Vol. 31, No. 2 June, 1964

THE JOURNAL OF DAIRY RESEARCH

CAMBRIDGE UNIVERSITY PRESS
Bentley House, 200 Euston Road, London, N.W. 1
American Branch: 32 East 57th Street, New York 22, N.Y.

Price:—THIRTY SHILLINGS *Net*
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The Journal of Dairy Research

was established in 1929 as a medium for the publication of the results of original research in dairy science and cognate subjects. It is published by the Cambridge University Press.

The subscription to the *Journal* is 80*s*. per volume, for orders placed in advance of publication, and covers three issues. Single copies may be purchased at 30*s*. each. Postage extra. Subscriptions may be sent to any bookseller or to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1. The subscription price in the U.S.A. is $13.50. Enquiries should be sent to the Cambridge University Press, American Branch, 32 East 57th Street, New York 22, U.S.A.

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VOL. 47 APRIL 1964 NO. 4

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J. Dairy Res. (1964), **31**, 137
With 2 plates

Some factors influencing stickiness of butter

By F. I. THOMASOS and F. W. WOOD
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(*Received* 14 *August* 1963)

Summary. The term stickiness, as used in the butter industry, refers to that property of butter which permits it to remain attached to solid surfaces. It is a phenomenon in which the components of force caused by adhesion and cohesion are inseparably involved and have been described by the term 'hesion'. Hesion measurements of commercial samples of conventionally-made butter were much higher than those of continuously-made butter. The results of experiments on conventional and laboratory continuously-made butter from the same butterfat source indicated that the characteristic crystal structure influenced the hesion values. Homogenization of Gold'n Flow and conventional commercial butters markedly increased hesion readings. When nitrogen gas was added to pre-crystallized continuously-made butter the hesion values decreased with increasing gas content. However, there was an increase in the amount of butter which remained on the adherend when it became detached from the butter surface. Limited experiments on the effect of gas content in conventional butter also indicated that an increase in gas content resulted in a decrease in hesion values with more butter remaining on the adherend. The results of this investigation indicated that the crystal structure was responsible for the adhesive property of butter and the gas content influenced the cohesive property. It would appear, then, that both the crystal structure and the gas content play an important part in causing stickiness of butter.

When butter adheres to and remains in contact with the surfaces of solid materials such as churns, printers, wrapping material, etc., the phenomenon is commonly referred to as 'stickiness'. This is a physical characteristic which involves both adhesion and cohesion. Claassens (1958) used the term 'hesion' in preference to stickiness because, in measuring the phenomenon by the application of a vertical pull at the adherend/butter interface, the combined effect of the forces resulting from adhesion and cohesion is estimated.

A number of workers (Richardson & Abbott, 1935; Coulter & Combs, 1938; Hunziker, 1940; Wilster, Jones & Haag, 1941) have drawn attention to the problem of sticky butter. These investigators have associated stickiness with variations in the butterfat composition and the technological treatments given to the butterfat. Wilster *et al.* (1941) have shown that the defect is more common during the winter months when the cows are fed dry roughage, especially alfalfa hay, which decreases the quantity of low melting-point glycerides in the milk fat. Most of the recommen-

dations for controlling stickiness involve the rate and temperature of cooling of the cream and the temperature of the wash water (Richardson & Abbott, 1935; Coulter & Combs, 1938; Wilster, Stout, Stein, Haag & Jones, 1942; Zottola, Wilster & Stein, 1961).

Claassens (1958) was the first to measure stickiness objectively, and this has led to a better understanding of the fundamentals of the phenomenon. He developed the 'hesion balance' with which he measured the hesion of butter to different materials and showed that it was dependent upon the time of contact, the nominal load, the temperature of the butter and the physico-chemical nature of the adherend surface (Claassens, 1959).

Jansen (1961) modified Claassens's apparatus and made it more convenient to use. He found that the relationship between hesion and extruder thrust, extruder friction and liquid fat index was dependent on temperature. Jansen also stated that repeated working of butter in a laboratory-scale blender showed an increase in hesion after the first working and a decrease after each of 6 further workings.

Reported investigations on stickiness have been conducted on conventional (churned) butter. However, it has been reported (deMan & Wood, 1958*b*) that Gold'n Flow, continuously-made butter (made by a process which is copyrighted by the Cherry-Burrell Corporation, Cedar Rapids, Iowa, U.S.A.), is unique in lacking the stickiness of conventional butter under normal handling conditions. This paper is a report of factors that influence the stickiness of both types of butter.

EXPERIMENTAL

Sources of butter

The commercial Gold'n Flow and conventional butters were obtained from local creameries. The butterfat concentrate for the manufacture of continuously-made butter in a laboratory continuous buttermaking machine, described by Wood & Thornton (1956), was obtained from 30-lb lots of conventional butter that were melted after removal of samples required for normal physical determinations. This permitted observations to be made on conventional and continuously-made butter from the same butterfat source. Butter made with this laboratory machine resembles Gold'n Flow butter and is referred to as non-precrystallized butter. With the addition of a precrystallization unit to the above laboratory buttermaker (McKnight & Wood, 1962), a precrystallized butter was obtained which was softer than normal continuously-made butter. The butters made with the laboratory equipment were extruded directly into cylindrical stainless-steel frames 2·9 cm in length by 4·7 cm internal diameter. The temperature of the extruded butter ranged from 42 to 47 °F. All samples were immediately placed in storage at 40 °F and held for 1 week before testing.

Commercial conventional and Gold'n Flow butters were stored in a tempering room at 40 °F for at least 1 week and then homogenized separately in lots of 150–300 lb in a Benhil Microfix butter homogenizer (Benz & Hilgers, Dusseldorf Nord, West Germany). After discarding the first few pounds of butter through the homogenizer from each lot, approximately 1-lb samples were cut from the extruded butter.

Samples of conventional and continuously-made butter from the same butterfat

source were re-worked in a laboratory-type blender without the application of vacuum. The blender, having a capacity of 1 kg, consisted of a strong metal vessel with a semi-cylindrical bottom into which were fitted two reverse acting sigmoid-shaped beaters. The butters were worked for varying lengths of time and samples were pressed into stainless-steel frames for testing.

Physical methods of analysis

Nitrogen gas in varying amounts was added to the precrystallized, continuously-made butter. In order to vary the gas content of conventional butter, butter granules from commercial churnings, after draining the buttermilk, were worked in the blender under various degrees of partial pressure. The gas content was measured by the direct method of deMan & Wood (1958*a*).

The macrotome used for preparing smooth level surfaces of butter was a modification of that designed by Claassens (1957); butter prints were prepared for measurements in the manner described by Claassens. An apparatus (Plate 1) similar to that described by Jansen (1961) was used for making hesion measurements. The stainless-steel adherend conformed to the specifications of Claassens (1958). The scale was calibrated to give hesion values in grams and a test of the apparatus showed a sensitivity of $\pm 2{\cdot}0$ g. The contact surface of the adherend was cleaned by repeated washing in acetone and carbon tetrachloride and wiped with clean cotton wool before measuring commenced and also between measurements. All measurements were made at 50 °F with a contact time of 60 sec. Measurements on 0·5-lb prints were made in 15 positions on each of 3 surfaces, while measurements of the butter in the cylindrical frames were made in 5 positions on each of 3 surfaces in duplicate samples. Fresh surfaces for the butter in the frames were obtained by forcing out the butter sufficiently to permit slices to be cut with a wire. When hesion measurements of butter in prints and in frames were compared there was essentially no difference in the values obtained and this was true for both conventional and continuously-made butters.

Hardness and oiling-off

The methods used were those described by Wood & deMan (1956) and deMan & Wood (1958*b*).

Polarized light photomicrographs

Uniform thicknesses of butter, prepared by the method of Herb, Audsley & Riemenschneider (1956) as modified by deMan & Wood (1959), were examined and photographed through a Zeiss–Winkel standard polarizing microscope.

