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Professor S. J. FOLLEY, Ph.D., D.Sc. (Manchester);
Hon. Dr Vet. Med. (Ghent); F.R.S.

Obituary

Professor S. J. Folley, Ph.D., D.Sc., Manchester; Hon. Dr Vet. Med., Ghent; F.R.S.

Professor S. J. Folley, F.R.S., died at the age of 64 on 29 June 1970 after a short illness. The National Institute for Research in Dairying has lost in him an eminent scientist whose distinguished career did much to enhance its reputation throughout the world. He graduated at Manchester University in 1927 and spent his early post-graduate years in the Departments of Physiology and Biochemistry of Liverpool University. He came to the Institute in 1932. Only recently he outlined his career at the Institute in a short article in the N.I.R.D. Biennial Reviews 1970. As he himself tells, he came to a Physiology Department which then "occupied a converted back bedroom in the Manor House"; at that time its staff consisted of a biochemist and a technical assistant. Professor Folley became Head of the Physiology Department in 1945; under his guidance the department rapidly expanded and in recent years its staff, including visiting workers, has at times exceeded 50.

Early in his career Professor Folley became interested in the endocrine control of lactation and, in particular, in the role of the hormones of the thyroid and anterior pituitary. During the Second World War he and his staff carried out extensive studies on the hormonal induction of mammary growth and lactation in sterile heifers and cows using synthetic oestrogens. These investigations led to more detailed and fundamental studies on the control of mammary growth and lactation in a number of species, and especially in the goat which proved to be an excellent experimental animal. Later, his interests turned towards neuroendocrine relationships, the physiology of the milk-ejection reflex, the assay of oxytocin in blood and factors affecting the release of prolactin and other hormones from the anterior pituitary. He was also greatly interested in the biochemistry of milk synthesis and pioneered early studies on the role of blood acetate as a substrate for the biosynthesis of milk fatty acids. Later, much research in his department was directed to the study of mammary enzymes and to the synthesis of lactose.

The outstanding importance of his work received wide recognition. He was awarded the degree of D.Sc. by his university in 1940 and was elected a Fellow of the Royal Society in 1951. In 1938 he was a Rockefeller Research Fellow at Yale University and in 1953 he was appointed Visiting Professor at the Collège de France where he gave a series of lectures which were later published in French, English and Russian. In 1964 he was given the title of Research Professor in Reading University and in the same year he was awarded an honorary doctorate by the University of Ghent. In 1969 he was presented with the Dale Medal of the Society for Endocrinology.

As a scientist he was original and resourceful and ideas flowed from his fertile mind. He had an infectious enthusiasm and the exhortation to 'drive on' became rapidly known to newcomers to his department. His enthusiasm was, however,

tempered by a keen criticism and he had little patience with the slipshod experiment or careless piece of writing. Professor Folley was beset with serious health problems throughout his career and in recent years he suffered from almost complete blindness. His success is an outstanding tribute to his great courage in meeting and overcoming these crises. The devotion of his wife Mary was a source of great strength and encouragement to him. She accompanied him to meetings and conferences and is herself a well-known and popular figure. Friends in many countries grieve with her at her loss and extend to her their affection and admiration.

A. T. COWIE

Printed in Great Britain

Local cooling of the mammary gland and milk production in the cow

By C. W. HOLMES
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(*Received* 27 *June* 1970)

SUMMARY. An experiment has been performed with 2 Jersey cows to determine the effect of a reduction in udder temperature on milk production. One half of the udder was covered while the other half was cooled by an increase in air movement at an air temperature of 6–10 °C; for closely clipped udders it was estimated that the cooling conditions might be equivalent to an air temperature of −10 °C with low air movement. Yields of milk, fat, protein and lactose were reduced significantly at the morning milking after 22 h cooling; the decreases which occurred at the afternoon milking after 9 h cooling, in all components except fat, were not significant. Wisconsin Mastitis Tests on the milk indicated that changes in udder health did not contribute to these effects.

Several workers have shown that local cooling of an area of skin in the sheep causes a reduction in wool growth (Doney & Griffiths, 1967; Downes & Hutchinson, 1969; Lyne, Jolly & Hollis, 1970). The possibility of a comparable effect on milk synthesis of changes in temperature within the udder has been mentioned by Johnson (1965), when discussing the overall effect of ambient temperature on milk production of cows. No work designed to test this possibility has been reported, although Ewbank (1968) induced an increase in the numbers of somatic cells in milk from an individual quarter of a cow's udder by local cooling of the quarter.

The present experiment was an attempt to determine the direct effect of local cooling of the udder on milk production and on the somatic cell counts in milk.

EXPERIMENTAL

Two Jersey cows, one 7 and the other 8 years old, were used when in the sixth month of lactation. They were each fed 11 kg dried grass daily during the experiments, in 2 feeds before milkings, and had constant access to drinking water. The cows were housed, side by side, in a controlled temperature room (± 1 °C), and restrained by tubular steel yokes. They were milked at 6.00 and 16.00 h with a bucket-type machine which enabled the milk from individual quarters to be collected separately. The whole surface of each udder was closely clipped, and cooling of one half was achieved by increasing the rate of air movement across its surface to 330–500 cm/s (7–11 m.p.h. approx.) by means of fans and a centrifugal blower; air movement was measured at a distance of 10–15 cm from various places on the udder with a silvered katather-

mometer. The other half of the udder was protected by a cover of cloth and sheep's fleece, held in place by elastic around the cow's girth and over her tail. Surface temperatures were measured by thermocouples (± 0·05 °C), fixed to each quarter of the udder with adhesive tape.

The complete experiment consisted of 4 periods, each of 5 days or 10 milkings. The cooling treatment, which was applied to one half of the udder of each cow during day 3, began after the morning milking at the end of day 2 and continued until the morning milking at the end of day 3. Air temperature was 12–14 °C on days 1, 2, 4 and 5, but it was between 6 and 10 °C on day 3; each cow was fitted with a cover during day 3. The plan is outlined below:

	Day									
	1		2		3		4		5	
Milking ...	pm	am	pm	am	pm	am	pm	am	pm	am
Treatment					↑ Cooling ↑					
Air temperature, °C	13		13		8		13		13	

During the first and third periods the left half of the udder of cow 1 and the right half of the udder of cow 2 were cooled, whereas in the second and fourth periods the opposite halves of each udder were cooled. Thus, each half of each udder was cooled twice during the experiment.

After each milking the milk from each quarter was weighed to within 1 g, stirred, and a 200–300 g sample taken for analysis; a smaller sample was also taken for determination of the cell count.

Samples were analysed for percentage contents of fat, protein and lactose with an Infra Red Milk Analyser (Grubb Parsons and Co. Ltd, Newcastle upon Tyne, England). The analyser was checked periodically against known standards and was accurate to ± 0·04 % for fat and protein and ± 0·02 % for lactose (Munford, 1968).

The cell content of the milk samples was assessed by the Wisconsin Mastitis Test (WMT) as described by Thompson & Postle (1964), except that a commercial reagent (Rapid Mastitis Test Reagent, I.C.I. (N.Z.) Ltd) designed for use in a modified California Mastitis test was used. The results of the WMT using this reagent have been found to be highly correlated with results obtained with the Prescott-Breed smear method (Mr R. C. W. Daniel, unpublished observations).

Milk samples from individual quarters were collected under aseptic conditions during only the third and fourth periods of the experiment; they were cultured on 10% sheep blood agar, incubated at 37 °C and examined at 24 and 48 h.

The yields of milk, fat, protein and lactose and the percentage composition of the 3 constituents were calculated for each udder half at each milking. The difference in yield between day 3 and days $(2+4) \div 2$ was calculated for both halves within cows and periods, and the effect of cooling determined by subjecting the 8 pairs of differences for each component to a 't' test, after suitable coding of the values in each case. Results from afternooon and morning milkings were analysed separately.

RESULTS

The cows remained in apparent good health, despite the relatively unsuitable housing. The procedures adopted for maintaining a temperature difference between

the 2 udder halves on day 3 seemed to be successful. The surface temperature on the cooled half was 20–25 °C and on the warmed half 34–36 °C; these values may be compared with those for both halves on days 1, 2, 4 and 5, which were 33–34 °C. However, it is likely that surface temperature on the cooled half was underestimated relative to that on the warmed half (Molnar & Rosenbaum, 1963) because of the rapid air movement over the thermocouples. After this experiment had been completed, measurements made with cow 2 and with another cow indicated that the cooling procedure might have caused a difference in intra-gland temperature of 0·2–0·4 °C between the 2 halves of the udder, measured with a thermocouple at a depth of 15 cm from the tip of the teat.

Table 1. *Yields of milk, fat, protein and lactose from cooled and warmed halves of the udder at morning and afternoon milkings, on days 2, 3 and 4. In addition the yield for day 3 is expressed as a percentage of the mean yield for days 2 and 4.*

(The P values denote the significance of the effect of cooling.)

Yield, g	Treatment of udder half	Day 2	Day 3	Day 4	$\frac{\text{Day } 3 \times 2}{\text{Days } 2+4} \times 100$, %	P
		Morning milkings				
Milk	Cooled	2460	2460	2630	96·5	< 0·005
	Warmed	2420	2530	2600	100·8	
Fat	Cooled	146	150	149	101·4	< 0·050
	Warmed	142	155	149	106·2	
Protein	Cooled	93	97	100	100·0	< 0·010
	Warmed	93	99	98	103·1	
Lactose	Cooled	122	121	129	96·0	< 0·025
	Warmed	120	126	131	100·0	
		Afternoon milkings				
Milk	Cooled	1810	1700	1810	93·9	< 0·20
	Warmed	1840	1730	1810	94·5	
Fat	Cooled	122	116	126	93·5	< 0·40
	Warmed	124	117	129	92·1	
Protein	Cooled	66	64	69	94·1	< 0·20
	Warmed	67	66	68	97·0	
Lactose	Cooled	89	83	89	93·3	< 0·20
	Warmed	90	85	89	94·4	

The mean results for milk, fat, protein and lactose yields from the cooled and warmed udder halves on days 2, 3 and 4 are presented in Table 1. In addition, the value (days 3×2/days $2+4) \times 100$ is given in each case so that a comparison of changes in the 2 udder halves can be made; the value of P referred to in each case was calculated as described in the Experimental section. It can be seen that for the morning milking the yields of milk and of all 3 components from the cooled half were reduced relative to those from the warmed half by about 3–5%; all these differences were significant. However, for the afternoon milkings the differences were smaller, and indeed for fat the yield from the cooled half increased relative to that from the warmed half by 1%. These results indicate the changes which took place in percentage composition; at morning milkings small increases in fat and protein content occurred on day 3 but little change in lactose, whereas, at afternoon milkings, the

fat percentage had fallen slightly on day 3 while the levels of lactose and protein were the same as at morning milkings. Milk from both the warmed and the cooled halves of the udder behaved very similarly with respect to these changes in percentage composition. One cow showed consistently larger effects of cooling than the other, although in general both showed similar effects. WMT scores tended to be higher on day 1 of each period than subsequently; they were apparently unaffected by cooling. An exception was milk from the left fore quarter of cow 1, which showed consistently high scores; nevertheless this quarter showed no consistent effect of cooling either.

The cultures of milk made during the third and fourth periods did not consistently reveal the presence of mastitis-producing bacteria in any quarters except the left fore quarter of cow 1, from which *Staphylococcus aureus* was consistently isolated.

DISCUSSION

It is not possible to compare accurately the cooling conditions which were used in these experiments with naturally occurring climatic conditions. However, using values of 0·05° and 0·12 °C/kcal m^2 h for the insulation of air at air movements of 9·6 and 0·6 m.p.h. respectively (Joyce & Blaxter, 1964), and a value of 0·05 °C/kcal m^2 h for the tissue insulation of the udder (Holmes, unpublished heat-flow disk measurements), it can be estimated that an air temperature of 10 °C with an air movement of 9·6 m.p.h. would be equivalent to an air temperature of − 10 °C with an air movement of 0·6 m.p.h. Interpretation of this type of calculation is complicated in that the hair covering the udder surface, which had been removed during these experiments, would make a contribution to local insulation in normal circumstances.

The results show that a marked increase in local heat loss and the consequent decrease in local temperature can cause a decrease in the rate of milk synthesis within the udder due possibly to reduced enzymic activity within the secretory cells or reduced blood flow in the cooled glands or both. Linzell (1950) showed that blood vessels in perfused mammary tissue are very sensitive to cooling, to which they react by vasoconstriction. The half udders had been cooled for 9 h before the afternoon milking and for 22 h before the morning milking on day 3, and this may explain the larger effect of cooling seen at the morning milking. This effect of cooling might be of practical importance under conditions of low air temperature, high wind speed and rain, which could lead to increased heat loss from the udder, particularly as cows tend to turn their hind quarters into wind and rain. Contact between the udder and an uninsulated concrete floor would also lead to rapid heat loss, and this has been discussed as a possible predisposing cause of mastitis (Ewbank, 1968), although no convincing evidence has been presented. The present results differ from those of Ewbank (1968), who induced an increase in the cell count of milk from cooled quarters, but his cooling technique differed from that used in the present experiment and may have been more severe. In any case the present results suggest that changes in udder health did not contribute to the decreases which occurred in milk production.

I acknowledge the help and advice of Mr R. C. W. Daniel of the Department of Veterinary Clinical Sciences; of Miss G. Bowman who performed the Wisconsin

Mastitis Tests, and of Professor R. E. Munford and Mrs S. Playne who carried out the analyses of milk samples.

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The specificity for κ-casein as the stabilizer of α_s-casein and β-casein

I. Replacement of κ-casein by other proteins

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(*Received* 15 *July* 1970)

Summary. The specificity of the interaction between κ-casein, α_s-casein and β-casein which forms the basis of micelle stabilization was studied by investigating the extent to which κ-casein could be replaced by other proteins. Of those tested, only gelatin replaced κ-casein and even it was only 2·5 % (w/v) as effective and required a long pre-incubation period. The micelles formed by each of κ-casein and gelatin with α_s-casein and Ca^{2+} were of a similar size to the casein–Ca complexes which compose natural micelles. Gelatin also formed complexes with α_s- and with β-casein at 30 °C in the absence of $CaCl_2$. Evidence was obtained that the interactions between gelatin and the caseins had a much stronger ionic component than had those between κ-casein and the other caseins. It was concluded that the interactions between κ-casein and α_s- and β-caseins which lead to micelle formation are highly specific and probably involve definite sites in each molecule.

The casein micelles of milk contain 3 major casein components, α_s-, β- and κ-caseins, which comprise respectively about 45, 33 and 11 % of the total casein present (Ribadeau Dumas, 1968). The micellar system is stabilized by κ-casein; if κ-casein is removed α_s- and β-caseins are precipitated by the Ca^{2+} normally present in milk (Waugh & von Hippel, 1956). κ-Casein also stabilizes α_s- and β-casein individually against precipitation by Ca^{2+}, micelles being formed in each case (Zittle, 1961; Zittle & Walter, 1963; Noble & Waugh, 1965). It is assumed in this paper that this involves the same kind of interactions between the casein molecules as occurs in natural micelle formation.

The precipitation of α_s- and β-caseins by Ca^{2+} must involve the aggregation of the molecules into very large units. In the presence of κ-casein aggregation still occurs, since micelles are formed, but it is less extensive. κ-Casein may act either relatively non-specifically as a protective colloid or detergent, or by interacting specifically at definite sites to form complexes that are not precipitable by Ca^{2+}. The main purpose of the present work was to differentiate between these possibilities by investigating the extent to which κ-casein could be replaced in the stabilization of α_s- and β-casein by proteins similar in size, charge or composition. This study also adds to evidence presently available on the mode of interaction between κ-casein and α_s- and β-caseins.

EXPERIMENTAL

Materials

Casein fractions. Casein fractions were prepared from the milk of individual, genetically typed Friesian cows from the Institute herd as described previously (Green, 1969), the crude κ-casein A and β-casein A being purified by treatment with calcium phosphate gel.

Proteins and peptides tested as stabilizers. Solutions of proteins at pH 7·0 were dialysed against 0·07 M-KCl, pH 7·0, before use, unless otherwise indicated. Crystalline bovine serum albumin was obtained from British Drug Houses Ltd, Poole, Dorset, and crystalline ovalbumin and swine skin gelatin were from the Sigma Chemical Co., St Louis, Mo., U.S.A. Blood serum was prepared by centrifugation of fresh cow's blood which had been allowed to clot. Egg albumen was obtained from a newly laid hen's egg. Milk serum was prepared from separated milk: $CaCl_2$ (0·2 M final concentration) was added and the casein micelles were removed by centrifugation at 75 000 *g* for 1 h. Soluble casein was removed by adjusting the supernatant liquid to pH 4·5 with N-HCl and centrifuging. The casein-free supernatant liquid was freed from Ca^{2+} by dialysis against 0·02 M-EDTA–0·07 M-KCl of pH 7·0, and then against 0·07 M-KCl of pH 7·0. The egg lipoprotein used was a mixture of lipovitellins and α-, β- and γ-livetins prepared by the $MgSO_4$ method of Bernardi & Cook (1960). Glycomacropeptide was prepared from whole casein as described by Alais & Jollès (1961) and dissolved in 0·07 M-KCl of pH 7·0, just before use.

Other proteins. Soyabean trypsin inhibitor was obtained from British Drug Houses Ltd, Poole, Dorset, and crystalline β-lactoglobulin was a gift from Dr R. L. J. Lyster.

Methods

Stabilization tests. The degrees of stabilization of caseins against precipitation by $CaCl_2$, by various test proteins, were normally determined by the methods of Zittle (1961) for α_s-casein and Zittle & Walter (1963) for β-casein. Unless otherwise indicated 0·07 M-KCl was present and this necessitated an increase in the concentration of $CaCl_2$ from 0·01 to 0·02 M.

Determination of protein concentrations. The concentrations of bovine serum albumin and ovalbumin were determined assuming $E^{1\%}_{279} = 6{\cdot}67$ (Luzzati, Witz & Nicolaieff, 1961) and $E^{1\%}_{280} = 6{\cdot}58$ (calculated from the data of Crammer & Neuberger, 1943) respectively. The concentration of swine skin gelatin was determined from the value of $E^{1\%}_{280} = 0{\cdot}775$ derived from measurements of the extinction and nitrogen content of a dialysed solution assuming that the material contained 18·3 % N (Eastoe, 1955). The concentrations of protein in blood and milk serum, egg albumen and lipoprotein solutions were determined from measurements of the extinctions at 280 nm and 260 nm (Layne, 1957). Other protein solutions were prepared directly from dry, salt-free material.

Determination of Ca^{2+} concentrations. The concentration of free Ca^{2+} in solutions was determined at 30 °C using a calcium electrode obtained from Corning Glass Works, Medfield, Mass., U.S.A., together with a Vibron electrometer (Electronic Instruments Ltd, Richmond, Surrey). The position of the readings on the scale was adjusted by application of a backing potential supplied by a 1·5 V Mallory alkaline

battery and a variable resistance. The electrode potential due to Ca^{2+} was taken as the difference between the potentials recorded with the test solution and a solution which was similar in all respects except that no Ca^{2+} was present.

Analytical ultracentrifugation. Sedimentation velocity analyses were made using a Beckman Model E analytical ultracentrifuge. Schlieren optics were used and runs were carried out at 30 °C at 59780 rev/min. Sedimentation coefficients were determined from the slope of a plot of the natural logarithm of the maximum ordinate against time. Viscosities were measured using an Ostwald viscometer at 30 °C and densities by means of a pycnometer.

Agarose gel filtration. A column (71 × 2·15 cm) of Sepharose 2B (Pharmacia, Uppsala, Sweden) was used at 23 °C. It was equilibrated before use with 0·07 M-KCl–0·02 M-$CaCl_2$–4 μg/ml penicillin of pH 6·9, and eluted with the same solution; 5 ml fractions were collected. The void volume of the column (V_0) and the effective internal volume (V_i) were taken as the elution volume of *Lactobacillus casei* cells (93 ml) and the difference between V_0 and the elution volume of lactose (335 ml) respectively. The Stokes' radii of the eluted fractions were calculated by the method of Ackers (1964); the effective pore radius of the gel (r) was determined by calibration of the column with 2 proteins. Thyroglobulin (Stokes' radius 8·5 nm, Andrews, 1970) had an elution volume of 263 ml, giving a pore radius of 115 nm. Fibrinogen (Stokes' radius 10·6 nm, calculated as described by Andrews (1970) from the diffusion coefficient determined by Shulman (1953)) had an elution volume of 261 ml, giving a pore radius of 136 nm. By averaging these, the pore radius was taken as 125 nm.

RESULTS

Stabilization tests using various proteins

Stabilization by κ-casein. The extent of stabilization of α_s- and β-caseins by various levels of κ-casein at 30 °C in both the presence and absence of KCl are shown in Figs 1 and 2. These provide the data with which those obtained with other proteins should be compared, since κ-casein is the natural stabilizer in the casein micelle in milk. The levels of α_s- and β-caseins remaining in solution (i.e. not centrifuged down at low speed) in the presence of $CaCl_2$ and the absence of stabilizer (abscissa = 0) provide the base lines which are equivalent to zero stabilization.

The stabilizing effect of κ-casein on α_s-casein was also tested at 0 °C, using the normal method except that KCl was absent and the incubation mixture was cooled to 0 °C before the addition of $CaCl_2$; incubation and centrifugation were then carried out at 0 °C. These results indicate that high concentrations of κ-casein stabilize α_s-casein as well at 0° as at 30 °C (Fig. 1), but the threshold level below which no stabilization occurs is higher at 0° than at 30 °C.

Stabilization by other proteins. None of the protein or peptide solutions shown in Table 1 stabilized either α_s- or β-casein at the levels indicated, under the standard conditions. Owing to the limitations of the assay method in which the proportion of casein 'in solution' was determined by subtracting the extinction at 280 nm of the test material from the total extinction, the concentrations of the proteins used could not be increased further.

It seemed likely that better conditions might be established for interaction of the

caseins with other proteins if the casein and protein were dissolved in concentrated urea, so as to break down some of the secondary structure, and the urea then slowly removed by dialysis. Such an experiment was tried with bovine serum albumin, ovalbumin and milk serum with both α_s- and β-casein. Solid urea was dissolved to 6 M final concentration in mixtures of solutions of casein and test protein in 0·07 M-KCl. The mixtures were incubated at 30 °C for 1 h before the urea was removed by

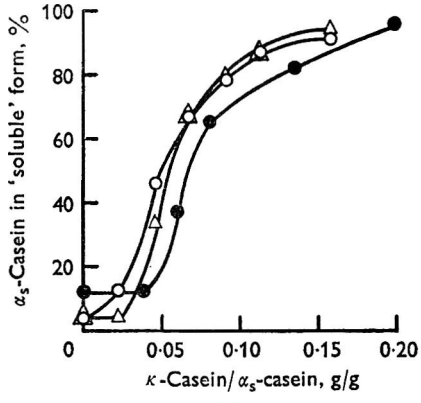

Fig. 1

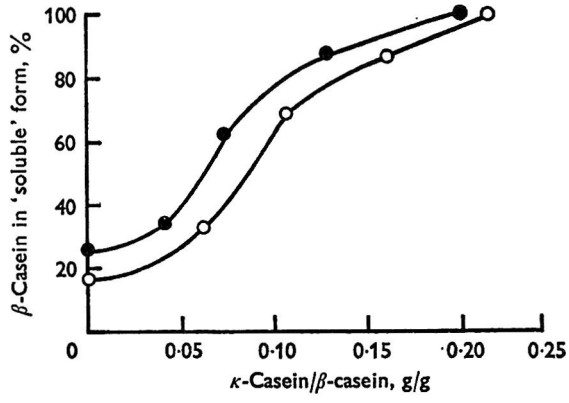

Fig. 2

Fig. 1. The stabilization of α_s-casein at 30 and 0 °C by κ-casein. ●, 0·07 M-KCl, 0·02 M-$CaCl_2$, 30 °C; ○, no KCl, 0·01 M-$CaCl_2$, 30 °C; △, no KCl, 0·01 M-$CaCl_2$, 0 °C.

Fig. 2. The stabilization of β-casein by κ-casein at 30 °C. ○, No KCl, 0·01 M-$CaCl_2$; ●, 0·07 M-KCl, 0·02 M-$CaCl_2$.

Table 1. *Protein and peptide solutions which did not stabilize α_s- or β-casein against precipitation by $CaCl_2$*

Protein solution	Highest value of protein/α_s- or β-casein (g/g) tested	
	α_s-casein	β-casein
Standard method		
Cow's blood serum	1·3	0·7
Egg albumen	2·5	1·5
Milk serum	1·9	1·5
Casein glycomacropeptide	1·0	2·0
Method involving urea treatment		
Bovine serum albumin	1·2	0·6
Ovalbumin	1·2	0·6
Milk serum	2·0	1·0

dialysis at 4 °C against 3 changes of 0·07 M-KCl of pH 7·0 over 24 h. The appropriate amount of each solution, determined from the extinction at 280 nm, was then taken and the stabilization test was carried out as described above. However, none of the protein solutions effected any stabilization when tested at levels up to those indicated in Table 1.

β-Casein was ineffective as a stabilizer of α_s-casein at a ratio of β-casein/α_s-casein = 0·7 under conditions where it was not itself precipitated by $CaCl_2$, i.e. at 0 °C. The

$CaCl_2$ level was raised to 0·025 M in the absence of KCl so as to leave a sufficient concentration of free Ca^{2+}, after binding to β-casein, to precipitate α_s-casein.

The evidence presently available suggests that the interactions between the caseins are mainly hydrophobic in nature and it has been suggested that κ-casein shows certain detergent-like properties, having discrete hydrophobic areas in the molecule (Hill & Wake, 1969). Therefore, it seemed possible that other proteins having discrete hydrophobic areas might replace κ-casein as stabilizers of α_s- and β-caseins.

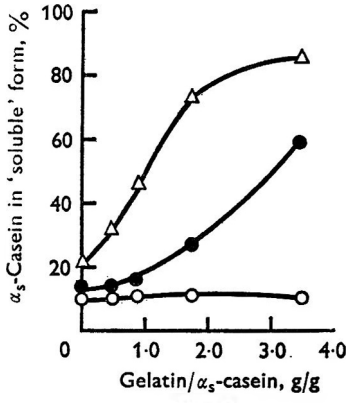

Fig. 3

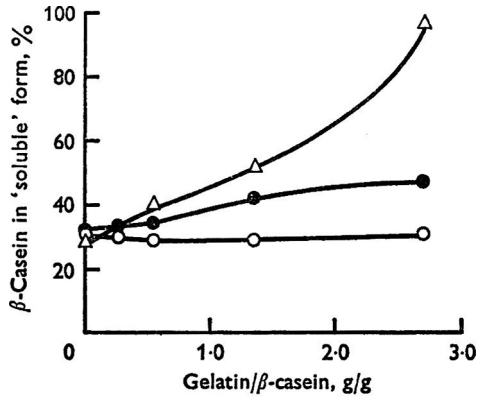

Fig. 4

Fig. 3. The effect of time on the level of stabilization of α_s-casein by gelatin. Gelatin and α_s-casein were pre-incubated at 30 °C in 0·07 M-KCl–2 μg/ml penicillin in a total volume of 4·9 ml. After the time indicated, 0·1 ml 1·0 M-$CaCl_2$ was added and the stabilization test was carried out in the standard manner. Pre-incubation time; ○, 0 h; ●, 6·5 h; △, 16 h.

Fig. 4. The effect of time on the level of stabilization of β-casein by gelatin. The pre-incubations and stabilization tests were carried out as described for Fig. 3. Pre-incubation time; ○, 0 h; ●, 6·5 h; △, 16 h.

Egg-yolk lipoprotein with the lipid partly removed was chosen as an example. A 300 mg sample of freeze-dried lipoprotein was extracted 3 times for 1 h each time with 10 ml ethanol–diethyl ether (3:2) (Evans, Bandemer, Heinlein & Davidson, 1968). The amount of lipid extracted, determined gravimetrically after evaporation of the solvent, amounted to 31 mg. Thus, assuming that the original material contained 24 % lipid, as found by Evans *et al.* (1968) for lipovitellin, 43% of the lipid had been extracted. Most of the protein residue dissolved in 0·07 M-KCl of pH 8·5, and this solution was tested as a stabilizer of α_s- and β-caseins both under the standard conditions and by the method involving solution of the protein and casein in 6 M-urea with subsequent removal of the urea by dialysis. No stabilization was obtained in either test up to a ratio of lipoprotein/α_s- or β-casein of 0·07. Higher ratios could not be tested because of the low solubility of the lipoprotein under the conditions of the experiment.

Like the other proteins tested, swine-skin gelatin did not stabilize α_s- or β-casein to any significant extent when the stabilization test was carried out in the standard manner. However, if the gelatin and casein were pre-incubated at 5 or 30 °C with or without pretreatment with urea, before the addition of $CaCl_2$, stabilization was obtained. At 30 °C the level of stabilization increased progressively with time

up to at least 16 h, when there was almost complete stabilization at the highest ratios tested (Figs 3, 4). Penicillin was added to the incubation mixtures to prevent bacterial growth, but this was shown to have no effect on the results.

Stabilization under conditions of controlled Ca^{2+} concentration

The results detailed above show that, under the conditions used, κ-casein and gelatin stabilize α_s- and β-caseins against precipitation by Ca^{2+}. However, the results do not differentiate between true stabilization in which a complex is formed which is not precipitable by Ca^{2+}, and apparent stabilization due to preferential binding of Ca^{2+} by the test protein leaving insufficient Ca^{2+} to precipitate the casein. It was therefore necessary to show to what extent the 'apparent' stabilizers compete with α_s- and β-casein for Ca^{2+} and to carry out stabilization tests at sufficiently high levels of Ca^{2+} to correct for this.

Table 2. *Stabilization of α_s-casein at a controlled Ca^{2+} concentration*

Test protein	Conc. $CaCl_2$, M	α_s-Casein 'soluble' in absence of test protein, %	Test protein/ α_s-casein, g/g	α_s-Casein 'soluble' in presence of test protein, %
κ-Casein	0·050	18	0·18	81
Gelatin (no pre-incubation)	0·063	18	5·5	15
Gelatin (25·5 h pre-incubation)	0·063	23	5·5	86

Table 3. *Stabilization of β-casein at a controlled Ca^{2+} concentration*

Test protein	Conc. $CaCl_2$, M	β-Casein 'soluble' in absence of test protein, %	Test protein/ β-casein, g/g	β-Casein 'soluble' in presence of test protein, %
κ-Casein	0·050	33	0·43	100
Gelatin (no pre-incubation)	0·035	33	5·4	30
Gelatin (25·5 h pre-incubation)	0·035	37	5·4	86

The 'apparent' stabilizers, at the concentrations used in stabilization tests, were titrated with $CaCl_2$ and the concentration of free Ca^{2+} determined with a calcium electrode. Thus, it was possible to determine the concentration of $CaCl_2$ required to give 0·02 M-Ca^{2+} in the presence of the protein, which maximally precipitated both α_s- and β-casein. Stabilization tests were then made under these conditions. Both Ca^{2+} determinations and stabilization tests were carried out in 0·07 M-KCl–0·05 M-imidazole-HCl buffer of pH 6·8 for α_s-casein and pH 7·0 for β-casein; the buffer was added to prevent small changes in pH, to which the calcium electrode was extremely sensitive. The concentrations of $CaCl_2$ required to give at least 0·02 M-Ca^{2+}, under the conditions used, and the extents of stabilization of α_s- and β-caseins at these $CaCl_2$ levels are shown in Tables 2 and 3 for κ-casein and gelatin.

