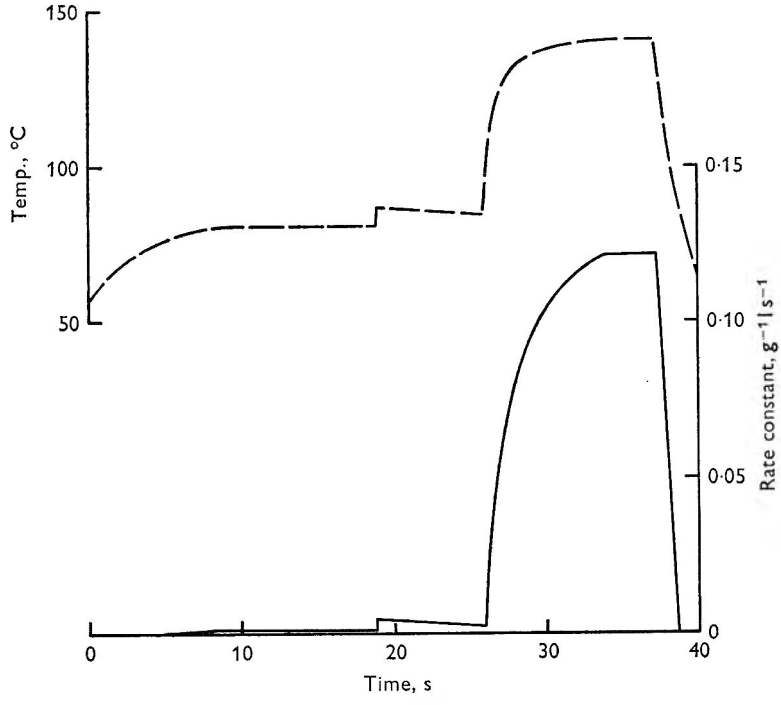


Fig. 1. The indirect heating arrangement. ——, Profile of the rate constant for $\beta$-lactoglobulin denaturation versus time; – – –, profile of the milk temperature versus time.

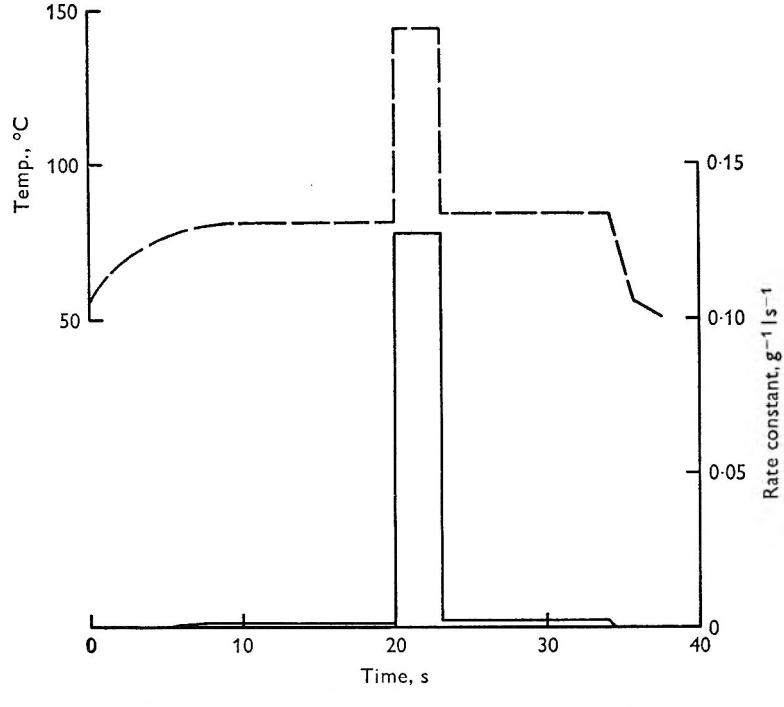


Fig. 2. The direct heating arrangement. ——, Profile of the rate constant for $\beta$-lactoglobulin denaturation versus time; – – –, profile of the milk temperature versus time.

the calculation. First, the area under the rate-constant profile is numerically integrated to find a value of $\int k(t)dt$ characteristic of that profile. Secondly, the value of the integral is used to replace the product $kt$ in equation (1) from which the expected concentration after heat-treatment can then be found. This method of calculation is in principle the same as that given by Burton (1958) for the bactericidal effectiveness of a UHT plant, simplified by omitting consideration of the distribution of residence times. The validity of the method depends on the assumption that consecutive heat-treatments at different temperatures are additive in their effect; for β-lactoglobulin denaturation, this assumption was tested and found to hold, within experimental error, by Lyster (1970).

Table 3. *Comparison of found and calculated percentage residual β-lactoglobulin in 3 milks after UHT treatment*

| Date of processing | Indirect heating | | Direct heating | |
|---|---|---|---|---|
| | Found | Calculated | Found | Calculated |
| 4 March | 21·2 | 24·0 | 34·9 | 47·6 |
| 18 March | 16·7 | 23·7 | 31·0 | 47·3 |
| 6 May | 16·5 | 25·1 | 28·9 | 49·2 |

The final results of these calculations are shown in Table 3 for 3 samples of milk, as percentage native β-lactoglobulin, together with the experimental results of the analysis of these milks, expressed in the same way.

It is seen that agreement is only fair, especially for the direct heating calculations; in all cases the expected amount of β-lactoglobulin is greater than that found analytically. However, the calculations are of value in indicating qualitatively the relative effects of the 2 heating methods, since they correctly predict that much less β-lactoglobulin should be denatured by the plant in the direct heating arrangement than in the indirect one.

There are several possible causes of the discrepancies between found and calculated amounts of β-lactoglobulin. For example, the assumption that consecutive heat-treatments are additive may be only approximately true; also it is possible that the very high turbulence in these plants enhances the rate of denaturation. Another consideration is that the time–temperature profiles used as a basis for the calculations are mean profiles; in reality, there is a distribution of milk residence times. How much this affects the calculation of the extent of reaction is not known. Finally, some parts of the milk must attain briefly temperatures that are considerably higher than those shown in the temperature profiles; for example, near the steam inlet in the direct heating arrangement, and in the layer of milk adjacent to the surface of the heat-exchanger plates in the indirect heating arrangement. In these calculations no attempt has been made to allow for these effects, but all of them might give rise to significant changes in the extent of denaturation.

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# Some aspects of the chemical composition of the milk fat globule membrane during lactation

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Summary. The protein, phospholipid and neutral lipid composition of a deoxycholate-soluble fraction (DOCM) from bovine milk fat globule membrane (FGM) was determined, at intervals during lactation, in 3 cows maintained at a constant level of food intake. It was suggested that the appearance of free fat in washed cream during the first 2 days post partum was related to membrane stability in colostral secretion. Differences between the cows in DOCM yield (mg/100 g cream lipid) and composition were greatest during the first 25 days post partum. DOCM yield increased markedly as lactation proceeded in one animal, and this was thought to be due to a decrease in globule size. Variation in DOCM composition was principally due to an alteration in neutral lipid content. There were few differences, however, between the quantities of phospholipid and protein in DOCM for all 3 animals, and similarly the percentage composition of the major DOCM phospholipids varied little, phosphatidyl choline being the predominant phospholipid.

Values so far reported for the composition of the bovine milk fat globule membrane (FGM) have been obtained using bulk milk samples from different breeds, and employing a variety of techniques for membrane isolation (Hayashi & Smith, 1965; Huang & Kuksis, 1967; Chien & Richardson, 1967; Swope & Brunner, 1970). These variables are generally accepted as being responsible, at least in part, for the observed differences in membrane composition (Prentice, 1969; Storry, 1970).

Comparative aspects of FGM composition, in terms of animal variation, breed, nutrition and stage of lactation, have not been fully investigated. Cheeseman & Mabbitt (1968), from studies on the properties of FGM in relation to fat retention in cheese curd, suggested that between-animal differences in membrane composition occur. Huang & Kuksis (1967) demonstrated quantitative changes both in individual lipids and in their component fatty acids when comparing FGM prepared from summer and winter milks.

There are elements in the structure of the FGM which are very easily removed by a variety of physical forces (Brunner, 1965*a*). It is likely that the outer layer is involved (Mabbitt & Cheeseman, 1967) and that this layer is important for the stability of the fat emulsion in milk (Prentice, 1969). Hayashi & Smith (1965) showed that when cream was treated with sodium deoxycholate (DOC) a fraction rich in phospholipid and representing approximately 40% of the total membrane weight

was released in a soluble form. They postulated that this material originated from the outer portion of the membrane.

The object of the present work was to determine whether the lipid and protein composition of DOC-soluble membrane (DOCM) varied with stage of lactation. Milk from individual cows, fed at a constant level of intake, was used as the source of membrane material. A preliminary account of the results for one cow, in early lactation, has been published (Anderson & Cheeseman, 1970).

## EXPERIMENTAL

### *Animals*

Three Friesian cows were used, each of which had completed 2 previous lactations. Feed intake was maintained constant irrespective of milk yield. The animals were housed and stall-fed throughout the experiment and given a ration consisting of 6–8 kg meadow hay plus 12 kg commercial dairy concentrate twice daily. The calving dates of the cows were as follows: cow 1, 25 October 1969; cow 2, 18 February 1970; cow 3, 21 March 1970. The day numbers used in subsequent paragraphs refer to the number of days after parturition.

### *Samples*

All the samples were obtained from the morning milking except those representing days 1 and 2 for cow 1. A 4·5-l subsample was taken from the total milk yield and placed in a vacuum flask immediately after milking. The time interval between milking and preparation of the membrane was 60–90 min, the temperature of the milk being maintained between 34 and 36 °C during that period.

Sampling frequencies were not identical for all 3 cows. Cow 1 was sampled daily for the first 14 days, and then once every 3 days for the next 14 days, once every 7 days for the following 14 days and then once every 40 days. Sampling frequencies were similar for cows 2 and 3 except that, in the light of the results for cow 1, sampling frequency was reduced during the first 28 days.

### *Cream preparation*

Cream was prepared from 4·5 l of uncooled, fresh, raw milk by separation in an Alfa Laval model AE 32 laboratory separator that had been prewarmed with deionized water at 40°C. The cream was washed 3 times in 5 volumes of 0·15 M-sodium phosphate buffer of pH 7·0, containing 0·15 M-sodium chloride and 0·25 M-sucrose (Hayashi & Smith, 1965) at 40 °C, the fat percentage of the cream being adjusted to 40 % between washes by addition of an appropriate amount of wash buffer. The effectiveness of the washing technique in removing residual milk proteins was monitored by checking the washings for their presence. Antibody–antigen precipitation tests were carried out on Ouchterlony agar diffusion plates (buffer 0·1 M veronal of pH 8·6 containing 0·2 M EDTA) using rabbit antisera to $\alpha_{s1}$-, $\beta$-, $\kappa$-caseins, $\alpha$-lactalbumin and $\beta$-lactoglobulin.

### *Membrane isolation*

The preparation of a membrane fraction (DOCM) soluble in the presence of sodium deoxycholate (DOC) was based on the method of Hayashi & Smith (1965). Washed

cream (40–70 ml) was adjusted to a fat content of 50 % by adding buffered sucrose, and shaken very gently for 1 h at 40 °C, after the addition of a solution of DOC to a concentration of 0·5 % (w/v). A stock solution containing 10 % DOC (w/v) was made up in 0·15 M-sodium phosphate buffer of pH 8·0, containing 0·15 M-sodium chloride and 0·25 M-sucrose. Treated cream was centrifuged in 100 ml tubes at 14000 ***g*** for 30 min at 35 °C and separated into an aqueous layer, a pellet ($P_1$), and a residual fat layer with a white fluffy low-density material attached to its lower surface. To facilitate removal of the layers, the fat was solidified by placing the centrifuge tube in crushed ice. The aqueous layer was carefully poured off through a hole made in the fat layer and was further centrifuged in 50 ml tubes at 50000 ***g*** at 4 °C for 90 min. A clear solution of the DOCM and a pellet ($P_2$) were obtained. The solution of DOCM was decanted from the pellet and dialysed for 48 h against 0·1 M-sodium phosphate buffer solution of pH 7·5 containing 0·15 M-sodium chloride. The total DOCM volume was determined and a portion removed for nitrogen analysis. The remainder was concentrated under nitrogen at 30 °C in a rotary evaporator.