RESULTS

Commercial butter

Comparative hesion measurements

Measurements made on commercial samples of conventional and continuously-made butter are summarized in Table 1 and show the marked difference in hesion values of these butters.

In the 20 measurements made, 10 for each type of butter, the hesion values for the Gold'n Flow butter, with one exception, were lower than those obtained for the conventional butters. The average hesion value of the Gold'n Flow butter was only 60 % of the average for the conventional butters.

Influence of homogenization

When commercial samples of conventional and continuously-made butter were homogenized, the hesion values increased substantially, an indication that the process produced a considerable change in the structure of the butter. Evidence of this change in the crystalline structure of Gold'n Flow butter is presented in Plate 2(*a*) (*b*). The data for hesion measurements are summarized in Table 2.

Table 1. *A comparison of stickiness of conventional and continuously-made butter on randomly selected commercial samples*

	Hesion measurements* (g)
Conventional	Continuously-made (Gold'n Flow)
74	38
81	47
86	48
87	57
91	59
102	60
114	63
119	68
126	73
135	87
Average 101	60

* Each value is the average of 45 measurements.

Table 2. *The effect of homogenization on stickiness in commercial conventional and continuously-made butter*

		Hesion values* (g)	
Butter type	Samples	Before homogenization	After homogenization
Conventional	1	21	50
	2	81	118
	3	88	123
Continuously-made	4	38	47
	5	47	150
	6	48	114
	7	60	75

* Each value is the average of 45 measurements.

Laboratory-made butter

Influence of butter types

Hesion and hardness determinations on conventional and 2 types of continuously-made butters prepared from the same butterfat source in the manner described above, are summarized in Table 3 for 8 different butterfat sources. The hardness values were

highest for the non-preerystallized butter which is comparable in physical structure to Gold'n Flow butter and lowest for the conventional butter. The precrystallized butter was in an intermediate position. These results are consistent with previous results reported from this laboratory. The results also show good negative correlation between hardness and hesion values for conventional and non-precrystallized butters; moreover, these results for hesion were similar to those reported in Table 2 for commercial butters of these types. The results obtained for the precrystallized continuously-made butter were not comparable with those obtained for the other butters.

Table 3. *A comparison of stickiness and hardness values of conventional and continuously-made butter from the same butterfat source*

			Continuously-made butter			
	Conventional butter		Non-precrystallized		Precrystallized	
Trials	Hesion (g)	Hardness (kg/4 cm²)	Hesion (g)	Hardness (kg/4 cm²)	Hesion (g)	Hardness (kg/4 cm²)
1	86	3·2	59	5·3	107	4·3
2	91	2·8	30	4·2	65	3·9
3	102	2·0	59	2·4	85	2·2
4	112	2·2	90	2·9	87	2·8
5	114	1·9	71	2·3	116	1·9
6	115	2·0	83	2·4	103	2·1
7	119	2·0	83	3·5	106	2·1
8	135	1·9	99	2·1	96	2·1
Average	109	2·2	72	3·1	96	2·7

Influence of re-working in a laboratory blender

Figs. 1 and 2 show the effect of re-working time on the hardness and hesion values of conventional and continuously-made butters. In both types of butter there was generally an increase in hesion values with a decrease in hardness and vice versa. In conventional butter there was a maximum reduction in hardness after 6 min of re-working, while the maximum hesion value occurred after 8 min of re-working. The hardness of the continuously-made butter decreased more slowly with continued re-working and there was a corresponding increase in hesion values. In other experiments not reported graphically, in which the working time was extended beyond 15 min, the maximum hardness reduction was only achieved after approximately 30 min of re-working. In these later experiments the maximum hesion value was attained after 20 min of re-working and this was followed by a rapid decrease. The oiling-off percentages found with samples of butter taken during the time of re-working are also shown in Figs. 1 and 2. These data show a decrease in the oiling-off percentages to similar minimum value for both types of butter, as the re-working progressed.

Influence of gas content

This section of the study was restricted to the laboratory continuously-made precrystallized butter to which nitrogen gas was easily added and to conventional butter worked in a blender under partial pressure to vary the gas content, as previously

described. The influence of gas content on the hesion and hardness of the continuously-made butter in a representative trial is shown in Fig. 3. The data for conventional butter are only available for initial and final gas contents and, consequently, graphical

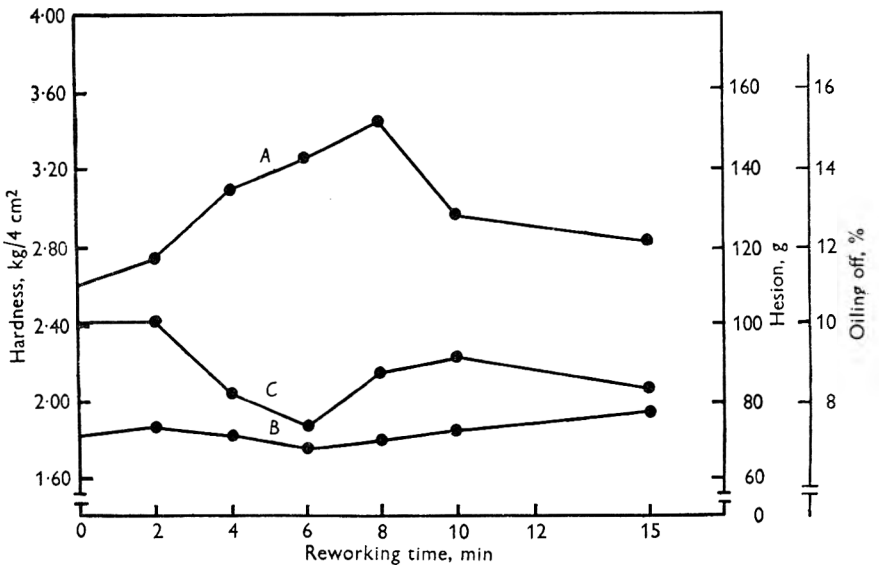

Fig. 1. The influence of re-working on the hardness, hesion and 'oiling off' of conventional butter. *A*, hesion; *B*, hardness; *C*, 'oiling off'.

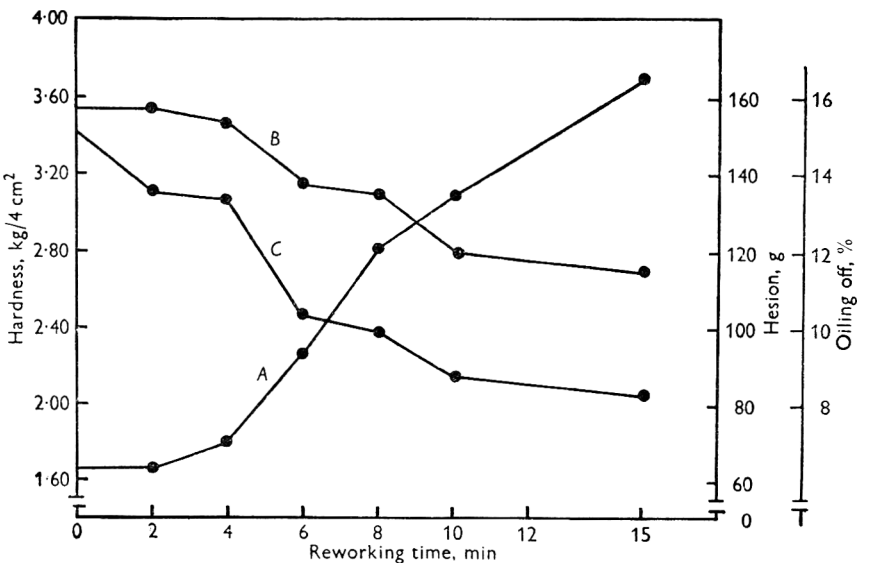

Fig. 2. The influence of re-working on the hardness, hesion and 'oiling off' of continuously-made butter. *A*, hesion; *B*, hardness; *C*, 'oiling off'.

presentation was not possible. These results are summarized in Table 4. There was a reduction in hardness with increasing gas content in both types of butter. In Fig. 3 curve *A* indicates that a small increase in gas content was paralleled by an increase in

hesion. However, when the gas content exceeded the normal range in butter (2–3 %), hesion values decreased. With the decrease in hesion values as a consequence of the increased gas content, there was an increase in the amount of butter that remained attached to the contact surface of the adherend when it became detached from the butter. In conventional butter, the contact surface of the adherend retained more butter when the butter was worked in the blender without vacuum than when worked under vacuum.