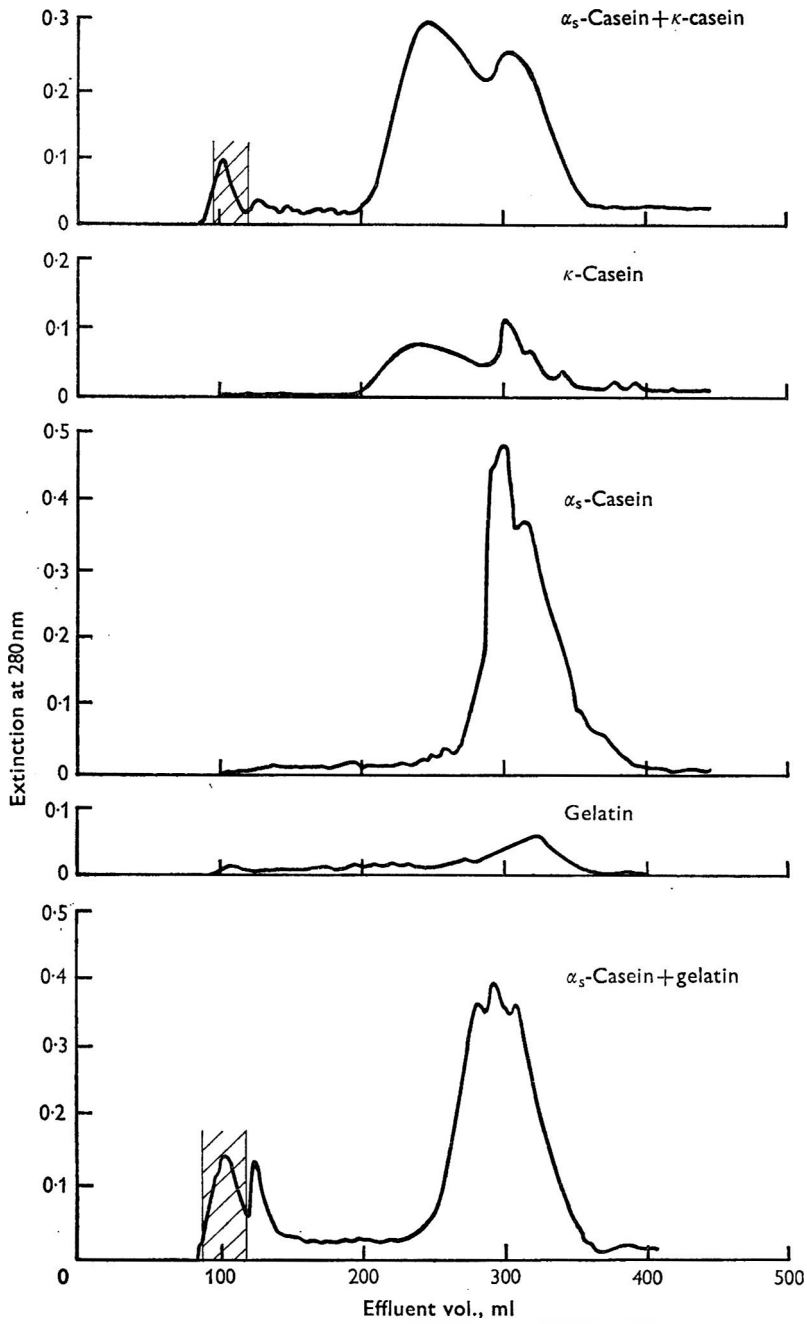

Fig. 5. Agarose gel filtration elution profiles of mixtures of κ-casein or gelatin with α_s-casein, and of the separate components. With the exception of α_s-casein alone, the samples applied to the column consisted of 10 ml of standard stabilization test incubation mixtures, containing 32·9 mg α_s-casein, 7·3 mg κ-casein and 105 mg gelatin, as appropriate, in 0·07 M-KCl–2 μg/ml penicillin–0·02 M-$CaCl_2$, which had been incubated at 30°C for 15 min, then centrifuged for 10 min. In the case of the α_s-casein–gelatin mixture and gelatin alone these were pre-incubated at 30 °C for 17 h before the addition of $CaCl_2$. For α_s-casein alone, $CaCl_2$ was omitted from the sample. Shaded areas represent fractions in which the effluent was cloudy. These fractions were clarified by the addition of 1 drop of N-NaOH before measuring the extinctions.

Gel filtration of mixtures of α_s-casein with κ-casein and gelatin in the presence of $CaCl_2$

The viscosities of gelatin solutions increase considerably with time on incubation at 30 °C (Davis & Oakes, 1922). The stabilization test depends on the separation of precipitated and micellar α_s-casein by centrifugation and this viscosity increase might invalidate the test by reducing the extent of aggregation and the rate of sedimentation of precipitated α_s-casein. Therefore, independent evidence for the formation of micelles in gelatin–casein mixtures was required.

Table 4. *Recovery and estimated size of material eluted from the agarose column in the range 95–130 ml*

Sample	Total recovery, %	Recovery in range 95–130 ml, %	Stokes' radius, nm	Upper limit for particle weight
α_s-Casein + κ-easein	92	4·2	66·8	$10·2 \times 10^8$
α_s-Casein + gelatin	100	9·7	65·0	$9·3 \times 10^8$
α_s-Casein	87	0·8	.	.
κ-Casein	100	0·0	.	.
Gelatin	70	5·4	.	.

Murphy, Downey & Kearney (1969) have described the fractionation of casein complexes by means of gel-filtration on Sepharose 2B columns. This technique was applied to incubation mixtures from stabilization tests of α_s-casein with both κ-casein and gelatin. The elution patterns compared with those of the components alone are shown in Fig. 5. From both incubation mixtures small peaks were obtained representing cloudy effluents, just beyond the void volume. The proportion of total material in each of these peaks and the calculated Stokes' radius for each aggregate, together with the particle weight calculated as described by Andrews (1970), are shown in Table 4. Of the total protein in the α_s-casein–κ-casein mixture 52 % was eluted as a peak earlier than would be expected from the behaviour of the separate components. The material in this second peak had a calculated Stokes' radius of 11·3 nm, giving an upper limit for the particle weight of $4·9 \times 10^6$. There was evidence of a small amount of an intermediate complex in the α_s-casein–gelatin mixture and there were indications that some of the material in the main peak was eluted earlier than would have been expected from the elution pattern of the separate components. Analytical starch-gel electrophoresis showed that all of the peaks obtained from each mixture contained both components.

Analytical ultracentrifugation

Gelatin–casein mixtures. Gelatin–casein mixtures were examined in the analytical ultracentrifuge in order to detect any complex formation in the absence of Ca^{2+} such as has been observed for κ-casein–α_s-casein mixtures under certain conditions (Noble & Waugh, 1965). α_s-Casein–gelatin and β-casein–gelatin mixtures were incubated at 30 °C for 30 min and 16 h before ultracentrifugation at 30 °C. α_s-Casein–gelatin mixtures were diluted with an equal volume of 0·07 M-KCl immediately before the run. The observed sedimentation coefficients in 0·07 M-KCl and the extent of

stabilization of the caseins against precipitation by $CaCl_2$ in the same mixtures are shown in Table 5. The results show that both caseins form complexes with gelatin.

Mixtures of gelatin with other proteins. In order to determine whether complex formation with gelatin is a general characteristic of proteins, ultracentrifugation of mixtures of gelatin with 3 non-casein proteins was carried out. Mixtures containing 4·3 mg/ml gelatin and 1·5 mg/ml protein in 0·07 M-KCl of pH 7·0 were incubated at 30 °C for 30 min and for 16 h before ultracentrifugation at 30 °C. In each instance only one schlieren peak was observed in the mixture; sedimentation coefficients are shown in Table 6.

Table 5. *Analytical ultracentrifugation of casein–gelatin mixtures*

Solution	Incubation time	Sedimentation coeff. (obs.)	Proportion in each fraction	α_s-Casein or β-casein in 'soluble' form, %
Gelatin (5·3 mg/ml)	30 min, 16 h	2·74	.	.
α_s-Casein (1·4 mg/ml)	30 min	4·12	.	.
β-Casein (1·5 mg/ml)	30 min	1·76, 16·46	30:70	.
α_s-Casein (1·4 mg/ml) +	30 min	7·64	.	10
gelatin (5·3 mg/ml)	16 h	6·57, 14·80	80:20	83
β-Casein (1·5 mg/ml) +	30 min	3·92, 14·89	80:20	16
gelatin (4·3 mg/ml)	16 h	3·43, 14·50	80:20	88

Table 6. *Analytical ultracentrifugation of gelatin–non-casein protein mixtures*

Solution	Incubation time	Sedimentation coeff. (obs.)
Trypsin inhibitor	30 min	2·38
Trypsin inhibitor + gelatin	30 min	3·67
	16 h	3·87
β-Lactoglobulin	30 min	3·94
β-Lactoglobulin + gelatin	30 min	3·67
	16 h	3·67
Bovine serum albumin	30 min	6·12
Bovine serum albumin + gelatin	30 min	4·28
	16 h	4·35

Effect of ionic strength on stabilization

The above results indicate that gelatin forms complexes with α_s- and β-caseins which, on addition of $CaCl_2$, aggregate into micelle-like structures. To this extent it behaves similarly to κ-casein. In an attempt to carry the comparison one stage further and shed light on the relative importance of different modes of protein–protein interaction involved in stabilization by each of the 2 proteins, the effect of ionic strength on the stabilization of α_s- and β-casein both by κ-casein and gelatin was determined. In each test the standard method was used except that the concentration of $CaCl_2$ was raised to 0·03 M and the ionic strength was varied by addition of KCl.

The results, shown in Tables 7 and 8, indicate that stabilization by gelatin is more sensitive to ionic strength than stabilization by κ-casein.

Table 7. *Effect of ionic strength on the stabilization of α_s-casein*

(The ratios κ-casein/α_s-casein (g/g) and gelatin/α_s-casein (g/g) were 0·19 and 5·1 respectively.)

	α_s-Casein in 'soluble' form, %		
Conc. KCl, M	No stabilizer	κ-Casein as stabilizer	Gelatin as stabilizer
0·07	22	63	78
0·09	24	48	67
0·11	30	45	54
0·15	30	46	49

Table 8. *Effect of ionic strength on the stabilization of β-casein*

(The ratios κ-casein/β-casein (g/g) and gelatin/β-casein (g/g) were 0·42 and 6·1 respectively.)

	β-Casein in 'soluble' form, %		
Conc. KCl, M	No stabilizer	κ-Casein as stabilizer	Gelatin as stabilizer
0·07	24	78	68
0·09	18	53	33
0·11	17	52	21
0·15	20	57	22

Properties of gelatin

It seemed that the apparent stabilizing action of gelatin might be further elucidated by comparing its properties with those of κ-casein on the one hand and of non-stabilizing proteins on the other. Since swine skin gelatin is less well characterized than most of the other proteins investigated, some of its properties were determined.

The carbohydrate content, determined as reducing sugar (Hassid & Abraham, 1957), was 1·4% (w/w), but the sialic acid content was below the level (0·02 %, w/w) measurable by the method of Warren (1959). The total phosphate content, determined by the method of Fiske & Subbarrow (1925), was 0·049% (w/w). The iso-ionic point was determined by gently stirring 20–70 mg gelatin in 0·07 M-KCl with 2 ml mixed bed resin prepared by mixing Amberlite IR-120 (H^+ form) and Amberlite IRA-400 (OH^- form) to pH 7·0. The gelatin–resin mixture was stirred until the pH value of the supernatant liquid had become constant. This value, pH 9·0–9·3, was taken as equal to the iso-ionic point of gelatin.

DISCUSSION

κ-Casein is the natural stabilizer of α_s-casein and β-casein in milk, enabling micelles to be formed. In contrast to other workers' results (Noble & Waugh, 1965), the present work indicates that κ-casein stabilizes α_s-casein at 0 °C nearly as well as at 30 °C, although complex formation is not observed in the absence of Ca^{2+} at 0 °C (Noble & Waugh, 1965).

Of the proteins tested as replacements for κ-casein in the stabilizing process, only gelatin was effective. This was so even under favourable conditions for complex formation, created by partial disruption of the secondary structure of possible stabilizers by urea followed by the slow removal of urea by dialysis. This procedure would 'open up' the protein molecule, enabling areas that are not exposed in the native molecule to become available for interaction with the casein. Moreover, gelatin was much less effective as a stabilizer than κ-casein, 40 times as much (w/w) and a prolonged pre-incubation period being required.

Evidence for interaction resulting in complex formation was obtained by analytical ultracentrifugation of gelatin–casein mixtures. However, these complexes were formed rapidly compared with the rate of increase in stabilization by gelatin and there is no indication that stabilizing power is associated with the formation of a particular complex. With α_s-casein there was some change in the sedimentation pattern with time. First a relatively small complex was formed, of higher sedimentation coefficient than either the α_s-casein aggregate or gelatin alone, and thus presumably of larger size. Later a small proportion of a much larger complex was detectable. The sedimentation coefficient of the smaller complex was close to that of the α_s-casein–κ-casein complex described by Noble & Waugh (1965). The sedimentation pattern of the β-casein–gelatin mixture did not change significantly with time. However, in contrast to the findings for α_s-casein, most of the large aggregate present in the β-casein solution was disrupted in the presence of gelatin, so that most of the material in the mixture was smaller than the original casein. This is similar to the action of detergents on caseins (Cheeseman & Jeffcoat, 1970), which was interpreted as being caused by disruption of the hydrophobic interactions in the aggregate with the formation of new hydrophobic bonds between the casein and the detergent. Thus, it may be that the mode of action of gelatin is similar to that of detergents.

Neither of the sedimentation patterns showed the presence of uncomplexed gelatin. This indicates that the complexes contained a greater proportion of gelatin than casein (w/w) in contrast to the α_s-casein–κ-casein complex which contains excess α_s-casein. This emphasizes a probable difference in the mode of action of gelatin and κ-casein. It may be that the casein molecules in the casein–gelatin complexes are occluded in a gelatin network, although the sedimentation data show that the network cannot be large. This hypothesis is consistent with the results of analytical ultracentrifugation of mixtures of gelatin with trypsin inhibitor, β-lactoglobulin and bovine serum albumin. All these proteins formed complexes with gelatin, indicating that this is probably a general characteristic.

However, the α_s-casein–gelatin complex behaved similarly to the α_s-casein–κ-casein complex in the presence of Ca^{2+}: aggregates eluted just beyond the void volume of a Sepharose 2B column were formed in both instances. The reason why such a small proportion of the material was present in the form of large aggregates is not clear, but it may be that the aggregates were in equilibrium with Ca^{2+} and the Ca-free complexes. Ca^{2+} would then be retarded on the column causing progressive dissociation of the aggregates. The particle weights of the aggregates cannot be ascertained with any certainty since the standards available, being much smaller, were eluted at relatively large volumes and there are no data available on the relation between particle weight and elution volume for Sepharose 2B columns. By comparison

Table 9. *Properties of some proteins tested as stabilizers compared with those of κ-casein*

	κ-Casein A	Swine skin gelatin	Bovine serum albumin	Ovalbumin	β-Lactoglobulin	β-Casein A
Phosphate content (%, w/w)	0·2 (b)	0·05	.	0·12 (j)	.	0·5 (b)
Carbohydrate content (%, w/w)	5·0 (b)	1·4	0 (g)	1·8 (j)	.	0·1 (b)
Molecular weight (monomer)	19 000 (b)	33 000 (e)	67 000 (h)	45 000 (j)	18 000 (k)	25 000 (k)
Iso-ionic point	3·7 (b)	9·0–9·3	4·7 (h)	4·9 (j)	5·4 (k)	4·9 (b)
Average hydrophobicity (cal/res.) (a)	1 200 (c)	939 (f)	1 120 (a)	1 110 (a)	1 230 (a)	1 320 (c)
Charge at pH 6	−0·25 (d)	+0·16 (f)	−0·33 (a)	−0·24 (a)	−0·28 (a)	−0·22 (k)
Proline and hydroxyproline content (residues %)	11·2 (d)	22·6 (f)	4·8 (i)	3·7 (i)	4·9 (k)	15·9 (k)
S—S bonds (number/mole)	0 (d)	0 (f)	16 (i)	1 (i)	2 (k)	0 (k)

(a) Calculated from amino acid analytical data as described by Bigelow (1967).
(b) Jollès (1966).
(c) Hill & Wake (1969).
(d) Calculated from data of Kalan & Woychik (1965).
(e) Estimated from sedimentation constant.
(f) Calculated from data of Eastoe (1955).
(g) Hughes (1954).
(h) Phelps & Putnam (1960).
(i) Tristram & Smith (1963).
(j) Warner (1954).
(k) McKenzie (1967).

with the standards, the calculated maximum particle weights were about 1×10^9. However, Murphy *et al.* (1969) have estimated the molecular exclusion limit of Sepharose 2B as about 1×10^8 which would give maximum particle weights of 1×10^7 to 1×10^8 for the aggregates. Thus, it is likely that the aggregates were of the same order of size as the casein–Ca complexes from which casein micelles are built up in the presence of calcium phosphate (Morr, 1967; Murphy *et al.* 1969). For the present purpose it is sufficient to say that the α_s-casein–κ-casein and α_s-casein–gelatin complexes were extensively aggregated in the presence of Ca^{2+} but that these aggregates were of limited, and approximately constant, size. It is clear that, whether or not α_s-casein was occluded in a gelatin network, it was not prevented from interacting with Ca^{2+} with the formation of aggregates although these were not large enough to precipitate.

The main conclusion that can be drawn from the present work is that the interactions of κ-casein with α_s- and β-caseins resulting in their stabilization in the presence of Ca^{2+} are too specific to be described as some kind of protective colloid mechanism. Some of the properties of the proteins tested as stabilizers are compared with those of κ-casein in Table 9. It is clear that stabilizing ability is not connected solely with any obvious gross properties of the proteins such as size, iso-ionic point, charge, phosphate content or average hydrophobicity. In fact, one point that stands out is the great difference in gross properties between κ-casein and gelatin, the only 2 proteins effective as stabilizers.

The question therefore arises as to whether κ-casein and gelatin form complexes with α_s- and β-caseins by the same mechanism. Before considering this it is necessary to note the characteristics of complex formation by caseins in the presence of Ca^{2+}. It is clear that complex formation in the absence of Ca^{2+} is not a prerequisite for micelle formation; complex formation between κ- and β-caseins in the absence of $CaCl_2$ has not been detected, and that between κ- and α_s-caseins does not occur in the absence of $CaCl_2$ under all conditions where there is stabilization on addition of $CaCl_2$ (Waugh & von Hippel, 1956; Noble & Waugh, 1965). It seems likely therefore that when micelles are formed either or both of the following types of interaction occur: (*a*) the individual caseins interact by means of intermolecular Ca-links, (*b*) Ca^{2+} neutralizes negative charges on the molecules enabling non-ionic interactions to occur. Either of these would be possible as both would reduce the ionic repulsion between the caseins, which are all negatively charged at the pH values at which micelle formation occurs.

The data on the effects of ionic strength on stabilization can now be considered in this light. In stabilization by κ-casein an increase in ionic strength, which has the effect of reducing charge–charge interactions, reduces the level of stabilization but does not eliminate it entirely. This indicates that although ionic interactions are important in maintaining the maximum level of stabilization they are not necessary for stabilization to occur. This suggests that the major role of Ca^{2+} is probably to neutralize charged groups so that non-ionic interactions occur although intermolecular Ca-links, which would probably be primarily electrostatic in nature (McAuley & Hill, 1969), may be formed. On the other hand, gelatin is positively charged at the pH value of the stabilization experiments, so ionic interactions may well be important. This is suggested by the observation that both α_s- and β-caseins form complexes

with gelatin in the absence of Ca^{2+} and is confirmed experimentally for β-casein in that the stabilization by gelatin was completely eliminated by raising the concentration of KCl from 0·07 M to 0·11 M. Changes in ionic strength had a less drastic effect on the stabilization of α_s-casein by gelatin, but the level of stabilization was reduced very considerably when the concentration of KCl was increased from 0·07 M to 0·15 M. Therefore, the evidence indicates that the casein–gelatin–Ca interactions forming the basis of the stabilization phenomenon have a strong ionic component. The interactions resulting in complex formation between gelatin and bovine serum albumin, β-lactoglobulin or trypsin inhibitor are also probably primarily ionic, since the last 3 proteins are all negatively charged at the pH value of the experiment (Table 9; Kunitz, 1947).

The results presented above emphasize the great specificity involved in the interactions between κ-casein and α_s- and β-caseins that lead to micelle formation. The only proteins known to replace κ-casein to any extent are gelatin, which is much less active and probably interacts in a different way, and para-κ-casein solubilized by coupling it to bovine serum albumin (Woychik & Wondolowski, 1967). Although quantitative data are not available, the finding that solubilized para-κ-casein stabilizes α_s-casein suggests that the primary area of the κ-casein molecule involved in the interactions leading to micelle formation is in the para-κ-casein moiety.

The available evidence indicates that the interactions of κ-casein with α_s- and β-caseins are probably primarily non-ionic. This is suggested by the like charge of the components, the effect of ionic strength on stabilization, the increasing aggregation of caseins with rise in temperature (Payens, 1966) and the ready formation of complexes with detergents (Noelken, 1966; Cheeseman & Jeffcoat, 1970; Green, 1971). The specificity of these interactions is reminiscent of that between enzyme and substrate, so that specific groups in each molecule are probably involved. This is indicated by experiments involving specific modification of amino acid residues in the casein molecules. For example, photo-oxidation, involving partial loss of histidine and tryptophan, removes the capacity of κ-casein to stabilize α_s-casein against precipitation by $CaCl_2$ (Zittle, 1964) and dephosphorylation of α_s-casein decreases its capacity to be stabilized by κ-casein (Pepper & Thompson, 1963).

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The specificity for κ-casein as the stabilizer of α_s-casein and β-casein

II. Replacement of κ-casein by detergents and water-soluble polymers

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Summary. The cationic detergent cetyltrimethylammonium bromide (CTAB), the anionic detergents sodium lauryl sulphate and sodium deoxycholate, and the non-ionic detergents Tween 20, Tween 80 and Brij 35, and lecithin, starch, glycogen, chondroitin sulphate and polyethylene glycol, were tested for their ability to replace κ-casein in stabilizing α_s- and β-caseins against precipitation by Ca^{2+}. Of the materials tested, CTAB and Tween 20 stabilized both caseins and Brij 35 stabilized only α_s-casein. Analytical ultracentrifugation of mixtures of CTAB with each casein and of Brij 35 with α_s-casein indicated that both detergents acted by disrupting the casein aggregates and complexing with the monomers. Addition of $CaCl_2$ did not aggregate the Brij 35–α_s-casein complex. It is concluded that the basis of the stabilization phenomenon involves specific interactions between κ-casein and α_s-casein or β-casein at mainly hydrophobic sites.

Green (1971) presented evidence that the stabilization of α_s- and β-casein against precipitation by Ca^{2+}, which is probably the basis of micelle formation, is extremely specific; of several proteins tested only gelatin replaced κ-casein and then only at relatively high concentrations. This indicates that κ-casein does not act simply as a protective colloid and suggests that stabilization results from specific interactions between the casein molecules. The present paper describes an extension of previous work (Green, 1971) designed to shed further light on the degree of specificity and types of bonding involved in interactions between caseins.

Hill & Wake (1969) suggested that stabilizing action may result from the detergent-like properties of κ-casein, and Cheeseman & Jeffcoat (1970) showed that caseins form complexes with certain detergents. In more general terms, Payens (1966) pointed out the strong possibility that hydrophobic interactions form the basis of interactions between different caseins. Green (1971) concluded that ionic interactions probably also play a minor part, but there is no information on the extent to which hydrogen bonding may be involved. These considerations led to the present study, in which detergents and water-soluble polymers were tested as stabilizers of α_s- and β-caseins against precipitation by Ca^{2+}.

EXPERIMENTAL

Materials

Polyoxyethylene sorbitan mono-oleate (Tween 80) and polyoxyethylene sorbitan monolaurate (Tween 20) were obtained from Honeywell and Stein Ltd, Carshalton, Surrey. Polyethylene glycol (mol. wt 300) was obtained from Koch–Light Laboratories Ltd, Colnbrook, Bucks, and polyethylene glycol (mol. wt 6000) from Union Carbide Ltd, London, W.1. Cetyltrimethylammonium bromide (CTAB), sodium lauryl sulphate, polyoxyethylene lauryl ether (Brij 35), sodium deoxycholate, sodium oleate, lecithin, starch, oyster glycogen and chondroitin sulphate were products of British Drug Houses Ltd, Poole, Dorset. Casein fractions were prepared as described by Green (1971).

Methods

These were as described by Green (1971).

RESULTS

Stabilization tests

Since κ-casein is the natural stabilizer of α_s- and β-caseins in the casein micelle, all the other materials tested were compared with it. The plots of the degree of stabilization (i.e. the proportion of α_s- or β-casein remaining 'soluble' and not centrifuged down at 2000 ***g*** for 10 min) against the amount of κ-casein added were shown in

Table 1. *Detergents as stabilizers of α_s- and β-casein*

	α_s-Casein		β-Casein	
Detergent	Detergent/α_s-casein, g/g	α_s-Casein in 'soluble' form, %	Detergent/β-casein, g/g	β-Casein in 'soluble' form, %
Cationic				
CTAB	1·40	93	1·81	93
Anionic				
Sodium lauryl sulphate	1·40	93	0·60	67
Sodium deoxycholate	0·27	0	0·53	10
Non-ionic				
Tween 80	13·9	7	26·7	42
Tween 20	13·4	12	6·3	48
Brij 35	14·6	46	25·0	28

Figs 1 and 2 of the previous paper (Green, 1971). These plots were sigmoid, indicating that stabilization by κ-casein is a co-operative phenomenon, which makes it difficult to express the results in a simple fashion. In the present paper they are normally expressed as the maximal level of stabilization obtained, together with the ratio of stabilizer to casein (w/w) required to give this, but where a closer comparison between the material tested and κ-casein has seemed of interest, more detailed data are given. The base lines, equivalent to zero stabilization, are provided by the levels of α_s- and β-caseins remaining 'in solution' in the presence of $CaCl_2$ and in the absence of stabilizer (abscissa = 0).

Detergents. Various detergents, cationic, anionic and non-ionic, were tested as stabilizers for α_s- and β-caseins with the results shown in Table 1. Tests were carried out in the presence of KCl except when sodium lauryl sulphate was used, in which case KCl was omitted as it precipitated the detergent. The low levels of the anionic detergents used were dictated by their low solubilities in the presence of $CaCl_2$. For

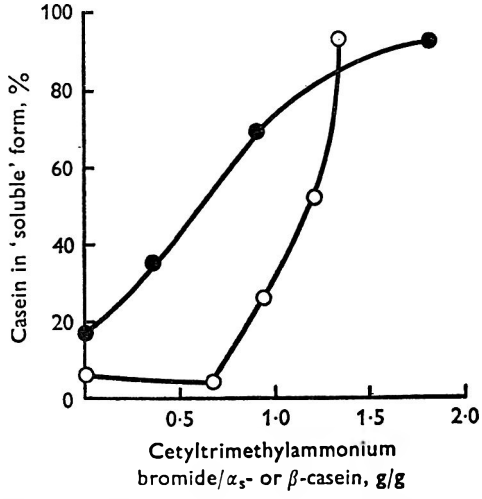

Fig. 1. The stabilization of α_s- and β-casein by CTAB in 0·07 M-KCl. ○, α_s-Casein; ●, β-casein.

Table 2. *Water-soluble, non-protein polymers as stabilizers of α_s- and β-caseins*

Polymer	α_s-Casein		β-Casein	
	Polymer/α_s-casein, g/g	α_s-Casein in 'soluble' form, %	Polymer/β-casein, g/g	β-Casein in 'soluble' form, %
Starch	6·0	5	13·0	17
Glycogen	6·0	2	13·0	10
Chondroitin sulphate	6·0	42	13·0	85
Polyethylene glycol (mol. wt 300)	1·4	0	2·5	20
Polyethylene glycol (mol. wt 6000)	13·1	4	29·0	15

CTAB, Tween 80 and Brij 35 the ratios of detergent to casein recorded in Table 1 were the highest tested. Tween 20 was tested at higher levels but without increasing the degree of stabilization of either casein. The effect of the concentration of CTAB on the level of stabilization obtained is shown in Fig. 1.

Lecithin. Lecithin, present in amounts of 6 and 14 times the weights of α_s- and β-casein respectively, effected no stabilization of either casein in the absence of KCl.

Water-soluble non-protein, polymers. The results of tests of various water-soluble polymers as stabilizers of α_s- and β-caseins are shown in Table 2. Only those tests with the polyethylene glycols were carried out by the standard procedure; the other

materials were tested in the absence of KCl and with 0·01 M-$CaCl_2$. Of these materials only chondroitin sulphate had any stabilizing action.

Effect of $CaCl_2$ concentration and ionic strength on the level of stabilization.

The results detailed above show that, under the conditions used, CTAB, sodium lauryl sulphate, chondroitin sulphate and possibly Tween 20, Tween 80 and Brij 35 appeared to stabilize both α_s- and β-caseins against precipitation by Ca^{2+}. However, as discussed previously (Green, 1971), it is necessary to carry out stabilization tests at a sufficiently high concentration of $CaCl_2$ to compensate for any binding of Ca^{2+} by the apparent stabilizer.

Table 3. *Effects of increased concentrations of $CaCl_2$ on the level of stabilization of caseins by apparent stabilizers*

	α_s-Casein		β-Casein	
Conc. $CaCl_2$, M	Stabilizer/ α_s-casein, g/g	α_s-Casein in 'soluble' form, %	Stabilizer/ β-casein, g/g	β-Casein in 'soluble' form, %
CTAB				
0·01	1·5	85	2·0	96
1·00	1·5	97	2·0	98
Tween 80				
0·01	33	9	63	20
0·20	31	10	28	22
Tween 20				
0·01	30	6	60	15
0·20	23	53	49	33
Brij 35				
0·01	33	15	66	8
0·20	3	58	66	20

Effect of increased concentration of $CaCl_2$ on the level of stabilization. When the $CaCl_2$ concentration in the stabilization test mixture was increased to 0·05 M, the apparent stabilization by sodium lauryl sulphate completely disappeared. Likewise the apparent stabilization by chondroitin sulphate at 0·01 M-$CaCl_2$ was completely absent at 0·1 M-$CaCl_2$. Therefore, it is probable that sodium lauryl sulphate and chondroitin sulphate are not true stabilizers but appear to stabilize caseins at low levels of $CaCl_2$ because they preferentially bind Ca^{2+}.

The effects of increasing the concentration of $CaCl_2$ in test mixtures containing the other apparent stabilizers are shown in Table 3. All the experiments were carried out in the absence of KCl. The results show no marked effect of $CaCl_2$ concentration on the extent of stabilization of either casein by CTAB. When Tween 20 was used with both caseins and Brij 35 with α_s-casein, the degrees of stabilization were increased at the higher concentration of $CaCl_2$. Tween 80 appeared to stabilize neither casein, and Brij 35 did not stabilize β-casein at either $CaCl_2$ concentration. These results indicate that CTAB and Tween 20 were true stabilizers of both α_s- and β-caseins and that Brij 35 stabilized α_s-casein only. It was not possible to confirm this as the calcium electrode gave unreliable results in the presence of detergents.