Pellets $P_1$ and $P_2$, both red-brown in colour, and the residual fat layer with its adherent layer of low density material were discarded.

### *Analytical*

DOCM was extracted with chloroform–methanol (2:1, v/v) and non-lipid material was removed by washing with 0·88 % (w/v) aqueous KCl according to the method of Folch, Lees & Sloan Stanley (1957). The lipid residue was dried to constant weight under nitrogen, and stored at −10 °C in 20–30 ml chloroform–methanol (2:1, v/v) containing 0·02 % butylated hydroxytoluene as an antioxidant.

Phospholipids were separated by thin-layer chromatography. Silica Gel G layers (0·4 mm) were prepared on 20 × 20 cm glass plates and activated for 2 h at 110 °C before use. About 10 mg lipid were spotted on to the plate which was then developed at room temperature in 150 ml of a solvent containing chloroform–methanol–28 % aqueous ammonia (130:50:11, v/v/v) in glass chambers measuring 23 × 23 × 7 cm. The running distance was 17 cm in approximately 1·25 h. Lipid bands were made visible by treatment with iodine vapour, and identified by reference to standard phospholipids. Individual phospholipids were isolated by scraping off the appropriate band into Quickfit tubes with size 19/26 stoppers, and extracted from the silica gel by shaking with 15 ml of methanol–HCl (90:10, v/v) for 2 min. The extract was clarified by centrifugation for 15 min at 1000 ***g***.

Phosphorus was determined by the method of Chen, Toribara & Warner (1956) and nitrogen by that of Lang (1958).

A factor of 25 was used to convert mg lipid phosphorus to mg phospholipid, and of 6·25 to convert mg nitrogen to mg protein.

## RESULTS

Cows 2 and 3 died during the experiments and no samples were obtained from them after days 82 and 45 respectively. Sampling from cow 1 was stopped at day 248 since this animal had persistent mastitis. When mastitic milk was used there was a tendency

for some oiling-off to occur during DOC treatment. With non-infected milk a 1-h incubation of 50% cream with 0·5% DOC (w/v) did not result in any free fat formation.

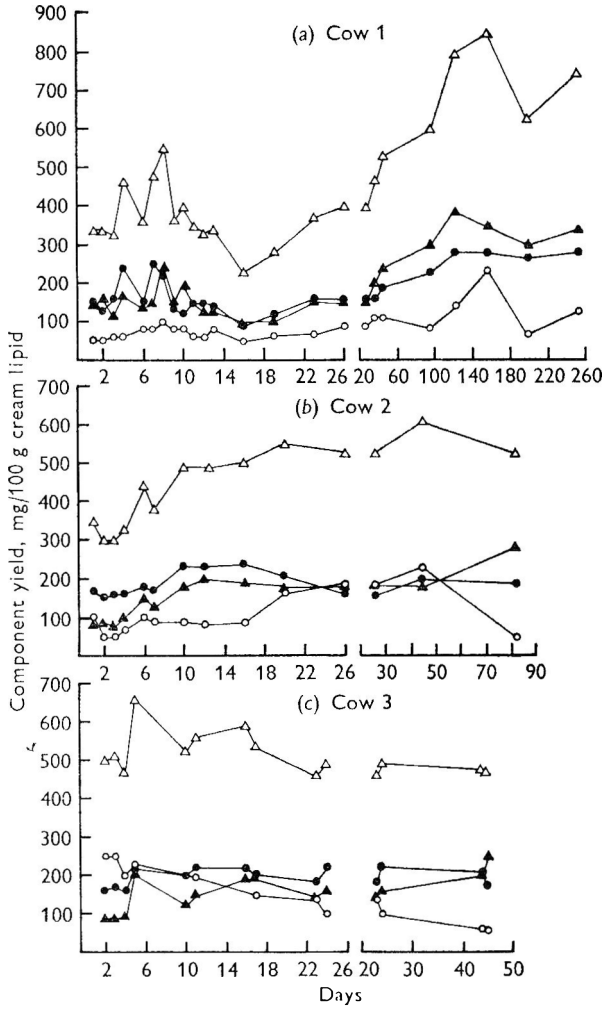


Fig. 1. Effect of stage of lactation on the quantity (mg/100 g cream lipid) of total DOCM (△), and DOCM protein (▲), phospholipid (●) and neutral lipid (○).

The results of the antibody–antigen diffusion precipitation tests showed that milk proteins were not detected, either in the washings of wash 3 or in the DOCM fraction, when the cream was diluted with 5 times its volume of buffered sucrose for each wash.

There was formation of some free fat in the separator and the appearance of a very small number of oil droplets in washed cream on days 1 and 2. The colour of the DOCM from these creams was also very pale but the intensity increased from day 2 to day 5 to a yellow-brown colour which was maintained throughout the experiment.

The milk fat percentage was more variable in cows 1 and 3 from days 1 to 10 (3·3–8·9 and 2·4–5·6 respectively) than during the rest of the lactation, when the

corresponding ranges were 2·5–3·8 and 2·6–3·6. The range for cow 2 for days 1 to 82 was 2·5–4·0.

The quantity of DOCM isolated from each of the 3 animals and the amounts of membrane neutral lipid, phospholipid and protein, expressed as mg/100 g cream

Table 1. *Gross composition of the DOCM fraction from cow 1*

| Days post partum | Neutral lipid, % | Phospholipid, % | Protein, % |
|---|---|---|---|
| 1 | 15·4 | 44·0 | 40·6 |
| 2 | 15·8 | 37·1 | 47·1 |
| 3 | 17·6 | 47·2 | 35·2 |
| 4 | 12·4 | 50·6 | 37·0 |
| 6 | 22·6 | 39·9 | 37·5 |
| 7 | 16·5 | 52·4 | 31·1 |
| 8 | 18·3 | 39·4 | 42·3 |
| 9 | 21·5 | 37·1 | 41·4 |
| 10 | 20·5 | 30·8 | 48·7 |
| 11 | 17·3 | 42·0 | 40·7 |
| 12 | 17·0 | 44·0 | 39·0 |
| 13 | 22·7 | 39·6 | 37·7 |
| 16 | 20·0 | 40·0 | 40·0 |
| 19 | 21·5 | 42·2 | 36·3 |
| 23 | 17·4 | 42·5 | 40·1 |
| 26 | 23·0 | 40·2 | 36·8 |
| 37 | 23·2 | 34·0 | 42·8 |
| 44 | 20·6 | 34·7 | 44·7 |
| 93 | 13·5 | 37·3 | 49·2 |
| 121 | 17·2 | 34·6 | 48·2 |
| 157 | 26·7 | 32·5 | 40·8 |
| 205 | 11·5 | 41·8 | 46·7 |
| 248 | 17·6 | 36·8 | 45·6 |

Table 2. *Gross composition of the DOCM fraction from cow 2*

| Days post partum | Neutral lipid, % | Phospholipid, % | Protein, % |
|---|---|---|---|
| 1 | 29·4 | 46·8 | 23·8 |
| 2 | 17·6 | 52·4 | 30·0 |
| 3 | 19·5 | 55·2 | 25·3 |
| 4 | 21·3 | 48·7 | 30·0 |
| 6 | 22·2 | 42·3 | 35·5 |
| 7 | 21·2 | 45·3 | 33·5 |
| 10 | 16·0 | 46·8 | 37·2 |
| 12 | 12·7 | 45·9 | 41·4 |
| 16 | 13·6 | 47·8 | 38·6 |
| 20 | 30·4 | 38·0 | 31·6 |
| 26 | 34·4 | 31·3 | 34·3 |
| 47 | 37·7 | 32·7 | 29·6 |
| 82 | 9·1 | 36·4 | 54·5 |

lipid, are shown in Fig. 1. The percentage composition of the DOCM is shown in Tables 1–3.

Preparation of DOCM was not usually done in duplicate, but duplicate preparations carried out from time to time during the experiment showed that replication was satisfactory.

Table 3. *Gross composition of the DOCM fraction from cow 3*

| Days post partum | Neutral lipid, % | Phospholipid, % | Protein, % |
|---|---|---|---|
| 2 | 50·2 | 31·7 | 18·1 |
| 3 | 48·5 | 33·5 | 18·0 |
| 4 | 44·7 | 34·9 | 20·4 |
| 5 | 34·6 | 33·8 | 31·6 |
| 10 | 37·4 | 38·6 | 24·0 |
| 11 | 34·0 | 39·5 | 26·5 |
| 16 | 32·1 | 36·5 | 31·4 |
| 17 | 28·4 | 35·6 | 36·0 |
| 23 | 29·7 | 39·8 | 30·5 |
| 24 | 20·4 | 46·2 | 33·4 |
| 44 | 14·3 | 42·7 | 43·0 |
| 45 | 12·0 | 36·6 | 51·4 |

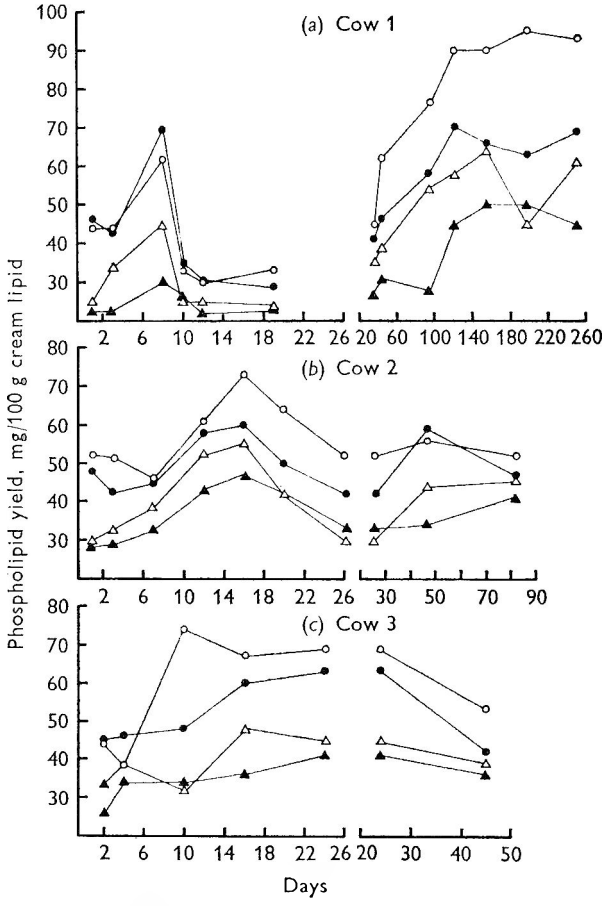


Fig. 2. Effect of stage of lactation on the quantity (mg/100 g cream lipid) of the major DOCM phospholipids, phosphatidyl choline (○), sphingomyelin (●), phosphatidyl ethanolamine (△) and phosphatidyl serine (▲).

It is well known that overall milk composition varies from day to day. In cow 3 samples were taken on successive days and showed that while day-to-day variation occurred (see Fig. 1*c*) it was small compared with the changes that occurred over a longer period.

During the first 45 days the amount of DOCM/100 g cream lipid isolated was different for the different cows. This was principally due to variation in the amounts of neutral lipid, especially in cow 3 from day 1 to day 25 and in cow 2 from day 20 to day 47. The level of neutral lipid was relatively constant in cow 1, representing 15% of the total DOCM on day 1 and 18% on day 248. Similar quantities of protein and phospholipid were found in the DOCM from all 3 animals during days 1–45 although there was a wider range in the values from cow 1.

Table 4. *Mean phospholipid composition of DOCM for individual animals as a percentage of the total phospholipid*

| | Cow 1* | Cow 2† | Cow 3‡ |
|---|---|---|---|
| Phosphatidyl choline | 29·7 | 29·5 | 29·7 |
| S.E. mean (±) | 0·74 | 0·72 | 1·74 |
| Sphingomyelin | 26·9 | 26·1 | 26·8 |
| S.E. mean (±) | 0·78 | 0·53 | 0·93 |
| Phosphatidyl ethanolamine | 20·1 | 21·1 | 20·5 |
| S.E. mean (±) | 0·56 | 0·73 | 1·12 |
| Phosphatidyl serine | 16·8 | 19·0 | 18·3 |
| S.E. mean (±) | 0·69 | 0·51 | 0·84 |

* Mean of 13 values. † Mean of 9 values. ‡ Mean of 6 values.