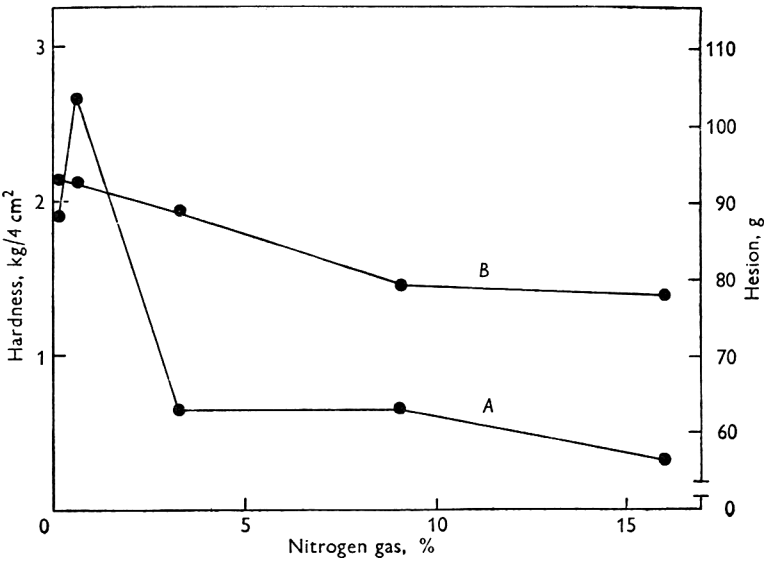

Fig. 3. The influence of gas content on hardness and hesion of continuously-made butter. *A*, hesion; *B*, hardness.

Table 4. *The influence of gas content on the stickiness and hardness values of conventional butter*

Trials	Gas content (%)	Hesion values* (g)	Hardness values (kg/4 cm²)
1	0·14	148	2·1
	1·49	130	2·0
2	0·18	135	2·9
	2·20	113	2·0
3	0·08	120	2·6
	1·65	89	2·2

* Each value is the average of 45 measurements.

DISCUSSION

The load dependence of the real area of contact between 2 smooth surfaces is attributed to the presence on the surface of numerous asperities of microscopic size (Bowden & Tabor, 1954). Consequently the real area of contact depends on the plastic deformability of the asperities. In measuring the hesion of butter the surfaces involved are hard and highly elastic stainless steel, and relatively soft and plastic butter. When these surfaces are pressed together the butter surface is deformed. The

softer the butter, e.g. freedom from asperities or other structures that may interfere with deformability, the greater will be the real area of contact and the greater will be the force required to detach the adherend from the butter.

Mulder (1949) drew attention to the influence of the method of manufacture, the structure of the materials from which the butter is made and the subsequent treatment on the physical structure of the product. Wood & deMan (1956) and deMan & Wood (1958*a*, *b*, *c*, 1959) have shown that Gold'n Flow continuously-made butter differs from conventional butter in its larger quantity of crystalline fat which results from rapid crystallization, its larger crystals of fat, its higher oiling-off percentage at 25–28 °C and its very low gas content.

More recently McKnight & Wood (1962) have shown that the introduction of a precrystallizer unit in the process greatly reduces the hardness of Gold'n Flow type butter by limiting the formation of mixed crystals (Mulder, 1953) and consequently reducing the total quantity of crystalline butterfat.

The hesion measurements (Tables 1 and 3) have confirmed previous visual observations of deMan & Wood (1958*b*) that, in general, Gold'n Flow butter does not adhere to surfaces so readily as conventional butter. The hesion measurements (Table 2) for both types of butter, before and after homogenization, indicate that homogenization causes structural changes in both butters which increase their stickiness. In the Gold'n Flow butter the polarized light photomicrographs of Plate 2 show a definite reduction of crystal size as a consequence of homogenization. Similar evidence of crystal size reduction by homogenization of conventional butter cannot be observed by this technique, as the fat crystals are normally only 1 μm or less in size compared with about 30 μm in Gold'n Flow butter. One could suppose that the physical changes in conventional butter are of a different nature. The increase in the temperature of the butter, as a consequence of the energy expended on the butter by the homogenizing process, might alter the size of the fat crystals sufficiently to interfere with normal setting. Some evidence that this may occur has been presented by deMan & Wood (1958*b*) who have shown that storage of butter at 20–22·5 °C reduces its hardness.

The re-working of both conventional and continuously-made butters for varying lengths of time appeared also to modify the structure of the butters and to influence stickiness. This was indicated by a decrease in hardness values of conventional butter after re-working for 6 min followed later by an increase in hardness. The decrease in hardness in the initial re-working is caused by a permanent modification of the primary structure of the fat (Mulder, 1949). Subsequent increases in hardness could have been caused partly by the recrystallization of the increased liquid fat content of the butter, resulting from the melting, by the heat of re-working, of the small crystals originally formed in the fat globules. Probably also responsible is the crystallization of the liquid fat released through disruption of the fat globules; the slow cooling conditions of these experiments would favour such crystallization. Similarly, in continuously-made butter, there was also a progressive reduction in hardness as the working progressed. In this butter, which has a greater hardness than conventional butter, the primary crystal structure predominates (deMan & Wood, 1958*c*) and, consequently, a more severe re-working was required to reduce the hardness to a minimum. Liquid fat, resulting from the melting of crystals as re-working progressed,

would also subsequently recrystallize into larger crystals because of the slow non-agitated cooling, and so increase the final hardness of the butter. In conventional butter the hesion values during re-working increased to a maximum value after 8 min and then decreased as the butter increased in hardness. In continuously-made butter there was a similar increase in hesion as hardness decreased. Longer re-working times (data not presented here) resulted in maximum hesion followed by a decrease. This decrease in hesion was accompanied by an increase in the hardness from a minimum value after 30 min re-working.

The fluctuation in the oiling-off percentage of the butters, which is a measure of the coarseness of the crystal structure, is also shown in Figs. 1 and 2. It should be noted that the initial oiling-off percentage is higher in the continuously-made butter but the minima for both butters on continued working are of similar magnitude.

It is evident from Fig. 3 that the gas content had a marked influence on hesion values. With a small increment in gas content, there was an increase in hesion which declined sharply as the gas content increased. A small increase in gas content, apparently, was enough to make the butter softer and improve the contact at the butter/adherend interface, but not enough to cause a weakening of the cohesive strength of the butter. At the higher gas contents, although the butter was softer and the contact between adherend and butter surface was greater, the hesion values decreased rapidly. Large increases in the gas content apparently lowered the cohesive strength of the butter to such an extent that a larger portion of the butter remained attached to the adherend. High hesion values occur when both the adhesive and cohesive strength of the butter are great, therefore a weakening of the cohesive strength causes lower hesion values without the adhesive strength being necessarily affected. Such butter, though having a relatively low hesion value, would still be regarded as sticky.

The results of this investigation suggest that the crystal structure is responsible for the adhesive property of butter. Evidence is offered that the gas content influenced the cohesive property. It would appear, then, that both the crystal structure and the gas content play an important part in the stickiness defect of butter as the term is used in the butter industry.

The authors gratefully acknowledge the financial assistance for this research provided by the Canada Department of Agriculture, Ottawa, Canada.