Effect of $CaCl_2$ concentration and ionic strength on the stabilization of α_s-casein by Brij 35. The phenomenon of the more effective stabilization of caseins by neutral

detergents at high than at low $CaCl_2$ concentrations was studied further for the stabilization of α_s-casein by Brij 35. The results of stabilization tests carried out at various $CaCl_2$ levels in the presence of KCl are shown in Fig. 2. They show that the level of stabilization at any given ratio of Brij 35 to α_s-casein and possibly also the maximal extent of stabilization obtainable increased with increasing $CaCl_2$ concentration. This may be either a specific effect of $CaCl_2$ or it may be due to the ionic strength. This was investigated by using various $CaCl_2$ concentrations, with KCl added to bring the ionic strength to a constant value of 1·5. The degree of stabilization by the same amount of Brij 35 was always the same, which indicates that it is dependent on the ionic strength rather than on the ionic components present.

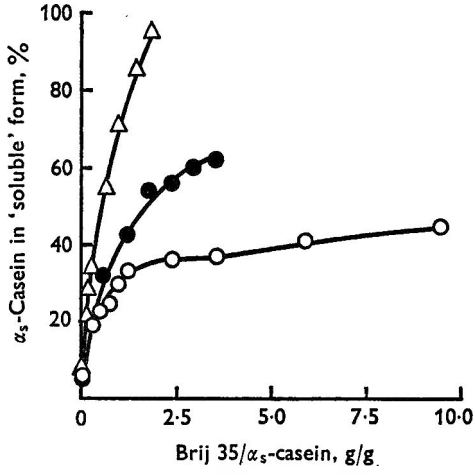

Fig. 2. Stabilization of α_s-casein by Brij 35 at various $CaCl_2$ concentrations. KCl was present at 0·07 M. ○, 0·02 M-$CaCl_2$; ●, 0·2 M-$CaCl_2$; △, 0·5 M-$CaCl_2$.

Analytical ultracentrifugation

Casein–CTAB mixtures. Ultracentrifuge runs of α_s-casein, β-casein and CTAB alone and mixtures of CTAB with each casein, at ratios giving at least 90 % stabilization under the conditions used, were carried out at 23 °C. The sedimentation coefficients in 0·07 M-KCl are shown in Table 4. The detergent formed a negative schlieren peak which floated, indicating that it was less dense than the solvent and had a lower refractive index. In the presence of the caseins this floating peak was absent although there was a negative gradient at the meniscus, indicating that some unbound detergent was present. The sedimentation coefficients of the caseins were much reduced in the presence of the detergent. In fact, the values for $s_{20,w}$ observed were less than those for the casein monomers (McKenzie & Wake, 1959). This suggests that the detergent acts by dissociating the casein polymers to smaller units and then forming complexes with them. It is not possible to estimate the size of the smaller units since the density and specific volume of the complex cannot be determined.

α_s-Casein–Brij 35 mixture. The sedimentation coefficients observed in ultracentrifuge runs at 23 °C of α_s-casein, Brij 35 and a mixture of the 2 giving 100 % stabilization of α_s-casein are shown in Table 5. In the mixture, the schlieren peaks

due to α_s-casein and Brij 35 were completely absent and a single peak, of the area expected from the sum of the 2 components, was present, showing that a complex was formed. The sedimentation coefficient of this complex suggests that the α_s-casein polymer was at least partially dissociated by the detergent. Also shown in Table 5 are the effects of 0·02 M and 0·5 M-$CaCl_2$ on the sedimentation constant of the complex; with neither strength of $CaCl_2$ was there any increase.

Table 4. *Analytical ultracentrifugation of casein–CTAB mixtures*

Solution	Sedimentation coeff. (obs.)	$s_{20,w}$
CTAB (4·0 mg/ml)	1·32 S (floating)	—
α_s-Casein (3·0 mg/ml)	4·95 S	4·67
α_s-Casein (3·0 mg/ml) + CTAB (4·0 mg/ml)	1·27 S	1·18
β-Casein (1·5 mg/ml)	14·31 S. 2·10 S	13·5 1·98
β-Casein (1·5 mg/ml) + CTAB (4·0 mg/ml)	0·93 S	0·86

Table 5. *Analytical ultracentrifugation of α_s-casein–Brij 35 in the presence and absence of $CaCl_2$*

(All solutions were prepared in 0·07 M-KCl, pH 6·8.)

Solution	Sedimentation coeff. (obs.)	$s_{20,w}$
Brij 35 (6·6 mg/ml)	1·5 S	1·4
α_s-Casein (3·3 mg/ml)	4·0 S	3·6
α_s-Casein (3·3 mg/ml) + Brij 35 (6·6 mg/ml)	2·2 S	2·0
α_s-Casein (1·4 mg/ml) + Brij 35 (6·6 mg/ml) + 0·02 M-$CaCl_2$	1·4 S	.
α_s-Casein (3·3 mg/ml) + Brij 35 (6·6 mg/ml) + 0·5 M-$CaCl_2$	0·5 S, 2·0 S	.

DISCUSSION

Of the large number of non-protein materials tested as replacements for κ-casein in stabilizing α_s- and β-caseins against precipitation by Ca^{2+}, only the detergents CTAB and Tween 20 were effective for both caseins, and Brij 35 was effective for α_s-casein only.

Definite conclusions as to the types of interactions occurring between κ-casein and α_s- and β-caseins can only be drawn from the results described if the stabilizing materials act in the same way as κ-casein. Two characteristics of κ-casein are relevant: it forms large complexes giving turbid 'solutions' with each of α_s- and β-casein in the presence of $CaCl_2$; and its stabilization curves (plots of level of stabilization versus amount of stabilizer added) with both α_s- and β-caseins are sigmoid in shape, indicating that the interactions occurring are co-operative (Hill, 1913), i.e. that the protection of a proportion of the α_s- or β-casein molecules facilitates protection of the rest.

The evidence available suggests that the detergents which stabilize caseins probably do not act in the same way as κ-casein. The stabilization curves were sigmoid

for CTAB with both caseins but not for Tween 20 with either casein or for Brij 35 with α_s-casein. In all instances, clear solutions were formed with casein–detergent mixtures after the addition of $CaCl_2$, i.e. there was no indication of micelle formation. This observation was clarified by the analytical ultracentrifugation results, which indicated that the positively charged detergent CTAB and the neutral detergent Brij 35 acted similarly in combining with at least partially dissociated forms of the casein polymers. The addition of $CaCl_2$ to the α_s-casein–Brij 35 complex did not apparently cause aggregation. These results contrast with those expected for materials acting similarly to κ-casein; in the presence of κ-casein and $CaCl_2$, α_s- and β-caseins complex in a polymeric form to give micelles of greatly increased sedimentation coefficient.

Thus, it must be concluded that, although α_s- and β-caseins interact with detergents, the interactions do not necessarily resemble those leading to micelle formation. However, the results confirm those of Cheeseman & Jeffcoat (1970), in indicating that the caseins readily form complexes based mainly on hydrophobic interactions. In comparison with the neutral detergents, the greater effectiveness of CTAB can be explained by additional ionic interactions between the negatively charged casein and the positively charged detergent, and the increase in stabilizing power of the neutral detergents with increase in ionic strength probably reflects screening of the charged groups in the casein by the ions in solution, enabling the casein to participate more readily in hydrophobic interactions.

On the other hand, the results indicate that hydrogen bonding, at least with α_s- or β-casein as the acceptor, is probably not a major factor in micelle formation. All the water-soluble polymers tested, most of them strong hydrogen-bond donors, were ineffective as stabilizers.

Payens (1966), observing that casein–casein interactions are favoured by increase in temperature, and Noelken (1966), who found that Brij 35 stabilizes α_s-casein in solution in the presence of $CaCl_2$, concluded that interactions between the different caseins are mainly hydrophobic in nature. The present results and those of Green (1971) lead to a similar conclusion, although they also point to a minor role for ionic interactions. Further, they emphasize the great specificity of the interactions forming the basis of micelle formation. However, the stabilization of α_s-casein by the carrageenans (Hansen, 1968) does not appear to fit into this pattern. These polysaccharide materials form stable micelles with α_s-casein, and the ester sulphate and 3,6-anhydrogalactose units appear to be involved in the interaction (Lin & Hansen, 1968). This points to the possible involvement of hydrogen-bond acceptor sites on α_s-casein and possibly some intermolecular bonding through Ca.

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Influence of the physical form of a barley grain and barley straw diet on nitrogen metabolism in sheep

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Summary. (1) Two wether sheep fitted with ruminal and duodenal re-entrant cannulas were used to study the influence of the physical form of a barley grain and barley straw diet and intraruminal addition of ammonium salts (mainly acetate) on digestibility of dietary constituents, the flow of digesta to the duodenum and the composition of digesta from the rumen and duodenum.

(2). Grinding and pelleting of the diet depressed the digestibility of crude fibre and increased that of the nitrogen-free extract and addition of ammonium salts increased the digestibility of crude fibre. The effects of the physical form of the diet on the composition of the short-chain fatty acids of rumen liquor were not consistent and the addition of ammonium salts produced changes over and above those attributable to the small amounts of acids in the mixture. The extreme values observed for the molar proportion of propionic acid were 12·3 and 38·1 % and the corresponding values for *n*-butyric acid were 28·1 and 9·0 %.

(3). Variations in the flow of nitrogenous materials to the duodenum were related more to the pattern of fermentation established in the rumen than to the experimental treatments. There was a highly significant relationship between the molar proportion of propionic acid and the abomasal output of nitrogen and also the abomasal output of α-ϵ-diaminopimelic acid and α-linked glucose polymers. The amino acid composition of duodenal digesta differed from that of the diet—in particular the proportion of glutamic acid was decreased and the proportions of aspartic acid, alanine, lysine and histidine were increased—but differences in composition between treatments and between animals were small.

(4). The faecal output of nitrogen differed little between animals and between treatments, but nitrogen retention was significantly increased during the intraruminal infusion of ammonium salts.

Alteration of the physical form of a diet, by grinding and pelleting, is known to influence the digestion of food materials in the rumen, and effects can be especially marked with diets containing a high proportion of concentrated foods. There are reports of effects on the composition of short-chain fatty acids in rumen liquor and the rate of passage of food particles (for references, see Moore, 1964; Shaw, 1961),

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the concentration of NH_3-N in rumen liquor (Alwash & Thomas, 1970), and the site of digestion in the gut of dietary constituents (for references, see Ørskov, 1969). The experiment reported here was undertaken to study the effect of the physical form of a barley grain and barley straw diet on the metabolism within the rumen of food N and of added non-protein N in sheep.

EXPERIMENTAL

Four wether sheep about 1 year old and fitted with permanent ruminal and duodenal re-entrant cannulas were used. The daily diet consisted of 700 g of barley grain and 300 g of barley straw, together with a complete mineral and vitamin supplement and 1 g of chromic oxide (Cr_2O_3) powder. The chromic oxide was premixed with the mineral and vitamin supplement. In the first 42-day period 2 sheep received the diet as rolled barley and chopped straw (about 1 in.), referred to henceforward as the 'chopped diet', and 2 received the diet in a finely ground, pelleted form. In the second 42-day period the diets were given in the reverse order. The composition of the diets is given in Table 1.

Table 1. *The dry-matter content and proximate composition of the foods and diets*

Food or diet	Dry matter, %	Proximate composition (% of dry matter)					
		Organic matter	Crude protein, N × 6·25	Ether extract-ives	Crude fibre	Nitrogen-free extract	Ash
Barley straw	88·3	94·3	2·9	1·3	43·2	46·0	5·7
Barley grain	86·5	97·4	11·3	2·3	5·6	78·5	2·6
Chopped straw + rolled barley diet*	87·0	96·6	8·6	1·9	16·2	69·9	3·4
Ground and pelleted diet	88·9	96·4	8·6	2·0	15·6	70·3	3·5

* Calculated from the values for the individual foods and corrected for the addition of minerals and vitamins.

Animals were maintained under constant lighting conditions and the daily diet was offered in 24 equal amounts at hourly intervals by means of an automatic feeder (Minson & Cowper, 1966). There was no access to drinking water but throughout the first 21 days of each 42-day period 2 l of water was given daily as a continuous intraruminal infusion, and in the second 21 days 2 l of a 4·2% (v/v) solution of a concentrated solution of ammonium salts was substituted for the water. This supplied 7 g N/day. The composition (%) of the concentrated solution of ammonium salts was N, 8·6; formic acid, 7·5; acetic acid, 24·9; propionic acid, 3·5.

Collection of samples

One member of each pair of sheep was used for experimental purposes, the other was used as a source of duodenal contents to replace those removed from the experimental animal during the sampling and measurement of flow.

In each 21-day subtreatment period balance studies were made over days 10–18. Quantitative collections were made of faeces, urine and food refusals and representative samples of foods, refusals, faeces, urine and infused materials were taken for analysis. Samples of duodenal digesta and measurements of the flow of digesta to the duodenum were taken on days 19–21 over 8-h periods from 10.00 to 18.00 h. The procedure was that described by Nicholson & Sutton (1969), except that 10 %, and not 20–30 %, of the contents was retained as a sample for analysis. Samples of rumen digesta were taken at $1\frac{1}{2}$-h intervals from 10.00 to 19.00 h on day 21. Weighted composite samples were prepared from the samples of duodenal contents collected during the 3 sampling periods and these, together with samples of rumen digesta, were stored at −20 °C for analysis.

Analysis of materials

Samples of the foods, refusals, and faeces were analysed for their proximate constituents and (faeces only) for chromic oxide (Christian & Coup, 1954), and samples of urine and the infused ammonium salts solution for N. The weighted composite samples of duodenal digesta were analysed for proximate constituents, chromic oxide (Christian & Coup, 1954), α-linked glucose polymers (MacRae & Armstrong, 1968), alcohol-soluble and insoluble N (Weller, Gray & Pilgrim, 1958) and, together with samples of rumen digesta, for total (Annison, 1954) and individual short-chain fatty acids (Storry & Millard, 1965). Free ammonia (McDermot & Adams, 1954) and pH were determined immediately on the fresh samples of duodenal and ruminal digesta.

Protein hydrolysates of the samples of foods and duodenal contents were prepared by the method described by Bidmead & Ley (1958), except that performic acid was removed in vacuo at low temperature. The free amino acids were determined with an automatic amino acid analyser of the type described by Spackman, Stein & Moore (1958) and manufactured by Evans Electroselenium Ltd, Halstead, Essex, using columns of Amberlite I.R. 120 resin.

RESULTS

The composition of rumen liquor

The effect of the physical form of the diet and intraruminal infusion of ammonium salts on the composition of rumen liquor is given in Table 2. Apart from the results for sheep 9 during the period when it received the chopped diet without addition of ammonium salts, pH was depressed by grinding and pelleting of the diet. There was no effect on rumen pH of addition of ammonium salts. The concentrations of NH_3-N and of total short-chain fatty acids were increased by grinding and pelleting, though not in all instances significantly, and to an even greater extent by the addition of ammonium salts. However, changes in the molar proportions of short-chain fatty acids of rumen liquor were much less consistent. The composition of acids for sheep 17 when it received the chopped diet was characterized by a very high proportion of propionic acid, whereas for sheep 9 with the same diet there was a much lower proportion of propionic acid and correspondingly higher proportions of acetic and *n*-butyric acids. Substitution of the ground and pelleted diet in both sheep caused a marked decrease in the proportion of propionic acid, and a compensating increase mainly in *n*-butyric acid. In sheep 9 this resulted in the exceptionally high proportion

Table 2. *Influence of the physical form of the diet and intraruminal infusion of 'ammonium salts' on the pH, concentration o ammonia nitrogen and the concentration and molar proportions of short-chain fatty acids in rumen liquor of sheep receivin diets of barley grain and barley straw*

					Short-chain fatty acids					
					Molar proportions, %					
Treatment	Sheep	pH	NH_3-N, mg/100 ml	Total concentration, mmoles/l	Acetic	Propionic	*n*-Butyric	Isobutyric	*n*-Valeric	Isovale
Chopped diet	9	5·9 ± 0·1	14·6 ± 0·9	84·5 ± 3·8	57·1	24·7	15·6	1·1	0·9	0·6
	17	6·3 ± 0·1	10·8 ± 0·7	96·7 ± 4·5	49·2	38·1	9·0	1·3	1·0	1·4
Chopped diet + ammonium salts	9	6·1 ± 0·1	57·7 ± 2·1	110·1 ± 1·8	62·5	19·0	15·5	1·0	0·9	1·2
	17	6·4 ± 0·1	42·1 ± 9·8	120·8 ± 1·9	63·6	17·3	17·1	0·7	0·9	0·4
Ground, pelleted diet	9	5·9 ± 0·1	28·1 ± 0·3	96·0 ± 2·6	58·1	12·3	28·1	0·7	0·7	0·3
	17	5·8 ± 0·1	14·4 ± 0·9	105·9 ± 1·9	55·2	23·5	18·6	1·1	1·1	1·1
Ground, pelleted diet + ammonium salts	9	5·9 ± 0·1	55·8 ± 2·8	119·5 ± 2·7	63·4	15·5	19·1	0·5	0·9	0·6
	17	5·8 ± 0·1	71·6 ± 12·5	154·4 ± 8·9	57·1	32·4	8·4	0·7	0·7	0·8

abomasal and faecal outputs of organic matter, crude fibre and nitrogen-free extract of sheep receiving diets of barley grain and barley straw

Treatment	Sheep	Organic matter			Crude fibre			Nitrogen-free extract		
		Intake, g/day	Output, g/day		Intake, g/day	Output, g/day		Intake, g/day	Output, g/day	
			Abomasal	Faecal		Abomasal	Faecal		Abomasal	Faecal
Chopped diet	9	838	391	244	147	94	83	596	193	130
	17	840	444	231	147	96	79	597	185	126
Chopped diet + ammonium salts	9	840	372	227	147	78	76	597	196	122
	17	803	431	235	130	97	68	579	193	123
Ground, pelleted diet	9	857	433	233	139	120	83	625	222	126
	17	858	463	249	139	87	86	626	218	127
Ground, pelleted diet + ammonium salts	9	857	454	235	139	168	82	625	186	127
	17	857	368	230	139	78	80	625	191	116

Table 4. *The influence of the physical form of the diet and intraruminal infusion of 'ammonium salts' on the intake, abomasal, faecal and urinary output, digestibility in the stomach and whole gut and retention of nitrogen of sheep receiving diets of barley grain*

Treatment	Sheep	Intake, g/day			Output, g/day			Digestibility, %		Nitrogen retention g/day
		Food	Ammonium salts	Total	Abomasal	Faecal	Urinary	Stomach	Overall	
Chopped diet	9	12·5	0	12·5	15·6	4·1	4·2	−24·8	67·2	4·2
	17	12·5	0	12·5	24·8	3·6	3·5	−98·4	71·2	5·4
Chopped diet + ammonium salts	9	12·5	7·3	19·8	15·0	4·1	9·8	24·2	67·2*	5·9
	17	12·3	7·2	19·5	15·9	3·7	7·3	18·5	69·9*	8·5
Ground, pelleted diet	9	12·2	0	12·2	12·8	3·4	5·7	− 4·9	72·1	3·1
	17	12·2	0	12·2	15·0	5·0	3·6	−23·0	59·0	3·6
Ground, pelleted diet + ammonium salts	9	12·2	7·6	19·8	14·7	3·8	7·7	25·8	68·9*	8·3
	17	12·2	7·5	19·7	20·4	4·8	6·7	− 3·6	60·7*	8·2

* Expressed as a percentage of the food nitrogen.

of *n*-butyric acid of 28 %. Addition of ammonium salts to the chopped diet produced a similar composition of acids in both sheep; acetic acid accounted, on a molar basis, for 63–64 % of the total acids and the molar proportions of propionic and *n*-butyric acids were roughly equal. However, the addition of the ammonium salts to the ground, pelleted diet was associated with small increases in acetic and propionic acids in sheep 9, but in sheep 17 there was a considerable increase in propionic acid.

The flow of materials to the small intestine

The flow of materials to the small intestine was calculated by reference to the flow of chromic oxide adjusted to give 100 % recovery of chromic oxide appearing in the faeces. The recovery in the faeces of the chromic oxide included in the diet averaged 70 % for the chopped diet and 88 % for the ground, pelleted diet. The low recovery with the chopped diet was due to partial separation of the chromic oxide from the main food materials during the delivery of the diet from the automatic feeder to the food box. The flow of chromic oxide to the duodenum ranged from 47 to 98 %, with an average of 77 %, of that appearing in the faeces.

Values for the flow to the duodenum of dietary constituents are given in Tables 3 and 4. There was no clear distinction between treatments for the abomasal output of organic matter, and there were irregular differences between sheep and treatments in the abomasal output of crude fibre. In sheep 9 when it received the ground, pelleted diet with the addition of ammonium salts, the measured abomasal output of crude fibre exceeded the dietary intake. However, the values for N-free extract showed much closer agreement between sheep and the digestibility in the stomach of this fraction was on average 2·7 percentage units lower for the ground, pelleted diet and 2·2 percentage units higher for the ground, pelleted diet with ammonium salts than the mean value (67·6 %) for the chopped diets.

Though the inclusion of ammonium salts increased the input of N by 60 %, there was no consistent effect either of physical form of the diet or of addition of ammonium salts on the abomasal output of N. Six of the 8 recorded values were within the range 12·8–15·9 g/day: the mean value of 15·0 g/day is an increase of 22 % over the intake of food N. However, for sheep 17 when it received the chopped diet and also the ground, pelleted diet with ammonium salts, the abomasal output of N was exceptionally high, with values of 24·8 and 20·4 g/day respectively. Alcohol fractionation of the nitrogenous compounds of the duodenal digesta gave the following values for sheep 9 and 17 respectively for the percentage of N in non-protein form: chopped diet, 38·5 and 36·9; chopped diet plus ammonium salts, 44·3 and 39·4; ground, pelleted diet, 42·0 and 43·7; ground, pelleted diet plus ammonium salts, 47·6 and 47·1. Values for the abomasal output of α-ϵ-diaminopimelic acid N and α-linked glucose polymers are given in Table 5. There was a close relationship between the abomasal output of α-amino-N and both α-ϵ-diaminopimelic acid N and α-linked glucose polymers, and for both constituents the 2 highest values were for sheep 17 on the 2 occasions when it showed an unusually high output of total N.

Table 5. *The influence of the physical form of the diet and intraruminal infusion of 'ammonium salts' on the abomasal output (g/day) of α-ε-diaminopimelic acid N and α-linked glucose polymers in the sheep*

Treatment	Sheep	α-ε-Diaminopimelic acid N	α-linked glucose polymers
Chopped diet	9	0·054	15·0
	17	0·073	27·4
Chopped diet +ammonium salts	9	0·045	16·5
	17	0·034	18·8
Ground, pelleted diet	9	0·027	7·5
	17	0·050	16·9
Ground, pelleted diet +ammonium salts	9	0·035	8·2
	17	0·056	22·1

The composition of duodenal digesta

Grinding and pelleting of the diet was associated with a small but significant ($P < 0·05$) depression in pH of duodenal contents of 0·2 percentage units (the mean value, with S.E., for the chopped diet was 2·7 ± 0·1) but addition of ammonium salts had no effect. Altering the physical form of the diet caused a reduction in the NH_3-N content of the digesta and addition of ammonium salts a slight increase. Mean values (mg/100 ml), with S.E., for the different treatments for sheep 9 and sheep 17 respectively were: chopped diet, 21·7 ± 1·5, 22·9 ± 0·2; chopped diet + ammonium salts, 24·7 ± 2·6, 24·7 ± 1·0; ground, pelleted diet, 13·6 ± 0·2, 10·7 ± 0·3; ground, pelleted diet + ammonium salts, 15·4 ± 0·8, 17·0 ± 0·9.

The amino acid composition of the diet, expressed as a percentage of the total measured α-amino nitrogen, was as follows: aspartic acid, 5·6; threonine, 5·0; serine, 6·9; glutamic acid, 20·6; proline, 4·1; glycine, 9·3; alanine, 7·6; valine, 7·3; cystine, 2·5; methionine, 3·9; isoleucine, 4·6; leucine, 8·5; tyrosine, 1·1; phenylalanine, 0·9; lysine, 5·2; histidine, 2·6; arginine, 4·4. The amino acid composition of the duodenal digesta differed characteristically from that of the diet. The mean changes, in percentage units, for acids which showed consistent effects were: glutamic acid, −8·9; proline, −2·0; methionine, −1·7; tyrosine, −1·0; α-ε-diaminopimelic acid, +0·6; isoleucine, +0·7; threonine, +1·2; histidine, +1·9; alanine, +2·6; lysine, +4·2; aspartic acid, +5·8. The physical form of the diet had only small effects. The ground, pelleted diet was associated with slightly higher values for the proportions of aspartic acid and glutamic acid and lower values for lysine and histidine than those obtained for the chopped diet, but the addition of ammonium salts tended to eliminate the differences between diets in the values for aspartic and glutamic acids.

Digestibility of dietary constituents and utilization of nitrogen

The effect of the physical form of the diet and intraruminal infusion of ammonium salts on the faecal output of organic matter, crude fibre and nitrogen-free extract is given in Table 3 and on faecal and urinary output and digestibility of nitrogen and on nitrogen retention in Table 4. Neither the physical form of the diet nor the addition of ammonium salts affected the overall digestibility of dietary organic matter, which averaged 72·1%, but the digestibility of crude fibre was reduced from 44·9 to 39·2% by grinding and pelleting and increased by 2·5–3·2 percentage units by the

addition of ammonium salts and to the greatest extent with the chopped diet. The digestibility of nitrogen-free extract was slightly higher in the ground and pelleted diet (mean value 79·8%) than in the chopped diet (mean value 78·5%) but the addition of ammonium salts had no effect.

Treatments had no clear-cut effect on overall digestibility of N, but for sheep 17 the digestibility of N was decreased by grinding and pelleting. The addition of ammonium salts had no effect on faecal output of N but significantly increased urinary N output and was associated with an increased N retention.

DISCUSSION

The present investigation was undertaken to assess the effects of the pattern of microbial fermentation on the utilization of N in the rumen.

The choice of a barley grain, barley straw (7:3) diet was made on the assumption that by changing its physical form marked changes in the pattern of digestion could be achieved without alteration of the chemical composition of the diet. Grinding and pelleting of the diet produced the expected effects of a decrease in the digestibility of crude fibre and a slight increase in that of the nitrogen-free extract. However, the

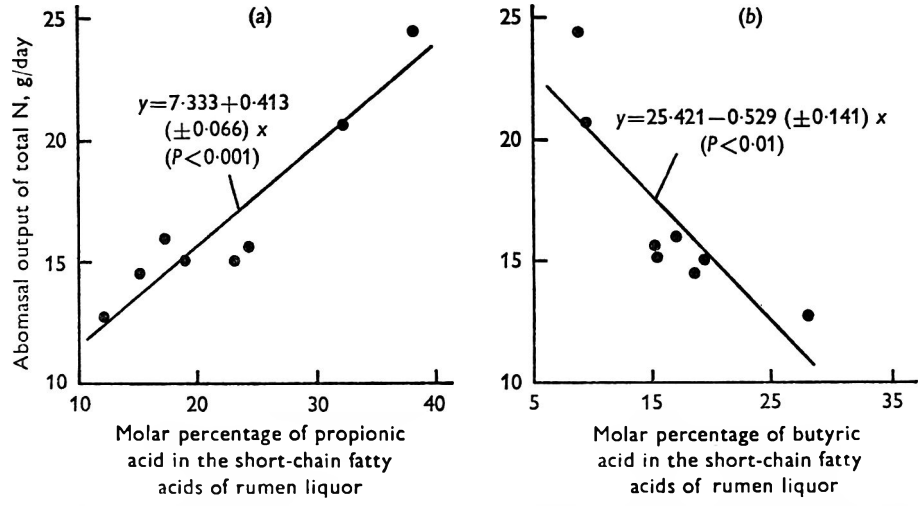

Fig. 1. The relationship between the abomasal output of total N and (*a*) the molar proportion of propionic acid and (*b*) the molar proportion of butyric acid in the short-chain fatty acids of rumen liquor in sheep receiving barley grain, barley straw diets.

effects of the physical form of the diet on the mixture of acids in rumen liquor were not consistent and the addition of ammonium salts produced changes over and above those directly attributable to the small amounts of acids included in the mixture. The influence of fermentation pattern on utilization of N in the rumen could not therefore be assessed in the direct way initially intended, but striking differences in the utilization of total dietary N were nevertheless apparent.

Grinding and pelleting of the diet in both sheep caused some reduction in the abomasal output of total N but these differences and those observed in response to

infusion of ammonium salts appeared to be related more to the pattern of fermentation established in the rumen than to the treatments themselves. There was a close direct relationship with the molar proportion of propionic acid and an inverse relationship with the molar proportion of butyric acid (Fig. 1). The relationship with the molar proportion of acetic acid was not significant ($P > 0{\cdot}05$). Thus, though the basal diet was comparatively low in N, the transfer of N to the duodenum was not directly influenced by the supply of additional non-protein N.

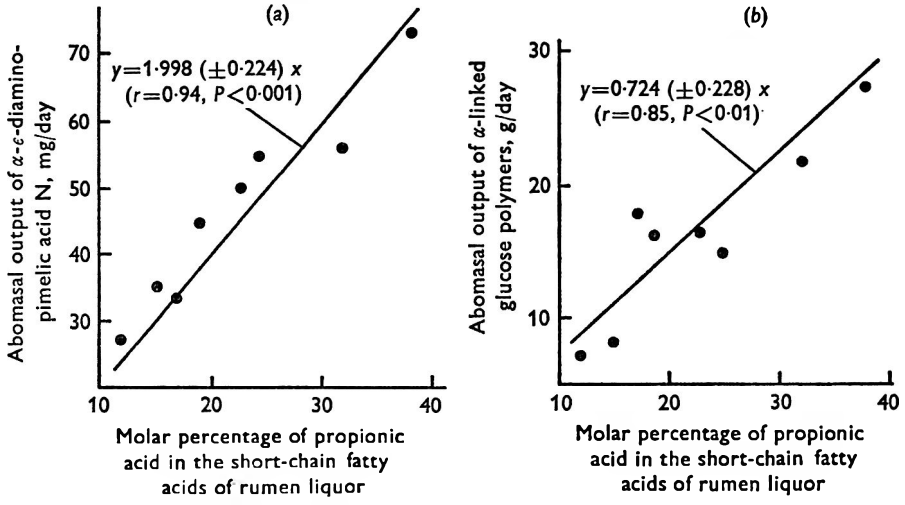

Fig. 2. The relationship between the abomasal output of total N and the abomasal output of (*a*) α-ε-diaminopimelic acid N and (*b*) α-linked glucose polymers in sheep receiving barley grain, barley straw diets.