With the exception of a high level of neutral lipid on day 1 in cow 2, cows 1 and 2 produced similar quantities of DOCM of comparable composition during the first 10 days. There appeared to be little change in DOCM composition, with any of the animals, at the end of colostral secretion.

Protein accounted for a greater proportion of the DOCM than did phospholipid in cow 1 between days 8 and 10 and from day 37 onwards. This was also noted with cow 2 at day 82.

The total yield of the major DOCM phospholipids is shown in Fig. 2, and the mean percentage composition for the DOCM from each animal is shown in Table 4. Phospholipid composition was determined only for every other sample during days 1–25. The missing values apparent from a comparison of Figs 1 and 2 were due to the fact that for some samples (3 from cow 1 and 1 from cow 2) there was insufficient material for phospholipid analysis. With all 3 cows phosphatidyl choline was quantitatively the most important phospholipid in the membrane, but in cows 1 and 3 sphingomyelin predominated in very early lactation.

## DISCUSSION

The most profound changes in milk composition over the lactation period occur during the transition from colostral to normal milk secretion. This appears to be particularly so for levels of fat secretion in the Friesian breed (Parrish, Wise, Hughes & Atkeson, 1950; Garratt & Overman, 1940). It might, therefore, be expected that

changes in FGM composition would be likely to occur at this time. The results from the present experiment suggest that changes in the amount and composition of DOCM were not associated with the cessation of colostral secretion. Values for DOCM yield and composition after parturition show that the differences between cows were greatest during the first 25 days of lactation, and thereafter became smaller.

Although the percentages of phospholipid and protein were different in DOCM (see Fig. 1 and Tables 1–3) the absolute quantities of these components were almost the same for cows 2 and 3 throughout the experimental period. Overall, the quantities of protein and phospholipid for cow 1 were slightly lower than for the other 2 cows during the first 25 days. It seems from the results in Fig. 1 and Tables 1–3 that for all 3 cows protein may well have become the predominant DOCM component after the first 6 weeks or so of lactation.

The DOCM prepared in the present experiment was a fraction essentially similar to that described by Hayashi & Smith (1965); a change in the composition of the stock DOC buffer was the principal difference in the 2 methods of isolation. Total yield of DOCM obtained by Hayashi & Smith (1965), from bulk-mixed Friesian–Jersey milk (833 mg/100 g cream lipid) was higher than that for most of the milks examined from cows 1, 2 and 3. In general, the composition of the Hayashi-Smith membrane (44 % protein, 43 % phospholipid, 13 % neutral lipid) showed a higher proportion of protein and a lower proportion of neutral and phospholipids than the DOCM composition given in Tables 1–3, particularly for the early part of lactation.

It is usually accepted that losses of membrane material occur during FGM preparation (Swope & Brunner, 1968). Such losses are likely to be accentuated by any decrease in FGM stability. Free fat was observed both in the separator and in washed cream in the preparations from all 3 cows on days 1 and 2. DOCM protein increased on day 3 and day 4 in cows 2 and 3 respectively. It seems probable that some of the membrane concerned with FGM stability was more easily removed, certainly on days 1 and 2, than with normal milk. The presence of alkaline phosphatase and xanthine oxidase in DOCM was observed by Hayashi, Erickson & Smith (1965), and since in our experiments there was an increase in the yellow colour of DOCM from day 1 to day 5, we suggest that at least part of the removed material was enzymic in origin as xanthine oxidase has a yellow colour in dilute solution (Avis, Bergel & Bray, 1955).

Kernohan & Lepherd (1969) indicated that the average globule size and the number of globules/g fat increased as milk fat percentage increased. Thus, the amount of FGM/unit fat would be expected to decrease. There were only very small changes in milk fat percentage from day 16 until day 157 in cow 1, during which time the DOCM yield increased by about 250 % with little compositional change. It appears likely that this increase was due to a decrease in fat globule size, although the published information on changes in globule size with stage of lactation is not definitive on this point (Brunner, 1965*b*). Variation between animals and between stages of lactation in DOCM composition was largely in the neutral lipid fraction, although shifts in phospholipid and protein did occur. The possibility arises that contamination of DOCM by residual membrane or by milk fat triglyceride might have occurred. The most likely source of contamination during the preparation of DOCM is the low-density material discarded with the residual fat layer described above (p. 411).

Compositional studies on this material (Anderson, Cheeseman & Shipe, unpublished) revealed that up to 50% of the dry matter was neutral lipid. However, large and varying amounts of contamination would be required to account for the large variation in the percentage of neutral lipid in the DOCM.

Contamination by milk-fat triglyceride can occur when a cooling step is introduced during FGM preparation (Vasic & deMan, 1964). In this way a systematic error would be introduced. However, neutral lipid variation in DOCM was not constant and usually proceeded in stages with occasional isolated peaks, suggesting that a consistent contamination by triglyceride had not occurred. It would appear likely that the variations in neutral lipid shown in Fig. 1 represent a true reflexion of variation in DOCM composition.

There were relatively few changes in percentage composition of the major DOCM phospholipids. Recent evidence (Kayser & Patton, 1970) suggests that the long-chain fatty acids ($C_{20}$–$C_{25}$) of sphingomyelin have a functional significance associated with FGM stability. It is of interest to note that at a time when the fat emulsion appeared to be least stable, sphingomyelin was the predominant phospholipid in the DOCM of cows 1 and 3.

The detailed analysis of a quantitatively significant fraction of the FGM clearly illustrates that variation in the total yield and composition of that fraction can occur both during a lactation and between individual cows of the same breed fed at a controlled dietary intake, although in the present work the quantities of protein and phospholipid in the fraction were similar between cows and over much of the lactation. Such variation could readily account for the differences in values for yield and composition of FGM reported by various workers.

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# Reviews of the progress of Dairy Science

## Section A. Physiology. Organ culture techniques and the study of hormone effects on the mammary gland

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## INTRODUCTION

The technique of organ culture involves the maintenance of whole organs or parts of organs (explants) in vitro, usually for periods of 1–2 weeks. Conditions are such that outgrowth is discouraged and the normal development, architecture and function of the tissue is maintained as far as possible. In these respects it differs from tissue culture, in which active outgrowth of cells occurs, often resulting in loss of the original organization of the tissue; and from cell culture, in which an agent such as collagenase is used to dissociate cells which are then grown as sheets on a suitable support or in suspension (Moscona, Trowell & Willmer, 1965).

The first development of the organ culture technique took place as long ago as the 1920s (Fell, 1964; Willmer, 1965), but extensive application to the study of the mammary gland has only occurred since 1950 (see reviews by Fell, 1964; Lasnitski, 1965; Dieterlen-Lièvre, 1970). The earlier studies were primarily histological, concerning the progressive development of the mammary gland and the effects of hormones on it. Since cultured tissues are dependent on diffusion for the exchange of

materials, the maximum size of explants is limited to a few cubic millimetres, and this has restricted their usefulness for biochemical studies. However, the development of suitable microtechniques has more recently enabled extensive biochemical investigations to be made (Turkington, 1968*a*, 1970*a*; Topper, 1970).

The use of an in vitro system has certain obvious advantages in isolating the tissue from the body and offering the possibility of placing it in a controlled environment. For reasons discussed in detail later (p. 439) organ culture offers a number of distinct advantages over the use of slices and over tissue and cell culture methods for the study of hormonal effects on the mammary gland. Any in vitro system is, however, to a greater or lesser degree abnormal and it is important that results obtained should be carefully compared with the results of studies performed in vivo.

The early studies on the mammary gland's response to hormones in vitro have been reviewed by Fell (1964), Lasnitski (1965) and Dieterlen-Lièvre (1970). These studies will therefore be considered only briefly and in relation to more recent work.

## METHODS

The methods which have been used to perform organ cultures of mammary gland vary widely in detail, but the basic principles remain the same. There are a number of essential requirements and these will be considered. Much invaluable practical information on setting up cultures is provided by Moscona *et al.* (1965) and Paul (1970). Culture and analytical methods which are especially applicable to the mammary gland have been reviewed by Rivera (1971).

### *Prevention of contamination*

This can only be accomplished by careful attention to sterile techniques. Apparatus is sterilized by dry heat or autoclaving, solutions by filtration. Antibiotics, especially penicillin and streptomycin, are often incorporated into the culture medium, but should not be relied on to prevent contamination. The toxic and metabolic inhibitory effects of many antibiotics on cells should always be considered (Bücher & Sies, 1969).

To avoid the introduction of toxic chemicals, care should also be taken over the selection and cleaning of apparatus (Paul, 1970).

### *Culture media*

Organ culture is carried out using either solid or liquid media, with or without the addition of some natural constituent such as serum or embryo extract. When it is possible to use defined components in a liquid medium, this is to be preferred, since natural constituents are of unknown and variable composition, while liquid media have the advantage of allowing free diffusion and being readily changed or added to as the conditions of culture or experimentation demand. Synthetic media used widely and successfully include medium 199 (Morgan, Morton & Parker, 1950) and medium MB 752/1 (Waymouth, 1959), though such complex mixtures may not always be essential.

In studying the very early stages of mammary gland differentiation a solid medium, such as a plasma clot or agar, appears to be necessary. Media should be changed every few days in prolonged experiments.

*Types of culture vessel*

In 1929, Fell & Robison described the use of a watch-glass in a Petri dish kept humid by a layer of moistened cotton wool, for the organ culture of chick embryo limb rudiments. Virtually all later techniques represent some modification of this basic system, which is illustrated in Fig. 1. In the original experiments, the explants were supported on cock plasma clotted by the addition of an equal volume of embryo extract, but various modifications have been introduced to allow the use of liquid media. The problem is to support explants at the surface of the medium, so that they can obtain nutrients from it without becoming water-logged, as mature organs in particular require a good oxygen supply to survive satisfactorily (Trowell, 1959, 1961). Chen (1954) used lens paper as a raft to support cultures and the use of rayon fibre has also been described (Shaffer, 1956). If rayon soluble in acetone is used, the explants can be supported on it without disturbance in the initial stages of histo-

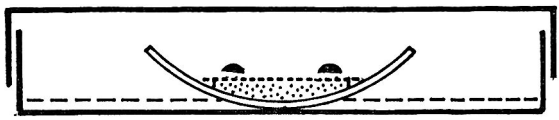

Fig. 1. A vessel for organ culture, after Fell & Robison (1929).

logical processing (Lasfargues & Murray, 1959). Such rafts, however, require very careful handling to avoid sinking and a more versatile method, with fewer technical problems, was introduced by Trowell (1954). In this a stainless-steel grid is used to support the cultures; expanded metal is the most suitable material for rigidity and ease of cleaning (Trowell, 1959). The grid may be overlaid with lens paper, agar, rayon or nylon fibre (Moscona *et al.* 1965) but lobulo-alveolar tissue of mammary gland survives satisfactorily if placed directly on the metal. A culture vessel of this type is illustrated in Plate 1 (*a*) and a simplified version of it, suitable for larger-scale cultures for biochemical studies, is shown in Plate 1(*b*) (Bolton, 1970; Bolton & Bolton, 1970).

For the culture of mammary gland, the gas phase is usually 95% oxygen with 5% carbon dioxide; the carbon dioxide enables the pH of the medium to be controlled. The gas supply may either be an open one, being continuously bubbled through distilled water into boxes containing the culture dishes (Mayne, Barry & Rivera, 1966; Takizawa, Furth & Furth, 1970*a*) or various types of closed container have been described (e.g. Trowell, 1954, 1959; Prop, 1961*a*). Special apparatus enables, for example, the respiration of organ cultures (Lucas, 1965) or their carbon dioxide production (Moretti & Abraham, 1966) to be studied.

Organ culture can also be carried out simply by suspending fragments in a liquid medium in vessels which are preferably shaken to improve oxygenation. This method has been particularly successful with skin slices which float and do not disintegrate on shaking (e.g. Medawar, 1948), but it can also be applied to mammary gland (e.g. Mishkinsky, Dikstein, Ben-David, Azeroual & Sulman, 1967). Mammary gland explants from small rodents contain a good deal of adipose tissue which assists flotation and mid-pregnant mouse gland survives remarkably well, without disintegration, in flasks shaken at 60 strokes/min for 3 days (Forsyth, unpublished).