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EXPLANATION OF PLATES

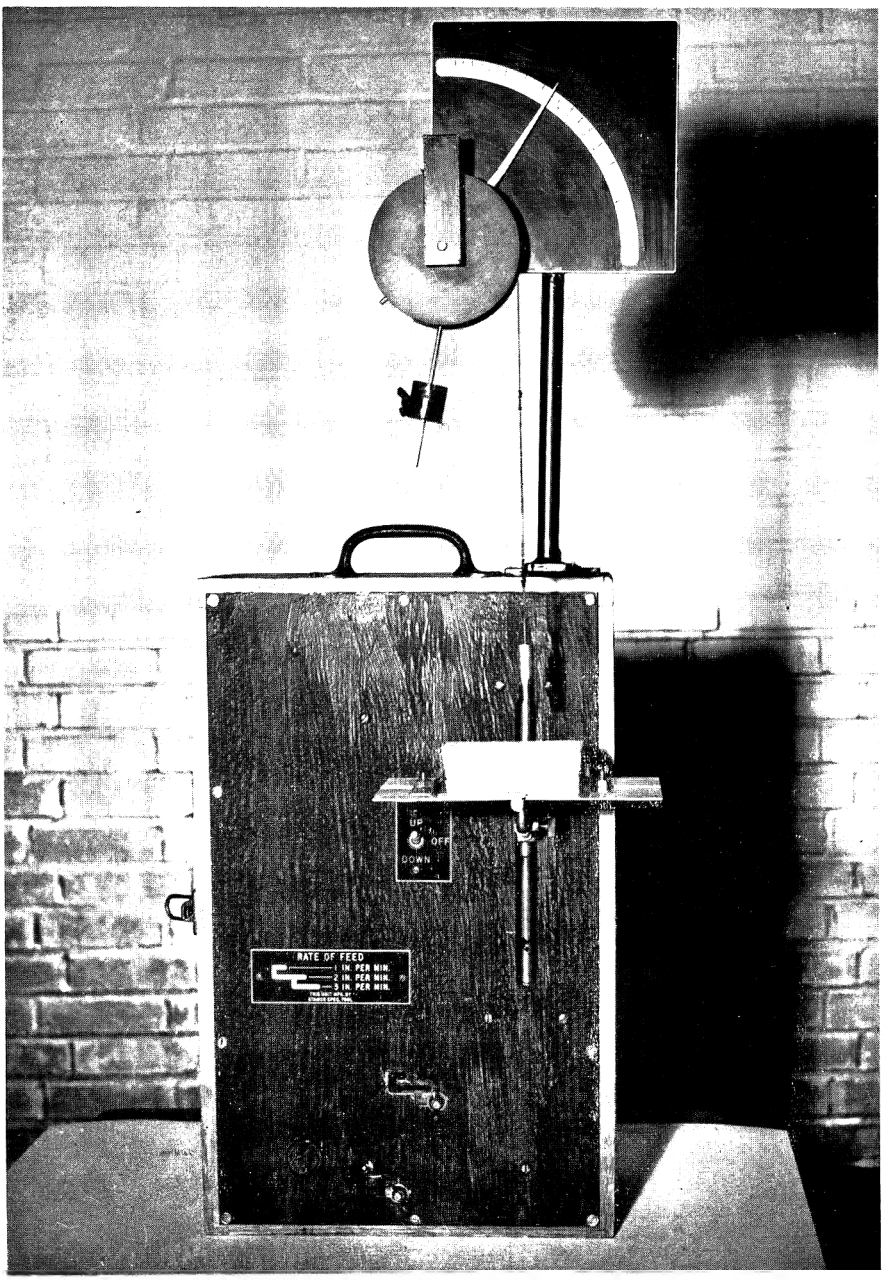
PLATE 1

Apparatus used for measuring hesion.

PLATE 2

Polarized light photomicrographs of fat crystals in Gold'n Flow butter. (*a*) Before homogenizing, (*b*) after homogenizing.

Printed in Great Britain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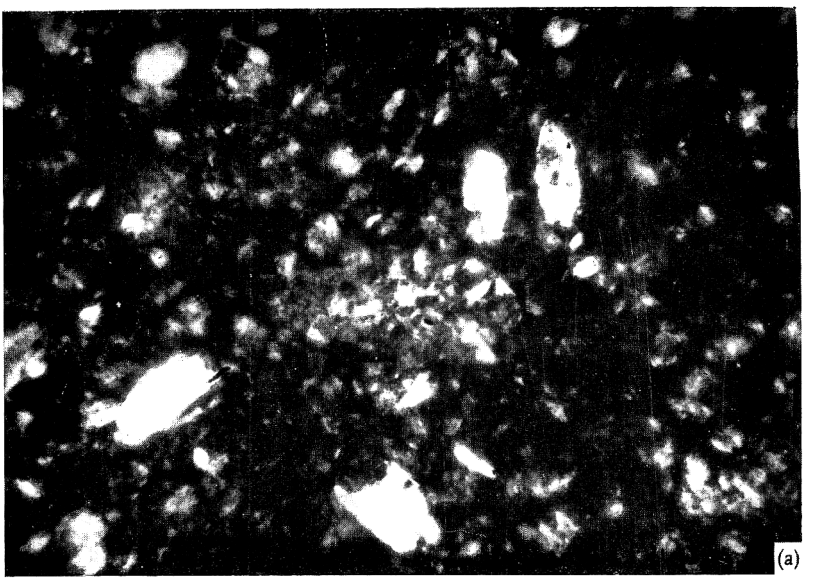
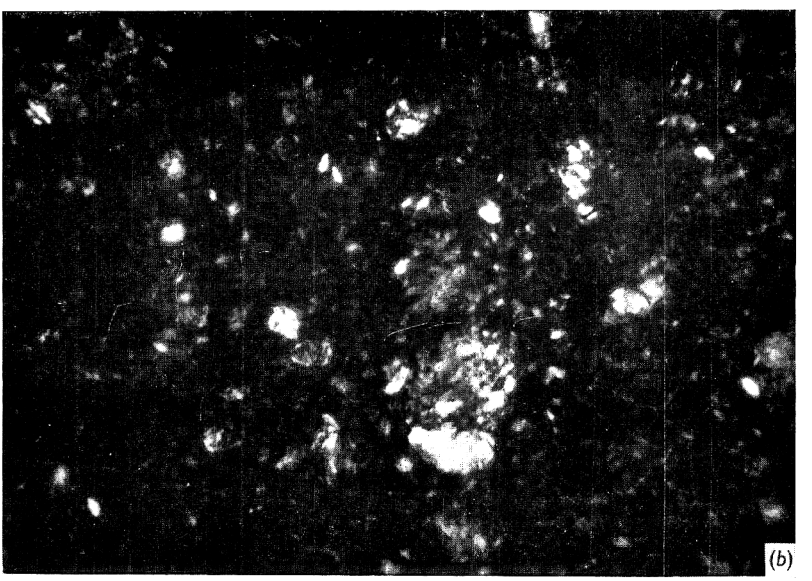

(a)


(b)

J. Dairy Res. (1964), **31**, 147

Characteristics of proteinases of 3 strains of *Staphylococcus lactis* isolated from Cheddar cheese*

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(*Received* 11 *November* 1963)

Summary. Proteinase systems of 3 strains of *Staphylococcus lactis* from Cheddar cheese have been studied. Proteinase of one organism was found in the culture supernatant, those of the other two were obtained in significant amounts only by disruption of the cells of the organisms. All 3 proteinases possessed maximum activity at alkaline pH (about 9·0–10·0). They were most active at relatively high temperatures (45–55 °C) and possessed temperature characteristics (μ) of 7000–8000 cals. Casein, β-lactoglobulin and haemoglobin were hydrolysed by the proteinases; casein most readily and β-lactoglobulin more readily than haemoglobin. Because of their characteristics, such proteinases are considered potentially capable of contributing to the ripening of Cheddar cheese.