The work of Eadie, Hyldgaard-Jensen, Mann, Reid & Whitelaw (1970) with hay, barley diets has demonstrated that a high proportion of butyric acid in the rumen liquor is associated with a large ciliate population, whereas a high proportion of propionic acid is associated with a reduction in ciliate numbers and an increase in bacteroides-type rods and in certain curved Gram-negative rods. Consistent with those observations, the abomasal output of α-ε-diaminopimelic acid (an amino acid peculiar to bacteria – see Smith, 1969) was closely related to the molar proportion of propionic acid, as was the passage to the small intestine of α-linked glucose polymers (Fig. 2), which may include both dietary starch and microbial polysaccharides. In vitro the extent of microbial growth and of protein synthesis/mole of ATP available from energy sources has been shown to vary with the microbial species, and Hobson & Summers (1967) have calculated that species such as *Bacteroides amylophilus* and *Selenomonas ruminantium* which are important organisms for the production of propionic acid in the rumen may produce up to twice the amount of bacterial protein/unit of energy consumed as do other species.

The material entering the duodenum showed the expected changes in amino acid composition as compared with that of the diet, namely a decrease in the proportion of glutamic acid and an increase in the proportions of aspartic acid, alanine, lysine and histidine, but neither experimental treatments nor fermentation pattern had

any marked effect. This relative constancy in the amino acid composition of the duodenal digesta is in line with the conclusions of Purser (1970) that the nature of the microbial species has little influence on the composition of microbial protein.

Though, from the limited information presented, it is not possible to judge the significance of these observations in terms of the nutrition of the host animal, an important consequence may be that the nature of the microbial species present in the rumen may influence not only the composition of the fermentation waste products, as is well known, but also the relative importance of the rumen and the intestine as sites for the uptake of nutrients.

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The effects of 2 methods of incorporating soybean oil into the diet on milk yield and composition in the cow

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Summary. In a feeding experiment with 6 cows in mid-lactation a portion of the starch in the concentrate part of the diet was isocalorically replaced by 8% soybean oil, and the effects on the yield and composition of the milk were studied. The oil was incorporated into the diet either as coarsely ground soybeans or by direct addition of the oil itself. The concentrate mixtures were given with a high-roughage diet that supplied 5·5 kg hay and 2·7 kg sugar-beet pulp/day.

When the soybean oil was included in the diet either in the form of soybeans or as the oil itself there was an increase in the yields of milk, solids-not-fat (SNF) and lactose, and an increase also in the percentage of lactose in the milk. The percentage of protein, however, was decreased.

When the oil was included in the form of soybeans there was an increase in the yield of fat but a decrease in the percentage of SNF in the milk.

When soybean oil was included in the diet, the yield of protein was increased but the yield of fat and the percentage of fat in the milk were both decreased.

With both methods of incorporating the oil there was an increase in the relative proportion of propionic acid and a decrease in that of butyric acid in the total volatile fatty acids of the rumen liquor. When the oil was added directly, but not when it was added as soybeans, there was a decrease in the proportion of acetic acid in the total volatile fatty acids. The implications of these findings are discussed.

It is known that the method by which unsaturated fats are incorporated into the diet of lactating cows can affect the yield and composition of the milk fat. For example, Moore, Hoffman & Berry (1945) showed that if 5–8 oz of cod-liver oil were given to lactating cows each day as a single dose the milk fat content was reduced. However, when the daily dose of cod-liver oil was divided into 12 small portions and these were given at different times throughout the day the milk-fat content was unaltered. Moore *et al.* (1945) also observed that when cod-liver oil was given as a single daily dose, the iodine value of the milk fat was greater than when the cod-liver oil was given in 12 smaller doses. In an earlier experiment Williams, Cannon & Espe (1939) showed that the method of incorporating soybean oil into the ration of lactating cows could affect the milk fat percentage as well as the fatty-acid composition of the milk fat. When the soybean oil was given as cracked soybeans to the cows the yield and content of fat in the milk were greater than when an equivalent amount of soybean oil was first extracted from the beans and then poured over the ration.

Williams *et al.* (1939) also reported that when the soybean oil was added directly to the diet of the cows, the Reichert Meissl and Polenske values and the linoleic acid content of the milk fat were lower but the oleic acid content of the milk fat was higher than when the cows were given cracked soybeans.

The experiments of Moore *et al.* (1945) and Williams *et al.* (1939) were conducted before it was known that unsaturated oils could alter the pattern of rumen fermentation and hence milk-fat synthesis, so an experiment was planned to investigate further the effects of the 2 methods of incorporating soybean oil into the diet of cows on the yield and composition of their milk, and to determine whether any differences that might arise could be related to changes in the pattern of rumen fermentation. The results of this experiment are now reported.

EXPERIMENTAL

Experimental animals

Six Ayrshire cows, which had calved on average 47 days before the beginning of the experiment, were used. During the experiment they were housed in a byre and yoked in stalls equipped for individual feeding. The cows were exercised in a concrete yard for 2 h each day. They were milked at intervals of 15 and 9 h. Their rations were divided into 3 portions each day and water was given *ad lib.*

Experimental diets

The cows were given 5·5 kg of a mature hay and 2·7 kg of sugar-beet pulp/day. The compositions (percentages of the dry matter) of the hay and sugar-beet pulp respectively were as follows: crude protein, 6·3, 12·0; crude fibre, 34·5, 16·2; ether extract, 1·6, 1·3; ash, 6·7, 5·9; nitrogen-free extractives, 50·9, 64·6. Three different concentrate mixtures were given to the cows and the mean daily intakes of each of the basal constituents are given in Table 1. The intakes of barley, molasses and minerals were the same for each concentrate mixture. The low-fat concentrate mixture contained 25% starch and in the 2 high-fat concentrate mixtures part of this starch was isocalorically replaced by soybean oil so that the oil constituted 8% by weight of the 2 high-fat concentrate mixtures. Coarsely ground soybeans were incorporated into one concentrate mixture and since the soybeans contained 16% oil they therefore constituted 50% by weight of this concentrate mixture. An equivalent amount of soybean oil was incorporated directly into the other concentrate mixture. Although this soybean oil was not extracted from the same batch of soybeans as that used in the other high-fat ration, it had a similar fatty-acid composition. The total fatty-acid contents (g/100 g dry matter) of the hay and sugar-beet pulp and of the low-fat and high-fat concentrate mixtures were 0·50, 0·49, 0·85 and 8·93% respectively.

The intakes of hay and sugar-beet pulp were kept constant throughout the experiment. The amount of concentrates given to each cow at the beginning of the experiment was calculated according to the average yield of milk produced in the 2 weeks before the experiment began. Thereafter, the amounts of concentrates were adjusted at the beginning of each period. This adjustment was made for each cow according to the average change in the yields of all the cows.

Table 1. *Mean daily intakes (kg) of each of the constituents of the concentrate mixtures*

	Low-fat concentrate mixture	Concentrate mixture containing:	
		Soybeans	Soybean oil
Barley	2·76	2·76	2·76
Starch	2·23	0·47	0·47
Molasses	0·36	0·36	0·36
Minerals	0·36	0·36	0·36
Extracted soybean meal	3·32	—	3·32
Soybeans	—	3·95	—
Soybean oil	—	—	0·63

Experimental design

The experimental design was two 3×3 Latin squares which were balanced for carry-over effects. The duration of each experimental period was 42 days and the change-overs between treatments were made abruptly.

Sampling and method of analysis

A 4-day composite sample of milk was taken on days 37–40 of each period. Fat, SNF, protein and lactose contents were determined by the methods described previously (Steele, 1969). On day 40 of each period samples of rumen liquor were taken from each cow by stomach tube 4 h after the morning feed (Steele & Moore, 1968). The proportions of the individual volatile fatty acids in the samples of rumen liquor were then determined according to the method of Cottyn & Boucque (1968).

Statistical treatment of results

The results were analysed statistically by methods outlined by Cochran & Cox (1957).

RESULTS

All the rations were consumed within 1 h of feeding.

Although the cows gained slightly in weight during the experiment, the different experimental treatments had no significant effects on the weights of the cows: the mean weights (kg) when they were given the low-fat concentrate mixture, the concentrate mixture containing soybeans and that containing soybean oil were respectively 465, 464 and 473; S.E. $\pm$ 5·1.

The mean daily yields of milk and the contents and yields of milk constituents produced in days 37–40 of each experimental period are given in Table 2. When soybean oil was included in the diet, either directly or as soybeans, the yields of milk, SNF and lactose and the content of lactose in the milk were increased but the protein content of the milk was decreased. The direct addition of soybean oil to the diet also increased the yield of protein in the milk whereas the inclusion of soybeans in the diet reduced the SNF content of the milk. When the cows were given the diet containing soybeans there was an increase in the yield of milk fat, in contrast to the reductions in the yield and content of milk fat observed when they were given soybean oil as a direct addition to the diet.

The concentrations of total steam-volatile fatty acids in the rumen liquor of the

cows and the relative proportions of the individual acids in that total on day 40 of of each experimental period are given in Table 3. The inclusion of the soybean oil in the diet either as soybeans or as a direct addition resulted in an increase in the

Table 2. *Mean daily yields and composition of the milk produced by the cows in days 37–40 of each period*

	Dietary treatments			
	Diet with low-fat concentrate mixture	Diet with concentrate mixture containing:		S.E.M. (±)
		Soybeans	Soybean oil	
Milk yield, kg	18·60	21·0**	23·0***	0·74
Milk-fat yield, g	704	785*	549***	30·5
SNF yield, kg	1·61	1·76*	1·96**	0·062
Lactose yield, g	878	1027***	1119***	35·3
Protein yield, g	615	620	679*	22·5
Milk-fat content, %	3·78	3·74	2·39***	0·124
SNF content, %	8·65	8·38**	8·52	0·103
Lactose content, %	4·72	4·89***	4·87*	0·038
Protein content, %	3·31	2·96**	2·96**	0·090

*, **, ***, Significantly different ($P < 0·05$, $P < 0·01$, $P < 0·001$ respectively) from the values obtained with the unsupplemented ration.

Table 3. *The composition (molar percentages) and the total concentrations (m-equiv./100 ml) of the total fatty acids in the rumen liquor of cows during day 40 of each treatment period*

	Dietary treatments			
	Diet with low-fat concentrate mixture	Diet with concentrate containing:		S.E.M. (±)
		Soybeans	Soybean oil	
Acetic	71·0	69·6	67·9**	0·70
Propionic	14·1	15·9*	17·8**	0·64
Iso-butyric	0·69	0·66	0·78	0·077
n-Butyric	13·0	12·3*	12·0**	0·23
Iso-valeric	0·57	0·56	0·64	0·072
n-Valeric	0·88	0·92	0·92	0·095
Total	8·67	8·62	8·09	0·847

*, **, Significantly different ($P < 0·05$, $P < 0·01$ respectively) from the values obtained with the unsupplemented ration.

relative proportion of propionic acid and a decrease in that of *n*-butyric acid. The direct addition of the oil to the diet also decreased the relative proportion of acetic acid in the mixture of steam-volatile fatty acids in the rumen liquor. None of the experimental treatments produced any significant change in the concentrations of total volatile fatty acids in the rumen liquor.

DISCUSSION

The results in Table 2 support those of Williams *et al.* (1939), who found that when cracked soybeans were incorporated in the diet of cows the fat content of the milk and the yield of milk fat were greater but the yield of milk was less than when the

cows were given a diet containing an equivalent amount of soybean oil. However, there were only 2 dietary treatments in the experiments of Williams *et al.* (1939) and no attempt was made to compare the effects of the diets containing cracked soybeans or soybean oil with the effects of a low-fat control diet. Our results show that the yield and percentage of fat in the milk of the cows given the diet containing soybean oil were markedly less than in the milk of the cows when they were given the low-fat diet. Compared with the results obtained with the low-fat diet, the addition of soybeans to the diet resulted in a small increase in the yield of milk fat but no change in the fat content of the milk. These different effects of dietary soybeans and soybean oil are at present difficult to explain. Storry, Hall, Tuckley & Millard (1969) have shown that when an emulsion of soybean oil was infused intravenously into cows, the percentage and yield of fat in the milk were increased. Moreover, the infusion of the soybean oil emulsion resulted in increased yields of linoleic and linolenic acids in the milk fat. Thus, it would appear that the mammary gland readily utilizes soybean oil fatty acids for milk-fat synthesis. It seems reasonable to suppose, therefore, that the effect of dietary soybean oil on milk fat secretion is an indirect one that might possibly involve some change in the pattern of rumen fermentation. It is well known that supplementation of the diet of cows with cod-liver oil reduces the milk-fat yield (e.g. Moore *et al.* 1945; Hilditch & Williams, 1964). Such changes have been shown to be associated with a pronounced decrease in the proportion of acetic acid and an increase in the proportion of propionic acid in the volatile fatty-acid fraction of the rumen contents (Shaw & Ensor, 1959; Nottle & Rook, 1963). When compared with the results obtained on the low-fat diet, the inclusion of soybean oil in the diet of the cows significantly decreased the proportion of acetic acid and significantly increased the proportion of propionic acid in the rumen volatile fatty acids (Table 3), but these changes in composition of the rumen volatile fatty acids were relatively small and could hardly be expected to account in themselves for a decrease in the yield of milk fat from 704 to 549 g/day. Although there were no significant differences in the concentration and composition of the volatile fatty acids when the cows were given the diets containing soybeans or soybean oil, the yield of milk fat on the dietary soybean treatment was somewhat greater than that when the cows were given the low-fat concentrate mixture, and much greater than that when they were given the diet containing soybean oil. The relatively small effect of dietary soybean oil on the relative proportions of the rumen volatile acids (Table 3) is in marked contrast to the very pronounced effect of dietary cod-liver oil. Shaw & Ensor (1959) reported that the addition of cod-liver oil to the diet of cows reduced the molar percentage of acetic acid in the total rumen volatile fatty acids from 71·1 to 56·1 and increased that of propionic acid from 14·1 to 28·2. Since the oil in the soybeans is located intracellularly, the rumen micro-organisms must digest the cell wall and other structural components of the bean tissues before the oil is liberated. This would result in a relatively slow release of the oil into the rumen fluid during the day. On the other hand, when the cows were given the diet containing the added soybean oil there would be a rapid release of the oil when the concentrate mixture entered the rumen and the initial concentration of oil in the rumen fluid would thus be relatively high. The precise mechanism by which these different situations in the rumen affect the yield of milk fat is a topic that requires further investigation.

The increases observed in the yield and content of lactose in the milk of the cows when they were given the diets containing soybeans or soybean oil (Table 2) were similar to the results of Steele (1969), who found that the addition of stearic acid or palmitic acid to the diet increased the yield of lactose in the milk. Blood glucose is recognized as being the precursor of milk lactose (Reiss & Barry, 1953) but it seems unlikely that the increase in the output of milk lactose could have resulted from an increase in the supply of blood glucose. The concentration of blood glucose was not determined in the present experiment, but it was found in the previous work (Steele, 1969) that the concentration of glucose in the plasma of cows given the low-fat control diet was about 53 mg/100 ml and that this concentration was unaltered when the diet was supplemented with stearic or palmitic acids. Moreover, Storry & Rook (1961) have shown that the synthesis of lactose in the mammary gland is not affected by variations in the plasma glucose concentrations within the range 20–80 mg/100 ml. According to Linzell (1968) the lactating mammary gland derives 30–50 % of its energy from the oxidation of glucose and 20–30 % from the oxidation of acetate. When the diet of cows is supplemented with various fats, oils or fatty acids, there is a reduction in the utilization of acetate for fatty acid synthesis in the mammary gland (Moore & Steele, 1968). Under these conditions, it is possible that a greater proportion of the blood acetate taken up by the mammary gland is oxidized to provide energy for the synthesis of milk constituents. This would mean that a greater proportion of the glucose taken up from the blood by the mammary gland could be utilized for the synthesis of lactose. Such an increase in the synthesis of lactose in the mammary gland might be the factor responsible for the increase in the yield of milk observed in the present work when cows were given the diets containing soybeans or soybean oil (Rook, Storry & Wheelock, 1965).

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The effects of dietary soybean oil on milk-fat composition in the cow

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Summary. The effects of the isocaloric replacement of starch in a low-fat concentrate mixture by 8% soybean oil on the yield and composition of milk fat were investigated in a feeding experiment with 6 cows in mid lactation. Two high-fat concentrate mixtures were given to the cows. In one the requisite amount of soybean oil was given by including 50% coarsely ground soybeans in the concentrate mixture. In the other an equal amount of soybean oil was added directly to the concentrate mixture. The concentrate mixtures were given with a high-roughage diet that supplied 5·5 kg hay and 2·7 kg of sugar-beet pulp/day.

When the 2 high-fat rations were given to the cows there were reductions in the percentages and yields of 10:0, 12:0, 14:0, 14:1, 16:0 and 16:1 fatty acids and increases in the concentrations and yields of 18:0, 18:1 and 18:2 fatty acids in the milk fat. The direct addition of the soybean oil to the diet also reduced the percentage and yield of 6:0 and 8:0 whilst the inclusion of the soybeans increased the percentage of 4:0 and the yields of 4:0 and 6:0 in the milk fat.

When both high-fat rations were given to the cows there were increases in the yields and concentrations of *cis*-9–18:1 in the milk fat, whilst inclusion of the soybean oil in the diet increased also the concentration and yield of *trans*-11–18:1.

The implications of these findings are discussed.

Williams, Cannon & Espe (1939) showed that the method of incorporating soybean oil into the diet of cows could affect the fatty-acid composition of the milk fat. They found that when the diet contained cracked soybeans the milk fat produced contained a higher content of linoleic acid and a lower content of oleic acid than when the diet contained an equivalent amount of soybean oil. However, the experiments of Williams *et al.* (1939) were somewhat limited in that the diets supplemented with soybeans or soybean oil were not compared with an unsupplemented control diet, and the concentrations of 18:1 and 18:2 in the milk fat were obtained indirectly from iodine and thiocyanogen values. In view of the importance of essential fatty acids (e.g. linoleic acid) in nutrition, it might be considered advantageous to increase the linoleic acid content of cow's milk. The work of Williams *et al.* (1939) certainly seemed to suggest that this might be possible, but the extent to which the linoleic acid content of milk fat might be increased will be limited by the extensive biohydrogenation of dietary unsaturated fatty acids that occurs in the rumen. There-

fore, the effects on the fatty-acid composition of the milk fat of supplementing the diet of the cow with soybeans or soybean oil were reinvestigated and the findings are now reported.

EXPERIMENTAL

Experimental animals

Six Ayrshire cows that had calved on average 47 days before the experiment began were used. The housing and management of the animals has already been described (Steele, Noble & Moore, 1971).

Experimental diets

Throughout the experiment the cows were given 5·5 kg of a mature hay and 2·7 kg of sugar-beet pulp/day. Three different concentrate mixtures were given. The low-fat control concentrate mixture was formulated so that when the cows were given the diet containing this concentrate mixture their daily intake of fatty acids was about 100 g. This low-fat concentrate mixture supplied 3·32 kg extracted soybean meal/day. The 2 high-fat diets were derived from the low-fat concentrate mixture by the isocaloric replacement of starch by 8 % soybean oil. In one of the high-fat concentrate mixtures (soybeans) the calculated amount of coarsely ground soybeans replaced the appropriate amount of starch and extracted soybean meal. In the other high-fat concentrate mixture (soybean oil) soybean oil replaced the appropriate amount of starch.

Table 1. *Mean daily intakes (g) of each fatty acid on the various dietary treatments*

	Dietary treatments		
		Diet with concentrate mixture containing:	
Fatty acids	Diet with low-fat concentrate mixture	Soybeans	Soybean oil
16:0	24·9	75·4	71·1
17:0	6·4	3·7	4·3
18:0	7·6	28·3	26·8
18:1	26·1	136·8	157·4
18:2	28·1	329·1	323·1
18:3 plus other C18 unsaturated acids	6·1	56·8	61·4
Total	99·2	630	644

The total intakes of each fatty acid are given in Table 1 and further details of the experimental diets have already been given by Steele *et al.* (1971).

Experimental design

The experimental design was two 3×3 Latin squares which were balanced for carry-over effects. The duration of each experimental period was 42 days and change-overs between treatments were made abruptly.

Sampling and methods of analysis

One 4-day composite sample of milk was obtained from each cow on days 37–40 of each period. The concentrations of total fat were determined as described previously (Steele & Moore, 1968). A sample of fat that had been extracted from the milk with chloroform:methanol (2:1, v/v) was hydrolysed by the method of Hutton & Seeley (1966). The proportions of the various fatty acids were then determined by gas–liquid chromatography with an instrument fitted with dual-flame ionization detectors and a temperature programmer (Pye-Unicam Ltd, Cambridge, England). The separations were carried out in glass columns (0·64 × 214 cm) packed with a stationary

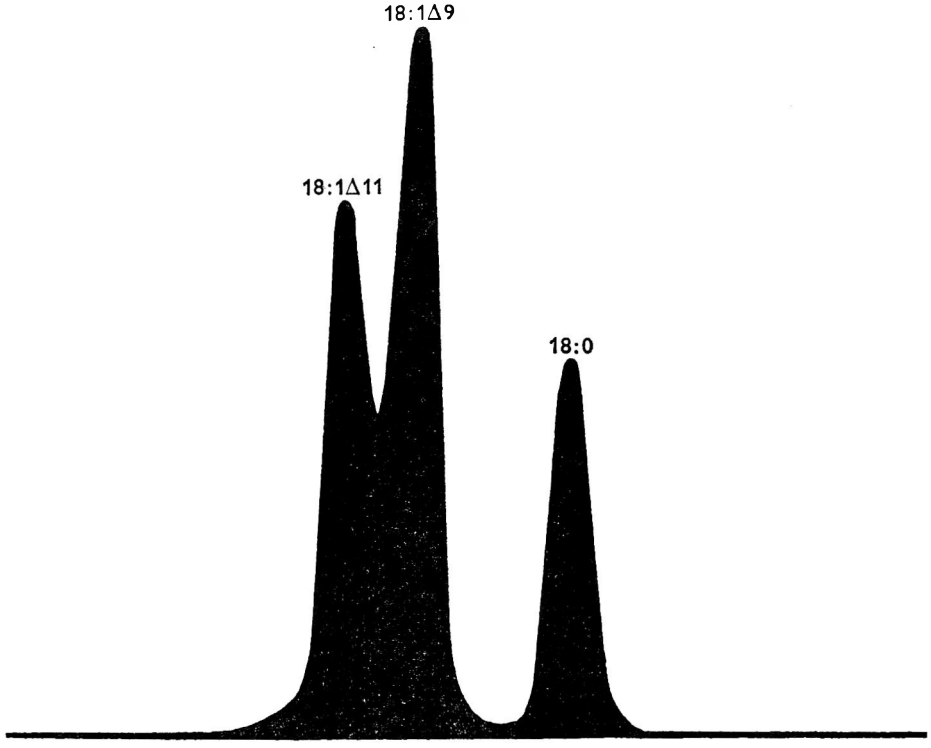

Fig. 1. Chromatogram showing the separation of 18:0, Δ *cis*-9–18:1 and Δ *cis*-11–18:1 on a DEGS-SCOT column (0·05 × 1525 cm): carrier gas, N_2; flow rate, 1·25 cm^3/min; temperature, 160 °C; detector, single-flame ionization (Perkin-Elmer Ltd, Beaconsfield, England).

phase of Carbowax 20 M terephthalic acid on a support of Chromosorb G (Perkin-Elmer Ltd, Beaconsfield, England). The 18:1 fatty acids of the milk fat were separated into total *cis*- and total *trans*- isomers by thin-layer chromatography on silica gel impregnated with silver nitrate (Morris, 1966). The proportions of the positional isomers in each of these fractions were determined on a gas–liquid chromatograph fitted with a single flame ionization detector and a support-coated open capillary column (0·05 × 1525 cm) containing a stationary phase of diethylene-glycol succinate (Perkin-Elmer Ltd, Beaconsfield England). The retention volumes of these isomers were identified by the use of authentic acids obtained from the Hormel Institute

(Austin, Minnesota, U.S.A.). Fig. 1 shows the gas-chromatographic separation of the Δ *cis*-9- and Δ *cis*-11-octadecenoic acids. The positional distribution of the double bonds was further checked by oxidation of the methyl ester fractions (Chang & Sweeley, 1962) and analysis of the resultant mono- and dicarboxylic acids by gas chromatography as described by Moore & Williams (1966).

Statistical treatment of results

The results were analysed statistically by the methods outlined by Cochran & Cox (1957).

RESULTS

The mean weight percentages and yields of the major fatty acids produced during days 37–40 of each period are given in Tables 2 and 3 respectively. When the soybeans were included in the diet there were decreases in the percentage of 12:0 and in the yields and percentages of the 10:0, 14:0, 14:1, 16:0 and 16:1 of the milk fat and

Table 2. *Mean weight percentages of the major fatty acids in the milk fat produced during days 37–40 of each period*

	Dietary treatments			
		Diet with concentrate mixture containing:		
Fatty acids	Diet with low-fat concentrate mixture	Soybeans	Soybean oil	S.E.M. (±)
4:0	3·00	3·69**	3·24	0·191
6:0	2·27	2·49	1·70*	0·200
8:0	2·13	1·53	1·13*	0·304
10:0	7·41	3·48*	2·70*	1·442
12:0	6·02	4·52*	3·00*	0·622
14:0	15·1	12·1**	9·74***	0·667
14:1	3·11	1·62**	2·08*	0·315
16:0	38·7	27·2**	24·0**	2·93
16:1	4·21	2·84*	3·09*	0·441
18:0	2·92	10·6***	6·79***	0·636
18:1	11·9	25·8**	37·8***	2·64
18:2	1·04	2·82***	1·94**	0·226
18:3 plus other C18 unsaturated acids	0·02	0·16	0·93*	0·254

*, **, *** Significantly different ($P < 0·05$, $P < 0·01$, $P < 0·001$ respectively) from the values obtained with the control ration.

increases in the yield of 6:0 and in the yields and percentages of 4:0, 18:0, 18:1 and 18:2. When the soybean oil was given as a direct addition to the diet of the cows there were reductions in the yields and concentrations of the 6:0, 8:0, 10:0, 12:0, 14:0, 14:1, 16:0 and 16:1 and increases in the yields and concentrations of 18:0, 18:1 and 18:3 and in the concentration of 18:2 in the milk fat.

The mean weight percentages and yields of the isomers of 18:1 in the milk fat produced during days 37–40 of each period are given in Table 4. Only the direct addition of the soybean oil to the diet increased the yield and concentration of the *trans*-11–18:1 in the milk fat. When either of the 2 high-fat diets was given to the cows, there were increases in the percentages and yields of the *cis*-9- and the *trans*-isomers (other than *trans*-11) of 18:1 in the milk fat.

Table 3. *Mean yields (g/day) of total and individual fatty acids in the milk fat produced during days 37–40 of each period*

	Dietary treatments			
		Diet with concentrate mixture containing:		
Fatty acids	Diet with low-fat concentrate mixture	Soybeans	Soybean oil	S.E.M. (±)
4:0	20·3	27·6**	17·4	1·66
6:0	15·2	18·6**	9·2***	0·835
8:0	14·0	11·4	5·8**	1·908
10:0	47·7	26·4*	13·2**	8·62
12:0	39·8	34·2	15·0***	3·91
14:0	101·1	90·0*	49·8***	3·61
14:1	20·7	12·0**	10·2**	2·02
16:0	260·9	203·1*	121·1***	19·78
16:1	28·5	21·1*	15·3**	2·99
18:0	19·7	78·3***	37·9*	5·54
18:1	78·3	192·4***	202·5**	28·37
18:2	7·48	19·8***	10·4	1·45
18:3 plus other C18 unsaturated acids	0·20	1·14	4·96**	1·200
Total	669	746*	521***	29·0

*, **, *** Significantly different ($P < 0·05$, $P < 0·01$, $P < 0·001$ respectively) from the values obtained with the control ration.

Table 4. *Mean weight and mean yields of the isomers of octadecenoic (18:1) acid in the milk fat during days 37–40 of each period*

	Dietary treatments			
	Diet with low-fat concentrate mixture	Diet with concentrate mixture containing:		S.E.M. (±)
Fatty acids		Soybeans	Soybean oil	
		Mean weight, %		
18:1 *cis*-9	10·4	24·0***	31·9***	2·37
18:1 *trans*-11	1·31	1·30	5·26***	0·585
Other 18:1 *trans*-isomers	0·10	0·50**	0·60**	0·101
		Mean yields, g/day		
18:1 *cis*-9	69·8	178·7**	171·1**	24·78
18:1 *trans*-11	7·83	10·1	28·3**	4·803
Other 18:1 *trans*-isomers	0·68	3·58**	3·12**	0·592

, * Significantly different ($P < 0·01$, $P < 0·001$ respectively) from the values obtained with the control rations.

DISCUSSION

There seems little doubt that when the 2 high-fat diets were given to the cows the resulting increases in the yields and concentrations of 18:0 and 18:1 in the milk fat were the direct result of an increased intake of each of these acids. It is also clear that the 18:0 and 18:1 in milk fat were not entirely of dietary origin. From Tables 1

and 3 it can be calculated that when the cows were given the control ration the daily intake of total C18 fatty acids was 67·9 g, whereas the daily output of 18:0 together with that of 18:1 in the milk fat amounted to 98·0 g. Since the different dietary treatments had no significant effect on the weights of the cows during this experiment (Steele *et al.* 1971) it seems unlikely that the additional 30·1 g of 18:0 + 18:1 in the milk fat of the cows given the low-fat diet was derived from the breakdown of adipose tissues. Annison, Linzell, Fazakerley & Nichols (1967) and Kinsella & McCarthy (1968) have shown that the C18 fatty acids of milk fat are not synthesized by the chain elongation of shorter chain fatty acids in the mammary gland. Therefore, it would appear that some of the 18:0 and 18:1 in milk fat is derived from *de novo* synthesis in tissues other than the mammary gland. It is also possible that a proportion of the 18:0 and 18:1 in milk fat is synthesized from acetate by the micro-organisms in the rumen.

Although the intakes of each of the C18 fatty acids were similar when the cows were given either of the 2 high-fat diets, the diet containing soybeans resulted in higher concentrations and yields of 18:0 and 18:2 and lower concentrations of 18:1 in the milk fat than did the diet containing soybean oil. It seems reasonable to suppose that the unsaturated acids in these 2 diets underwent differing degrees of hydrogenation by the bacteria of the rumen. Consequently, the amounts of each of the C18 fatty acids that were absorbed into the blood and taken up by the mammary gland would be different when the cows were given either of the 2 high-fat diets. It is known that the rate at which free linoleic acid is released into the rumen affects the extent to which it is hydrogenated by rumen micro-organisms. Moore, Noble, Steele & Czerkawski (1969) showed that when esterified linoleic acid was infused into the rumen of sheep more 18:0 and less 18:1 were formed than when free linoleic acid was given as an intraruminal infusion.

In the present experiment, when the cows were given the soybeans the rate of release into the rumen of the intracellular oil would be slower than when the cows were given the soybean oil as a direct additive to the diet. From the results of Moore *et al.* (1969) it might be expected that when the diet containing soybean oil was given, more 18:1 and less 18:0 would be produced from 18:2 in the rumen than when the diet containing the soybeans was given. This would therefore explain the difference in the output of these 2 acids in the milk fat when the 2 high-fat diets were given to the cows. Ward, Scott & Dawson (1964) have shown that *trans*-18:1 is an intermediate in the biohydrogenation of C18 polyunsaturated fatty acids in the rumen. From the results in Table 4 it can be calculated that *trans*-18:1 acids accounted for 9 and 23% respectively of the increases in the concentration of total 18:1 in the milk fat when the diets containing soybeans and soybean oil were given to the cows. This is consistent with the view that the biohydrogenation of linoleic acid in the rumen was much less complete when the diet of the cows contained soybean oil than when it contained soybeans.