*Preparation of tissues*

A crucial step is the preparation of the explants which must be done using sterile techniques and with a minimum of damage to the tissue. The methods used will depend on the stage of development of the gland studied. Thus, mammary rudiments from mouse, rat or rabbit embryos can be explanted as whole organs, and the partially developed glands of virgin female mice and rats have also been studied in this way. More developed glands and the glands of larger animals should be cut into suitably sized pieces, usually 1–2 $mm^3$. The microtome described by Stadie & Riggs (1944) has been used to prepare slices of mammary tissue which can then be cut with a scalpel into small squares. This method, however, involves some compression, and where possible dissection under a binocular microscope using fine watch-maker's forceps and spring scissors is to be preferred.

*Histological examination*

Fell (1964) emphasizes the importance of careful histological examination in all organ culture work, including biochemical studies. This is essential, both to determine the response of the cultures to substances administered and to ensure that conclusions are based only on cultures in which survival is good.

## DEVELOPMENT OF THE FOETAL MAMMARY GLAND IN VITRO

Three species – the mouse, the rat and the rabbit – have been studied.

*Differentiation in the absence of hormones*

Early work by Balinsky (1950) and Hardy (1950) showed that ventral body wall explants taken from 10-day-old mouse embryos, before the appearance of the mammary bud on day 11, would differentiate the mammary bud in vitro when cultured on a plasma clot consisting of equal parts of cock blood plasma and chick embryo extract. Balinsky (1950) observed only limited differentiation, even if explants were taken from 11- to 14-day embryos, and could observe no effects of adding oestrogens or mouse pituitary extract to the culture medium. Hardy (1950), however, obtained differentiation to a stage similar to that in the 7-day-old mouse, with the formation of primary, secondary and later ducts, which became canalized, and of a streak canal. The rate of differentiation was similar in vivo and in vitro and cultures were continued successfully for as long as 25 days. Lasfargues & Murray (1959) observed duct development in mouse mammary anlagen transplanted on to agar containing only the synthetic medium 199. It thus appears that the early stages of mammary differentiation can occur in the mouse in the absence of any hormonal stimulation.

In all these experiments the sex of the embryos providing the explants was not determined. Hardy (1950) saw no nipple development and found it impossible to divide the glands which developed in vitro into male and female types, although she noted that almost all glands showed some form of opening at the skin surface. Development in male mice, however, involves detachment of the mammary bud from the epidermis. It has been shown by experiments in vivo that androgens from the foetal testis induce the male type of development and that if the gonads are destroyed

in embryos of either sex the female type of mammary development occurs (see Raynaud, 1969). A female type of development might therefore be expected in vitro in the absence of androgenic hormones. This has been confirmed in recent experiments by Kratochwil (1971). Mammary-gland rudiments from 13-day-old male mouse embryos undergo a female type of development in vitro. Culture of 12- to 14-day-old female mammary rudiments with the testes of 13-day-old male embryos or with very low concentrations of testosterone ($10^{-9}$ M) leads to the male type of regressive changes but explants from 15-day-old female embryos are no longer responsive. Thus, androgens act directly and over quite a short time period to induce the male type of mammary gland development and the genetic sex of the gland is not involved in its response.

*Ectodermal–mesodermal interactions*

The interactions between the epithelium and underlying mesenchyme which are involved in mammary differentiation in the mouse have been studied by Kratochwil (1969), using plasma clots and medium containing horse serum and chick embryo extract. Many of the experiments were carried out on filter assemblies of the type described by Grobstein (1956), which enable the epithelium to be separated from the mesenchyme by cellulose ester filters preventing cytoplasmic contacts between the tissues. Dissociation of epithelium and mesenchyme was achieved either enzymically, using crude trypsin plus pancreatin, or mechanically by peeling the skin from the underlying tissues. When mammary gland rudiments from 12- to 14-day-old mouse embryos were cultured in vitro they developed to the stage seen in 7-day-old female mice; a nipple, nipple sheath, duct system and adipose tissue were formed. The observation of Hardy (1950) that rates of development in vivo and in vitro were similar was also confirmed. Active outgrowth in vitro occurred only after a resting phase, the length of which roughly corresponded with the length of the similar phase occurring between the first appearance of the rudiment in 11-day embryos and outgrowth of the primary sprout on day 16. Combination of older epithelium with 12-day mammary mesenchyme showed that the length of the resting phase was determined by the age of the epithelium, and that normal morphogenesis was achieved in these conditions.

Isolated mammary epithelium failed to develop in the absence of mesenchyme or in the presence of too little mesenchyme. When mammary epithelium was combined with the mesenchyme from salivary gland rudiments of 12-day embryos, a pattern of development similar to that seen in the salivary gland occurred. However, combined with jaw mesenchyme such mammary epithelium developed fairly normally. Mammary mesenchyme was unable to induce morphogenesis in salivary epithelium. Exposure of either epithelium to both salivary and mammary mesenchyme simultaneously strongly inhibited development.

Mammary rudiments from 16-day-old embryos largely failed to develop although the epithelium developed when recombined with mesenchyme from younger embryos. It is suggested that by 16 days the embryonic mesenchyme has become dependent on systemic influences (Kratochwil, 1969).

Similar results were obtained in the rabbit by David & Propper (1964) and Propper & Gomot (1967). Differentiation in vitro was observed in the absence of hormones;

explants of latero-ventral body wall from 12-day embryos formed the mammary ridge, mammary buds and primary sprouts. With prolonged culture degeneration ultimately set in and explants of older embryos (25–29 days) tended to dedifferentiate. Using trypsin to separate epidermis and mesoderm, Propper & Gomot (1967) showed that mammary mesoderm from 12- to 14-day embryos could induce mammary differentiation in indifferent epidermis. Combining mammary epidermis with indifferent mesoderm, they showed that normal development occurred on or after day 13; from this time the mammary epithelium could differentiate on indifferent mesoderm and also influence the latter's development, indicating that induction had occurred.

*The effect of hormones*

All these experiments suggest autonomous early development of the mammary gland, but do not exclude susceptibility to hormonal influences. Lasfargues & Murray (1959) advanced the development and differentiation of mouse mammary glands in vitro by the addition of hormones; the best development of both the mammary gland and the surrounding stroma was achieved with a combination of oestrogen + progesterone + ovine prolactin + bovine growth hormone. By studying these hormones and cortisol, alone and in various combinations, their individual effects were analysed. While oestrogen alone was inhibitory, and progesterone even more so, when combined in a ratio of 1:1000, they no longer inhibited development. Active growth of mammary epithelium was achieved with growth hormone, prolactin increased the degree of differentiation, while cortisol induced duct distension and enabled secretory activity to occur in response to prolactin.

The effects of hormones in vitro on the mammary gland of the 17-day-old rat foetus have been extensively investigated by Ceriani (1969, 1970*a*, *b*). Morphology, mitotic indices and ability to synthesize a casein-like material were studied and the results obtained by the various techniques were found to correlate well. Waymouth's agar medium was used and culture continued for 3, 6 or 9 days. In the absence of any hormones the mammary anlagen grew and branched once, the ducts ultimately developed lumina but did not penetrate the mesenchyme, tissue maintenance became poor and no casein-like material was synthesized. Insulin, which is necessary in vitro for the survival and development of the mammary gland of the adult mouse (Elias, 1959; Rivera & Bern, 1961) and rat (Barnawell, 1965), had marked effects on rat mammary anlagen, improving maintenance and growth. The ducts formed now penetrated the mesenchyme which appeared as a well-developed fat pad; small amounts of casein-like material were synthesized. Ovine prolactin stimulated growth and proliferation of mammary anlagen giving rise to ducts with terminal accumulations of cells, its effect being dependent on dose and on the presence of insulin. Neither bovine growth hormone nor bovine serum albumin could replace prolactin; growth hormone produced necrosis. Aldosterone stimulated the formation of ductules, its effect being most marked in the presence of insulin + prolactin, when the branching ductules lined by a single layer of cells were seen to contain secretion, and synthesis of casein-like material was stimulated. This combination of hormones also most effectively maintained the mitotic index at 9 days. The addition of progesterone further increased growth and secretory activity. The casein-like material formed no

longer showed a single fast-moving band on polyacrylamide gel electrophoresis, but 3 bands, compared with 4 bands of casein from rat milk. Oestrogen to some extent mimicked the effects of progesterone but was toxic at high concentrations and a combination of the 2 hormones proved even more harmful. Testosterone inhibited both mammary differentiation and the synthesis of casein-like material.

Electron microscopy of cultured mammary anlagen (Ceriani, Pitelka, Bern & Colley, 1970) showed complementary changes. In the presence of insulin + aldosterone + prolactin, the anlagen from 17-day-old foetal rats were transformed to a stage resembling that seen in the new-born female. After 17 days of intra-uterine life the epithelial cells of the mammary anlagen are largely undifferentiated, showing only sparse rough endoplasmic reticulum, a small Golgi apparatus, small mitochondria, occasional fat droplets but no other evidence of secretory activity. After 9 days culture with the 3 hormones, not only had extensive development of a duct system with a lumen and connective tissue sheath taken place, but the epithelial cells also showed marked development of cell organelles. Their polarization was less developed than in the new-born rat, but the Golgi apparatus sometimes appeared more active, the Golgi vesicles containing secretory granules. However, the lumina contained no secretory products other than occasional fat droplets.

Even in the complete absence of hormones some growth and limited organelle development in epithelial cells occurred, but the basal lamina disappeared and growth was disorganized. The principal effect of insulin alone appeared to be the maintenance of the basal lamina and organization of the growth of epithelial and stromal tissue. Insulin + prolactin resulted in better organization of duct growth but in neither case was there any marked development of epithelial cell organelles.

Some differences are evident between the results of Ceriani (1970*a*) in the rat and Lasfargues & Murray (1959) in the mouse, in particular with respect to the effects of growth hormone, and of oestrogen + progesterone + anterior pituitary hormones. However, comparisons are difficult since Ceriani (1970*a*) used insulin in all his hormonal combinations and took anlagen at a later stage of development.

## LOBULO-ALVEOLAR DIFFERENTIATION IN VITRO

### *Requirement for in vivo priming in immature mice*

In vitro studies of post-natal mammary gland development in immature and virgin females have mainly been made using the mouse. The results obtained depend on the age and strain of the animal from which explants are taken. Ichinose & Nandi (1964, 1966) have found that the mammary glands of 3- to 4-week-old BALB strain female mice explanted as whole glands on a synthetic medium degenerate in the absence of hormones. A combination of insulin, oestradiol-17$\beta$, progesterone, ovine prolactin and bovine growth hormone maintained the gland, but there was no evidence of normal development. However, if the mice were treated with oestradiol + progesterone in vivo, duct growth was stimulated and end-buds proliferated, sometimes forming alveoli, but no lobules. These glands were then able to respond to hormones in vitro and full lobulo-alveolar differentiation to the extent seen in mid to late pregnancy was induced by a combination of insulin, oestrogen, progesterone, aldosterone, prolactin and growth hormone. Insulin was found to be essential for

maintenance of epithelial tissues and lobulo-alveolar differentiation was also achieved with insulin + the anterior pituitary hormones + aldosterone, but not in the absence of aldosterone. Prolactin, combined with insulin and aldosterone, is effective in the absence of growth hormone in this strain (Singh & Bern, 1969).

The responses of 7 different strains of mice have been compared by Singh, DeOme & Bern (1970). The length of in vivo pretreatment with oestrogen and progesterone required for lobulo-alveolar development to occur subsequently in vitro varied from 5 to 15 days. The strains also differed in the minimal doses of insulin, aldosterone, prolactin and growth hormone which were effective for development in vitro, but there was no correlation between the in vivo and in vitro requirements. In this study the effect of prolactin or growth hormone in the absence of the other was not examined.

*Lobulo-alveolar differentiation in vitro without priming*

Lobulo-alveolar development can be induced in vitro without hormonal pretreatment, if the explants are taken from older mice. Using 5-, 6- and 7-week-old CBA mice, and a medium containing serum, Prop (1966) found that the response to hormones improves with age, so that a combination of insulin, prolactin, progesterone and cortisol gave only 50% alveolar development in tissue from 5-week-old mice but abundant development when explants were taken from 7-week-old mice.