An important change occurring during cheese ripening is the hydrolysis of casein to soluble products. At least part of this change has been attributed to the action of micro-organisms and indeed many bacteria found in cheese, including micrococci and staphylococci, are capable of hydrolysing casein (Baird-Parker, 1963; Baribo & Foster, 1952; Brandsaeter & Nelson, 1956; Vanderzant & Nelson, 1953). The development of the characteristic flavour of Cheddar cheese has also been attributed to the action of micro-organisms and their enzymes, and probably depends upon the formation of a large variety of compounds that arise through hydrolysis and metabolism of carbohydrates, fats and proteins. Hydrolysis of casein yields peptides and amino acids and these have been considered as important flavour constituents (Mulder, 1952; Stadhouders, 1960). Because some micrococci are proteolytic, and because some have been implicated in cheese-flavour production (Alford & Frazier, 1950*b*; Franklin & Sharpe, 1963; Harris & Hammer, 1940; Robertson & Perry, 1961), the present investigation was undertaken to obtain more information about the proteolytic systems of micrococci isolated from Cheddar cheese.

MATERIALS AND METHODS

Two samples of Canadian Cheddar cheese purchased locally at different times were plated in agar medium containing 0·5% (w/v) Tryptone (Difco) plus 0·5% (w/v) yeast extract (Difco) and in α-bromopropionic acid agar (Robertson, 1960). Surface colonies were picked and 25 catalase-positive cocci were isolated. Three of these were

* Issued as N.R.C. No. 7937.

proteolytic and were characterized by the methods of Baird-Parker (1963) and Abd-el-Malek & Gibson (1948).

For proteinase production, organisms were grown in a medium containing 0·5 % (w/v) vitamin-free casein (Nutritional Biochemical Corp.) and 0·2 % (w/v) yeast extract adjusted to pH 7·0 with NaOH. Erlenmeyer flasks (1 l) with indented baffles and containing 250 ml of broth were inoculated and incubated at room temperature (about 23 °C) for 20–24 h on a rotary platform shaker operating at 150 oscillations/min. Crude proteinase preparations were obtained either from culture supernatants or from disrupted cells. Cultures at a final pH of 8·5 were adjusted to pH 4·6 with concentrated HCl and centrifuged at 12800 *g* for 15 min at 4 °C to remove precipitated casein and cells. The supernatants were adjusted to pH 8·5 with 40 % (w/v) NaOH and tested for proteinase activity. Cells were harvested by centrifuging 500 ml of culture at 12800 *g* for 15 min at 4 °C and were suspended in 50 ml of water. These suspensions, maintained at about 5 °C, were treated in a Raytheon 10 KC sonic oscillator for 10 min, and were then centrifuged at 12800 *g* for 15 min at 4 °C. The supernatants (cell extracts) were tested for proteinase.

Proteinase activity was generally determined at 35 °C by mixing 3·0 ml of casein substrate at pH 9·0 with 3·0 ml of proteinase preparation (i.e. culture supernatant or cell extract). Immediately after the addition of enzyme and again after an incubation period, samples were taken and added to equal volumes of 0·6 N-trichloroacetic acid (TCA). The mixtures were allowed to stand for 10–15 min and were then filtered through Whatman No. 2 filter paper. Tyrosine in the filtrate was determined by Anson's (1938) method (10 ml of 0·5 N-NaOH + 1·0 ml of TCA filtrate + 4·0 ml of water + 3·0 ml of diluted (1 : 3) phenol reagent (Folin & Ciocalteu, 1927)). This method detected proteinase activity but did not indicate whether more than one enzyme was present. Casein substrate was prepared as follows: 12 g vitamin-free casein (Nutritional Biochemical Corp.) was dissolved in 200 ml of 0·025 N-NaOH, heated in a boiling water bath for 10 min and cooled. Boric acid (9·2 g) and 7·0 mg of Merthiolate (Lilly) were added and the volume made to 300 ml. This stock solution was adjusted to the desired pH with NaOH or HCl and diluted 1 : 2 before use. Crystallized bovine plasma albumin (Armour Laboratories), crystallized β-lactoglobulin (Pentex, Inc.) and haemoglobin (Fisher Scientific Co.) were prepared as 2 % (w/v) solutions in 0·9 % (w/v) NaCl. With these substrates, reaction mixtures consisted of 3·0 ml of substrate + 2·0 ml of buffer (0·1 M-KH_2PO_4 + 0·1 M-H_3BO_3, pH 9·0) + 1·0 ml of enzyme preparation.

RESULTS

The cheese samples examined gave plate counts of about $1{\cdot}5 \times 10^7$/g on Tryptone-yeast extract agar and $5{\cdot}7 \times 10^6$/g on α-bromopropionic acid agar. These results suggested that the samples had a relatively large 'non-lactic' flora. Of 25 catalase-positive cocci isolated from the 2 cheeses, 3 cultures were proteolytic but were difficult to classify (Table 1). They could be placed in group I (*Staphylococcus*) as defined by Baird-Parker (1963) but it was not possible to assign them to a subgroup. When the scheme of Shaw, Stitt & Cowan (1951) was used, the organisms fell into subgroup 3 and therefore, for ease of discussion in this paper they are referred to as strains of *Staphylococcus lactis*.

When the 3 strains (8, 19-5-1, and 19-5-9) chosen because they were capable of causing clearing of skim-milk in agar, were grown in liquid medium containing casein, the soluble tyrosine content of the cultures increased by about 150 μg/ml in 40 h cultures of strain 19-5-9 and by about 50 μg/ml in 40 h cultures of strains 19-5-1 and 8, indicating that casein was indeed being hydrolysed. When cultures of 19-5-9 in casein medium were centrifuged, supernatants were actively proteolytic and cells, disrupted by sonic oscillation, had only slight activity (about 10 % of that in supernatants). Culture supernatants of strains 19-5-1 and 8 were inactive or had only slight proteolytic activity whereas disrupted cells were actively proteolytic. These results suggested that the proteinase of strain 19-5-9 is an extracellular enzyme system and that the proteinases of strains 19-5-1 and 8 are 'cell-bound' (Pollock, 1962).

Table 1. *Characteristics of micrococci isolated from Canadian Cheddar cheese*

Characteristic*	8	19-5-1	19-5-9
Pigment	Yellow	Yellow	Sl. orange
Coagulase	0	0	0
Phosphatase	0	0	0
Glucose (aerobic)	+	+	+
Glucose (anaerobic)	+	+	+
Mannitol (aerobic)	0	0	0
Mannitol (anaerobic)	0	0	0
Maltose (aerobic)	0	0	+
Lactose (aerobic)	0	+	±
Cellobiose (aerobic)	0	0	0
Arabinose (aerobic)	0	0	0
NO_3 reduction	0	+	+
NH_4 from arginine	+	+	+
Growth at 45 °C	0	0	0
Growth at 10 °C	+	+	+
Growth in 5 % NaCl	+	+	+
Growth in 15 % NaCl	0	0	0
Growth in NH_4HPO_4 after 3rd serial transfer	0	+	0
MR	+	0	0
VP	0	0	0
Survived 60 °C for 30 min†	+	+	0

* Determined by methods of Baird-Parker (1963); + = positive, 0 = negative, ± = variable reaction.
† Heat resistance determined by the method of Abd-el-Malek & Gibson (1948).

Characteristics of the proteinases in culture supernatants of strain 19-5-9 and cell extracts of strains 8 and 19-5-1 were determined. Samples of casein substrate were adjusted to various pH levels and mixed with supernatants or extracts. The pH of these mixtures was determined at the end of the reaction period because the pH changed only by about 0·1 pH unit during incubation. Proteinases of these organisms hydrolysed casein more rapidly at alkaline than at acid or neutral pH (Fig. 1). The extracellular proteinase system of strain 19-5-9 was active over a broad pH range with optimum activity at about pH 9·7. At pH 6·1, activity was about 50 % of maximum. The 'cell-bound' proteinase systems of strains 8 and 19-5-1 were most active at about pH 9·3; above and below this pH, activity was reduced sharply. Proteinases of strains 8 and 19-5-1 had about 50 % of maximum activity at pH 6·4 and 6·6, respectively.