The increases in the percentage and yield of 18:2 in the milk fat when the cows were given the diet containing soybeans (Tables 2, 3) are probably a reflexion of the delayed contact between the rumen bacteria and the intracellular oil. Before the hydrogenating micro-organisms could reach the intracellular oil of the soybeans the cell walls would have to be digested. Thus, it seems reasonable to suppose that before

all the cells of the soybeans had been attacked in the rumen, some would have passed farther along the digestive tract where the unmodified oil would be liberated and absorbed. Hence, more 18:2 would be absorbed when the diet containing soybeans was given to the cows and consequently more 18:2 would be secreted in the milk.

From the values given in an earlier publication (Steele *et al.* 1971) and from those given in Table 3 it can be calculated that when the cows were given the control diet and the diets containing soybeans and soybean oil the 18:2 in the milk fat constituted 0·5, 1·2 and 0·7% respectively of the total calories in the milk. According to Holman (1960) the requirement for dietary linoleate by the human infant is approximately 1% of the dietary calories. However, it must be remembered that of the various 18:2 isomers, only *cis*-9, *cis*-12–18:2 (linoleic acid) possesses essential fatty-acid activity and that *cis*-9, *cis*-12–18:2 accounts for only about 65% of the total 18:2 in cow's milk fat (Moore, 1966). Thus, even when the cows were given the diet containing soybeans, the milk would appear to be somewhat low in essential fatty acids.

The decrease in the synthesis of the shorter-chain fatty acids (C8–C16) in the milk fat (Table 3) that resulted from the increased intake of dietary fat is in agreement with earlier observations (Moore & Steele, 1968; Noble, Steele & Moore, 1969). It was shown previously (Steele *et al.* 1971) that the inclusion of soybeans or soybean oil in the diet of the cows had little effect on the concentrations of the various volatile fatty acids in the rumen content. Moore & Steele (1968) have suggested that an increased uptake of long-chain fatty acids from the blood leads to increased concentrations of long-chain fatty acids and their CoA derivatives in the mammary gland. Increased concentrations of fatty acids or their CoA derivatives lead to inhibitions of acetyl CoA carboxylase (Howanitz & Levy, 1965; Smith & Dils, 1966; Hibbit, 1966) which catalyses the rate-limiting step in fatty-acid synthesis in the bovine mammary gland (Ganguly, 1960). Table 3 shows also that when soybean oil was given to the cows the yield of 4:0 in the milk fat was unaffected and when soybeans were included in the diet the yield of 4:0 and 6:0 were both increased. A possible explanation for the different effects of dietary long-chain fatty acids on the secretion in the milk fat of 4:0 and 6:0 on one hand and the fatty acids from 8:0 to 16:0 on the other may be found in the work of Becker & Kumar (1965). They have shown that in the goat mammary gland the synthesis of 4:0 and 6:0 is by a pathway that is independent of malonyl CoA and hence does not depend on acetyl CoA carboxylase.

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The relationship between plasma lipid composition and milk fat secretion in cows given diets containing soybean oil

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Summary. The effects on the composition of the plasma lipids of the isocaloric replacement of starch in a low-fat concentrate mixture by 8% soybean oil were investigated in a feeding experiment with 6 cows in mid-lactation. Two high-fat concentrate mixtures were given: in one, the soybean oil was incorporated into the diet by the inclusion of the appropriate amount of coarsely ground soybeans; in the other, the soybean oil was incorporated directly in the concentrate mixture. The concentrate mixtures were given with a high-roughage diet that supplied daily 5·5 kg hay and 2·7 kg of sugar-beet pulp.

When the cows were given the 2 high-fat diets there were increases in the concentrations of total plasma fatty acids and in the concentrations of fatty acids circulating in the plasma as phospholipids and cholesteryl esters. There were increases in the concentrations of plasma unesterified fatty acids and triglyceride fatty acids when the cows were given the diet containing soybeans but there were decreases in the concentrations of these 2 fractions when the cows were given the diet containing soybean oil.

The inclusion of soybeans in the diet increased the concentration of 18:0 in the plasma triglycerides and unesterified fatty acids whilst the inclusion of soybean oil in the diet increased the concentrations of 18:0 and 18:1 but decreased the concentration of 18:2 in the plasma unesterified fatty acids.

When the cows were given the 2 high-fat diets there were decreases in the concentrations of 16:0 and 16:1 and increases in the concentration of 18:2 in the plasma cholesteryl esters. The inclusion of soybeans in the diet also reduced the concentrations of 18:1 and 20:3 in the plasma cholesteryl esters but increased the concentrations of 18:0 and 18:2 in the plasma phospholipids.

The major fatty acid circulating in the plasma of the cows was 18:2 (46·4–55·3%) on all diets but only about 1% (0·40–1·04%) of the total plasma 18:2 occurred in the plasma triglycerides.

The results are discussed in relation to the changes in the composition of the milk fatty acids produced by the cows when they were given the experimental diets.

In a previous report (Steele, Noble & Moore, 1971*b*) an experiment was described in which a study was made of the effects of 2 methods of feeding soybean oil on the composition of the milk fat of cows. In this experiment blood samples were taken

from the cows on the various dietary treatments. The plasma lipids have now been analysed and the results of these analyses together with a discussion of the relationship between the compositions of the plasma and milk lipids are now reported.

EXPERIMENTAL

Full details of the cows, experimental diets and procedures have been given (Steele, Noble & Moore, 1971*a*). Briefly, 6 Ayrshire cows were given 5·5 kg of hay and 2·7 kg of sugar-beet pulp/day. Three different concentrate mixtures were given; one of these was of low-fat content, and when the diet containing this mixture was given to the cows their daily intake of total fatty acids was less than 100 g. The other 2 mixtures were constituted by isocalorically replacing part of the starch in the low-fat concentrate mixture by 8% by weight of soybean oil. In one mixture, the appropriate amount of oil was incorporated by adding coarsely ground soybeans and in the other by the direct addition of the soybean oil. The fatty-acid contents and compositions of the various dietary components are given in Table 1. The amounts of concentrate mixture given to each cow were adjusted for milk yield. The experimental design was two 3 × 3 Latin squares which were balanced for carry-over effects. The duration of each experimental period was 42 days. Blood samples were taken from the subcutaneous abdominal vein on day 40 of each period. The lipids were extracted from the samples of plasma by the method of Nelson & Freeman (1959) and the plasma lipids were analysed by the methods described by Moore, Noble & Steele (1968). The results were analysed statistically by the methods outlined by Cochran & Cox (1957).

Table 1. *Total fatty-acid contents and fatty-acid compositions of the fats in the various dietary constituents*

Fatty acid	Hay	Sugar-beet pulp	Low-fat concentrate mixture	Soybeans	Soybean oil
	Weight percentages of the total				
16:0	28·6	26·1	23·4	10·6	9·4
17:0	8·8	3·9	5·9	—	—
18:0	6·9	15·4	6·6	4·1	3·7
18:1	9·6	14·1	34·2	21·9	24·7
18:2	25·0	32·3	28·6	54·5	52·6
18:3	20·2	5·5	1·1	8·9	9·6
	Total fatty acid content, g/100 g dry matter				
	0·50	0·49	0·85	16·2	93·0

RESULTS

The concentrations of the total plasma fatty acids and the fatty acids contained in the various lipid fractions in the plasma of the cows given the different diets are presented in Table 2. The concentrations of the total fatty acids, the phospholipid fatty acids and the cholesteryl ester fatty acids in the plasma were increased when the cows were given either of the 2 high-fat diets. Although the concentrations of the triglyceride fatty acids and unesterified fatty acids in the plasma did not vary

significantly with dietary treatment, the concentrations of these 2 fractions tended to increase when the cows were given the diet containing soyabeans and tended to decrease when the cows were given the soybean oil as a direct addition to the diet. When the cows were given the 2 high-fat diets there were slight increases in the relative proportions of the cholesteryl ester and phospholipid fractions in the plasma lipids and relative decreases in those of the triglyceride and unesterified fatty acid fractions.

Table 2. *Mean concentrations (mg/100 ml) of total fatty acids and of the fatty acids present in the individual lipid fractions of the plasma of the cows on each dietary treatment*

	Diet with low-fat concentrate mixture	Diet with concentrate mixture containing:		S.E.M. (±)
		Soybeans	Soybean oil	
Total fatty acids	145	337***	338***	24·3
Triglyceride fatty acids	5·46	6·60	3·97	1·98
Unesterified fatty acids	5·73	6·28	5·27	2·17
Phospholipid fatty acids	85	198***	206***	17·3
Cholesteryl ester fatty acids	49	124***	122***	8·82

*** Significantly different ($P < 0·001$) from the value obtained with the low-fat concentrate mixture.

Table 3. *Mean weight percentages of the major fatty acids in the plasma triglycerides of the cows on each dietary treatment*

Fatty acid	Diet with low-fat concentrate mixture	Diet with concentrate mixture containing:		S.E.M. (±)
		Soybeans	Soybean oil	
16:0	65·2	48·2	55·5	9·50
16:1	2·24	1·73	1·96	0·538
18:0	16·3	31·0*	21·9	5·64
18:1	8·95	9·44	14·5	2·89
18:2	6·91	8·74	5·29	2·04

* Significantly different ($P < 0·05$) from the value obtained with the low-fat diet.

The fatty-acid compositions of the plasma triglycerides are given in Table 3. The only significant change in the composition of the triglycerides was an increase in the concentration of 18:0 when the cows were given the diet containing soybeans. Nevertheless, there was a tendency for the concentrations of 18:0 and 18:1 to increase and for the concentrations of 18:2 and 16:0 in the plasma triglycerides to decrease when the cows were given the soybean oil as a direct additive to their diet. There was also a tendency for the concentration of 18:2 to increase and for that of 16:0 to decrease in the plasma triglycerides when the cows were given the diet containing soybeans.

The compositions of the plasma unesterified fatty acids of the cows are given in Table 4. When the cows were given the high-fat diets there was an increase in the concentration of 18:0 and a decrease in that of 16:0 in the plasma unesterified fatty acids. The direct addition of the oil to the diet also produced an increase in the concentration of 18:1 but decreased that of 18:2 in the plasma unesterified fatty acids

The composition of the plasma cholesteryl esters are given in Table 5. When the cows received the high-fat diets there were decreases in the concentrations of 16:0 and 16:1 and an increase in the concentration of 18:2 in the plasma cholesteryl esters. The greater increase was observed when the cows were given the diet containing soybeans. The inclusion of the soybeans in the diet also caused the concentrations of the 18:1 and 20:3 in the plasma cholesteryl esters to be reduced.

Table 4. *Mean weight percentages of the major fatty acids in the plasma unesterified fatty acids of the cows on each dietary treatment*

Fatty acid	Diet with low-fat concentrate mixture	Diet with concentrate mixture containing: Soybeans	Diet with concentrate mixture containing: Soybean oil	S.E.M. (±)
16:0	39·7	27·2**	19·2***	3·43
16:1	2·48	1·41	2·80	1·42
18:0	22·7	35·5***	36·8***	1·74
18:1	15·3	17·0	27·7***	1·85
18:2	18·0	17·7	12·5*	1·99

*, **, *** Significantly different ($P < 0·05$, $P < 0·01$, $P < 0·001$ respectively) from the values obtained with the low-fat diet.

Table 5. *Mean weight percentages of the major fatty acids present in the plasma cholesteryl esters of the cows on each dietary treatment*

Fatty acid	Dietary treatments: Diet with low-fat concentrate mixture	Diet with concentrate mixture containing: Soybeans	Diet with concentrate mixture containing: Soybean oil	S.E.M. (±)
16:0	6·57	3·87***	3·90***	0·207
16:1	2·51	1·08***	1·47***	0·126
18:0	0·75	0·74	0·70	0·203
18:1	4·04	2·40**	3·49	0·308
18:2	75·4	84·0***	80·5***	0·861
18:3	6·87	5·39	7·96	0·971
20:3	0·83	0·25**	0·31	0·148
20:4	1·17	0·78	0·76	0·341

, * Significantly different ($P < 0·01$, $P < 0·001$ respectively) from the values obtained with the ow-fat diet.

The fatty-acid compositions of the plasma phospholipids are given in Table 6. In the plasma phospholipids the concentrations of 18:0 and 18:2 were increased and that of 20:3 decreased when the cows received the diet containing soybeans. Although a decrease in the concentration of the 20:3 was the only significant change that occurred in the plasma phospholipids when the soybean oil was given as a direct addition to the diet of the cows, there was a tendency for this dietary treatment to result in an increase in thc concentrations of 18:0, 18:1 and 18:2 in the plasma phospholipids.

The total concentrations of the individual fatty acids in the plasma of the cows are given in Table 7. When the 2 high-fat diets were given to the cows there were increases in the amounts of all the major fatty acids in the plasma. When the soybean diet was given to the cows the total concentrations of 16:0, 18:0 and 18:2 in the plasma tended to be higher and that of 18:1 tended to be lower than when the diet contained the soybean oil as a direct additive.

Table 6. *Mean weight percentages of the major fatty acids present in the plasma phospholipids of the cows on each dietary treatment*

	Dietary treatments			
	Diet with low-fat concentrate mixture	Diet with concentrate mixture containing:		
Fatty acid		Soybeans	Soybean oil	S.E.M. (±)
16:0	14·2	14·5	12·3	0·51
18:0	20·7	23·7**	21·3	0·79
18:1	12·4	10·0	13·9	1·66
18:2	34·1	40·7**	37·8	1·67
18:3	2·43	1·56	2·31	0·867
20:3	4·91	2·56***	3·09**	0·329
20:4	2·46	1·78	2·17	0·324

, * Significantly different ($P < 0·01$, $P < 0·001$ respectively) from the value obtained with the low-fat diet.

Table 7. *Mean total concentrations (mg/100 ml) of individual fatty acids in the plasma of the cows on each dietary treatment*

	Dietary treatments		
	Diet with low-fat concentrate mixture	Diet with concentrate mixture containing:	
Fatty acid		Soybeans	Soybean oil
16:0	21·1	38·4	33·3
16:1	1·49	1·54	2·02
18:0	20·2	52·1	47·5
18:1	13·9	24·5	34·9
18:2	67·3	186·4	176·9
18:3	5·43	9·77	14·5
20:3	4·58	5·38	6·74
20:4	2·66	4·49	5·40

From Table 7 it can be seen that 18:2 was the major fatty acid circulating in the plasma on each dietary treatment. This essential fatty acid accounted for 46·4, 55·3 and 52·3% of the total plasma fatty acids when the diets contained respectively the low-fat concentrate, soybeans and soybean oil as a direct addition. It should be noted that on these respective diets only 0·81, 1·04 and 0·40% of the total plasma 18:2 occurred in the triglycerides.

DISCUSSION

Comparison of the yields of total fatty acids in the milk of the cows on the various dietary treatments (Steele *et al.* 1971*b*) with the results in Table 2 showed that the yield of total milk fatty acids was not related to the concentration of total plasma

fatty acids or to the concentrations of fatty acids circulating in the plasma as phospholipids or cholesteryl esters. This comparison showed also that as the concentration of triglyceride fatty acids and unesterified fatty acids in the plasma increased, the yield of total milk fatty acids increased (cf. Moore, Steele & Noble, 1969). The direct relationship between total milk fatty acids and the concentration of plasma triglycerides supports the work of Barry, Bartley, Linzell & Robinson (1963) and Annison, Linzell, Fazakerley and Nichols (1967), who found that a portion of the milk fat of cows is derived from the plasma triglycerides that circulate as chylomicrons and low-density lipoproteins. The results of West, Annison & Linzell

Table 8. *Comparison of the ratios of certain fatty acids in the plasma triglycerides and milk fat of the cows on each dietary treatment*

	Dietary treatments		
	Diet with low-fat concentrate mixture	Diet with concentrate mixture containing:	
Ratio		Soybeans	Soybean oil
18:0/18:1			
Milk fat	0·25	0·41	0·18
Plasma triglycerides	1·82	3·28	1·50
16:0/16:1			
Milk fat	9·2	9·6	7·8
Plasma triglycerides	29·1	27·9	28·3
(18:0+18:1)/18:2			
Milk fat	14·3	12·9	23·1
Plasma triglycerides	3·65	4·63	6·89

(1967) and those of Bishop, Davies, Glascock & Welch (1969) suggest that in ruminants the plasma triglycerides are taken up by the mammary gland where they are substantially or completely hydrolysed. As discussed in detail by Moore, Steele & Noble (1969), the positive relationship between the concentration of unesterified fatty acids in the plasma and the yield of total milk fatty acids is probably a reflexion of the increased pool size of unesterified fatty acids in the mammary gland. The results of West *et al.* (1967) show that the plasma unesterified fatty acids are in equilibrium with the unesterified fatty acids in the mammary gland.

The values given in Table 8 are in agreement with those of Moore, Steele & Noble (1969) and are consistent with the views that the mammary gland contains a desaturase that actively converts 18:0 to 18:1 and that this desaturase has a relatively low specificity for 16:0 (Lauryssens *et al.* 1960; Bickerstaffe & Annison, 1968). It is also interesting to observe that, irrespective of dietary treatment or the amount of 18:0 taken up from the blood triglycerides by the mammary gland, the 18:0/18:1 ratio in the plasma triglycerides was, on average 7·9 times greater than the corresponding ratio in the milk fat (Table 8). At present, no explanation for this observation can be put forward. The ratio of (18:0+18:1)/18:2 in the blood triglycerides was much less than in those of the milk (Table 8). These findings add further weight to the argument (Moore, Steele & Noble, 1969) that there is a specific distribution of 18:2 amongst the triglycerides of the different plasma lipoproteins, and that the 18:2 content of the triglycerides in the chylomicrons and low-density

lipoprotein triglycerides that supply precursor fatty acids for the synthesis of milk fat in the mammary gland is less than the 18:2 content of the total plasma triglycerides.

The diet containing soybeans resulted in higher concentrations and yields of 18:0 and lower concentrations and yields of 18:1 in the milk fat than did the diet containing soybean oil (Steele *et al.* 1971*b*). These differences in milk-fat composition are reflected by the differences in the fatty acid composition of the plasma triglycerides of the cows given these 2 diets (Table 3). This supports the suggestion put forward by Steele *et al.* (1971*b*) that when the cows were given the diet containing soybeans the proportion of the dietary 18:2 that was completely hydrogenated to 18:0 in the rumen was greater than when the cows were given the diet containing soybean oil. Although the rate of hydrolysis of triglycerides by rumen micro-organisms is fairly rapid (Moore, Noble, Steele & Czerkawski, 1969) the rate of release of intracellular oil from the soybeans into the rumen will be relatively slow. Thus, when the cows were given the diet containing soybean oil, the initial concentration of free 18:2 in the rumen contents would be considerably greater than that in the rumen contents when the cows were given the diet containing soybeans. Experiments in vivo and in vitro (Moore, Noble, Steele & Czerkawski, 1969) have shown that when the initial substrate concentration is low, the main product of the hydrogenation of 18:2 by rumen micro-organisms is 18:0; when the initial substrate concentration is high, the main product of the hydrogenation of 18:2 is 18:1.

The results given in Table 7 show that when soybeans or soybean oil were included in the diet of the cows the total concentration of 18:2 in the plasma was increased by about 114 mg/100 ml. Virtually all of this increased amount of 18:2 circulating in the plasma was found in the cholesteryl ester and phospholipid fractions. This finding is in agreement with the results of Noble, Steele & Moore (1969) and Moore, Noble & Steele (1969), which showed that when sheep were given intraruminal or intra-abomasal infusions of maize oil, the absorbed 18:2 was selectively incorporated into the plasma phospholipids and cholesteryl esters and only to a very limited extent into the plasma unesterified fatty acids and triglycerides. Although it is generally accepted that the reason for the low concentrations of 18:2 in bovine milk fat is the extensive hydrogenation of dietary 18:2 that takes place in the rumen (reviewed by Storry, 1970), the results of the present investigation and those of earlier studies (Moore, Steele & Noble, 1969; Moore, Noble & Steele, 1969; Noble *et al.* 1969) emphasize that the 18:2 that does escape hydrogenation is selectively incorporated into those lipid fractions in the blood that cannot donate fatty acids to the mammary gland for milk fat synthesis.

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Changes in the composition of bovine milk fat during milking

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SUMMARY. Fat analyses were carried out on fore-milk and residual milk samples to elucidate the significance of the changes in fatty-acid composition of milk fat during milking previously reported by other workers. The levels of phospholipid in fore-milk samples per unit weight of fat were considerably higher than those of residual milk samples. The fatty acid composition of the triglyceride in fore-milk and residual milk samples was similar. These results suggest that the differences in fatty acid composition of milk fat collected during milking can be attributed to decreases in the relative amount of phospholipid-rich globule membrane associated with increasing globule diameter.

It has been clearly shown that there is an increase in the average diameter of fat globules during the course of milking of dairy cows (Jenness & Patton, 1959; Brunner, 1965; Kernohan & Lepherd, 1969). Since the volume of a sphere is proportional to the cube of its diameter and the surface is proportional to its square it is clear that for a given weight of emulsified fat the total surface area of the fat globules is inversely proportional to the average globule diameter (Sommer, 1951).

On the assumption that the globule is coated with a uniform layer of phospholipid and protein (Morton, 1954; King, 1955; Patton, Durdan & McCarthy, 1964; Hayashi & Smith, 1965) it would be expected that during the course of milking the relative content of phospholipid to total fat would decrease as the average diameter of the globules increased. This could possibly account for the changes in fatty-acid composition of milk fat during milking reported by some authors (Lemus, 1902; Mulder, 1945; Aristova, 1961). Observations are presented in the first part of this paper which throw some light on this point.

The theory suggested by Russian workers (Zaks, 1962) to explain the small changes in iodine number of milk fat collected during successive phases of milking, and following thermal stimulation and injection of oxytocin, implies that triglyceride of different fatty-acid composition is secreted by individual sections of the gland. For this reason the triglyceride fatty-acid composition of milk fat in fore-milk and residual milk samples was also examined.

METHODS

Fore-milk and residual milk samples were collected by means of a teat siphon inserted into one quarter of each of 5 average yielding cows of various breeds. The fore-milk sample (200 ml) was taken prior to washing the udder and the residual milk was collected after the end of normal milking following the intra-muscular injection of 30 i.u. Pitocin (Parke-Davis and Co. Ltd, Sydney).

The milk-fat content of the samples was then determined by the Babcock method (Davis, 1959). Lipid for phospholipid estimation was first extracted with ethanol–ether (3:1, v/v) and a second extraction was carried out on a portion of the first extract. The second extraction involved the addition of ether and water in a suitable ratio, shaking the mixture, removing the ether phase and evaporating it to dryness. Lipid phosphorus was determined on the dried extract by the procedure described by Zilversmit & Davis (1950).

Total lipid was extracted in chloroform–methanol (cf. Hartmann & Lascelles, 1964) and triglycerides separated from phospholipids by adsorption of the latter on silicic acid (Carroll, 1963). Butyl esters were prepared from the triglyceride solution by dialkyl carbonate-induced transesterification which is specific for glycerides (Wadsworth, 1968). The butyl esters were separated on DEGS (polyester of diethylene glycol succinate) columns which were temperature programmed from 70 to 180 °C at 4 degC/min. (see Wadsworth, 1968). Peak areas were measured by multiplying peak height by retention time (Bartlet & Iverson, 1966).

RESULTS

Changes in phospholipid and milk fat content during milking

The milk fat and phospholipid content of the milk samples from 5 cows are presented in Table 1.

Table 1. *Total fat and phospholipid content of fore-milk and residual milk samples from 5 cows*

(Values presented are means and standard errors of duplicate determinations.)

Cow	Sample	Total fat, g %	Phospholipids, mg %	Phospholipid/ total fat, mg/g
(1) Friesian	Fore-milk	0·9*	65·3 ± 17·5	72·5
	Residual	7·8	108·9 ± 3·5	13·9
(2) Jersey	Fore-milk	1·1	28·5 ± 5·3	25·9
	Residual	14·8	131·2 ± 0·8	8·8
(3) Australian Illawarra Shorthorn	Fore-milk	1·8	57·1 ± 16·0	31·7
	Residual	8·2	126·8 ± 8·9	15·5
(4) Friesian	Fore-milk	1·0	10·2 ± 1·1	10·2
	Residual	9·6	37·7 ± 0·0	3·9
(5) Ayrshire × Guernsey	Fore-milk	4·0	13·2 ± 1·1	3·3
	Residual	11·4	24·5 ± 1·9	2·1

* S.E. of all total fat samples ≦ 0·05 g %.

It may be seen that the levels of phospholipid per gram of fat in the fore-milk samples were 1·5–4 times greater than the comparable values for residual milk.

Changes in composition of triglycerides during milking

The results of the analysis of the percentage composition of the major fatty acids of triglycerides in samples from cows 1, 2 and 3 are presented in Table 2. In cow 1 no differences were observed in unsaturated fatty acid content of fore-milk and residual milk samples while in cows 2 and 3 there was a slight increase in the percentage of 18:1, 18:2 and 18:3 in residual samples. It is doubtful, however, whether these differences are meaningful.

Table 2. *Fatty-acid composition of triglyceride from fore-milk and residual milk samples*

(Results (weight % of total fatty-acid butyl esters) are expressed as means ± standard errors of duplicate samples.)

	Cow 1		Cow 2		Cow 3	
Fatty acids	Fore-milk	Residual	Fore-milk	Residual	Fore-milk	Residual
10:0	1·3 ± 0·07	1·6 ± 0·03	3·9 ± 0·01	3·7 ± 0·08	3·5 ± 0·21	4·2 ± 0·65
12:0	1·5 ± 0·00	1·7 ± 0·06	4·1 ± 0·12	3·9 ± 0·03	7·6 ± 2·32	4·7 ± 0·77
14:0	6·4 ± 0·06	6·7 ± 0·14	10·3 ± 0·11	9·8 ± 0·16	14·5 ± 0·25	13·2 ± 1·44
16:0	26·0 ± 0·04	25·7 ± 0·13	26·2 ± 0·21	26·2 ± 0·04	33·7 ± 0·65	33·4 ± 2·50
18:0	10·8 ± 0·12	10·6 ± 0·06	8·2 ± 0·13	8·2 ± 0·01	4·9 ± 0·19	5·2 ± 0·40
18:1	41·6 ± 0·67	41·0 ± 0·12	34·1 ± 0·49	36·4 ± 0·14	20·1 ± 0·58	23·1 ± 0·18
18:2	3·2 ± 0·01	3·3 ± 0·09	2·9 ± 0·01	3·4 ± 0·07	2·1 ± 0·02	2·5 ± 0·05
18:3	1·8 ± 0·22	2·0 ± 0·03	0·6 ± 0·38	1·4 ± 0·03	0·9 ± 0·04	1·8 ± 0·13
Remainder	7·4	7·4	9·7	7·0	12·7	11·9

DISCUSSION

The phospholipid content of whole milk is generally accepted as being 0·03 % (Mohr & Moos, 1932; Horrall, 1935; Holm, Wright & Deysher, 1936; Rhodes & Lea, 1958). The values reported here were of this order but ranged widely from 0·009 to 0·06 %. This wide range is understandable considering the differences in fat content of these samples.

The relatively high phospholipid levels in the fore-milk samples and the much lower levels in the residual milk samples are consistent with an increase in globule size during milking (Kernohan & Lepherd, 1969) with the consequent decrease in phospholipid-rich fat globule membrane per gram of fat. Considering the highly unsaturated nature of the fatty acids of phospholipids (Smith & Jack, 1959; Smith & Lowry, 1962), it is suggested that the relative decrease in phospholipid content described above could explain the lowered iodine values in residual milk reported by Aristova (1961) and probably those observed by others (Lemus, 1902; Mulder, 1945) for samples collected towards the end of normal milking.

The results obtained for the fatty-acid composition of the milk-fat triglycerides are in accord with the values for whole milk obtained by other authors (Herb, Magidman, Luddy & Riemenschneider, 1962; Jensen, Gander & Sampugna, 1962;

Gander, Jensen & Sampugna 1962; Patton & McCarthy, 1963; Jensen, Quinn, Carpenter & Sampugna, 1967), with high levels of 18:1 and 16:0 and lower levels of 18:0, 14:0, 18:2 and 18:3. The fatty-acid composition of fore-milk and residual milk are remarkably similar (Table 2) with only a suggestion of an increase in long-chain unsaturated fatty acids in residual milk. Thus, it is clear that the small decreases in iodine number reported by Russian (cf. Zaks, 1962) and other workers (Lemus, 1902; Mulder, 1945) during milking cannot be attributed to changes in the fatty-acid composition of triglycerides. On this basis the proposition that separate areas of mammary-gland parenchyma produce milk of different fatty-acid composition cannot be sustained.

It is suggested that the differences in fatty-acid composition of milk during milking reported previously could be attributed solely to changes in globule diameter.

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Purification of rennin

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Summary. A method based on salt fractionation, iso-electric precipitation and gel-filtration chromatography is described for the purification of the enzyme rennin (E.C. 3.4.4.3).

The enzyme rennin which is present in the stomach of the young calf is responsible for the hydrolysis of κ-casein in the coagulation of milk (Alais, Mocquot, Nitschmann & Zahler, 1953). There is, therefore, considerable interest in studying the mechanism of rennin action. Obviously for such work it is essential to have a pure preparation of the enzyme and in particular a preparation which is free from pepsin (E.C. 3.4.4.1) because in some respects pepsin has a similar specificity to that of rennin (Foltmann, 1966). The method of purification devised by Berridge (1955) is widely used but its success is dependent on the starting material used. We have been unable to obtain satisfactory results with this method and so we have developed an alternative one which is described here.

EXPERIMENTAL AND RESULTS

Step 1. Sodium chloride fractionation. Five litres of commercial rennet (Chr. Hansen, Reading, Berks) was saturated with sodium chloride at 20 °C to precipitate the proteins (Berridge, 1955). The mixture was filtered through a Whatman No. 3 paper. The precipitate and filter paper were shredded, suspended in 1 l of water, dialysed against tap-water at 4 °C until the water was free from chloride ions and then filtered. The filtrate was retained.

Step 2. Iso-electric precipitation. The iso-electric point of rennin is pH 4·5 (Foltmann, 1966) and that of pepsin is *c.* pH 1·0 (Young, 1963). To precipitate the rennin the dialysed solution was therefore adjusted to pH 4·5 with 0·1 M-HCl. A heavy cloudy precipitate was obtained which contained most of the clotting activity of the original solution. The mixture was centrifuged for 10 min at 2000*g* to obtain the precipitate, which was washed with 0·1 M acetate buffer of pH 4·5. The rennin-containing precipitate was suspended in 80 ml water and rendered soluble again by increasing the pH to 6·8 with 0·1 M-NaOH.

Step 3. Sodium chloride fractionation. Step 1 was repeated.

Step 4. Iso-electric precipitation. Step 2 was repeated. The filtrate obtained from the iso-electric precipitation was shown to be free from pepsin by the method of Rick (1963). As most of the pepsin would remain in solution at pH 4·5 it was concluded that the rennin precipitate was therefore free from pepsin. The enzyme assay could

not be performed on the precipitate because rennin is insoluble at the pH used in the assay.