The effects of hormones in vitro on whole mammary glands of 5-week to 3-month-old virgin mice have also been studied by Prop (1960, 1961*a*, *b*), Koziorowska (1962*a*, *b*), Chapekar & Ranadive (1963), Ranadive & Chapekar (1964) and Gadkari, Chapekar & Ranadive (1968) all using media containing serum, and by Rivera (1964*a*) using a defined medium, with or without rat serum. Insulin was necessary for maintenance of the gland, which regressed in the complete absence of hormones (Prop, 1961*a*; Koziorowska, 1962*a*, *b*; Rivera, 1964*a*). Actively growing terminal ducts and end buds were more sensitive to the absence of insulin than were the larger ducts (Rivera, 1964*a*). Consistent with this is the degeneration of isolated end-buds cultured in medium 199 alone and their maintenance by insulin (Elias, 1962) while isolated primary ducts showed less marked regression, though insulin again gave good maintenance and stimulated proliferation of epithelial and stromal elements (Rivera, 1963).

The minimal hormonal combination for inducing lobulo-alveolar development in vitro in unprimed mice was generally found to be insulin, progesterone, cortisol and prolactin (Prop, 1960, 1961*a*; Ranadive & Chapekar, 1964), although Lasfargues (1960) reported slight lobulo-alveolar differentiation in fragments of gland from 3-month-old females in response to oestrogen + progesterone in the absence of any other hormones. The results of Prop (1963) suggest that the role of growth hormone is to increase sensitivity to prolactin.

Rivera (1964*a*) notes that the response to insulin, oestrogen, progesterone, aldosterone, prolactin and growth hormone added to a synthetic medium involves the formation of lateral buds, not normally a feature of differentiation in vivo. Alveolar differentiation occurred when 5% rat serum was also included, though this was ineffective in the absence of added hormones.

Detailed comparison between different studies is made difficult by the wide variety

of media and hormone combinations employed. In addition, strain differences in response have been detected by Prop (1960), Ranadive & Chapekar (1964) and Gadkari *et al.* (1968).

Mammary tissue from 3- to 4-week old female rats of various strains was shown by Dilley & Nandi (1968) to form small lobules of alveoli in the absence of steroids when cultured on a synthetic medium containing insulin + prolactin. Addition of oestradiol + progesterone + aldosterone increased the degree of lobulo-alveolar differentiation obtained. By contrast with mice of similar age, no in vivo priming was required to obtain this result (cf. Ichinose & Nandi, 1964, 1966). However, Mishkinsky *et al.* (1967) could not obtain lobulo-alveolar differentiation in vitro in tissue from mature virgin female rats in the absence of steroids. Strain differences or a lower hormonal requirement of immature mammary tissue have been invoked to explain this discrepancy (Dilley & Nandi, 1968).

*Comparison with in vivo studies*

In general, the results obtained by in vitro methods agree very well with in vivo studies on hypophysectomized, adrenalectomized, ovariectomized animals. Thus, lobulo-alveolar differentiation has been obtained in such triply operated rats with large doses of prolactin + growth hormone (Talwalker & Meites, 1961), or with ovarian and adrenocortical hormones + lower doses of prolactin and growth hormone in rats (Lyons, 1958) and mice (Nandi, 1958). The role of insulin is somewhat uncertain and is discussed later (p. 431). Also, in mice, lobulo-alveolar development can be induced in vivo in the absence of adrenal steroids, but in vitro these appear to be necessary. The reasons for this can only be conjectured (see Ichinose & Nandi, 1966).

*Effect of thyroxine*

Singh & Bern (1969) have used organ culture to investigate the role of thyroxine in lobulo-alveolar development. Using 3- to 4-week-old BALB mice, pretreated with oestrogen + progesterone, lobulo-alveolar differentiation was achieved in vitro on a synthetic medium with insulin + aldosterone + sheep prolactin. In the presence of an optimal prolactin concentration (5 $\mu$g/ml) thyroxine inhibited development. However, at prolactin levels of 3 $\mu$g/ml or below, thyroxine increased the degree of lobulo-alveolar development at low concentrations, whereas higher concentrations were again inhibitory. These findings may explain conflicting reports on the influence of the thyroid on mammary development (for references see Singh & Bern, 1969).

## THE INITIATION AND MAINTENANCE OF SECRETORY ACTIVITY IN VITRO

*Morphological studies*

A number of different species have now been examined and the hormonal requirements for initiation of secretion in vitro on natural and synthetic media are well known, studies on the mouse being again the most extensive. Lobulo-alveolar tissue has been taken from mice of different strains and at various stages of pregnancy (e.g. Rivera & Bern, 1961; Lasfargues, 1962; Rivera, 1964*b*, 1966; Stockdale, Juergens & Topper, 1966), from pseudopregnant rabbits (e.g. Brumby & Forsyth, 1969; Denamur, 1969, 1971), from guinea-pigs (Gerritsen, 1960) from pregnant rats

(Uyldert & Villaudy, 1967; Uyldert, Peters & Ariëns, 1969), from rats, guinea-pigs, hamsters, rabbits and dogs primed with oestrogen and progesterone (Barnawell, 1965) and from women (e.g. Prop, 1969).

As in other studies of mammary tissue explanted postnatally, the presence of insulin in the culture medium is necessary for good maintenance (Elias, 1959), though Barnawell (1965) found that there were species variations in the ability of lobulo-alveolar mammary tissue to survive in the absence of hormones. An adrenal corticoid further improves survival in the mouse (Elias, 1959) and in the rat, guinea-pig, hamster, rabbit and dog (Barnawell, 1965). In C3H mice the requirement for cortisol in addition to insulin increased with the degree of development towards secretory activity (Rivera & Bern, 1961) and with length of time in culture. Mayne & Barry (1970) found that corticosterone had little effect on survival in 48-h cultures of mid-pregnant C3H mouse mammary gland. Similarly, El-Darwish & Rivera (1970) obtained good survival of Swiss albino mouse mammary gland for 3 days with insulin alone, but by 5 days insulin + corticosterone was required.

Non-secretory lobulo-alveolar tissue, cultured in the presence of insulin or insulin + an adrenal corticoid, undergoes little change other than an increase in the size of the alveolar and duct lumina (Elias, 1959; Rivera & Bern, 1961; Rivera, 1964*b*; Stockdale *et al.* 1966; El-Darwish & Rivera, 1970). When stainable secretion is already present in the lumina, as in explants from late pregnant mice (17–19 days of pregnancy), it tends to disappear during culture with these 2 hormones (Rivera & Bern, 1961). Prolactin, added to insulin and an adrenal corticoid, initiates or maintains secretion (Elias, 1959; Rivera, 1964*b*). There are species variations in sensitivity of response to sheep prolactin (Barnawell, 1965); the dog and the rabbit are particularly sensitive, but the guinea-pig is relatively unresponsive. In mice, parity increases sensitivity to prolactin (Rivera, 1964*b*).

None of the species studied by Barnawell (1965) showed any lactogenic response to ox growth hormone. However, some strains of mice, notably C3H, will show lactogenic responses to ox growth hormone in vitro (Rivera, 1964*b*, 1966; Hallowes & Wang, 1971), reflecting similar responsiveness in vivo (Rivera, 1964*b*, 1966). In vitro responses to sheep and pig growth hormones have also been shown in C3H mice (Rivera, Forsyth & Folley, 1967). Minimal hormone concentrations are required to demonstrate these specific responses. Thus, in vivo strain A mice are refractory to the mammogenic and lactogenic effects of ox growth hormone while C3H mice are highly responsive (Nandi, 1961). Experiments in vitro, however, using high levels of hormones (insulin, 100 $\mu$g/ml, + cortisol, 8 $\mu$g/ml, + prolactin or growth hormone, 200 $\mu$g/ml) failed to demonstrate differential responsiveness in the 2 strains (Rivera, 1964*b*). Not until the minimal hormonal requirements for the maintenance of mammary gland structure in vitro were determined (insulin 5 $\mu$g/ml + corticosterone 1 $\mu$g/ml for both strains) did their differential response to growth hormone become apparent. Maximal secretion was induced in explants from nulliparous C3H mice by 1 $\mu$g/ml sheep prolactin or 2 $\mu$g/ml ox growth hormone, while strain A mice responded maximally to 5 $\mu$g/ml sheep prolactin. At higher levels of growth hormone (10–15 $\mu$g/ml) strain differences became less apparent (Rivera, 1964*b*, 1966; Rivera *et al.* 1967), perhaps in part due to prolactin contamination of the growth hormone preparations. It is also possible that the structural similarities recently reported

between the 2 molecules are concerned (Aloj & Edelhoch, 1970; Bewley & Li, 1970; Niall, Hogan, Sauer, Rosenblum & Greenwood, 1971).

The only other purified hormones shown to be capable of initiating secretion in vitro are human growth hormone (Rivera *et al.* 1967) and human chorionic somatomammotrophin (human placental lactogen) (Turkington & Topper, 1966; Forsyth, 1967*a*; Turkington, 1968*b*; Forsyth & Folley, 1970). Both these hormones show prolactin-like activity in other bioassays (for review see Forsyth & Folley, 1970).

Rivera (1964*c*) found that in C3H mice secretion was initiated in vitro by corticosterone or aldosterone or cortisol in combination with insulin + prolactin + growth hormone. 11-Deoxycorticosterone was, however, less effective. Similar results were obtained by Barnawell (1965) and the steroid structural requirements have been investigated in more detail by Turkington, Juergens & Topper (1967) by comparing histological and biosynthetic responses to insulin and prolactin, with and without steroid. At a concentration of $3 \times 10^{-7}$ M, the 16 compounds examined were found to fall into 3 groups; they failed to support initiation of secretion or supported it to a greater or lesser extent. The important features of the 5 most effective steroids (21-deoxycortisol, aldosterone, cortisone, cortisol and prednisolone) were a 20-keto group, an 11$\beta$-hydroxyl (or 11-keto group) and a 17$\alpha$-hydroxyl or aldehyde group at position 18. At a higher concentration ($15 \times 10^{-5}$ M), 11$\beta$-hydroxyprogesterone and corticosterone became highly effective, indicating that an oxygen containing group at position 17 or 18 was no longer necessary. The contribution to these results of factors such as cell permeability, rates of interconversion and breakdown is not known, but the results suggest that both the main steroids secreted by the mouse adrenal, aldosterone and corticosterone, may play a part in mammary gland function.

In the rabbit, in vivo results indicate that there may be no absolute requirement for steroids in lactation. Thus, prolactin alone initiates lactation in adrenalectomized and ovariectomized–adrenalectomized rabbits (Cowie & Watson, 1966; Denamur, 1969), re-initiates or maintains lactation in hypophysectomized lactating rabbits (Cowie, Hartmann & Turvey, 1969) and initiates lactation in hypophysectomized or triply operated pseudopregnant rabbits (Denamur, 1969, 1971). Similarly in vitro there is evidence of initiation of lactation in explants from pseudopregnant rabbit mammary glands with insulin + prolactin, even when the tissue is taken from hypophysectomized or adrenalectomized animals (Denamur, 1969, 1971). The further addition of cortisol is, however, stimulatory. Secretory activity has been initiated in Swiss albino mouse mammary gland in vitro with insulin + prolactin (El-Darwish & Rivera, 1970).

A few reports have appeared of the effects of hormones on the human mammary gland in vitro. Barker, Fanger & Farnes (1964) observed hyperplasia of ductal cells with insulin in the presence of 20% human male serum. Mioduszewska, Koszarowski & Gorski (1969) have reported effects on growth and secretory activity in carcinoma tissue with prolactin, human growth hormone, cortisol, oestrogen, testosterone and progesterone.

Human secretory mammary tissue maintained on a medium containing 5% human male serum + insulin showed regression (Prop, 1969), but the addition of sheep prolactin stimulated further secretion and this effect was more marked in the presence of cortisol. Progesterone inhibited these changes. On the other hand, human chorionic

somatomammotrophin maintained structure and increased cell size but did not stimulate secretory activity, in contrast with its effect on the mouse mammary gland (Turkington & Topper, 1966; Forsyth, 1967*a*).

The effects of hormones in vitro on the ultrastructure of lobulo-alveolar mammary tissue have also been examined. In a study by Wellings, Cooper & Rivera (1966) using 12- to 14-day pregnant C3H mice the effects on alveolar cells of insulin + corticosterone, with and without prolactin and/or growth hormone were compared. In the absence of anterior pituitary hormones, the explants underwent little change; cell organelles were generally poorly developed, there were few microvilli on the luminal surface of the alveolar cells and the lumina contained negligible secretory material. Prolactin and growth hormone produced identical effects in this strain of mouse (see also Rivera, 1964*b*), and many mammary cells were stimulated to resemble those of normal mice lactating in vivo. There was an increase in the amount of rough endoplasmic reticulum, which was disposed at the base of the cell and the sides of the nucleus, the Golgi apparatus was enlarged and contained secretory material, microvilli on the luminal cell surface were increased and both protein granules and lipid droplets could be identified at the apex of the cell and in the alveolar lumina.