The proteinase systems were active at relatively high temperatures; that from strain 19-5-9 was most active at 55 °C whereas those in cell extracts of strains 8 and 19-5-1 were most active at about 45 °C (Fig. 2). In other experiments, activity of proteinases of strains 8 and 19-5-1 at 15 °C was about 15% of that at the optimum temperature, indicating that these enzymes have considerable activity at low temperatures. Temperature characteristics (μ) were calculated from Arrhenius plots of the data (Fig. 2); 19-5-9 proteinase (4 points) had μ = 6900 cals, 8 and 19-5-1 proteinases (3 points each) had μ = 6900 and 7800 cals, respectively. These low values indicate that the enzymes are relatively active at low temperatures.

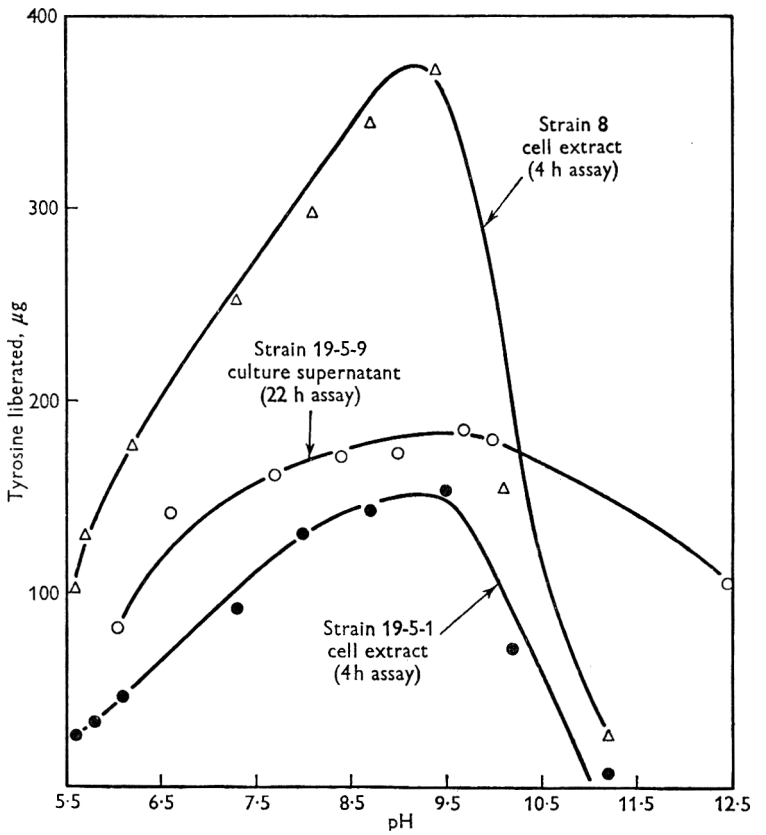

Fig. 1. Effect of pH on hydrolysis of casein by proteinases of *Staph. lactis* strains from Cheddar cheese. 3·0 ml casein solutions adjusted to a given pH and incubated at 35 °C with 3·0 ml proteinase preparation. The pH of the reaction mixture was determined at the end of the experiment.

Proteinase systems appeared to lose activity rapidly at temperatures above their optima. The least thermolabile proteinase was that from strain 19-5-9 (Fig. 3). Proteinases of the other 2 organisms were quite heat sensitive; that from strain 8 being slightly less resistant than that from 19-5-1.

The ability of the proteinases to hydrolyse various proteins was studied. Of the substrates tested, casein was hydrolysed most readily; β-lactoglobulin was hydrolysed

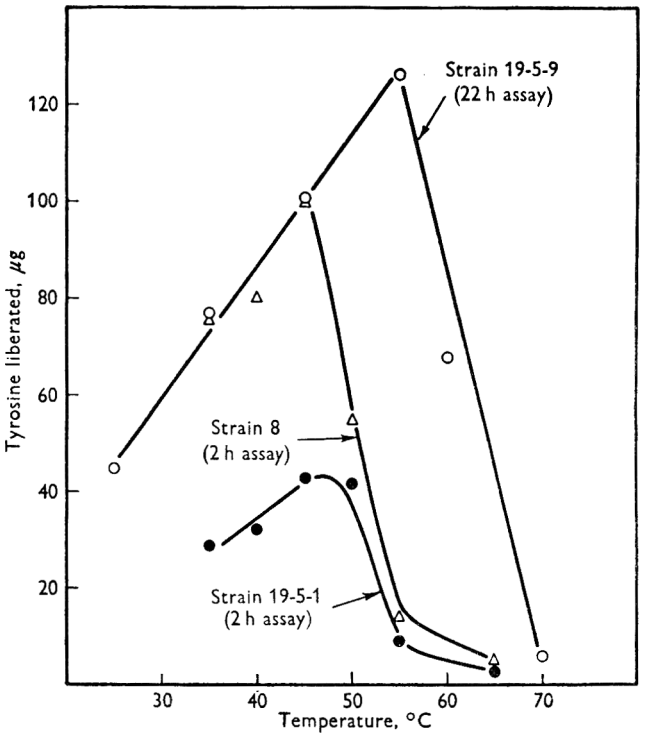

Fig. 2. Effect of temperature on activity of proteinases of *Staph. lactis* strains from Cheddar cheese; 3·0 ml casein solution, pH 9·0 incubated with 3·0 ml proteinase preparation for 22 h (strain 19-5-9) or 2 h (strains 8 and 19-5-1) at different temperatures.

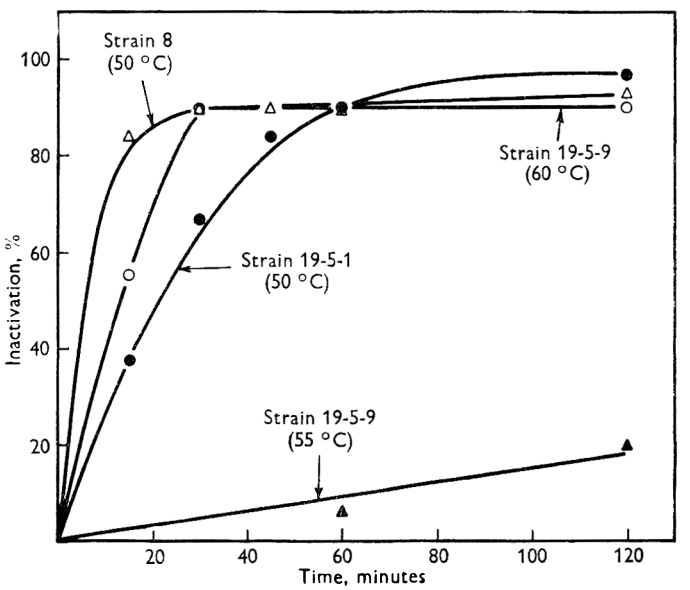

Fig. 3. Heat inactivation of proteinases of *Staph. lactis* strains from Cheddar cheese. Proteinase preparation (20 ml) + 0·1 M-borate buffer, pH 9·0 (20 ml) heated in a water bath; samples removed at intervals, cooled and assayed. Assay: 3·0 ml casein solution, pH 9·0 + 3·0 ml proteinase preparation incubated at 35 °C for 20 h.

more readily than haemoglobin; bovine plasma albumin was hydrolysed very slowly (Table 2). These results were not altered if these substrates had been denatured by heating in boiling water for 10 min.