Step. 5. Gel filtration. A 20 ml portion of the solution containing the rennin was subjected to gel-filtration chromatography on a column of Sephadex G-200 (Pharmacia, Uppsala, Sweden). A typical elution profile is shown in Fig. 1. The clotting activity was contained in the major peak eluted. The first peak eluted was in the void volume and may have contained a small proportion of polymerized rennin (de Koning, 1968), which would account for the clotting activity, in addition to impurities. There were also some impurities eluted after the main rennin fraction.

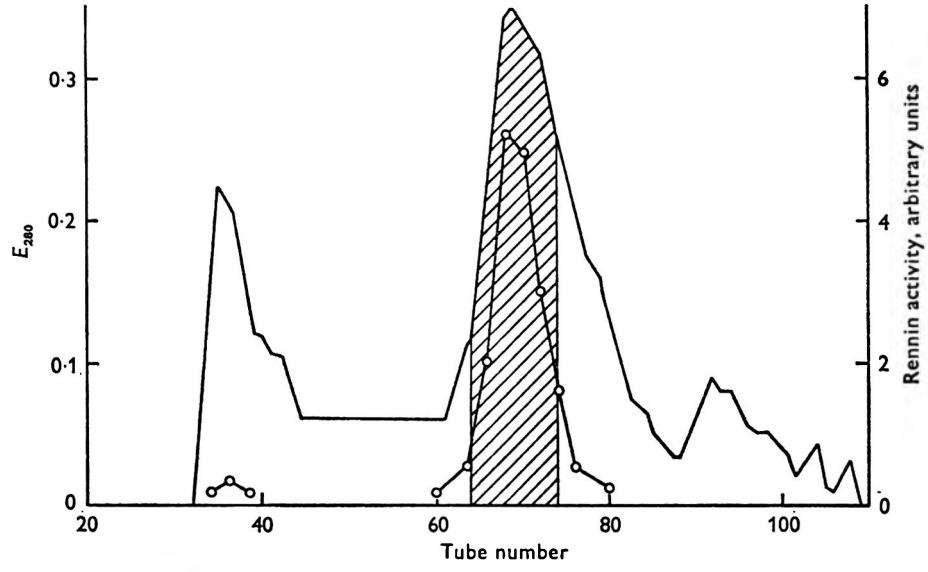

Fig. 1. Gel-filtration chromatography of the rennin-containing solution (step 5). The chromatography was done at 4 °C on Sephadex gel G-200 using a column 90 cm long by 6 cm diam., with a flow-rate of 50 ml/h. The buffer was 0·005 M-KH_2PO_4 adjusted with 0·1 M-NaOH to pH 5·6. ——, Absorbance at 280 nm; O—O, activity.

Step 6. Preparation of rennin crystals. The fractions indicated in Fig. 1 were combined and used in this step. Sodium chloride was added very slowly at 4 °C by using a dialysis bag filled with salt which was suspended and rotated in the rennin-containing solution until a white protein precipitate was obtained. The solution containing this precipitate was centrifuged at 2000*g* for 10 min and the precipitate dissolved in 10 ml water. Crystals were obtained from this solution by freezing and thawing.

Alternatively the rennin in the eluate was precipitated at 1·8 M-NaCl. The mixture was centrifuged at 2000*g* for 10 min and the supernatant discarded. Rennin crystals and a small amount of NaCl crystals were obtained by slowly drying the protein over P_2O_5 at 4 °C.

Specific activity. The rennin crystals had an activity of 80 units/mg protein, where 1 unit is the amount of protein required to clot 10 ml milk in 1 min at 30 °C. A commercial preparation of rennin (Sigma Chemical Co. Ltd, London) had a corresponding specific activity of 42 units/mg protein.

Ion-exchange chromatography of the rennin crystals. The purity of the preparation was checked by ion-exchange chromatography during which crystalline rennin resolves into 3 enzymically active components. The elution profile (Fig. 2) shows that there was a small amount of material eluted first, which was probably pro-rennin, and a major peak which contained all the clotting activity. There was some

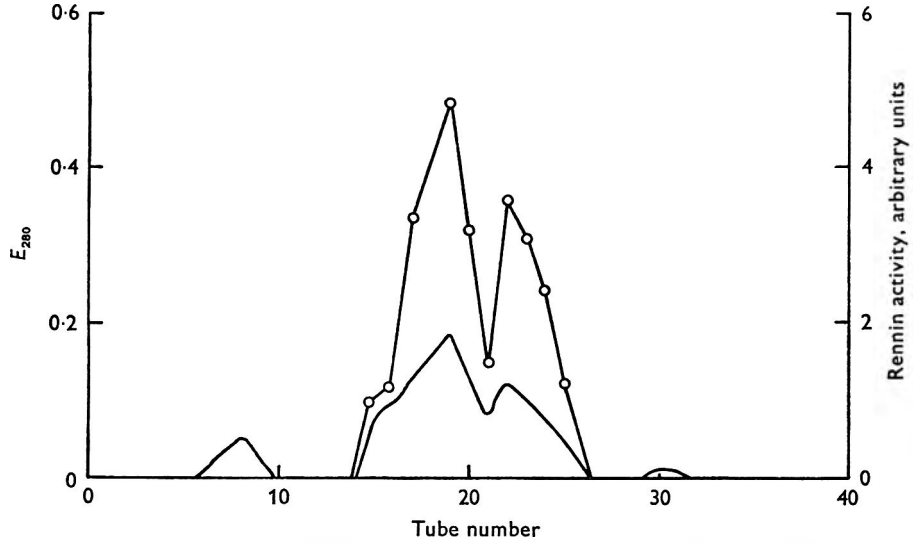

Fig. 2. Ion-exchange chromatography of crystalline rennin. The chromatography was performed on DEAE-cellulose (W. and R. Balston, Ltd, Maidstone, Kent). The column was 26 cm long by 2 cm diam. and was eluted with buffer and pH gradient (300 ml 0·1 M phosphate, pH 5·8 and 300 ml 0·4 M phosphate, pH 5·5). ——, Absorbance at 280 nm; O—O, activity.

resolution of this main peak into 3 components. The ratio of specific activities of rennin A : B : C was 3 : 2·5 : 1, which is in good agreement with the corresponding values obtained by Foltmann (1966).

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The effects of increasing amounts of dietary coconut oil on milk-fat secretion in the cow

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Summary. (1). A study is reported on the effects of 4 levels of coconut oil, added to a basal diet low in fat, on the secretion in cow's milk of fat and its component fatty acids. (2) A significant reduction in the yield of milk fat occurred at the highest level of supplementation. In terms of individual fatty acids the yields of lauric and myristic acids increased progressively with increased intake, maximum yields being obtained with the 7 % level of coconut oil. Conversely the yields of caproic, caprylic, capric and palmitic acids progressively decreased with increased coconut oil intake. The yields of C18 acids were unchanged.

Earlier studies have shown that addition of either coconut, red palm or groundnut oil to a basal diet low in fat increased the secretion in cow's milk of both total fat and the major fatty acids characteristic of these oils (Storry, Rook & Hall, 1967). In further studies in which the basal diet was supplemented with 3 levels of red palm oil, the yields in milk of total fat and of the fatty acids contained in the oil supplement were positively correlated with the dietary intakes, except for linoleic acid which was hydrogenated in the rumen before its absorption and secretion in milk (Storry, Hall & Johnson, 1968). The present paper describes the effects on the secretion of milk fat of supplementing a low fat diet with 4 levels of coconut oil.

EXPERIMENTAL

Animals and their management. Five Friesian cows in the second month of lactation were fed a diet of meadow hay (5 kg), sugar-beet pulp (5 kg) and a basal concentrate mixture. The basal concentrates consisted of barley (21·8 %), rice (16·3 %), tapioca (16·3 %), decorticated groundnut meal (32·7 %), blood meal (10·9 %) and minerals (2·0 %). To the basal concentrates coconut oil was added at approximately 2·0, 4·0, 7·0 and 10·0 % (w/w) to give 4 experimental mixtures. The ether extract values for the basal and the 4 oil-supplemented concentrate mixtures were 0·83, 2·34, 4·04, 6·90 and 10·30 % respectively and the corresponding gross energy contents, as determined by bomb calorimetry, were 3·92, 4·03, 4·20, 4·32 and 4·48 kcal/g. Ether extract values for the hay and sugar-beet pulp were 1·24 and 0·37 % respectively.

The basal concentrate mixture was fed according to yield at the rate of 0·4 kg/kg milk and the oil supplemented mixtures at corresponding isocaloric rates.

Procedure. The experimental design was a 5 × 5 Latin square with periods of 22 days. The effects of the oil supplements on the content and yield of total milk fat during the first 10 and last 12 days of each period and on the yields of individual fatty acids during the last 12 days of the feeding periods were analysed by analysis of variance (Snedecor, 1950).

The content and composition of milk fat were determined as described previously (Storry *et al.* 1967) and the content and composition of total fatty acids in the hay, sugar-beet pulp and concentrate foods as described by Sutton, Storry & Nicholson (1970).

RESULTS

Yields of milk and milk fat. The effects of the coconut-oil supplements on the yield of milk and on the percentage and yield of milk fat are given in Table 1. During the first 10 days there was no significant effect other than a fall in milk yield at the

Table 1. *Effect of dietary supplements of coconut oil on the percentage and yield of milk fat and on the yield of milk by cows*

Oil supplement, %	Milk yield, kg/day	Milk fat content, %	Yield of milk fat, g/day
	First 10 days of each period		
0	21·4	3·6	764
2	21·1	4·2	858
4	20·5	4·2	847
7	21·4	4·3	907
10	20·1*	4·2	842
	Last 12 days of each period		
0	20·4	4·0	805
2	20·5	4·2	847
4	20·1	4·1	811
7	20·3	4·0	806
10	19·7	3·6	701*

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

highest level of supplementation, although the contents and yields of fat in milk tended to be higher on all the supplemented diets when compared with those on the basal diet. During the last 12 days there was little effect of the treatments other than a significant fall in the yield of milk fat at the 10% level of supplementation.

Secretion of fatty acids in milk. The content and composition of fatty acids in the hay, sugar-beet pulp and concentrate foods are given in Table 2 and the mean dietary intakes of fatty acids on each of the diets in Table 3. The coconut-oil supplements resulted in major increases in the dietary intake of lauric, myristic and palmitic acids and slight increases in the dietary intakes of capric, stearic and oleic acids. The yields of fatty acids in milk during the last 12 days of each period are given in Table 4 and the responses in yields of individual fatty acids differed from one another. With the exception of the yield of butyric acid, which increased with the lower levels of coconut oil, the yields of other short-chain acids, namely caproic, caprylic and capric, decreased progressively with increased level of coconut oil. On

Table 2. *Content of total fatty acids (g/kg) and composition (%) of major fatty acids in hay, sugar-beet pulp and concentrate foods*

Constituent	Hay	Sugar-beet pulp	Concentrate foods 0%	2%	4%	7%	10%
Total fatty acid...	10·6	6·8	11·9	21·7	33·6	55·6	78·9
Fatty acid†							
10:0	—	—	—	0·1	0·6	1·3	2·2
12:0	0·5	0·4	0·3	19·0	30·6	38·8	41·5
14:0	1·4	1·2	0·4	12·7	17·0	19·3	20·2
16:0	32·7	30·7	18·6	16·6	15·1	13·8	13·1
18:0	7·4	1·4	3·4	4·3	3·9	3·4	3·4
18:1	14·3	11·4	38·7	24·5	18·5	14·2	12·7
18:2	19·5	48·6	35·6	21·4	13·8	9·2	6·8
18:3	14·9	6·2	—	—	—	—	—

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

Table 3. *Mean fatty acid intake (g/day) on the basal and 4 coconut-oil supplemented diets*

Fatty acid	Diet 0%	2%	4%	7%	10%
10:0	—	0·2	1·5	5·3	10·4
12:0	0·7	34·0	77·1	158·6	197·1
14:0	1·5	23·6	43·7	79·8	96·7
16:0	45·6	55·3	64·2	80·6	86·4
18:0	7·6	11·6	13·7	17·5	27·2
18:1	49·6	53·8	56·9	67·9	61·7
18:2	61·9	63·8	58·7	62·4	61·6
18:3	9·6	9·0	9·3	8·4	16·9
Total	176·5	251·3	325·1	480·5	558·0

Table 4. *Effect of dietary supplements of coconut oil on the yields (g/day) of the major fatty acids in milk of cows*

Fatty acid	Level of coconut-oil supplement, % 0	2	4	7	10
4:0	22·8	31·3*	28·0	24·0	20·7
6:0	16·2	16·3	15·9	14·3	11·9
8:0	10·0	10·5	9·8	7·0*	6·7*
10:0	29·8	29·8	28·1	23·2*	18·1***
12:0	44·5	57·6	70·5**	91·3***	89·5***
14:0	118·1	132·6	134·8	137·8*	129·4
16:0	319·0	336·9	308·5	267·2*	207·3***
18:0	20·7	23·2	21·1	24·0	20·4
18:1	99·1	93·9	97·6	94·1	94·0
18:2	5·6	3·7	4·3	3·6	2·9

* $P < 0{\cdot}05$; ** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$.

the other hand, the yields of lauric and myristic acids, the major constituents of coconut oil, increased progressively with the amount of coconut oil fed and the responses in these acids appeared to reach a maximum at the 7 % level of supplementation. Palmitic acid decreased in yield at the higher levels of coconut oil intake, in spite of the fact that the dietary intake of palmitic acid almost doubled. The yields of C18 acids showed no significant changes.

DISCUSSION

Although the basal low fat diet in the present experiment was similar to that used in our previous studies (Storry *et al.* 1967) the present experiment differs in that the oil supplement was given for 22 instead of 14 days and at various levels ranging from 2 to 10 % of the concentrate food instead of at the single level of 2 %. These differences should be remembered when comparing the results of the 2 experiments, especially as an effect of time on the response in milk fat yield to dietary fat supplements has previously been observed (Steele & Moore, 1968*a*; Storry *et al.* 1968; Rohr & Okubo, 1968). The increased content and yield of fat in milk during the first 10 days of feeding in the present experiment, although just failing to reach statistical significance, thus tends to support our earlier findings of an increased content and yield of milk fat when coconut oil was fed for 14 days at a level of 2 %. However, during the last 12 days of the feeding periods there was little change in the content and yield of milk fat with levels of coconut oil below 7 % and a significant fall in milk fat yield at the 10 % level of supplementation. These findings thus support the decreased content and yield of fat in milk of cows observed by Steele & Moore (1968*d*), who fed 10 % myristic acid or 5 % lauric acid for 20 days and by Rohr & Okubo (1968) who fed 5 % coconut oil for 36–45 days.

The reason for the lack of a sustained increase or for the depression in yield of total milk fat with feeding of coconut oil is more apparent when the yields of individual fatty acids are considered. The increased yields of lauric and myristic acids in association with an increased dietary intake of these acids fully confirms previous observations that the mammary gland can utilize these intermediate chain acids when they are provided in the diet (Storry *et al.* 1967; Steele & Moore, 1968*c*; Storry, Tuckley & Hall, 1969). At the same time, however, the increased yields of lauric and myristic acids were offset by decreased yields of caproic, caprylic, capric and particularly of palmitic acids, so that the yield of total fatty acids in milk would depend on the balance of these 2 effects. Similar depressions in the intramammary synthesis of fatty acids with the feeding of a variety of fatty acids and oils have been observed elsewhere (Steele & Moore, 1968*a–d*; Storry *et al.* 1968; Noble, Steele & Moore, 1969) and this effect probably results from changes in the production of rumen volatile fatty acids and possibly also from inhibited *de novo* synthesis of fatty acids within the alveolar cell (see Storry, 1970). Rumen volatile fatty acids were not determined in the present experiments, but decreases in the proportion of acetate and increases in the proportion of propionate, with variable responses in the proportions of butyrate, have been reported by others in the rumen of cows fed myristic and lauric acids (Steele & Moore, 1968*d*) or coconut oil (Rohr & Okubo, 1968).

An interesting feature of the present experiments is that the yield of butyric acid

was increased or maintained as the dietary intake of coconut oil increased in contrast to the progressive reduction in the yields of other short-chain fatty acids synthesized within the mammary gland. This differential response of butyric acid has been observed previously (Storry *et al.* 1967, 1968; Noble *et al.* 1969) and may reflect increased synthesis of butyrate by non-malonyl pathways (see Storry, 1970).

It may be concluded that although feeding coconut oil to cows increases the yield in milk of the major fatty acids contained in the oil, the concomitant depressed yields of other acids which also occur makes it an unsuitable oil supplement for increasing the content and yield of total milk fat.

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Reviews of the progress of Dairy Science

Section E. Diseases of dairy cattle. Thiamine deficiency, with particular reference to cerebrocortical necrosis – a review and discussion

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(*Received* 19 *October* 1970)

CONTENTS

INTRODUCTION

There is a vast literature on thiamine deficiency in monogastric animals and its biochemistry, reviewed by Wolstenholme & O'Connor (1967) and by Peters (1963), but relatively little has been known until recently of the occurrence of thiamine deficiency in the ruminant. This literature is reviewed and discussed with particular reference to cerebrocortical necrosis.

CEREBROCORTICAL NECROSIS

The name cerebrocortical necrosis (CCN) was suggested by Terlecki & Markson (1959) for a disease of sheep and cattle involving the central nervous system. The condition was recognized as a distinct clinico-pathological entity after they had examined a large number of cases over a period of years.

Clinical signs

Sheep. Clinical signs of the disease followed a regular pattern, beginning with wandering and circling, staggering and swaying, or standing precariously with feet wide apart and head drawn back in convulsive, extensor spasms. The animals

appeared to be blind and they eventually fell over with legs extended, kicking intermittently until exhausted. Handling usually resulted in violent galloping movements ending with extensor spasms, opisthotonus and nystagmus. Most untreated animals died after an illness lasting from a few hours to several days (Terlecki & Markson, 1961; Spence, Stevens, Saunders & Harris, 1961).

Cattle. The early signs of circling were not seen in cattle; the syndrome began with dullness and ataxia, leading to collapse and convulsive struggling. Hyperaesthesia, opisthotonus, amaurosis, nystagmus, trismus and tonic extensor spasms were prominent symptoms as in sheep. Death usually ensued in a state of coma after an illness lasting 2–6 days (Terlecki & Markson, 1961).

Pathology

At post-mortem examination the only consistent gross lesions were in the brain, and in a high proportion of cases the lesions were visible to the naked eye, consisting of areas of yellowish discoloration in the cerebral cortex, while histological examination showed characteristic lesions of neuronal necrosis, and microglial phagocytic reaction with a recognizable pattern of distribution which was bilateral though not necessarily symmetrical. Some gyri were swollen. The disseminated necrosis of the cerebral cortex was the only constant finding in the disease. Further study and more detailed analysis of the cerebral lesions suggested that the pathological basis was cellular anoxia (Terlecki & Markson, 1961, Spence *et al.* 1961). Clegg (1966) reported hypokalaemia in affected animals but the significance and interpretation of this observation is uncertain.

Distribution

In the United States a disease called polioencephalomalacia (PEM) was described by Jensen, Griner & Adams (1956). The disease, characterized by a focal necrosis disseminated throughout the cerebral cortex, differed from CCN only in that the mortality rate was lower. It has been reported to occur both in feedlot and grazing cattle and sheep and is known in Wyoming as 'blind staggers' and in Colorado as 'forage poisoning'.

Post-mortem examination of affected animals revealed that the primary significant lesions were limited to the brain, where multiple yellowish foci of necrosis were found in the cerebral cortex. A similar condition has been described by Hartley & Kater (1959) in lambs in New Zealand. As PEM and CCN are similar in so many respects, they will be treated as synonyms in this review.

CCN has been reported to occur in Australia (Nicol, 1961–2), Canada (Loew, Radostits & Dunlop, 1969), Switzerland (Fankhauser, 1962), Germany (von Sandersleben, 1966), South Africa (Pienaar & Thornton, 1964) and France (Tournut, Labie & Espinasse, 1967). The latter authors describe the incidence in France, and have also identified similar conditions in goats and in the antelope.

Incidence and causal agents

Spence *et al.* (1961) described 21 outbreaks in sheep and 12 in calves, critically examining factors of incidence such as sex, breed and management, but were

unable to implicate any particular factor. The age incidence in sheep varied from lambs of 6 weeks of age to ewes of 7 years or more, but the majority of cases were in lambs 2–7 months old. The flock morbidity rate in sheep ranged from 1–6 % and the mortality rate was usually 100 %. In cattle, the age incidence ranged from 10 weeks to 11 months but the disease was most common in calves 3–7 months of age. Incidence varied from 1 to nearly 50 % in a group of calves; a few animals appeared to recover but the majority died.

Various transmissions of tissue from affected animals into animals of the same species or into mice failed to produce the disease or to show the presence of bacteria or viruses.

Hartley (1956) suggested that clostridial enterotoxins might be implicated in the pathogenesis of PEM. This possibility was carefully examined by Spence *et al.* (1961) and by Terlecki & Markson (1961), but they were unable to substantiate it. Jensen *et al.* (1956) considered the possibility of selenium as a toxic agent but could not find any evidence for it. This was also the conclusion of Howell (1961). Lead, arsenic, mercury and plant poisons have been considered as causal agents but have not been shown to produce CCN (Howell, 1961; Jensen *et al.* 1956). Various aspects of the aetiology of CCN have been reviewed by Markson & Terlecki (1968).

Thiamine and CCN

Davies, Pill, Collings, Venn & Bridges (1965) showed the involvement of thiamine in CCN after investigating reports that multivitamin preparations were beneficial for treatment of CCN. This was confirmed by Hentschl, Walton & Miller (1966), who treated PEM with vitamin B complex. Examination of tissues from affected animals showed them to be low in thiamine. Other known biochemical aberrations of classical thiamine deficiency, such as raised blood pyruvate and lactate and lowered erythrocyte transketolase activity, were also found (Pill, 1967; Edwin, 1970; Loew, Dunlop & Christian, 1970). Plasma pyruvate kinase levels have been shown to rise considerably (Edwin, 1970).

Treatment of CCN

Reports indicate that treatment of affected animals, particularly calves, with large doses of thiamine preparations administered parenterally is effective if given before widespread neuronal damage has occurred.

Davies *et al.* (1965) treated an affected calf, in the final comatose phase, with 100 mg thiamine in aqueous solution intravenously; 12 h later the animal was standing, but relapsed after 72 h. A response followed further injections of thiamine but recovery was not complete. Pill (1967) reported complete recovery in 9 calves (3–6 months old, Ayrshire, Friesian or Hereford cross) treated with 200 mg thiamine intravenously plus 200 mg intramuscularly. Hentschl *et al.* (1966) successfully treated Holstein steers of 400 lb liveweight by intramuscular injection of 10 ml of a multivitamin preparation containing 1 g thiamine hydrochloride. They reported an improvement in 4 h; the treatment was repeated the next day.

BIOCHEMISTRY OF THIAMINE DEFICIENCY

The biochemical role of thiamine was first revealed by the classical studies of Peters (1936), who demonstrated its implication in carbohydrate utilization. As its pyrophosphate ester, cocarboxylase, thiamine is known to act as a coenzyme in 3 areas of carbohydrate metabolism. In the glycolytic pathway it catalyses the decarboxylation of pyruvate to acetyl coenzyme A, and of α-ketoglutarate to succinyl coenzyme A in the tricarboxylic acid cycle. Further, it is a coenzyme for the transketolase (E.C. 2.2.1.1) in the pentose pathway which mediates in the formation of sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate from ribose-5-phosphate and xylulose-5-phosphate; and of fructose-6-phosphate and glyceraldehyde-3-phosphate from xylulose-5-phosphate and erythrose-4-phosphate. Brin, Shohet & Davidson (1958) have demonstrated that transketolase activity and the concomitant restoration of activity by the in vitro addition of thiamine pyrophosphate (TPP) to erythrocyte haemolysates would constitute an early and sensitive index of thiamine deficiency.

TPP is a coenzyme in over 24 enzyme systems (Sauberlich, 1967).

Apart from its coenzyme function thiamine has been shown to have an independent role in nerve conduction. Von Muralt (1962), working with isolated nerve fibres, demonstrated the release of small but measurable quantities of thiamine on excitation. Further, Itokawa & Cooper (1969) have demonstrated the localized presence of thiamine triphosphate in neuronal membrane, and have suggested for it a role in ion transport. Cooper, Itokawa & Pincus (1969) have also shown that thiamine triphosphate is virtually absent from the brains of patients suffering from subacute necrotizing encephalomyelopathy.

Thiamine deficiency in monogastric animals

The effects of thiamine deficiency on the central nervous system have been reviewed by Dreyfus & Victor (1961). In monogastric animals dietary deficiency of thiamine is followed by a definite sequence of events. Brin (1964) recognizes 5 stages of deficiency, depending upon the rate of intake of thiamine. In man, a preliminary stage is discernible within 5 days on a thiamine-low diet. This is characterized by reduced urinary excretion of thiamine. Secondly, a biochemical phase is seen within 10 days when erythrocyte transketolase activity is depressed with a positive TPP effect of about 15 %. The third is a physiological phase which is seen in 21–28 days and is shown by a fall in bodyweight, loss of appetite, general malaise, insomnia and general irritability. Urinary thiamine during this phase falls to 0–25 μg daily and the TPP effect is raised to 30 %. This is followed in 30–300 days by a clinical phase with increased malaise, loss of bodyweight, polyneuritis, bradycardia, peripheral oedema, cardiac enlargement and ophthalmoplegia. Urinary thiamine excretion is negligible and the TPP effect is in excess of 40 %. Finally an anatomical phase is recognized. This is characterized by cardiac hypertrophy, degeneration of the granular layer of the cerebellum, perivascular cerebral haemorrhages with degeneration of neurones, swelling of microglia and proliferation of astrocytes. The TPP effect is in excess of 50 %.

In experimental thiamine deficiency depletion of thiamine proceeds at different

rates in different tissues. Lowry (1952) demonstrated that rats on a thiamine-deficient diet showed a marked fall in urinary thiamine, followed by reduced thiamine levels in blood, liver, kidney, heart and finally brain, in that order. Using ^{35}S-labelled thiamine Balaghi & Pearson (1966) found that the rate of decrease of thiamine is highest in liver and lowest in brain. Thus, it is evident that brain is particularly resistant to depletion of thiamine and this may be important in considering the pathogenesis of CCN. The concentration of thiamine in brain does not fluctuate a great deal, and on depletion, nervous symptoms only appear when it has fallen to 20% of its normal value.

The thiamine-dependent mammalian enzymes transketolase, pyruvate and α-ketoglutarate dehydrogenases show different degrees of sensitivity to thiamine deficiency, and the biochemical lesions would be manifest with different degrees of severity. The restriction in keto-acid oxidation results in a rise in the concentration of these substances in the blood stream. This is shown by the sudden rise in blood pyruvate when thiamine-deficient animals are given glucose. Bueding, Wortis & Stern (1942) treated human subjects showing Wernicke's syndrome, a thiamine-responsive condition, with glucose at the rate of 10 mmols/kg bodyweight, and showed that blood keto-acids rose sharply. The rise could be soon reversed by the administration of thiamine. Apart from pyruvate, levels of other keto-acids such as α-ketoglutarate, acetoacetate, and also of glyoxalate are elevated in the blood stream and urine.

Although thiamine deficiency can be produced readily in monogastric animals, ruminants have been shown to synthesize members of the B group of vitamins at an adequate rate, and hence to be independent of an extraneous supply in the diet.

Thiamine deficiency in pre-ruminant animals

Thiamine deficiency has been produced in the pre-ruminant animal by feeding thiamine-low diets. Johnson, Hamilton, Nevens & Boley (1948) fed such a diet to new-born dairy calves, and demonstrated the onset of chronic or acute symptoms, according to the body stores of thiamine. The urinary thiamine excretion fell to a level below 10 μg/day, and blood and urine pyruvate levels were above normal. Clinical signs developed, characterized by weakness, incoordination, convulsions and head retractions. The convulsions and polyneuritic symptoms responded immediately to thiamine therapy.

A combination of physiological, clinical and enzymic criteria was used by Benevenga, Baldwin & Ronning (1966) to characterize the onset of thiamine deficiency in calves fed a semi-purified diet. Blood pyruvate, lactate levels and urinary pyruvate excretion increased, and liver transketolase and the pyruvate dehydrogenase system decreased significantly concomitantly with the appearance of overt deficiency symptoms.

Draper & Johnson (1951) studied the effect of omitting thiamine from the diet of the lamb. Here also a uniform pattern was seen, characterized by anorexia in 2–3 weeks and a fall in growth rate followed in the fourth to fifth week by convulsions including opisthotonus. These tetanic spasms were similar to manifestations of thiamine deficiency in other species, and disappeared when thiamine was given. In these studies, however, the brains were not examined. Lewis, Terlecki, Markson, Allcroft & Ford (1967) fed a thiamine-deficient diet to lambs from 48 h of age. The

animals reached a crisis in 18–30 days and were killed *in extremis*. A fall in urine thiamine and rise in blood pyruvate was noted. The brain showed no gross lesions, but histological examinations revealed slight changes in the cortex and thalamus resembling those found in field cases of CCN.

The 3 studies mentioned here have all been carried out on very young ruminants, at a stage when they were still dependent on an extraneous source for their thiamine requirement. It can be assumed that this requirement is high in a rapidly growing animal. In the ruminating animal, with its full complement of thiamine-producing symbiotic micro-organisms, it would be difficult to induce a state of thiamine deficiency by purely dietary means.

THIAMINE ANTAGONISTS

Effects in monogastric and pre-ruminant animals

Thiamine deficiency can be induced in monogastric animals not only by feeding thiamine-low diets but by giving thiamine antagonists. These compounds may be structurally similar to thiamine and act as true antimetabolites by competing with thiamine in several of its co-enzyme functions. Their mode of action is reviewed by Steyn-Parvé (1967). Pyrithiamine, oxythiamine and amprolium are 3 examples which have been studied extensively. Administration of these compounds to experimental animals such as rats, mice or pigeons is followed by a fall in growth rate and the manifestation of a number of effects which are also seen in nutritional thiamine deficiency. These are true antimetabolites of thiamine since the deficiency symptoms can be reversed by the administration of thiamine.

The various thiamine antagonists have different potencies and may interfere with thiamine utilization at different points in the metabolic pathway and/or compete with thiamine at different membrane sites.

Pyrithiamine. When pyrithiamine is given to animals TPP is reduced in the tissues, even though the diet may have contained free thiamine. The rate of disappearance of TPP was greater and the survival time markedly shortened when the animals (mice, rats, pigeons) were kept on a thiamine-deficient diet (De Caro, Rindi, Perri & Ferrari, 1958; Koedam, 1958). The rate of disappearance of TPP depends on the pyrithiamine:thiamine ratio in the diet (De Caro *et al.* 1958; Koedam & Steyn-Parvé, 1959). The nature of the diet was also shown to have a profound effect on the rate of TPP depletion. A high carbohydrate diet was similar to a thiamine-deficient diet in this respect. The disappearance of TPP from tissues was accompanied by an increase in urinary excretion of thiamine. The behaviour of the brain is particularly interesting in pyrithiamine-treated animals. Although on a thiamine-deficient diet the brain loses its thiamine at a slower rate than other tissues, the thiamine loss is particularly rapid from the brain in animals treated with pyrithiamine or pyrithiamine in combination with thiamine (De Caro *et al.* 1958; Koedam, 1958). In rodents De Caro has shown that a single dose of pyrithiamine has a lasting effect. Rindi & Perri (1961) found that pyrithiamine accumulates in the rat brain. In in vitro experiments with rat liver, pyrithiamine has been shown to inhibit thiamine kinase (E.C. 2.7.6.2) (cocarboxylase synthetase) in the formation of TPP (Eich & Cerecedo, 1954; Koedam & Steyn-Parvé, 1960).