More recent studies by Mills & Topper (1970) on the effect of hormones on the ultrastructure of mammary cells in vitro will be considered in relation to theories of hormone action (see p. 436).

### *Biochemical changes*

The ability of the mammary gland to respond to hormones in vitro with the production of secretory changes, which at the light and electron-microscopic levels resemble the normal changes accompanying initiation of secretion in vivo, has proved a powerful tool for analyzing functional differentiation of the mammary cells. The secretory changes involve a variety of hormone-influenced specific syntheses (Topper, 1968, 1969, 1970; Turkington, 1968*a*, 1970*a*; Denamur, 1969, 1971).

#### *DNA synthesis*

In the presence of insulin alone, mammary epithelial cells of mature virgin and mid-pregnant mice initiate DNA synthesis in vitro (Stockdale *et al.* 1966; Stockdale & Topper, 1966; Lockwood, Voytovich, Stockdale & Topper, 1967; Turkington, 1968*c*; Wang, Amor & Bulbrook, 1970) and divide, as shown by rises in mitotic indices (Prop & Hendrix, 1965; Stockdale *et al.* 1966; Stockdale & Topper, 1966) and a doubling of the average number of cells per alveolar cross-section (Mills & Topper, 1970). Autoradiographic studies have shown that the number of cells incorporating [$^3$H]thymidine is increased but that the rate of replication per cell is not detectably affected (Turkington, 1968*c*). Nuclei of mammary fat pad cells did not incorporate [$^3$H]thymidine during a 48-h incubation and only 20% of fibroblasts did so compared with 70% of alveolar epithelial cells (Stockdale *et al.* 1966).

The maximum rate of DNA synthesis in response to insulin, measured in 4-h incubations with [$^3$H]thymidine, was reached at 24 h in tissue from mid-pregnant C3H mice. However, tissue from 3-month-old virgin mice did not respond maximally until 48–72 h after the start of culture (Stockdale & Topper, 1966). Friedberg, Oka & Topper (1970) have shown that this time-lag phase reflects an insensitivity to insulin,

and that insulin-sensitivity is acquired by the explanted mammary gland of virgin mice independently of exogenous insulin, perhaps as a response to the stimulus of being explanted. Mammary epithelium of immature 3-week-old C3H mice synthesizes DNA and undergoes mitosis in vitro in the absence of added insulin (Voytovich & Topper, 1967).

Other hormones may, however, be involved in DNA synthesis. Thus, Mayne & Barry (1970) found that prolactin prevented the decline in rate of DNA synthesis by mid-pregnant mouse mammary gland, which otherwise occurred in their cultures between 12 and 24 h, and maintained the insulin-stimulated rate to 48 h. In cultures prolonged to 5 days, El-Darwish & Rivera (1970) found that DNA content was best maintained at 2 days by insulin + prolactin and at 3 and 5 days by the further addition of corticosterone. Maximum rates of DNA synthesis occurred at 24 h and were produced effectively by insulin alone, the decline thereafter being rather slower if the 3 hormones were present (see also Rivera & Cummins, 1970). Using autoradiography, Prop (1961*b*) showed that the addition of prolactin strongly stimulated mitosis in whole-mount cultures of virgin female mouse mammary gland, by comparison with glands cultured with insulin, progesterone and cortisol in a medium containing serum, while Dilley (1971) found that prolactin was required to maintain initial mitotic and DNA-synthetic activity in the presence of insulin in immature female rat mammary glands in vitro.

A factor from the submaxillary salivary glands of mice which stimulates growth of epidermis has been shown to initiate DNA synthesis and cell division in mammary explants (Turkington, 1969*a*), while ox and sheep growth hormones also stimulate DNA synthesis to a limited extent (Turkington, 1968*c*). The initiation by insulin of DNA synthesis in mammary gland explants can be prevented by both androgens (Turkington & Topper, 1967) and oestrogens (Turkington & Hilf, 1968) and can be delayed by lithium or ammonium ions (Turkington, 1968*d*). The effect of androgens increases with dose over the range $3{\cdot}5 \times 10^{-8}$ to $3{\cdot}5 \times 10^{-5}$ M, while oestradiol-17$\beta$ was found to be inhibitory at concentrations of $4 \times 10^{-12}$ M and $4 \times 10^{-8}$ M, but not at a concentration of $4 \times 10^{-10}$ M.

The mechanisms involved in these effects are largely unknown. The minimal effective concentration of insulin (0·5 $\mu$g/ml or $4 \times 10^{-8}$ M) is considerably higher than that obtaining in vivo, though the effective levels of growth hormone are within the physiological range (Turkington, 1968c). However, Friedberg *et al.* (1970) have found that the addition of 2·5% bovine serum albumin to synthetic medium 199 minimizes loss of insulin by adsorption on to glassware so that it becomes effective at a concentration of $10^{-9}$ M. The effect of insulin is independent of its effect on glucose transport, since it occurs when fructose is substituted for glucose in the medium (Lockwood, Stockdale & Topper, 1967). Nevertheless, Palmiter (1969*a*) has suggested that insulin may have a permissive, rather than a direct role, in stimulating DNA synthesis. Even if the in vitro finding of insulin as a sufficient stimulus for DNA synthesis by the mammary gland can be substantiated in vivo, it is clear that other influences must modulate its effect since mammary proliferation occurs only at certain times despite the continuous presence of endogenous insulin. Tissue sensitivity perhaps produced by other hormonal events may be involved (Friedberg *et al.* 1970). The importance of ovarian steroids and their interaction with anterior pituitary

hormones has been emphasized by Bresciani (1971) and his views are consistent with older in vivo studies on hypophysectomized–ovariectomized–adrenalectomized animals (e.g. Lyons, 1958; Nandi, 1958). The interaction observed in vitro between insulin and oestrogens affecting rates of DNA synthesis (Turkington & Hilf, 1968) could be of physiological significance. It is apparently impossible to initiate more than one cycle of cell division in the mammary gland in vitro (Lockwood, Stockdale & Topper, 1967; Mills & Topper, 1970; Topper, 1970) and this may reflect the absence of some systemic influence or a hormonal interaction so far overlooked. (For further discussion and references on the role of insulin see Denamur, 1969, 1971.)

Experiments with puromycin and cyclohexamide, which inhibit protein synthesis, indicate that the initiation of DNA synthesis by insulin and the completion of DNA replication both require that protein synthesis takes place. Autoradiography showed that both the number of cells synthesizing DNA and the rate of DNA synthesis were affected. The effect of puromycin was at least partially reversible (Turkington, 1968*c*). Prior synthesis of DNA polymerase may be required for DNA synthesis (Lockwood, Voytovich, Stockdale & Topper, 1967). The activity of the enzyme is very largely associated with the fibroblasts in fresh mammary tissue from virgin female C3H mice and the large increase in its activity produced by insulin may represent de novo synthesis by epithelial cells. In 48-h cultures the rate of DNA synthesis and the activity of DNA polymerase closely paralleled each other. However, DNA synthesis virtually ceases in vitro by day 3–4 (Lockwood, Stockdale & Topper, 1967), while DNA polymerase activity continues at an elevated level.

Marzluff, McCarty & Turkington (1969) report co-ordinated synthesis of DNA and histones in response to insulin.

*RNA synthesis*

Hormonal stimulation of total RNA synthesis by mouse mammary gland in vitro has been reported by a number of workers; the most marked and sustained stimulation was achieved by a combination of insulin + prolactin with or without an adrenal corticosteroid (Mayne *et al.* 1966; Stockdale *et al.* 1966; Mayne & Barry, 1967, 1970; Mayne, Forsyth & Barry, 1968; Turkington, 1968*b*).

The roles of each of these hormones have been analysed further by Green & Topper (1970), using mid-pregnant BALB mice, a strain relatively free of mammary tumour RNA-virus. By destroying the mammary anlagen unilaterally in immature mice (DeOme, Faulkin, Bern & Blair, 1959) they were able to compare the responses of the mammary fat pad with and without mammary epithelium. Dissociation of epithelium and fat cells was also achieved after culture. Explants were cultured for 4 days with insulin or insulin + cortisol and then exposed to these hormones and prolactin in various combinations for a further 24 h, in the presence of [$^3$H]uridine. Prolactin stimulated uridine incorporation into total RNA by epithelial but not fat cells. By contrast, cortisol depressed RNA synthesis by fat cells but had no detectable effect on epithelial cells. The RNA synthesis of mammary gland previously incubated in insulin + cortisol was stimulated both by insulin and prolactin, but it was concluded that it is prolactin-linked RNA synthesis which may be important in the transformation of non-secretory to secretory cells. Mammary gland explants incubated with insulin alone for 4 days were also able to respond to insulin + prolactin with

increased RNA synthesis on day 1 but by day 2 the stimulation had declined, and instability of RNA in the absence of cortisol may account for this. Such insulin + prolactin incubated explants do not synthesize casein (see p. 433 below and p. 436).

The species of RNA stimulated in vitro have been studied in a series of papers by Turkington and co-workers (see Turkington, 1969*b*, 1970*b*; Turkington & Ward, 1969; Turkington & Riddle, 1970*a*). They show that insulin and prolactin stimulation result in the formation of multiple classes of RNA. The effects reported include an early stimulation of rapidly labelled nuclear RNA (which may in part represent messenger RNA), stimulation of the proportion of transfer to total RNA and of the activity of transfer RNA-methylating enzymes, of the formation of ribosomes and their organization into polysomes and of the activity of RNA polymerase. It is concluded that hormonal stimulation of casein synthesis (see p. 433 below) involves first an increase in transcriptional activity which is later coupled to translational activity. The substantiation of these conclusions must depend on future work.

Recent in vitro experiments by Turkington (1970*c*) using insulin and prolactin co-valently bound to large polymers of Sepharose suggest that both hormones can exert their effects on RNA synthesis without entering the mammary cell. It has not, however, been shown conclusively that the hormones remain attached to the Sepharose under the conditions of incubation.

*Protein and enzyme synthesis*

The initiation of specific milk protein (casein) synthesis by hormones in vitro was first observed by Juergens, Stockdale, Topper & Elias (1965). A combination of insulin, cortisol and prolactin was needed to stimulate the formation of 'casein-like' phosphoproteins by mid-pregnant C3H mouse mammary tissue, in agreement with earlier histological studies (Stockdale *et al.* (1966) and p. 427). By contrast, the synthesis of non-milk proteins required only insulin (Lockwood, Turkington & Topper, 1966). It was later shown that the casein produced in vitro was electrophoretically indistinguishable from that in normal mouse milk (Turkington, Juergens & Topper, 1965). The rate of casein synthesis is maximal at 48 h, reaching about 50% of the rate observed in tissue from mice on day 10 of lactation (Topper, 1968).

Stimulation in vitro of whey protein synthesis requires the same hormonal combination (Lockwood *et al.* 1966; Turkington, Lockwood & Topper, 1967). The whey protein, $\alpha$-lactalbumin, is a component of the enzyme lactose synthetase which catalyses lactose synthesis and this is considered further on pp. 434–5.

The activity of a number of other enzymes has been stimulated by hormones in vitro. Using mid-pregnant C3H mice, Jones & Forsyth (1969) obtained significant stimulation of glucose-6-phosphate dehydrogenase, malic enzyme (E.C. 1.1.1.40), UDPG pyrophosphorylase, ATP citrate lyase, lactate dehydrogenase, but not malate dehydrogenase, in the presence of insulin, corticosterone and prolactin. There was no stimulation in the absence of prolactin.

However, Leader & Barry (1969) obtained marked stimulation of the activity of 2 enzymes of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase, with insulin alone in cultures of mammary gland from C3H mice on days 12 and 19 of pregnancy. The further addition of corticosterone and prolactin maintained glucose-6-phosphate dehydrogenase activity

to 45 h in the cultures from 19-day pregnant mice and was stimulatory at 22 and 45 h in the cultures from 12-day pregnant animals. Inhibition of DNA synthesis (with hydroxyurea) had little effect on the increase in enzyme activity, while inhibition of RNA (with actinomycin D) and of protein synthesis (with cyclohexamide or puromycin) prevented it. However, the later addition of actinomycin D and cyclohexamide indicated that all essential RNA and protein syntheses were complete by 3·5 h and 12 h respectively. It is suggested that the large increases in the activity of these enzymes, which occur also at parturition, are induced, not by insulin itself, but by an increase in intracellular glucose concentration produced by removing tissue from the anti-insulin action of a placental lactogenic hormone.