Table 2. *Hydrolysis of various proteins by proteinases of strains of* Staph. lactis *from Cheddar cheese*

	Tyrosine liberated,† μg		
Substrate*	Strain 8	Strain 19-5-1	Strain 19-5-9
Casein	174	86	200
β-Lactoglobulin	94	52	117
Plasma albumin	10	7	12
Haemoglobin	47	33	64

* 2% (w/v) solutions in 0·9% (w/v) NaCl.

† Reaction mixtures. 3·0 ml substrate + 2·0 ml buffer (0·1 M-KH_2PO_4 + 0·1 M-H_3BO_3, pH 9·0) + 1·0 ml enzyme preparation. Incubation at 35 °C for 20 h.

DISCUSSION

Proteinases of 3 strains of *Staph. lactis* isolated from Canadian Cheddar cheese, when tested *in vitro*, hydrolysed casein and β-lactoglobulin. Although the enzymes were most active at alkaline pH, the data showed the enzyme systems had considerable activity at pH 5·6–6·0 and by extrapolation of the data would appear to have a small amount of activity at pH 5·3–5·4. The enzymes were most active at high temperatures but at least two (those from strains 8 and 19-5-1) had considerable activity at the low temperatures at which cheese matures (about 15 °C). Therefore, proteinases of these strains of *Staph. lactis* although not acting at their optimum pH or temperature could contribute to overall protein hydrolysis during the ripening process. The degree to which such organisms actually do contribute to protein hydrolysis and cheese ripening would depend upon the numbers present as well as upon their proteolytic activity. Relatively high counts of non-lactic organisms in cheese were obtained in this and other investigations (Alford & Frazier, 1950*a*; Feagan & Dawson, 1959), and even if only a few of these organisms (10–20%) were proteolytic micrococci and staphylococci, they could account for a considerable amount of protein hydrolysis in Cheddar cheese during the long ripening period.

Micrococci have been shown to enhance flavour of Cheddar cheese when certain strains were added to cheese milk (Alford & Frazier, 1950*b*; Robertson & Perry, 1961). The effect may have been due to the proteolytic and lipolytic (Franklin & Sharpe, 1963) activity of these organisms since optimum flavour development is thought to be dependent upon both these processes (Kristoffersen & Gould, 1960). Although Stadhouders (1960) using one strain of *Staph. lactis* concluded that added micrococci did not affect protein hydrolysis in Dutch cheese, the present results and those obtained by Baribo & Foster (1952) suggest that micrococci could influence casein hydrolysis during cheese ripening.

Proteinases of 2 strains of *Staph. lactis* (8 and 19-5-1) were not liberated into the medium as the organism grew but remained associated with the cells. This suggested the organisms produced proteinase systems that, in the terminology employed by Pollock (1962), were completely 'cell-bound'. When these organisms were grown in

media containing casein, they caused hydrolysis of the casein and this indicated that although the proteinases were 'cell-bound' they were accessible to their substrate. It seems possible that the presence of such organisms in cheese could result in hydrolysis of casein without prior autolysis of the cells.

Robertson & Perry (1961) showed that a strain of micrococcus enhanced Cheddar cheese flavour and suggested, as one possibility, that enzymes of this organism may have influenced flavour. The authors point out that if extracellular enzymes were responsible for the improved flavour they could have been produced by the viable but non-proliferating cells and if intracellular enzymes were responsible they would have been liberated by cell autolysis. Franklin & Sharpe (1963) concluded that their study confirmed the influence of bacterial activity on cheese flavour. They suggested that the influence may be the result of the action of bacterial enzymes released during heat treatment of milk and subsequently active in the cheese, of the release of proteolytic and lipolytic enzymes by autolysis of micro-organisms during ripening, or of the metabolic activity of multiplying strains. While exoenzymes and endoenzymes, released by autolysis of cells, undoubtedly contribute to the cheese ripening process, a possibility not considered by Robertson & Perry (1961) or Franklin & Sharpe (1963) is that 'cell-bound' enzymes which are accessible to their substrate, such as the proteinases of *Staph. lactis* strains 8 and 19-5-1, might also contribute to the ripening process. It would appear, therefore, that any consideration of the influence of bacterial enzymes in cheese ripening should include the possibility that 'cell-bound' enzymes may be as important as the more commonly discussed intracellular and extracellular enzymes.

The author wishes to thank Mrs Alice K. Chambers for her valuable technical assistance during this work.

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Printed in Great Britain

The manufacture of 'normal' and 'starter-free' Cheddar cheese under controlled bacteriological conditions

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(*Received* 9 *December* 1963)

Summary. A relatively simple method is described for the manufacture of Cheddar cheese under controlled bacteriological conditions. This method is shown to give the required degree of asepsis and to permit the manufacture of cheeses of uniform and normal composition.

The composition, bacteriology and flavour of 28 'aseptic' cheeses and of 18 'non-aseptic' control cheeses made by this method are discussed. Thirty-nine of these cheeses were made using singly 3 strains of *Streptococcus cremoris* and 2 strains of *Str. lactis* as starter and 7 were made using gluconic acid lactone in place of starter. The effect of inoculating the milk with a strain (25·2) of '*Lactobacillus plantarum-casei*' and a strain (L_1) of micrococcus was also investigated.

The role of micro-organisms in the development of the characteristic flavour of Cheddar cheese is already well recognized. There is, however, little precise information about the types of organisms responsible or the stage in the ripening process at which their influence is exerted. It has been found that individual strains of starter streptococci may have an effect on the flavour of cheese (Perry, 1961). The effects of other types of organisms are more difficult to define because of their relatively low numbers and their uncontrollable access to the milk during cheese-making. Where attempts have been made to determine the effect of particular organisms on cheese flavour, the usual technique has been to add to normal cheese milk a sufficiently heavy inoculum of the test organisms to dominate the existing flora, and so minimize the effect of adventitious organisms. This type of experimental approach has, for example, given results suggesting that lactobacilli, although always present in large numbers in mature Cheddar cheese, may contribute little to the characteristic Cheddar flavour (see Mabbitt, 1961), whereas various strains of micrococci, although not increasing in numbers in the cheese, may markedly enhance the flavour of the cheese at maturity (e.g. Robertson & Perry, 1961).

The significance of such findings is limited, however, by the wide range of flavours normally encountered in the control cheeses and the fact that in the experimental cheeses the effect of adventitious organisms, particularly those leading to flavour defects, cannot be completely eliminated. Clearly, the manufacture of cheese under completely aseptic conditions from sterilized milk inoculated with the organism or

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organisms under investigation, would provide the answer to many flavour problems. Unfortunately, complete heat sterilization of milk renders it unsuitable for cheese-making while other methods of controlling bacterial growth, such as chemical sterilization or the addition of antibiotics, also have an adverse effect on the curd or influence the maturing of cheese. Furthermore, quite apart from the problems of destroying organisms present in the milk to be used for cheese-making, the difficulties thought to be associated with cheese-making under aseptic conditions have apparently deterred many workers.

A few years ago Mabbitt, Chapman & Sharpe (1959) described an aseptic cheese vat and a method of manufacture by which they were able to attain a satisfactory degree of asepsis in 3 out of 6 cheeses. In planning to exploit the aseptic cheese-making technique it seemed to us that certain modifications to the vat itself and to the processes employed might simplify and shorten the methods of pasteurizing and cheese-making, and might remove some of the possible sources of contamination. The present communication describes the system finally developed for the manufacture of Cheddar cheese, both with and without starter, under controlled bacteriological conditions and also gives data on the composition, bacteriology and flavour of the cheeses.

EXPERIMENTAL

Consideration of the problems involved and preliminary experiments on the manufacture of Cheddar cheese under aseptic conditions led to a number of modifications to the techniques described by Mabbitt *et al.* (1959). In our attempts to repeat their work, the main difficulty encountered was contamination of the milk and curd from the factory atmosphere when temperature changes within the vat, or even movements of hands and arms in the gauntlets, resulted in non-sterile air being sucked into the vat presumably through the main seal. This was overcome by maintaining the vat at a slight positive pressure with sterile air.