Pyrithiamine has also been shown to displace thiamine from isolated nerve tissue. Cooper (1968) and Itokawa & Cooper (1969) suggested that this action might result in interference with ion transport and consequently of nerve conduction.

Oxythiamine. The anti-thiamine potency of oxythiamine is not as great as that of pyrithiamine; its effects are reviewed by Steyn-Parvé (1967).

Urinary excretion of thiamine is not increased to the same extent as with pyrithiamine treatment and the effect on the brain is not so profound. However, high doses of oxythiamine can be toxic. Although oxythiamine does not inhibit thiamine kinase activity in the rat liver (Eich & Cerecedo, 1954), it has been shown that oxythiamine pyrophosphate is a powerful antagonist in this reaction. Oxythiamine pyrophosphate has a much greater affinity for holotransketolase than TPP and can displace TPP from it.

Yonezawa & Iwanami (1966) have studied thiamine deficiency in nervous tissue using tissue culture techniques. The application of pyrithiamine or oxythiamine produced degenerative changes, either acute or chronic, depending on the concentration of the antimetabolites.

Amprolium. Amprolium (Merck, Sharp and Dohme, Hoddesdon, Herts), a coccidiostat, is a much milder antagonist of thiamine than pyrithiamine or oxythiamine (Rogers, 1962). Brin (1964) has shown that in the normal rat amprolium at a dietary level of 10000 ppm depressed transketolase activity in erythrocyte haemolysates. Thiamine-deficient rats showed a similar but more severe effect.

Pill, Davies, Collings & Venn (1966) gave pyrithiamine by twice daily injections of 10 mg over a period of 9 days to a pre-ruminant calf fed on a thiamine-low semi-purified diet. After this treatment amprolium was given orally for a further 11 days. A condition indistinguishable from CCN was produced.

Markson, Terlecki & Lewis (1966) produced CCN in 4 pre-ruminant calves by administering amprolium in a diet of cow's milk. The effects of pyrithiamine, oxythiamine and amprolium given parenterally to pre-ruminant calves have been compared by Markson, Lewis, Terlecki & Edwin (in preparation).

Effects in ruminants

The use of thiamine antagonists has been studied by a number of workers. Lewis, Terlecki & Ford (1967, unpublished) failed to produce evidence of thiamine depletion or CCN in ruminating lambs by administering oxythiamine or neopyrithiamine orally for 5 months and subsequently by intramuscular injection daily for 10 weeks. However, the 6 animals fed amprolium at levels increasing from 0·5 to 1·5% of the total dry-matter intake developed characteristic clinical and histopathological signs of CCN, indistinguishable from the naturally occurring condition, after between 5 weeks and 7 months on experiment.

In the ruminant calf, Markson, Edwin, Lewis & Richardson (in preparation) have induced CCN by giving amprolium by rumen fistula. These animals developed the typical signs of CCN and were killed *in extremis*. The characteristic lesions of the disease were seen on histopathological examination of the brain.

THIAMINASES

Sources

Thiamine-destroying factors of natural occurrence are known (for a review see Murata, 1965). An early example was associated with a nervous disease of silver foxes called 'Chastek' paralysis. The animals had been fed a diet of raw carp and were shown to be severely thiamine-deficient; the condition could be rapidly reversed by giving thiamine (Green, Carlson & Evans, 1941). Similar outbreaks have been described in Norway (Ender & Helgebostad, 1945). The cause of the thiamine destruction was shown to be an enzyme—thiaminase— which was present in the raw carp (Woolley, 1941, 1952; Sealock, Livermore & Evans, 1943). Thiaminases have since been shown to be present in many species of fish, such as garfish and ide. Ender & Helgebostad (1945) have shown that feeding these fish in the raw state to foxes or rats resulted in a condition similar to beri beri, which responded to thiamine treatment.

Geraci (1969) reviewed the literature on diet-induced thiamine deficiency in captive marine animals and recently White (1970) has reported thiamine deficiency in an Atlantic bottle-nosed dolphin (*Tursiops truncatus*) on a diet of raw fish.

Many shell fish, such as mussels, clams and oysters, are potent sources of thiaminase (Fujita & Matsukawa, 1942). It has also been shown by Japanese workers to be present in several species of bacteria, yeasts and fungi (Ozawa, Nakayama & Hayashi, 1957). In the plant kingdom bracken, equisetum, celosia and certain oil seeds have also been shown to contain it (Okamoto, 1950; Evans, Jones & Evans, 1950; Bhagvat & Devi, 1944*a*, *b*).

Chemistry of reactions catalysed by thiaminases

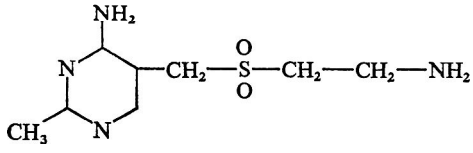
Two types of thiaminase designated I and II have been recognized (Fujita, Nose & Kuratani, 1953). By paper chromatographic separation of reaction products the mode of action of thiaminase II has been found to be a simple hydrolytic fission of the pyrimidine (Pyr) and thiazole (Thz) moieties of thiamine, at the methylene bridge

$$\text{Pyr—Ch}_2\text{—Thz} \rightarrow \text{Pyr—CH}_2\text{—OH} + \text{Thz} + \text{H}^+.$$

Thiaminase I mediates in a base exchange reaction and requires an amine as a co-substrate. In the presence of a suitable base, cleavage of thiamine results in free thiazole and a new product comprising the pyrimidine part of thiamine and the activating amine (Sealock & Davis, 1949):

$$\text{Pyr—CH}_2\text{—Thz} + \text{Base} \rightarrow \text{Pyr—CH}_2\ \text{base} + \text{Thz} + \text{H}^+.$$

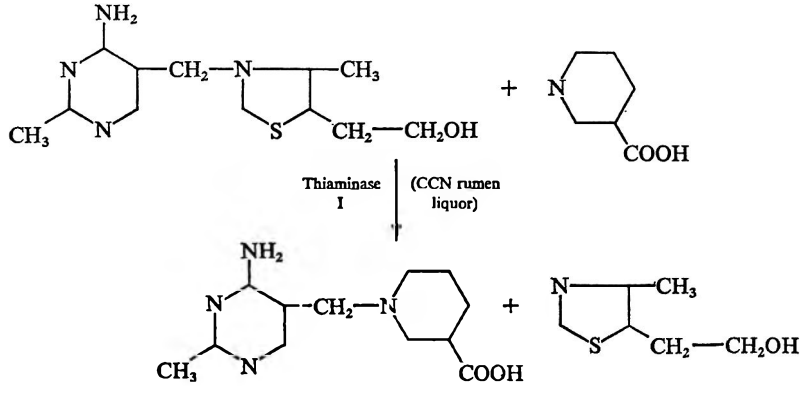
A number of aromatic amines and heterocyclic compounds have been shown to activate thiaminase I to varying degrees, depending on the amine and the source of the enzyme. Barnhurst & Hennessy (1952) found that for fish and clam thiaminase, hypotaurine was a specific activator and they isolated and characterized icthiamine.



Thiaminase in CCN

After examining the various factors involved in the aetiology of CCN Edwin, Lewis & Allcroft (1968) suggested that a thiaminase of type I might be involved. They showed that an antithiamine compound similar to amprolium might be formed from thiamine and α-picoline (a compound known to be present in bracken) in the alimentary tract of affected animals.

The ruminal contents of calves and sheep affected with CCN have been examined and found to contain significant amounts of a thiaminase (Edwin, Spence & Woods, 1968). Further work confirmed the presence of the enzyme in every case of CCN examined in this laboratory. Its presence was also demonstrated in intestinal contents and unvoided faeces. Faecal samples from some in-contact animals were also shown to contain thiaminase (Edwin & Lewis, 1970). The thiaminase was shown to be of type I (Edwin & Jackman, 1970) and to need nicotinic acid or nicotinamide as a specific activator. The resulting compound, *N*-(2′-methyl-4′-aminopyrimidyl-(5′)-methyl-3-carboxy-pyridinium chloride hydrochloride, was isolated by in vitro incubation of thiamine and centrifuged rumen liquor from CCN cases.



Assay of thiaminases

Thiaminases have been assayed by a number of different procedures. Sealock *et al.* (1943) showed that enzymic cleavage of thiamine by the Chastek paralysis factor was a hydrolytic reaction in which a hydrogen ion was released

$$R'CH_2\overset{+}{N}R_3 + H_2O \rightarrow R'CH_2OH + NR_3 + H^+.$$

In bicarbonate buffer the hydrogen ion and thus the concentration of enzyme was measurable by the yield of carbon dioxide.

Watkin, Thomas & Evans (1953) employed essentially the same method for studying the nature of the thiaminase from bracken and found that DL-proline and L-hydroxyproline were active as cofactors in their in vitro system.

Fujita (1954) estimated thiaminase activity by incubating known amounts of thiamine with the enzyme under specified conditions and measuring the rate of decomposition. In certain samples—especially bacterial suspensions—an apparent decomposition due to phosphorylation of added thiamine was also measured, by

treating the incubation mixtures with diastase. A heat-inactivated preparation was included as a control. Thiamine was measured by the thiochrome method.

Gnaedinger (1965) devised an improved procedure for determining thiaminase activity in white fish.

Kenten (1957) measured thiaminase activity by spectrophotometric determination of heteropyrithiamine formed by the action of thiaminase on thiamine using pyridine as a cofactor. He utilized this procedure for the measurement of thiaminase in bracken. Murata & Ebata (1959) studied this method and found that the amount of heteropyrithiamine produced was directly proportional to the amount of thiaminase I over a wide range.

Douthit & Airth (1966) studied the thiaminase from *Bacillus thiaminolyticus* by spectrophotometric determination of the rate of formation of anilothiamine.

Edwin & Jackman (1970) assayed thiaminase activity by incubating ^{14}C-thiazole-labelled thiamine with the enzyme and measuring the amount of free radioactive thiazole formed, by scintillation counting.

THIAMINE-RESPONSIVE NERVOUS DISORDERS

A nervous disorder in farm dogs, possibly associated with thiamine deficiency, was reported by Mayhew & Stewart (1969). The dogs were fed a prepared dog food with a very high fat content. Four animals died; a fifth affected dog was treated with thiamine intramuscularly and recovered. Similar cases were reported by Stirling (1969) who also noted a response to thiamine, given in a multivitamin supplement.

Newman (1970) reported a nervous syndrome in dogs and humans after eating a fish, a large red snapper, *Lutjanus bohar*. All recovered after parenteral administration of thiamine.

Loew, Martin, Dunlop, Mapletoft & Smith (1970) recorded 5 clinical cases of thiamine deficiency in cats fed a commercial cat food of low thiamine content. The signs of ataxia, subnormal righting reflexes and anorexia disappeared rapidly after administration of multiple B vitamin preparations.

A nervous disorder in pigs associated with suspected thiamine deficiency has been described by Newman (1969); it was connected with movement between farms and change of diet. Animals responded to parenteral thiamine therapy. Newman suggested that the clinical signs and histological appearance of nervous tissue from a single case were probably similar to those of CCN in ruminants.

Differential diagnosis

The differential diagnosis of CCN from other nervous disorders in which similar clinical signs are exhibited must be by bacteriological, histopathological and biochemical examination.

Spence *et al.* (1961) included a case of listeriosis in their series of animals with suspected CCN, and Howell (1961) also reported an instance of listeriosis presented to him as a case of PEM.

Other cases of suspected CCN have been confirmed at post-mortem examination and by biochemical analysis or histopathological examination to be cases of lead poisoning or cerebral coenurosis. However, to date, in the present authors' labora-

tory, abnormally high levels of thiaminase activity have only been found in the rumen contents from animals in which CCN has been confirmed by histopathological examination (Edwin & Lewis, unpublished).

CONCLUSIONS

It is now evident that CCN must be regarded as a particular form of thiamine deficiency, in which the ruminal supply of thiamine is cut off by the action of a thiaminase; the compound produced from the pyrimidine part of the thiamine molecule and the activating amine may itself have potent anti-thiamine activity which would exacerbate the condition. This is probably the reason for the early involvement of cerebral functions in the disease, which does not occur in frank thiamine deficiency.

It is also clear that CCN must be classed with other diseases which are caused by thiaminases, such as Chastek paralysis—the nervous disease of animals fed raw fish, and the thiaminase disease of man which is endemic in Eastern countries.

To date, the source of the high concentration of thiaminase in the rumen of affected animals is not known, although there is increasing evidence that this is of microbial origin.

These findings may have far-reaching ramifications in the field of animal production, particularly in the greater need for thiamine when animals are fattened on diets of high carbohydrate content. The young animal is particularly vulnerable and its thiamine reserves are likely to be lower and more rapidly depleted than are those of older animals.

The fact that thiaminase has been detected in the faeces of in-contact animals suggests that there may be many subclinical cases of CCN which pass unnoticed, and from which the animals recover spontaneously. However, this may well cause an interruption in their weight gain curve.

This concept of thiamine deficiency has wide implications of economic importance in regard to ruminant species and requires further investigation.

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Reviews of the progress of Dairy Science

Section B. The survival of pathogens in cheese and milk powder

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CONTENTS

INTRODUCTION

Because milk is a suitable medium for the growth of many bacterial species including pathogens and because these may contaminate it either directly from the cow or from the milk handler, occasional outbreaks of disease traceable to dairy products do still occur in spite of advances in dairy manufacturing processes.

The isolation of pathogens from foods is often a more complex problem than isolation from medical materials because the organisms are frequently not evenly distributed throughout the product, are often low in number and are slow to revive after they have been exposed to debilitating factors during processing and storage. In recent years there have been significant advances in the bacteriological analysis of food, and research in this field continues throughout the world.

A. SOURCE AND INCIDENCE

Cheese

Even today most cheese is made by traditional methods, frequently from raw milk, and is much handled by the cheesemaker and other factory personnel. Reliance on the effects of starter competition and of low pH to eliminate pathogens often leads to careless practices. Low pH is not completely effective in destroying pathogens, and in addition there is no certainty that all the cheese reaching the market has developed the pH level characteristic of the variety. Some may never reach the low pH required because the cheese starter has failed and the fermentative processes responsible for the drop in pH were not completed. Furthermore, in the mould-ripened or mould-infected cheese, and in cheese with surface bacterial smear, there are pockets or layers in or on the cheese where the pH tends to be neutral or alkaline. Similar pockets might also result from the incidental presence of bacteria

which produce alkaline conditions. For these reasons it would be unsafe to rely on the pH of the cheese generally as a factor eliminating the survival of either viruses or bacteria. Sharpe, Neave & Reiter (1962) and Reiter, Fewins, Fryer & Sharpe (1964) showed that pathogenic staphylococci multiplied rapidly in cheese in the manufacture of which the starter had been phaged. The presence of antibiotic residues and the contamination of milk with antibiotic-resistant staphylococci can lead to massive populations of pathogenic staphylococci in cheese due to selective multiplication of staphylococci and the inhibition of starter cultures (Thatcher & Ross, 1960).

As in most biological processes, there are a number of interwoven factors which will influence the survival of pathogens in cheese. Each species of pathogen may vary from strain to strain in its sensitivity to those properties of cheese which are thought to bring about its destruction. Many of the investigations into the survival of pathogens in cheese have been carried out using cheese milk artificially inoculated with pathogens. Such investigations are valuable but must be interpreted with caution. Walker, Harmon & Stine (1961) showed that pathogenic staphylococci naturally occurring in milk were better adapted to withstand the cheese-making and curing processes than are laboratory cultures. Besides, it is difficult to predict the degree of contamination which might occur naturally and to simulate this by artificial contamination. The effect of environment during storage on the response of the organism to isolation procedures should not be overlooked.

Many species of pathogens, both bacterial and viral, can find their way into milk and hence into cheese. Fabian (1947) tabulates outbreaks of disease traced to the consumption of infected cheese of many varieties. Pathogenic staphylococci, *Salmonella typhi*, *S. typhimurium*, various other *Salmonella* species and *Brucella* were the most common pathogens responsible. *Mycobacterium tuberculosis* is also known to survive for long periods in cheese (Hammer & Babel, 1957). While rickettsial and virus infections are known to be spread by milk and milk has a stabilizing effect on them (Cliver, 1969), their dissemination by cheese has not been reported.

Pathogenic staphylococci

In recent years outbreaks of staphylococcal food poisoning due to cheese in Canada, United States and Britain have resulted in a number of surveys being made to investigate the problem throughout the world (Takahashi & Johns, 1959; Thatcher, Comtois, Ross & Erdman, 1959; Keogh & Hansen, 1963; Murray, 1963; Donnelly, Black & Lewis, 1964; Sharpe, Fewins, Reiter & Cuthbert, 1965). The incidence of pathogenic staphylococci, particularly in raw milk cheese, is widespread.

The increased incidence of staphylococcal mastitis in dairy herds since the advent of antibiotics (Munch-Petersen, 1960) has made the presence of pathogenic staphylococci in cheese more likely (Thatcher, 1958). Added to this, when one considers the high rate of nasal carriers of pathogenic staphylococci in the normal community (30–50 % of healthy individuals (Williams, 1963)), one realizes the opportunities for post pasteurization contamination of a product still being processed largely by hand. The nasal carrier rate in hospitals, among staff and patients, is 60–80 % (Angelotti, 1969). A similarly high rate among dairy factory personnel in constant contact with infected milk might be expected. No data on the carrier rate among dairy factory personnel are available. The possibility of contamination of foods by persons

suffering from boils, carbuncles, salt sores and other suppurative lesions is often ignored. Thatcher, Simon & Walters (1956) isolated pathogenic staphylococci from a large proportion of cheeses made from pasteurized milk, and Zehren & Zehren (1968*b*) found cheese made from pasteurized milk in a particular factory in the United States to be contaminated with enterotoxin. The source of contamination by staphylococci was thought to be dirty equipment, indicating that poor sanitation can be a contributing factor to the incidence of pathogenic staphylococci in cheese. The frequent introduction of such staphylococci into milk factories through raw milk probably leads to a high incidence of environmental contamination. There is no doubt that pathogenic staphylococci find their way into cheese, and while their numbers decline during maturing of cheeses, such as Colby and Cheddar, when the manufacturing processes have proceeded normally (Mattick, Neave & Chapman, 1959; Roughley & McLeod, 1961; Reiter *et al.* 1964), there are cases where pathogenic staphylococci survived many months (Hendricks, Belknap & Hausler, 1959; McCleod, Roughley & Richards, 1962; Keogh & Hansen, 1963).

While consumption of cheese containing living pathogenic staphylococci helps to disseminate many strains of the organism in the community and contaminated cheese may lead to contamination of other foods, the actual presence of these organisms in cheese is perhaps not of such importance in relation to health as the presence of their enterotoxins. If enterotoxin is formed at any stage either in the milk or during manufacture it may remain many months in maturing cheese of low pH (Minett, 1938; Kréjaković-Miljković, 1960). Cheese should be examined within 3 weeks of manufacture for the presence of pathogenic staphylococci in order to reduce the risk that the decline in numbers may proceed to extinction before testing and yet the enterotoxin may remain. Zehren & Zehren (1968*a*, *b*) examined 2112 vats of cheese and found 59 vats to contain enterotoxin. They pointed out that the degree of acidity at early stages of manufacture was more important for the prevention of staphylococcal multiplication and enterotoxin production than was the pH in the final cheese. Jezeski, Tatini, Olson & Casman (1969) examined enterotoxin production in experimentally contaminated cheese and found that when starter failure had occurred, even with low initial contamination, extensive growth of staphylococci and production of enterotoxin resulted. Enterotoxin was found in Colby and Cheddar even with normal starter activity. Thatcher *et al.* (1959) demonstrated enterotoxin in 8 of 149 cheese samples 30–60 days old. Very little information is available concerning the activity of enterotoxin in cheese apart from indirect evidence that cheese many months old has been known to cause food poisoning. It is only recently that serological methods have been devised for the detection of enterotoxin (Angelotti, 1969), thus permitting more reliable studies.

While most experimental work and surveys of pathogenic staphylococci in cheese have been carried out for Cheddar and Colby, other types have been investigated and the presence of pathogenic staphylococci in them has been reported (Legler, Burkhardt & Wendrich, 1957; Mickelsen, Foltz, Martin & Hunter, 1963). Thatcher *et al.* (1959) examined a number of cheeses of different types imported into Canada. Most of them were made from pasteurized milk and no staphylococci were found. Mickelsen, Foltz, Martin & Hunter (1961) surveyed the incidence of pathogenic staphylococci in many varieties of cheese available in the United States and found

that 7·2 % contained coagulase-positive staphylococci. They reported a relationship between the presence of coliforms and *Staphylococcus aureus* in cheese.

Salmonella

The literature on the occurrence of *Salmonella* in dairy cattle has been reviewed by Bowmer (1965), Gibson (1965) and Schroeder (1967) and leaves no doubt that salmonellosis is quite prevalent in dairy herds. While salmonellae are rarely, if ever, excreted in the milk, they contaminate the milk during milking and subsequent handling. Salmonellae, however, are readily destroyed by pasteurization so that any occurrence of these organisms in pasteurized dairy products would be from external sources such as water, flies, dust, birds, rodents, humans and the raw materials added to the milk. Reviewers have pointed out that in recent years, while there has been a decrease in the incidence of *S. typhi* infections, there has been a marked increase in salmonellosis (Bowmer, 1965; Marth, 1969). Contributing to this increase are the changing eating habits of the general population with the introduction of pre-cooked and partially heat-treated frozen foods, and a higher incidence of human carriers among food handlers compared with the general population (Galton & Steele, 1961; Lang, 1968). The decrease in the incidence of typhoid fever traced to food has possibly resulted from better public health measures in isolating the organisms and tracing carriers as well as improved hygiene and the pasteurization of milk. Fabian (1947) and Marth (1969) report the occurrence of *S. typhi* infection attributed to cheese prior to 1947 and since that time reference to its occurrence due to cheese has been rare. One must not become too confident however, because, unlike most other salmonellae, the infective dose of *S. typhi* is extremely small and the organism does not need to multiply in the milk product to reach sufficient numbers to initiate the disease.

The many varieties of cheese which have been involved in gastroenteritis outbreaks due to salmonellae and the survival of salmonellae in these types of cheese have been reviewed by Marth (1969). Because many of the soft cheeses are consumed after short periods of maturing, sometimes only days, the possible survival of these organisms for even a short time should be regarded with concern. McDonough, Hargrove & Tittsler (1967) showed that salmonellae present in cottage cheese outlive the shelf life of the product. Recently, in cheeses made from artificially contaminated milk, salmonellae were found to survive for 9 months (Hargrove, McDonough & Mattingly, 1969). Goepfert, Olson & Marth (1968) found that for a 10000-fold decrease in numbers of salmonellae in Cheddar cheese at 13 °C a period of 10–12 weeks was required, and at 7·5 °C a period of 14–16 weeks. Mocquot, Lafont & Vassal (1963) in experiments with blue-veined cheese reported that the percentage survival of *Salmonella* in a 6-day-old cheese was 0·01.

Hargrove, McDonough & Mattingley (1969) manufactured 65 lots of Cheddar cheese and 7 lots of Colby cheese with artificially contaminated milk. The test organisms used were *Salmonella seftenberg*, *S. typhimurium*, *S. newbrunswick* and *S. newport*. Investigating the factors affecting the survival of salmonellae in Cheddar cheese and Colby cheese, they came to the conclusion that while the pH of the cheese and the starter had the greatest influence, their study revealed no single variable which could ensure that these types of cheese could not be a source of salmonellosis.

Salmonellae are known to survive high salt concentrations. Hargrove, McDonough & Mattingley (1969) found that salt concentrations of 1·80–2·63 % in cheese had no effect on the survival of these organisms. The growth of salmonellae in various concentrations of salt at different temperatures was investigated by Matches & Liston (1969) who found that salmonellae would grow at salt concentrations of 8 % at 37 °C. At temperatures below 37 °C they found 3 % NaCl to be slightly stimulatory. Goepfert *et al.* (1968) reported that the addition of 3 % NaCl at a pH of 4·9 had no detrimental effect on the multiplication of *S. typhimurium* other than to lengthen the lag phase and 4·5 % NaCl at the same pH did induce the death of the organism. Park, Marth & Olson (1969) studied the behaviour of *S. typhimurium* in low-acid cheese and observed that it grew rapidly during manufacture and additional growth occurred during the first 2 weeks of maturing at 13 °C, after which the numbers declined but viable organisms survived 3 months regardless of the ripening temperature (7 or 13 °C) and in one case more than 4 months. White & Custer (1969) artificially contaminated 52 vats with Salmonella strains. Three test strains, *S. newport*, *S. newbrunswick* and *S. infantis*, were used. Preliminary results indicated that the majority of cheeses contaminated with *S. newport* and *S. newbrunswick* yielded viable salmonellae for a period exceeding 120 days. No mention was made of any abnormality in manufacture. *S. typhimurium* has been shown by Goepfert *et al.* (1968) to multiply rapidly during manufacture of Cheddar cheese until the curd was salted. These authors also pointed out that it is hazardous to assume that the salmonella problem does not arise when the food is relatively acidic in nature.

The antagonistic effects of short-chain fatty acids on salmonellae in meat was reported by Khan & Katamay (1969). Evidence that the production of volatile fatty acids in curd may be responsible for the decline in numbers of salmonellae in cheese was reported by Goepfert *et al.* (1968). A decline in numbers of salmonellae was observed with 0·1 % acetic acid, together with 4·5 % NaCl at pH 4·9 in sterile milk; it was more rapid than that observed under the same conditions without acetic acid. Sorrells & Speck (1970) reported the inhibition of *S. gallinarum* by acetic acid produced by *Leuconostoc citrovorum*. On the other hand Hargrove, McDonough & Mattingley (1969) report finding no inhibitory effect of acetic acid on salmonellae. Volatile fatty acids, however, may contribute to the more rapid decline of salmonellae in blue-veined cheese (Mocquot *et al.* 1963).

Shigella

There are few reports of the presence of *Shigella* species in dairy products and foods in general compared with the occurrence of salmonellae. They occur less commonly in the community than do salmonellae and are not involved in cattle infections. A few outbreaks have been attributed to milk and reports of these cases have been reviewed by Bryan (1969). A case of *Shigella sonnei* infection from Saint Marcelin cheese led Rubinsten (1964) to investigate the survival of this organism in Saint Marcelin cheese kept under refrigeration. *Sh. sonnei* was isolated after 6 days storage but not after 7, 10 or 14 days.

Brucella

It has been shown that *Brucella abortus* survives ordinary cheese-making processes, and the long survival of *Brucella* strains in various types of cheese is well known (Harris, 1950; Gilman & Marquardt, 1951; Hammer & Babel, 1957). The survival of *Br. abortus* in cheese for 6 months was reported by Gilman, Dahlberg & Marquardt (1946). Rammel (1967) reviews the more recent published work on the survival of *Br. abortus* and *Br. melitensis* in cheeses of many varieties. *Br. melitensis* was isolated after periods of 4–16 days in Feta and up to 90 days in Pecorino.

In spite of the long survival of *Br. abortus* in Cheddar cheese, until 1947 (Gilman *et al.* 1946; Fabian, 1947) there had been no proven cases of undulant fever traced to this product, and since that time no cases attributable to cheese have been reported in the literature. *Br. abortus* does occur in cheese which reaches the consumer, as shown by Thatcher *et al.* (1956) who demonstrated its presence in 4 of 6 samples tested. The isolates were from Limburger, Romadur and "fromage-en-grain" (packed Cheddar curd). Unlike enterotoxigenic disorders and gastro-enteric diseases, the onset of which is rapid, the onset of undulant fever due to *Br. melitensis* and *Br. abortus* can vary from one week to several months, so that cheese as a source could escape detection. Epidemiological studies cited by Spink (1956) indicated that there was a high incidence of *Br. abortus* in bovine disease, and according to serological evidence a high number of people have been infected, yet there was a very small number of cases of clinical illness. The potential dangers associated with *Br. melitensis* in goat's milk however are greater, as this organism is the most infective for man of the 3 species, *Br. abortus*, *Br. suis*, *Br. melitensis*, while *Br. abortus* is the least. Fresh cheese made from goat's milk has undoubtedly played some part in human infection with *Br. melitensis*. Freshly prepared cheeses produced from unheated milk are consumed in many parts of the world and constitute an important source of infection of the populations concerned (Harris, 1950, Dalrymple-Champneys, 1960). Fortunately, *Brucella* strains are readily destroyed by pasteurization.

Mycobacterium tuberculosis

Improved control measures, which have almost eradicated tuberculosis in cattle in some countries, and the pasteurization of milk have reduced the importance of milk as a carrier of bovine tuberculosis to the human. In those countries where tuberculosis of cattle remains a problem there still exists the danger of transmission of *M. tuberculosis* by unpasteurized milk and dairy products.

Unless antibiotics produced in cheese by some of the ripening organisms or moulds (Meyer, Touillier & Malgras, 1952; Grecz, 1964) play a part, the long survival of *M. tuberculosis* can be expected. The organism, which is as heat-sensitive as most non-spore-forming bacteria, is however very resistant to chemical disinfectants, including acids and alkalis, and would therefore be perhaps less likely than other pathogens to be affected by the pH of the cheese.

Hammer & Babel (1957) reviewed the reported survival of *M. tuberculosis* in a number of varieties of cheese and came to the conclusion that the type of cheese was a factor which influenced survival. Cheese contaminated with *M. tuberculosis* has been shown to be infective after 220 days (Cheddar), 305 days (Tilsit) and

3 months (Camembert). Frahm (1959) also reported that the viability of *M. tuberculosis* varies greatly with different cheeses. Virulent organisms were present in Camembert and Edam after 2 months and more. In Emmental there was a decline in numbers of tubercle bacilli after heating the curd to 55 °C. Hahn (1959) examined 6 Emmental cheeses made from artificially infected milk, one of which produced tuberculous lesions in one of 8 guinea-pigs injected with it after 3 months of maturation of the cheese. Quarg infected with very low numbers of tubercle bacilli produced the disease when fed to guinea-pigs. Ĭotov & Todorov (1959) found virulent tubercle bacilli in Bulgarian white cheese, made from artificially infected milk, after 120 days storage. Meyer, Touillier & Malgras (1952) investigating the decline in numbers of tubercle bacilli in the first 4 weeks of maturing of Emmental and Gruyère cheese reported that rapidly growing propionic acid bacteria produced a bactericidal substance active on tubercle bacilli.