Some stimulation of these enzymes by insulin alone in cultured mammary gland from mid-pregnant Swiss albino mice has also been observed by Rivera (1969) and Rivera & Cummins (1971). Maximal stimulation, however, as in the experiments of Leader & Barry (1969) required insulin + corticosterone + sheep prolactin or prolactin-like hormones (human growth hormone or human chorionic somatomammotrophin) (Rivera, 1969; Rivera & Cummins, 1969). By contrast, glucosephosphate isomerase, an enzyme of the glycolytic pathway for glucose metabolism, was strongly stimulated by insulin alone over the period of 24 to 72 h in culture (Rivera & Cummins, 1971). These results accord well with those of Bolton & Bolton (1970) who obtained stimulation of pentose phosphate pathway activity in rabbit mammary gland with prolactin in the presence of insulin + corticosterone (see below).

*Carbohydrate and lipid metabolism*

Insulin strongly stimulates glucose uptake by explants of mid-pregnant C3H mouse mammary gland (Moretti & DeOme, 1962; Mayne & Barry, 1970). The further addition of corticosterone and/or prolactin had no effect in this species (Mayne & Barry, 1970). However, prolactin stimulates glucose uptake by pseudopregnant rabbit mammary gland explants in the presence of insulin + corticosterone (Bolton, 1970).

Insulin also stimulates the formation of carbon dioxide and fatty acids from glucose by mouse mammary gland explants (Moretti & Abraham, 1966), but the stimulated rates were better maintained at 2 days by insulin + corticosterone + prolactin than by insulin alone (Mayne & Barry, 1970).

In mammary gland explants from pseudopregnant Dutch rabbits, prolactin in the presence of insulin has been shown to stimulate glucose oxidation via the pentose phosphate pathway (Bolton & Bolton, 1970) and total lipid synthesis. Both these effects were maximal after 2 days in culture and the extent of stimulation was related to the dose of prolactin (Bolton, 1970). Corticosterone had no effect in the absence of prolactin, but increased the metabolic response to insulin + prolactin (Bolton, 1970). In addition, the pattern of fatty acids synthesized in vitro in response to prolactin quite closely resembles that present in rabbit milk (Strong, Dils & Forsyth, unpublished).

The induced synthesis of lactose from [$^{14}$C]glucose by rabbit mammary gland in vitro has recently been demonstrated (see Denamur, 1971). It required insulin + prolactin, and the further addition of cortisol increased the rate of synthesis.

As mentioned previously (p. 433), the final step in the synthesis of lactose is

catalysed by the enzyme lactose synthetase, which is composed of 2 subunits, the A and the B proteins (Brodbeck & Ebner, 1966). The A protein is a galactosyltransferase which catalyses the reaction:

$$\text{UDP-galactose} + N\text{-acetyl-glucosamine} \rightarrow N\text{-acetyl-lactosamine} + \text{UDP}.$$

There is evidence that in the mammary gland it is attached to the Golgi membranes (see Brew, 1969).

In the presence of the B protein (the whey protein, $\alpha$-lactalbumin), the specificity of the A protein is modified so that the reaction now catalysed is:

$$\text{UDP-galactose} + \text{glucose} \rightarrow \text{lactose} + \text{UDP}.$$

The hormonal stimulation of this enzyme complex has been studied in mouse mammary-gland explants by Turkington, Brew, Vanaman & Hill (1968) and by Palmiter (1969*b*). Induction of maximal activity required insulin + cortisol + prolactin – that is, the same hormonal combination required for casein synthesis, both the A and B proteins appearing at about the same time. This contrasts with the in vivo situation in rats and mice, in which the A protein activity rises linearly from mid-pregnancy to reach a maximum before parturition, whereas the sharp rise in B protein activity does not occur until just after parturition (Kuhn, 1968; Turkington *et al.* 1968). It is possible that progesterone may be responsible for this difference. It has been reported selectively to inhibit synthesis of $\alpha$-lactalbumin in mouse mammary explants (Turkington & Hill, 1969) and to inhibit secretory responses observed histologically in dog mammary-gland explants (Barnawell, 1967) and human mammary gland tissue (Prop, 1969). Changes in progesterone metabolism at parturition resulting in a fall in plasma levels have been reported in the rat (Kuhn, 1969). However, progesterone was not inhibitory in the foetal rat mammary gland (Ceriani, 1970*a*, *b*).

Moreover, recent results of Jones (1971) indicate that the concentration of $\alpha$-lactalbumin within the Golgi vesicles of mouse mammary gland does not increase abruptly at parturition and that previous measurements of total $\alpha$-lactalbumin may have been misleading in terms of lactose synthetase activity and the initiation of lactose synthesis.

### *Theories of hormone action*

As a result of in vitro studies it has been possible to gain some insight into the ways in which hormones produce their effects on the mammary gland. The experimental techniques which have been of particular importance in this connexion are: (1) the use of isotopically labelled precursors; (2) sequential culture in different hormonal environments; (3) use of mice of different ages and stages of mammary development; (4) the use of inhibitors, for example of mitosis (colchicine), of DNA synthesis (hydroxyurea, testosterone), of RNA synthesis (actinomycin D) and of protein synthesis (cyclohexamide, puromycin); (5) electron-microscopic studies.

In 1966, Stockdale & Topper proposed a model for the hormone-dependent functional differentiation of the mammary epithelial cells in vitro. They suggested that there are 2 cell populations in the mid-pregnant mouse mammary gland at the time of explantation. One of these does not divide, synthesizes casein at a rate uninfluenced by hormones and is responsible for the observed base-line of casein synthesis. The other population of non-secretory cells synthesizes DNA and divides

in response to insulin. If cortisol and prolactin are absent at this time the daughter cells do not synthesize casein. However, if these hormones are present daughter cells arise with the capacity to synthesize casein.

This hypothesis has been developed in the light of subsequent work, including that on the ultrastructure of the cultured gland (Mills & Topper, 1970). Mid-pregnant mouse mammary tissue contains some alveoli with cells showing the ultrastructural characteristics of secretory activity and these, as noted above, are presumed to be responsible for base-line casein synthesis. The majority of alveoli contain relatively unspecialized cells most of which divide in response to insulin, giving daughter cells of similar structure – that is, with a central nucleus, primitive Golgi apparatus lateral to the nucleus, sparse rough endoplasmic reticulum and an absence of secretory granules. Such cells exposed to cortisol show one crucial difference, the development of abundant rough endoplasmic reticulum. Initially it was thought that cortisol could produce this effect only if present at the time of mitosis (Lockwood, Stockdale & Topper, 1967) but it now appears that it can act post-mitotically, if sufficient length of time in incubation is allowed (Mills & Topper, 1970).

The insulin–cortisol cells are subsequently acted on by both prolactin and insulin to convert them to fully differentiated secretory cells and some aspects of this transformation have been recognized (see Mills & Topper, 1970). It involves a redistribution of organelles within the cell so that the nucleus and rough endoplasmic reticulum are basal while the enlarged Golgi lies above the nucleus. Secretory granules are now present in the Golgi and the apical cytoplasm and lipid droplets are also seen.

It thus appears that adrenal corticoids are responsible for the development of rough endoplasmic reticulum and that this is required for the synthesis of secretory proteins although not for synthesis of non-secretory proteins (see p. 433 and Topper, 1970). The post-mitotic actions of prolactin and insulin are not fully understood but involve redistribution of organelles, prolactin-promoted RNA synthesis, which can occur in the absence of rough endoplasmic reticulum, and probably the completion of enzyme complements, including, for example, the A protein of lactose synthetase on the already formed rough endoplasmic reticulum (Keenan, Saacke & Patton, 1970; Topper, 1970).

It is still not entirely clear whether, as originally suggested by Stockdale & Topper (1966), mitosis has an essential role in the process of functional differentiation. Much of the evidence rests on the use of inhibitors and has been critically discussed by Wessells (1968). Undoubtedly many of these inhibitors are toxic at the concentrations used and can lead to mammary cell necrosis and death (Forsyth & Jones, unpublished). Recent experiments by Turkington, Majumder & Riddle (1971) would appear less open to criticism on these grounds. They exposed explants of mid-pregnant C3H mouse mammary gland to 5-bromo-2′-deoxyuridine and this, incorporated into DNA as a structural analogue of thymidine, prevented the prolactin induced synthesis of casein and $\alpha$-lactalbumin. There was little effect on total protein synthesis, on the induction of lactose synthetase A protein, or on the formation of transfer RNA, total rapidly labelled nuclear RNA or ribosomal RNA, but prolactin-stimulated polysome formation was inhibited. The latter is required for casein synthesis and such polysomes may contain the requisite messenger RNA (Turkington & Riddle, 1970*a*). The effects of 5-bromo-2′-deoxyuridine could be completely reversed by providing excess thymidine

in the medium and it had no effect when added post-mitotically, so that it appears to be inhibitory only when incorporated into DNA and not through non-specific side effects. The 90% inhibition of casein synthesis achieved with 5-bromo-2′-deoxyuridine in one cycle of cell division suggested to the authors that only one daughter cell can differentiate and that the other remains as a stem cell. This view is, however, rather difficult to reconcile with the histological appearance of the secreting gland in which all mammary epithelial cells of a given alveolus appear active (Turkington, 1969*c*; Keenan *et al.* 1970).

In recent years a great deal of interest and experiment has been stimulated by the suggestion that cyclic AMP (adenosine 3′,5′-monophosphate) may act as a 'second messenger' translating hormonal messengers received at the cell membrane into intracellular changes within the target cell (see Pastan & Perlman, 1971, for review). There is, as yet, no clear evidence as to what role this system may play in the mammary gland.

## MAMMARY GLAND ORGAN CULTURE AS A BIOASSAY FOR PROLACTIN

It is evident that under suitable conditions prolactin exerts specific effects on the mammary gland in vitro. It does so at relatively low doses, so that the system forms a suitable basis for a bioassay and it has been exploited as such in various ways. To date, most workers have used a histological end-point for the bioassay (e.g. Prop, 1963; Nicoll, Bern & Brown, 1966; Mishkinsky *et al.* 1967; Brumby & Forsyth, 1969; Frantz & Kleinberg, 1970; Nicholson, 1970*a*, *b*; Forsyth & Myres, 1971). However, a more objective assessment of the responses is desirable and it is possible that some biochemical parameter may prove suitable. Loewenstein, Mariz, Peake & Daughaday (1970) have recently reported an assay based on measurement of the activity of the enzyme, *N*-acetyllactosamine synthetase, which catalyses the reaction:

$$N\text{-acetylglucosamine} + \text{UDP-galactose} \rightarrow N\text{-acetyllactosamine} + \text{UDP}.$$

### *Assay of hormone production by tissues*

The production and release of prolactin, as of other anterior pituitary hormones, is regulated by the hypothalamus (for review and references see Yates, Russell & Maran, 1971). In all vertebrate classes except Aves, the principal influence of the hypothalamus on prolactin production is an inhibitory one, mediated by a prolactin-inhibiting factor (see Meites & Nicoll, 1966), though evidence has also been advanced of a prolactin-stimulating factor in turtles (Nicoll, Fiorindo, McKennee & Parsons, 1970) and rats (Mishkinsky, Khazen & Sulman, 1968; Nicoll *et al.* 1970).

Organ culture of pituitaries has proved a useful method for the study of prolactin synthesis and release (see Rivera & Kahn, 1970). In most experiments prolactin production has been monitored using the pigeon crop assay. However, Nicoll *et al.* (1966), in studies on the evolutionary history of prolactin, cultured pituitaries from representatives of 6 classes of vertebrates, 2 teleost fish, a lungfish, a salamander, a turtle, the domestic pigeon and 3 mammals (rat, hamster and guinea-pig). They then assessed the mammotrophic activity of the medium from the pituitary cultures by using it to support mammary gland explants from mid-pregnant strain A mice.

Medium from all the tetrapod pituitaries was as effective as ovine prolactin in initiating secretion in the mammary gland, while the teleost and lungfish pituitaries had only a slight effect. A hormone with ability to stimulate the mammary gland thus emerged long before the evolution of that end organ.