All this evidence points to the need to pasteurize milk for cheese, and thereby to destroy all pathogens, if outbreaks of cheese-borne disease are to be avoided; other procedures allowed at present in some countries, e.g. minimum maturing periods of 60, 90 or 120 days before sale, seem unjustifiable in the light of present knowledge. It cannot be assumed that such storage eliminates the risk in all cheeses for the consumer as there is sufficient evidence that pathogens and enterotoxin can survive for even longer periods. Walker *et al.* (1961) have pointed out that the reduction in numbers of staphylococci that occurs during storage for 60 days (the period usually permitted as an alternative to pasteurization) may not make the cheese less dangerous because of the possible survival of the enterotoxin, and similarly, Hammer & Babel (1957) have cautioned against the holding of cheese made from raw milk for a given period as a reliable safety measure. Other possibilities such as heat-treatment at a temperature lower than pasteurization may slow down the multiplication of pathogens during manufacture, but may not destroy them nor necessarily increase their sensitivity to those factors which can contribute to their inactivation. The H_2O_2–catalase treatment of milk for cheese manufacture, popular in the United States, does not ensure the destruction of pathogens (Keogh, 1964). Thus, procedures other than pasteurization may reduce the risk of cheese-borne outbreaks of disease but will not eliminate it. No procedure will destroy enterotoxins formed in the milk before cheese-making, and pasteurization will not destroy the organisms which may contaminate the cheese during manufacture. As far back as 1912, Sammis & Bruhn strongly recommended pasteurization of milk for cheese manufacture and this has since been reiterated by many others (Harris, 1950; Hammer & Babel, 1957; Thatcher *et al.* 1959; Sharpe *et al.* 1962; Keogh & Hansen, 1963; Czulak, 1965).

The survival of all potentially pathogenic contaminants must be considered. It would be unwise to accept recommendations based on tests concerning the survival of any one bacterial species or strain.

The fact that cheese of more uniform quality can be made from pasteurized milk (Czulak, 1965) is surely confirmed by the practice in those countries where pasteurization of milk for cheese manufacturing is now either compulsory or widely used. There is therefore no valid reason for not using pasteurization as an effective preventive treatment.

Milk powder

Modern processing methods for the manufacture of milk powder are designed to render the product more acceptable in flavour and solubility, and because of them the product has become more vulnerable to contamination and to the growth of contaminants during manufacture. The milder heat treatments during spray-drying compared with those used in roller-drying afford little protection against the survival of pathogens. The instantization process in which the powder is wetted and re-dried introduces a new loophole for contamination. Modern packaging techniques, designed to prevent chemical deterioration, allow longer survival of micro-organisms. Unfortunately, modern processing techniques are not always coupled with improved hygienic practices.

Today, milk powder is used as an additive to other foods and in the manufacture of recombined products such as sweetened condensed milk and evaporated milk (Muller, 1965; Muller & Kieseker, 1965). For these purposes some purchasers require milk powder made from milk which has received a mild heat-treatment (sometimes only marginal H.T.S.T. pasteurization) to fulfil the specification for whey protein nitrogen values. At the same time, however, powders with low bacterial counts are specified. This does not necessarily ensure the absence of pathogens since they can be present in low-count milks or can occur as a result of post pasteurization contamination. Such powders are intended for products which receive further heat-treatment during manufacture, and they should not be used for other purposes, nor should they be used if the time elapsing between reconstitution and heat-treatment is sufficiently long to allow bacterial multiplication. The danger exists, however, that contaminated powder in a factory may cause the infection of other powders or foods prepared there.

The stages during processing and the means by which milk powder may be contaminated and contaminants may multiply and produce toxins have been described by Crossley & Johnson (1942), Crossley (1945), Anderson & Stone (1955), Hawley & Benjamin (1955), Crossley & Campling (1957), Keogh (1966), Schroeder (1967), Galesloot & Stadhouders (1968). Contamination can occur at a number of stages after the preheating of the milk. It may be from the milk handler, dirty equipment, water, dust, rodents, birds, vermin and from infected powder carried in air currents. Heldman, Hall & Hedrick (1968) have illustrated diagrammatically the stages in processing of milk powder where the product is in contact with air. In the production of instantized powder the process entails such contact at 9 stages. Air filtration at all stages would be a safeguard provided the filters are efficient. Collins *et al.* (1968), following an inter-state outbreak of *S. newbrunswick*, reported that results of a survey carried out in a factory, whose milk was implicated, pointed to the instantizing process as the source of contamination.

McDonough & Hargrove (1968) studied the heat resistance of salmonellae and the effects of storage on survival in dried milk. Their study showed that while certain heat and moisture combinations will aid in lessening the chances of salmonellae surviving during the drying operation, they cannot be relied upon for complete control. It was also shown that while pasteurization destroys salmonellae in liquid milk, higher temperatures are required to kill them in concentrates. This is an

important point to consider since it is the practice in some areas to intership concentrates among plants for further processing.

LiCari & Potter (1970*b*) showed that, while spray-drying at temperatures usually employed commercially killed substantial numbers of *Salmonella* in skim-milk, in no case in their investigations did it render the milk powder completely free from *Salmonella*. In further work with agglomeration and redrying of the contaminated powder, significant reduction in salmonellae was not achieved by the heat of redrying.

Storage at different temperatures of milk powder contaminated with salmonellae showed little reduction in the numbers of viable salmonellae in 15 weeks. The high storage temperatures which bring about destruction of the salmonellae in powder would be expected to affect adversely the flavour of the product (McDonough & Hargrove, 1968). This was confirmed by LiCari & Potter (1970*c*).

That pathogens and their toxins survive in milk powder is evidenced by the outbreaks of staphylococcal food poisoning in Britain in 1953 (Anderson & Stone, 1955; Crossley & Campling, 1957) and in Puerto Rico in 1956 (Armijo, Henderson, Timothée & Robinson, 1957) and of gastro-enteritis due to *Salmonella* in Britain in 1950, Bulgaria in 1945–1950, in the United States in 1966 and 1967 (Marth, 1969) and in Newfoundland in 1968 (Julseth & Deibel, 1969). Schroeder (1967) reported that a survey carried out in the United States of America, in which 3315 samples of dried milk from 200 factories in 19 States were examined by health authorities, revealed that 1 % of these were contaminated with salmonellae and that 8·2 % of 1475 environmental samples contained salmonellae. Twenty-one different serotypes were isolated. Relatively small numbers of salmonellae in a food product which is not heat-treated before consumption can be significant. Low numbers in milk powder grow rapidly upon rehydration and incubation (Julseth & Deibel, 1969).

It is clear that personnel involved at any stage in the production of milk powder must be alert to all the bacteriological hazards and be vigilant about hygiene if food poisoning caused by contaminated milk powder is to be eradicated.

B. Bacteriological analysis

Staphylococci

In the last decade many new media have been developed for the isolation of pathogenic staphylococci from clinical sources and from foods (Carter, 1960; Innes, 1960; Vogel & Johnson, 1960; Finegold & Sweeney, 1961; Hopton, 1961; Baird-Parker, 1962*a*; Deneke & Blobel, 1962; Carantonis & Spink, 1963; Crisley, Angelotti & Foter, 1964; Blair, Emerson & Tull, 1967; Elliot, 1968; Hobbs, Kendall & Gilbert, 1968; Holbrook, Anderson & Baird-Parker, 1969 and others). These media are selective because of their salt concentrations ranging from 6·5 to 10 % or because they incorporate other substances such as potassium tellurite, glycine, lithium chloride, sodium azide or antibiotics. Various differential agents such as mannitol, phenolphthalein phosphate, and egg yolk are sometimes included. Most of the media are modified versions aimed at ensuring less inhibition of pathogenic staphylococci or better differentiation between coagulase-positive and coagulase-negative strains than the original media. Many have been found satisfactory for the isolation of pathogenic staphylococci from clinical sources but less so for the

isolation of pathogenic staphylococci from foods. Hobbs *et al.* (1968) list 8 different selective media for coagulase-positive staphylococci and the countries in which they are widely used. There is a lack of comparative data on the isolation of pathogenic staphylococci from dairy products, especially cheese and milk powder, which are the products most frequently implicated in food poisoning outbreaks in recent years. Many of the comparative tests have been made on cultured isolates from these products rather than using contaminated products themselves. Such comparisons are valuable but suffer from the disadvantage that they do not reflect the effects of the initial numbers of organisms present and the effects of the debilitating influence of processing and environment on particular strains of the contaminants and their subsequent growth on the various selective media. The previous cultivation history of the organism influences its growth on selective media (McDivitt & Topp, 1964; Ordal, 1970). Drying, heat-treatment, pH, salt content and oxygen tension of the food could all affect the recoverability of these organisms. Some of these factors have been shown to have this effect (Baird-Parker & Davenport, 1965). Iandolo & Ordal (1966) found that after sublethal heat-treatment at 55 °C for 15 min, 99% of the viable population of *Staph. aureus* were unable to reproduce on media containing 7·5% sodium chloride. Also, the contaminated product itself and the other types of contaminants may have some effect on the selectivity of the particular medium (Joshi, Blackwood & Dale, 1963; Jay, 1963; Crisley, Peeler & Angelotti, 1965).

Table 1 shows the results of some of the comparative tests with various media for the isolation of pathogenic staphylococci from foods. It could well be that less complex and possibly a less inhibitory medium such as sheep blood agar containing 7·5% salt (Keogh & Hansen, 1963) might be more reliable although, perhaps, less selective. This medium has not been compared with some of the more complex ones. Sheep blood cells are not lysed by the presence of 7·5% sodium chloride. Reiter *et al.* (1964) and Baird-Parker & Davenport (1965) showed that the recovery of heat-shocked cells of *Staph. aureus* was better on blood agar than on Baird-Parker's medium. Rammel & Howick (1967) used blood agar for the isolation of coagulase-positive staphylococci from cheese. Baird-Parker & Davenport (1965) found that media such as S110 medium and phenolphthalein phosphate agar (Barber & Kuper, 1951), which are poor for the isolation of *Staph. aureus* cells that have been subjected to heat or drying (Busta & Jezeski, 1961; Sharpe *et al.* 1962; Mickelsen *et al.* 1963; Baird-Parker & Davenport, 1965), can be improved by the addition of pyruvate. They suggest that blood and pyruvate have in common the ability to break down hydrogen peroxide, which may account for the fact that media containing these substances are best for the recovery of air-dried, freeze-dried and heat-shocked cells of *Staph. aureus*.

Baird-Parker's medium (1962*a*) has been favoured by a number of authors and has been thoroughly investigated using foods in general and pure cultures (Table 1). However, this medium is rather unstable and fails to inhibit *Proteus* which gives a reaction similar to that of coagulase-positive staphylococci. Recently, a more stable version of this medium has been developed and sulphamezathine has been added to inhibit *Proteus* (Holbrook *et al.* 1969). Such comprehensive comparative studies for the use of Baird-Parker's medium—or indeed any other media for the

isolation of pathogenic staphylococci from milk-powder and cheese—have not been reported but on the little evidence available Baird-Parker's appears to be the medium of choice. There has been some uncertainty as to the relationship between the egg-yolk reaction and coagulase production. Koskitalo & Milling (1969) used Carter's (1960) medium in which egg-yolk was added to S110 medium and found that the egg-yolk reaction failed to differentiate between coagulase-negative and coagulase-positive staphylococci from Cheddar cheese. Gare (1969) found that of 1000 coagulase-positive staphylococci isolated from nasal swabs and clinical smears 21·8% were egg-yolk negative, and later Gare (1970) observed differences between phage groups with egg-yolk reaction. The validity of the egg-yolk reaction for the recognition of coagulase-positive staphylococci has been questioned also by Jay, 1966. Baird-Parker (1962) commented on the interference of fermentable carbohydrate, including mannitol, with the typical egg-yolk reaction.

Enrichment methods are probably not always necessary for the isolation of staphylococci from foods which are suspected as being agents in food poisoning, because these organisms may be present in large numbers. On the other hand, the numbers may have declined and bear no relationship to the presence of enterotoxin. Enrichment methods are important in quality control tests for coagulase-positive staphylococci in foods since even low numbers may be of ultimate significance and those that are present may not be easily revived. The literature is lacking in comparative data for enrichment methods for dairy products. The most commonly used enrichment media are listed in Table 2.

Giolitti & Cantoni (1966) developed an anaerobic glycine–tellurite-broth medium which they compared with Chapman's (1945, 1946) mannitol salt agar, Buttiaux & Brogniart's (1947) medium and blood agar for the isolation of coagulase-positive staphylococci from foods, including milk products. They claimed that it was superior to these media, its selectivity being due to the presence of lithium chloride and tellurite together with glycine. The inhibition of micrococci is balanced against the enhancement of staphylococci by the creation of anaerobic conditions. A further advantage of the medium was that it was possible to inoculate it with larger samples than is usual if other media are used.

Chou & Marth (1969) have compared direct plating and enrichment methods for the isolation of coagulase-positive staphylococci from frozen foods. Greater recovery using enrichment procedures was observed. Results of the comparative tests were greatly influenced by the type of agar medium used to recover the staphylococci from the enrichment medium and also by the time of incubation.

Carter (1960) reported that direct plating of frozen foods was essentially as efficient as selective enrichment prior to plating but Gilden, Baer & Franklin (1966) found better recovery when prior enrichment was used for the recovery of staphylococci from frozen foods.

In instances where foods have been frozen or the staphylococci have been subjected to low heat-treatment or drying, enrichment media might be expected to give better recovery of these organisms. However, for purposes of estimating numbers by the enrichment method it is necessary to use the 'Most Probable Number' technique of estimation.

The mode of infection of a product such as cheese will influence the distribution

Table 1. *Synoptic comparison of media for the isolation of coagulase-positive staphylococci*

Authors	Media compared	Product	Remarks
Baird-Parker (1962*a*)	A. Egg–tellurite–glycine–pyruvate agar (ETGPA) (1) B. Phenolphthalein-phosphate agar (2) C. Tellurite–glycine agar (11) D. Tellurite–glycine agar (9) E. Tellurite egg-yolk agar (7)	Meat Fish	ETGPA favoured
Baird-Parker (1962*b*)	A. ETGPA (1) F. Peptone yeast extract glucose agar G. Blood agar	Pure cultures	ETGPA was better than peptone yeast extract glucose agar but gave slightly lower counts than on blood agar. Author tabulates comments from other laboratories for testing various foods
Sharpe *et al.* (1962)	H. Modified mannitol salt agar, with only 6·5 % NaCl, and mannitol omitted (4) B. Phenolphthalein–phosphate agar (2) A. ETGPA (1)	Milk and cheese	ETGPA favoured
Jay (1963)	J. S 110 + egg-yolk K. Mannitol salt agar (3) C. Tellurite–glycine agar (11) L. Polymyxin agar (6) M. Vogel-Johnson's medium (10) N. Mannitol sorbic acid broth (8)	Meat	S 110 + egg-yolk most effective
Marshall, Neighbors & Edmondson (1965)	C. Tellurite–glycine agar (11) M. Vogel-Johnson's medium (10) K. Mannitol salt agar (3) O. S 110 medium (4) P. Tellurite polymyxin egg-yolk agar (TPEY) (5)	Dry milk and pure cultures	Tellurite–glycine agar and Vogel-Johnson's medium too inhibitory for enumerating small numbers. Favoured TPEY Mannitol salt agar allows bacilli to grow
Crisley *et al.* (1965)	P. TPEY (5) A. ETGPA (1) E. Tellurite–egg-yolk agar (7) O. S110 medium (4) Q. Trypticase soy agar (as control)	Staphylococci isolated from various foods	TPEY favoured
Thieulin *et al.* (1966)	K. Mannitol salt agar (3) A. ETGPA (1)	Milk and fresh cheese	ETGPA favoured
De Waart, Mossel, ten Broeke & Van de Moosdijk (1968)	A. ETGPA (1) R. Horse blood agar K. Mannitol salt agar (3) S. Mannitol salt agar + sodium azide	Staphylococci isolated from various foods	ETGPA favoured
Holbrook *et al.* (1969)	T. Modified ETGPA A. ETGPA (1)	Meat and pure cultures	Modified medium was more stable and eliminated *Proteus*
Munch-Petersen (1970)	U. Ox blood agar V. Neave's medium A. ETGPA (1) O. S110 medium (4)	Milk	Ox blood agar and ETGPA favoured

Table 1 (*cont.*)

Authors	Media compared	Product	Remarks
Chou & Marth (1969)	K. Mannitol salt agar (3) O. S110 medium (4) P. TPEY (5) M. Vogel-Johnson's medium (10) C. Tellurite–glycine agar (11)	Frozen feeds of animal origin	Mannitol salt agar and S110 recovered more coagulase-positive staphylococci than the other media

References: (1) Baird-Parker, 1962*a*, *b*; (2) Barber & Kuper, 1951; (3) Chapman, 1945; (4) Chapman (1946); (5) Crisley *et al.* 1964; (6) Finegold & Sweeney, 1961; (7) Innes, 1960; (8) Raj & Liston, 1961; (9) Sevel & Plommet, 1960; (10) Vogel & Johnson, 1960; (11) Zebovitz, Evans & Niven, 1955.

of staphylococci within a cheese and within the cheeses from a vat. It is possible that if no statistical basis of sampling is adopted (which is often the case), high counts obtained by including a single colony by chance in a sample of cheese may lead to erroneous interpretation of the incidence of staphylococci, for the one cheese or for cheeses from a vat. Alternatively, absence of staphylococci in a sample may also lead to erroneous interpretations. Rammel & Howick (1967) showed a significant difference in the variance between each of 5 cheeses from a given vat in 7 of 13 vats examined. Enterotoxin also could be in localized regions. This was taken into account by Zehren & Zehren (1968*a*).

The reliability of coagulase production as a criterion of pathogenicity and toxigenicity has been questioned. Thatcher & Simon (1956) reported that some enterotoxigenic staphylococci found to be coagulase-negative on first isolation became coagulase-positive later, after subculturing for 5 months, and were shown to belong to phage Group III. Silverman *et al.* (1961) also questioned the correlation between coagulase production and enterotoxigenicity. The occurrence of coagulase-negative staphylococci as a causative agent in disease has also been reported (Smith & Farkas-Himsley, 1969).

Table 2. *List of enrichment media for the detection of pathogenic staphylococci*

Medium	Reference
Cooked meat medium + 10 % NaCl	Maitland & Martyn (1948)
Trypticase soy broth + 10 % NaCl	Baer (1966)
Anaerobic glycine tellurite broth	Giolitti & Cantoni (1966)
Lactose salt broth	Buttiaux & Brogniart (1947)
Brain heart mannitol salt broth	Wilson, Foter & Lewis (1959)
Sorbic acid mannitol broth	Raj & Liston (1961)

Grossgebauer, Schmidt & Langmaack (1968) suggest that lysozyme is a better indicator of pathogenicity than coagulase, especially in strains of animal origin and that it could be used to differentiate between pathogenic and non-pathogenic coagulase-negative staphylococci. Victor, Lachica, Weiss & Deibel (1969) found a good correlation between the presence of heat-stable deoxyribonuclease and enterotoxin production. The use of the DN-ase test (Jeffries, Holtman & Guse, 1957) alone or together with coagulase to differentiate between pathogenic and non-pathogenic strains of staphylococci has gained some popularity in recent years (DiSalvo, 1958; Jacobs, Willis & Goodburn, 1963, 1964; Thieulin *et al.* 1966; Blair *et al.* 1967).

Some enterotoxin considerations

Advancement in knowledge of staphylococcal enterotoxin has been considerable in the past decade. Methods have been developed for serological detection and identification of enterotoxin in foods and culture filtrates. The extraction, concentration and serological testing of foods and culture filtrates for enterotoxin has been reviewed by Angelotti (1969). The serological methods for the detection and identification of enterotoxin include gel diffusion, precipitation, immuno-fluorescence and haemagglutination techniques, and, in addition, a flotation antigen-antibody system has been described (Hopper, 1963). Before gel diffusion techniques are used the enterotoxin must be extracted and concentrated. Casman (1967) describes a technique for the extraction and concentration of enterotoxin from high protein foods including cheese. Zehren & Zehren (1968*a*) also describe the technique for the extraction and concentration of enterotoxin from cheese and its detection by microslide precipitation diffusion.

Friedman & White (1965) demonstrated cell-associated enterotoxin. Genigeorgis & Sadler (1966*a*, *b*) described an immuno-fluorescence technique for the detection of enterotoxins which confirms this observation. The demonstration of enterotoxin was based on the presence of morphologically specific fluorescent precipitates around the bacterial cells and not on the presence of fluorescent cells alone. A similar immuno-fluorescence technique has been described by Stark & Middaugh (1969), who claim that the technique, because of its sensitivity, eliminates the necessity for extraction and concentration of the enterotoxin. Genigeorgis & Sadler (1969) reported the elimination of non-specific staining in immuno-fluorescence methods by neutralizing the cell reactive sites using normal rabbit serum.

Silverman, Knott & Howard (1968) described 'reverse passive haemagglutination' for the detection of enterotoxin B. Because of the sensitivity of the test no concentration of the enterotoxin in extracts and culture filtrate is required. Results of the test are available within a few hours.

It is now known that there are at least 4 serologically distinct staphylococcal enterotoxins (A, B, C, D) and there is at least one unclassified (Bergdoll, 1969).

The effects of growth conditions on the production of enterotoxin have been investigated by a number of workers. It was shown that enterotoxin was produced in the latter part of the exponential phase of growth and could be produced by non-replicating cells (Markus & Silverman, 1968). Later it was reported by other workers (Morse, Mah & Dobrogosz, 1969) that enterotoxin production occurred in the transition period from the exponential to the stationary phase. Aeration of the culture appears to be a part of many methods for the production of enterotoxin and has been shown to increase enterotoxin production (Stark & Middaugh, 1969). Genigeorgis & Sadler (1966*b*), investigating enterotoxin production in a medium using a range of sodium chloride concentrations from 2 to 16% and with the initial pH of the medium varying from 5·1 to 6·9, found that growth of a *Staph. aureus* strain and its enterotoxin production was better when the pH was increased and the salt concentration decreased. There was an interaction of these 2 factors. Similar observations on the effects of sodium chloride and pH were made by Reiser & Weiss (1969). Drye & Mah (1969) investigated the effects of casein hydrolysates on

enterotoxin B production. Of 4 different casein hydrolysates tested, protein hydrolysate powder produced the highest differential rate of enterotoxin to total protein synthesis by the staphylococci. The component responsible for the stimulation was dialysable and was also present in a methanol extract.

Methods for the production of enterotoxin by staphylococci have been reviewed by Angelotti (1969). For the production of enterotoxin A, B, and C, Jarvis & Lawrence (1970) used on the one hand the cellophane-over-agar technique of Hallander (1965) with brain heart infusion agar and with casein hydrolysate agar, and on the other hand aerated cultures using brain heart infusion and casein hydrolysate. The effects of the 2 media were marginal but the concentration of enterotoxin was 4–8 times greater using the cellophane technique than in aerated cultures.

From the foregoing it appears that there is still much to be learned about the relation of various characteristics of staphylococci with their pathogenicity and enterotoxigenicity. At present no single medium seems to be completely reliable for the isolation and identification of enterotoxigenic staphylococci without confirmation by some other test. However, in the light of present progress in this field it may not be long before such a single selective medium becomes available.

Salmonella

It is now established that foods have a marked effect on the selectivity of media for the isolation of *Salmonella* (Hurley & Ayres, 1953; Silliker & Taylor, 1958). This effect varies in extent with different foods and is thought to be due to the water-soluble components of the food. The need to determine the optimum selective medium for each particular food is apparent.

The problems associated with the detection of *Salmonella* in foods have been discussed by Silliker & Greenberg (1969) and earlier literature was reviewed by McCoy (1962). The reliability of media for the isolation of *Salmonella* is assessed not only by the number of positive isolations made but also by the number of *Salmonella* serotypes isolated, because some selective media do not permit the isolation of all types (Banwart & Ayres, 1953). It is usual practice to employ at least 2 selective enrichment media and several selective solid media for the isolation of salmonellae for both clinical and food investigations. Because of the debilitated state of the organisms in foods it is the practice in some laboratories to include a pre-enrichment method to rejuvenate the organisms before subjecting them to selective media, and this has proved effective (North, 1961; Taylor & Silliker, 1961; Taylor, 1961). The efficiency of the pre-enrichment medium will be influenced by the total flora and particularly the coliform content of the food being tested. It is therefore advisable to inoculate the food sample initially into both pre-enrichment and enrichment media. In some cases the food itself when suspended in a diluent may be used as a pre-enrichment medium (McCoy, 1962). Poelma, Wilson, Romero & Padron (1969) reported the results of collaborative studies on artificially contaminated milk powder samples. The samples were reconstituted with distilled water (100 g/l) and 2 ml of 1 % aqueous brilliant green solution was added. The mixture was incubated at 35 °C for 24 h, subcultured into selenite cystine broth, incubated and streaked on selective agar plates. Good agreement between collaborative results was

obtained. In experiments involving spray-dried milk powder, LiCari & Potter (1970*a*) found the use of lactose broth (North, 1961) as a pre-enrichment medium to have no advantage over the method of dissolving milk powder in sterile distilled water containing 0·002% brilliant green dye and incubating for 18–24 h at 37 °C. They found tetrathionate broth with 0·001% brilliant green dye and 1·25 ppm sodium sulphathiazole (to inhibit *Proteus*) as the enrichment medium and bismuth sulphite agar as the selective solid medium to be satisfactory. They claimed that by applying a MPN technique to this method of isolation they were able to recover known levels of *Salmonella* quantitatively from spray-dried milk powder.

Different periods of incubation of the pre-enrichment and enrichment tests have been advocated. A temperature of 37 °C is most commonly used. Harvey & Thomson (1953) suggested a temperature of 43 °C as being more selective but the advantage of the higher temperature of incubation is in doubt (Hobbs, 1962). North (1961) advised incubation of lactose broth pre-enrichment cultures for 24 h at 37 °C while Montford & Thatcher (1961) recommended a 5-h incubation at this temperature. It is advisable that most selective enrichment media are subcultured on solid media after both 24 and 48 h incubation to cover the possibilities of slow growth of the salmonellae or overgrowth by contaminants in the selective enrichment. Taylor & Silliker (1961) compared methods of enrichment for the isolation of *Salmonella* from foods. Pre-enrichment in lactose broth was superior to reconstitution of egg albumin in water, followed by inoculation into both selenite and tetrathionate broths. The effect of thermal injury on the recovery of *S. typhimurium* was investigated by Clark & Ordal (1969). When injured cells were placed in trypticase soy broth, nutrient broth, lactose broth or lauryl tryptose broth they recovered and grew at a rate equal to that of normal cells. Recovery of injured cells occurred in tetrathionate broth and selenite F broth but these were not quite satisfactory growth media for the organism.

Improved isolation of *Salmonella* from foods without using lactose broth enrichment is claimed by Alford & Knight (1969). Shaking the sample in a basal medium for 4 h at 37 °C before adding selenite and cystine, and then incubating for 24 h resulted in a time-saving of 24 h and the use of one less medium.

In addition to the lengthy and expensive procedures entailed in the detection of *Salmonella* and the biochemical and serological identification of the organism, it is necessary to test many large samples of the food because of the possible uneven distribution of the salmonellae which may be present in very low numbers. Since the recovery of *Salmonella* from milk powder in the United States in 1966 and 1967, a demand for salmonella-free milk powder and dairy products of other types has highlighted the urgency of establishing the reliability of existing methods for the detection of *Salmonella* when applied to dairy products and ideally of developing more rapid and less costly methods for routine control purposes. It has been the practice to use methods applicable to dried egg-yolk and other foods to dairy products. It would seem that the reliability of such methods for individual dairy products has not been thoroughly investigated.

Read & Reyes (1967) compared 8 enrichment procedures for the isolation of *Salmonella* from milk. They reported that tetrathionate broth with iodine and tetrathionate broth with iodine and brilliant green incubated at 41·5 °C for 24 h were

most effective. Lang (1968) compared the efficiency of potassium tetrathionate metachromatic yellow broth, potassium tetrathionate crystal violet broth and selenite broth for the testing of milk. He favoured selenite broth and strongly advised against the use of potassium tetrathionate metachromatic yellow broth.

It is frequently necessary, when testing cheese or milk powder, to emulsify these products with the help of sodium citrate. There appear to be no data available as to the effect of this step on the subsequent concentration of the organisms by enrichment media.

It is not intended here to list the selective enrichment or the selective solid media currently used. These have been reviewed and discussed by Silliker & Greenberg (1969) and the media are listed in standard texts.

The need to establish more rapid and less expensive routine methods for the detection of *Salmonella* in foods has been well recognized by some researchers and work is progressing. Silliker & Greenberg (1969) have expressed the view that for routine control purposes 'a single selective medium may produce more "total positives" and therefore may effect better "total control".' Whether pre-enrichment is practiced and which selective medium is chosen would be dictated by the nature of the food and previous experience.

Some new methods for the isolation of *Salmonella* from foods, although not as yet applied to dairy products, will be mentioned here. Some of them need more extensive assessment on foods which are naturally contaminated. They also need to be assessed for use on the individual dairy products. Silliker & Taylor (1958) have pointed to the hazards that might arise from borrowing methods applied to one type of product and applying them to another without question.

A possible future solution may be in immuno-fluorescence techniques which at this stage are not without problems. The application and reliability of these techniques have been reviewed by Goepfert & Insalata (1969). Immuno-fluorescence techniques still require concentration of the cells by pre-enrichment or selective enrichment. Reamer, Hargrove & McDonough (1969) reported that inclusion of trypsin in the pre-enrichment medium increased the sensitivity of both a standard culture method and an immuno-fluorescence method for the detection of *Salmonella* in non-fat dried milk. Preliminary experiments indicated that coliforms were affected by the trypsin but there was no adverse effect on *Salmonella*.

Hargrove, McDonough & Reamer (1969) have developed a medium which they claim permits the pre-enrichment step to be omitted. It is sufficiently selective to inhibit the growth of contaminating organisms. The medium is based on the ability of *Salmonella* to decarboxylate lysine or produce H_2S or both. Novobiocin is included to inhibit Gram-positive contaminants, and trypsin to digest casein. Dairy products, when added to selective media, may become clotted and there is often difficulty in attaining complete emulsification of some of them, e.g. young cheese, so that *Salmonella* trapped within the clot or lump might be missed. The addition of trypsin would reduce this risk. The medium has been used successfully for the isolation of *Salmonella* from artificially contaminated raw milk, skim-milk powder, cottage cheese and Cheddar cheese.

Dulcitol-selenite broth in a motility flask was used for the detection of *Salmonella* from food. A drop in pH in the enrichment broth indicated the presence of *Salmonella*

which was confirmed by a fluorescent staining method (Abrahamsson, Patterson & Riemann, 1968). The authors claimed that the technique permitted the detection of even small numbers of *Salmonella* in 1–2 days.

Banwart & Kreitzer (1969) also employed a glass apparatus for the detection of *Salmonella* from egg noodles, cake mixes and candies. The system included motility, biochemical, and serological tests and the authors claimed that one *Salmonella* cell inoculated into the sample could be detected in 48 h.

An accelerated procedure for the detection of *Salmonella* in dried foods and feeds was described by Sperber & Deibel (1969). This method still required the use of pre-enrichment and enrichment cultures but the plating step was omitted and serological tests were made after culturing in a post-enrichment broth medium. They claimed the procedure to be rapid and accurate in comparison with traditional methods (Fantasia, Sperber & Deibel (1969).

It would seem that in the present state of knowledge relating to the routine testing of dairy products for *Salmonella* it would be unwise to place too much reliance on the methods used for the testing of other foods. More work needs to be done to assess methods in relation to the individual dairy products. At the same time bacteriologists in the dairy industry should continue their efforts to develop more rapid, reliable and less expensive methods for routine control purposes.

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ERRATA

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P. 13:

In Table 4, the values for 2 % sodium citrate should be 115·0, 179·5, 0·55 instead of 6, 25, 35.

P. 365:

In Table 2, read α_s- for $\alpha_s=$ throughout.

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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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