Fractions of mouse and human pituitary glands, prepared by polyacrylamide gel electrophoresis, have been assayed for lactogenic activity using mammary gland organ culture (Nicholson, 1970*a*, *b*).

The technique of co-culture has been used to study hormone production by the placenta. There has long been evidence that the rodent placenta produces a prolactin-like hormone (for references see Ray, Averill, Lyons & Johnson, 1955; Matthies, 1967; Kohmoto & Bern, 1970) and it is now well known that the human placenta produces a material (human chorionic somatomammotrophin) which partially cross-reacts immunologically with human growth hormone and has prolactin-like and growth hormone-like activity (see Selenkow, Saxena, Dana & Emerson, 1969, for review). Recently Kohmoto & Bern (1970) co-cultured strain A mouse placenta and mammary gland, demonstrating a mammotrophic effect on the mammary gland with placentae explanted after day 5 of pregnancy. Using a similar technique the goat placenta has been shown to produce a prolactin-like hormone (Forsyth, 1972).

*Assay of lactogenic activity in plasma*

Radioimmunoassay methods are now available for the measurement of blood levels of prolactin in a number of species; for example, the rat (Kwa & Verhofstad, 1967; Niswender, Chen, Midgley, Meites & Ellis, 1969), the mouse (Kwa, Verhofstad & van der Bent, 1967), the sheep and goat (Arai & Lee, 1967; Bryant & Greenwood, 1968; Johke, 1969) and the cow (Johke, 1969; Schams & Karg, 1970). These methods, which depend upon the preparation of specific antisera to the hormone, measure immunologically reactive material which is not necessarily biologically active (see Greenwood, 1967). It is therefore desirable that there should be means of checking the results of radioimmunoassays against a suitably specific and sensitive bioassay. The classical methods for the assay of prolactin, in particular that using the pigeon, have proved rather unsatisfactory in measuring blood levels of the hormone, largely because of interference by non-specific inflammatory factors in the measurement of the response (see Meites & Nicoll, 1966; Forsyth, 1967*b*). However, the organ culture method is proving very suitable for this purpose. Plasma to be assayed can be incorporated directly into the medium on which mammary gland explants are then maintained. By this means Brumby & Forsyth (1969) detected elevated levels of lactogenic activity in the plasma of goats at parturition. Subsequently good agreement has been noted between the bioassay and a radioimmunoassay for prolactin levels in the plasma of goats during a suckling episode post partum (Forsyth, 1972; Buttle & Forsyth, in preparation). During the second half of pregnancy, the bioassay indicated the presence of lactogenically active material not measured by the radioimmuno-assay. This material is apparently secreted by the placenta (see also p. 438 above).

In primates, including man, a radioimmunoassay for prolactin has only very recently become a possibility. There has been considerable controversy about the nature of human prolactin and even its existence as a hormone separate from primate growth hormone, which has intrinsic prolactin-like activity (see Boot, 1970; Forsyth

& Folley, 1970). Progress is now being made towards the isolation of prolactin in primates (see Guyda & Friesen, 1971). Meanwhile, mammary-gland organ culture has provided additional evidence for the existence of human prolactin and is being used to measure it in human plasma (Forsyth, 1970; Frantz & Kleinberg, 1970; Forsyth & Myres, 1971; Forsyth, Besser, Edwards, Francis & Myres, 1971). Elevated prolactin levels have been detected both in women lactating normally post partum and in patients with abnormal lactation of diverse aetiology.

## MISCELLANEOUS STUDIES

### *Organ culture of bovine teat canal*

Rapp & Rickard (1961*a*) have established suitable conditions for the maintenance of bovine teat canal (epithelium + connective tissue) in vitro. They found that the original epithelium sloughed off, but that it was replaced by a similar stratified, squamous keratinizing epithelium within a few days. Vitamin A and hormones are known to produce marked effects on epithelia (Lasnitski, 1965) and have been investigated with respect to teat canal epithelium by Rapp & Rickard (1961*b*). Vitamin A was found to inhibit keratinization, but no effects of oestrogens, progesterone, testosterone, adrenal corticoids, thyroxine or bovine pituitary extracts could be detected. It is possible that such studies could be exploited further in relation to the role of the teat canal as a barrier to mastitis. In this connexion the application of organ culture techniques to the study of immunoglobulin synthesis by the mammary gland could be considered. Interesting results have already been obtained in this field using short-term tissue culture (Lawton, Asofsky & Mage, 1970).

### *Culture of neoplastic mammary gland*

Reports on organ culture of neoplastic mammary tissue and comparison of its responses with that of the normal gland are quite numerous. Consideration of this topic is outside the scope of this review but for information and references the reader is referred to Michelson & Haguenau (1969), Stoll (1970), Takizawa, Furth & Furth (1970*a*, *b*) and Turkington & Riddle (1970*b*, *c*). The general topic of the application of organ culture techniques to neoplastic tissues is considered by Easty (1970).

## COMPARISON WITH OTHER IN VITRO TECHNIQUES

Use of an in vitro system has certain obvious advantages in isolating the tissue from the body and offering the possibility of placing it in a controlled environment. Slices of mammary tissue have been used for many years in biochemical studies, but remain viable for a few hours at best and this limits their usefulness, especially in the study of hormones, which in the main act over longer periods of time to produce their effects. Insulin is the only hormone which has been shown consistently to have an effect on tissue slices (Abraham, Cady & Chaikoff, 1960; McLean, 1960). Tissue and cell cultures are viable for much longer periods, but they tend to dedifferentiate and lose their specific function. Bovine mammary cell cultures lose the ability to synthesize lactose in 1–2 days, although synthesis of $\beta$-lactoglobulin persists for 2–3 weeks (Twarog & Larson, 1964), while lipid synthesis also declines after 2 days (Kinsella, 1968).

Direct comparisons between cell and organ culture techniques are very few. Andersen & Larson (1970) have compared the ability of hormones to maintain $\beta$-lactoglobulin and $\beta$-casein synthesis in organ and dispersed cell cultures of lactating bovine mammary gland; having observed more synthesis per cell in cell than in organ cultures, and comparable effects of hormones, they concluded that cell cultures offer some advantages. However, the use of only one very high level (20 $\mu$g/ml) of prolactin and the absence of histological checks on the survival of the bovine tissue under the conditions of organ culture used limit the significance of this study. Moreover, it related to maintenance of existing function rather than to the initiation of new syntheses. Turkington (1970*c*) has reported stimulation of rapidly labelled nuclear RNA synthesis in dispersed cell cultures of mouse mammary epithelial cells by insulin (5 $\mu$g/ml) and prolactin (5 $\mu$g/ml). It is not, however, known whether, as in organ culture conditions, the entire series of biochemical events culminating in casein, $\alpha$-lactalbumin, milk lipid and lactose synthesis can occur in the isolated cell. A minimum of cellular organization or architecture appears to be necessary for certain co-ordinated syntheses to occur. For example, broken cell preparations of rabbit mammary gland do not make the characteristic pattern of milk lipids (E. M. Carey & R. Dils, personal communication). It remains to be seen to what extent tissue structure is also important. The observation by Mills & Topper (1970) of redistribution of organelles within the mammary cell may indicate that some form of orientation is required. In relation to mammary morphogenesis the importance of epithelial-mesenchymal interactions is clear (see also Lasfargues, 1961).

However, the extent of stimulation produced by hormones in vitro is rarely as great as that seen at parturition and normally declines after about 2 days. This is not related simply to a failure to survive, since, for example, the ability to respond to prolactin is shown by explants cultured previously with insulin or insulin + cortisol for 4 days (Mills & Topper, 1970). Since the organ culture system is essentially a static one this may represent, in part, the occurrence of product-inhibition and the onset of changes characteristic of involution in the normal gland. Ultrastructural changes suggestive of this were noted by Wellings *et al.* (1966) in 5-day cultures of mouse mammary gland.

## CONCLUSIONS

Although caution must be exercised in interpreting results obtained in vitro and applying them to events occurring in vivo in the course of mammary gland development and function, it is clear that organ culture has proved an important experimental technique leading to new concepts in mammary physiology. It has so far been applied to only a very limited range of species, the overwhelming number of studies being on rodents and lagomorphs. It will be of great interest to see studies of this type extended to other species and in particular to ruminants.

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## EXPLANATION OF PLATE

Typical organ culture dishes. (*a*) Using a 9 cm plastic Petri dish, watch-glass and stainless steel grid to support 4–6 explants at the surface of 1 ml of liquid medium. (*b*) Using a 5 cm Petri dish and stainless steel grid to support up to 30 explants at the surface of 5 ml liquid medium.

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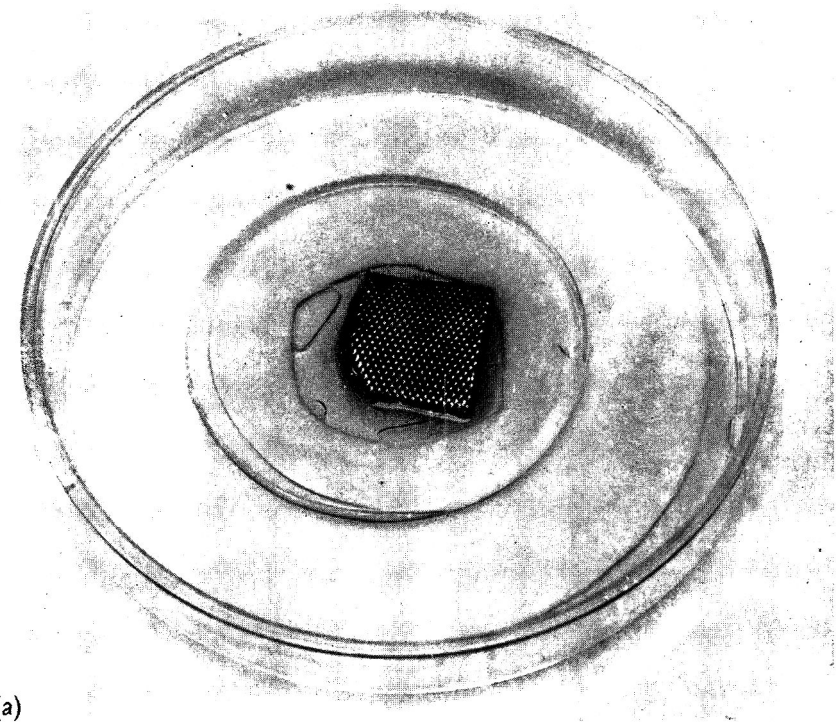
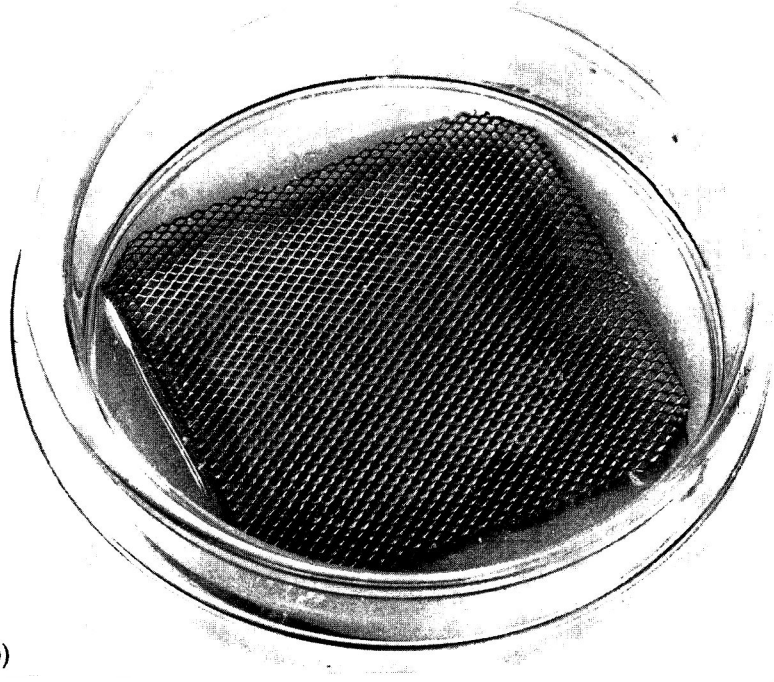


(a)



(b)

ISABEL A. FORSYTH

(*Facing p.* 444)

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