Some of the manipulative difficulties encountered were due to the necessity of reaching to all corners of the vat and were therefore directly related to the size of the vat; also in order to speed up the operations of heating and cooling it seemed desirable to use the minimum quantity of milk consistent with the production of Cheddar cheeses of uniform quality. Ten to eleven gal of milk in a vat of about 18 gal total capacity was finally adopted as a reasonable compromise. Standard methods of cheese-making could be followed in a vat of this size, yet the scale was small enough to avoid some of the problems in the handling of larger quantities of milk. The milk was drawn aseptically from cows selected for low-count milk and was pasteurized in the vat.

To simplify construction, mechanical stirrers were dispensed with. Some modifications were made in the hooping arrangements.

The apparatus and methods finally adopted were as follows.

Cheese vat and cover

The design of the stainless-steel vat and cover is shown in Fig. 1. The rectangular, jacketed vat was fitted with a narrow trough in which the vat cover rested. The vat cover was fitted with: (1) two 6-in diam. stainless-steel flanges over which rubber gauntlets

were attached by means of adhesive tape; (2) a window consisting of a sheet of $\frac{3}{16}$ in clear Perspex cemented and clamped in place; (3) short lengths of 1-in diam. stainless-steel tubing to serve as inlets for inoculating, for steaming the vat during sterilization, and for sterile air; (4) a dial thermometer; and (5) a perforated tray for holding cheese-making equipment and sample bottles.

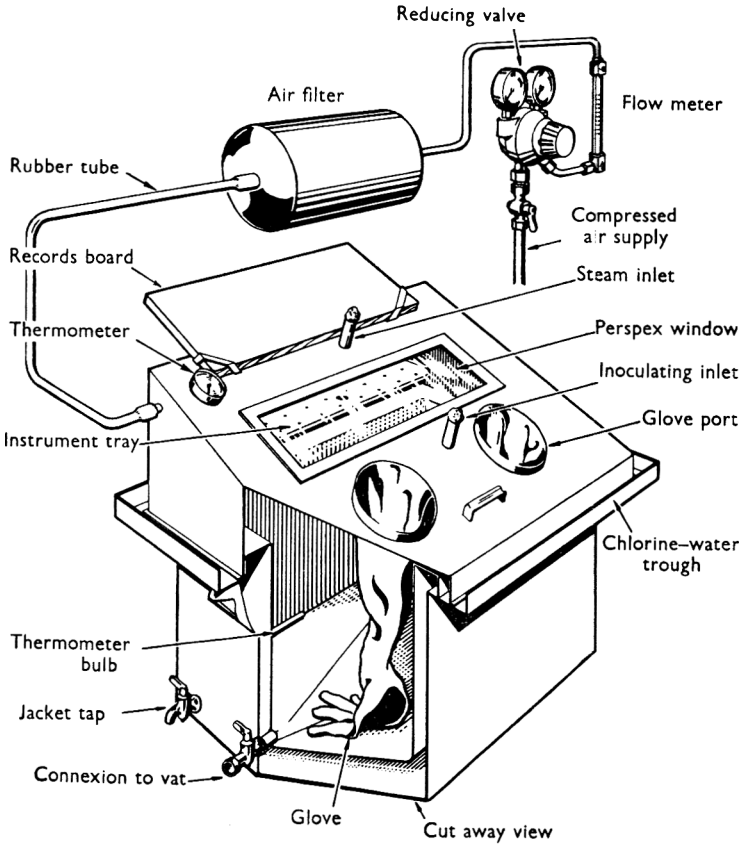

Fig. 1. 'Aseptic' cheese vat.

Cheese-making equipment

The following equipment was found to be adequate:

(1) A small curd knife (12 in × 5 in) with vertical blades giving a $\frac{3}{8}$ in cut.
(2) A laboratory palette knife for cutting and 'milling' the cheddared curd.
(3) A strainer to fit over the inside of the vat outlet.
(4) A wide-mouthed bottle for salt.
(5) A plastic bag to hold the 'milled' curd at the end of the making process.

Milk

Prior to the collection of milk for cheese-making, small samples were drawn aseptically from individual cows and examined bacteriologically. Only those cows whose milk was consistently found to contain less than about 100 bacterial cells/ml when freshly drawn were used to provide the aseptic milk for cheese-making.

Sets of teat cups, milking clusters and pipe lines were specially cleaned and were sterilized by immersion overnight in a 1:1000 solution of Zepharin (10% aqueous alkyl-dimethyl-benzyl-ammonium chloride, Bayer Pharma (Sydney) Ltd). Milk sampling buckets with sealed lids were sterilized by autoclaving at 20 lb for 30 min. At milking the udders and teats of the selected cows were thoroughly washed with 1:1000 Zepharin solution, and, using the sterilized cups, 100–120 lb milk was drawn into the sterilized sampling buckets.

Cheese-making technique

The day before the cheese was to be made the vat and cover were thoroughly cleaned and wiped over with hypochlorite solution (80 ppm. available chlorine). The vat and trough were then filled with hypochlorite solution (80 ppm. available chlorine) and left to stand overnight with cover attached. Next morning the vat was emptied and sterile water introduced aseptically via one of the inlets to enable traces of residual chlorine to be removed. Fresh chlorine water was placed in the trough and the vat was steamed for 30 min with all inlets open. During this steaming the temperature in the vat was 200–210 °F. The gauntlets were immersed in the chlorine water overnight and were suspended in the vat during rinsing and steaming. Towards the end of the steaming period the sterile air line was attached to the vat and throughout the cheese-making process air was passed through the vat at the rate of about 20 l/min.

After steaming, the sterilizing and inoculating inlets were plugged with sterile cotton wool; the cover was then raised briefly while the milk was poured as aseptically as possible into the vat and the previously autoclaved cheese-making equipment and sample bottles were placed on the tray. After closing the lid into the hypochlorite solution, steam was blown through the vat for 10 min to re-sterilize the airspace above the milk.

Steam was passed through the jacket of the vat to raise the milk to a temperature of 155 °F. This temperature was maintained for 5 min and the milk was then cooled to a setting temperature of 93 °F by running cold water through the jacket.

The method of cheese-making was essentially the 'fast make' as described by Whitehead & Harkness (1959). Starter, any other micro-organisms to be added to the vat, and Seitz-filtered rennet were introduced through the inoculating inlet using a hooded, sterile filter-funnel. All normal cheese-making operations, including the manual agitation of the milk during heat treatment and cooling, and of the curd and whey during cooking, were performed using the rubber gauntlets. Samples of milk and curd for bacteriological examination were taken within the vat using the sterile bottles already provided. Whey samples for acidity determinations were taken at the outlet tap.

After salting, the curd was placed in the sterile plastic bag to protect it from contamination when the vat cover was raised momentarily to introduce a dressed and sterilized 12-lb loaf cheese hoop. After allowing about 15 min for the displacement of non-sterile air, and again working through the gauntlets, the curd was transferred from the plastic bag to the hoop. The closed hoop was then removed from the vat and pressed in the normal manner.

Cheeses were stored at 55 °F for 14 days, waxed, and thereafter held at 45 °F.

Cheese manufactured (see Table 1)

(*a*) *Using starter.* Cheeses were made under controlled bacteriological conditions using singly 3 strains of *Str. cremoris* (HP, C_{13}, KH) and 2 strains of *Str. lactis* (ML_3, ML_8). The effect of the addition to the milk of a strain (25·2) of '*L. plantarum-casei*' (Sherwood, 1939) and a strain (L_1) of micrococcus, previously shown to have a beneficial effect on cheese flavour (Robertson & Perry, 1961) was also investigated.

With each starter cheeses were manufactured in groups of six. As far as possible the cheeses within each group, both control and experimental, were made on successive days and the milk for each group of experimental cheese was drawn from the same cows. Each group included 4 cheeses made under aseptic conditions from milk inoculated with (1) starter only, (2) starter plus lactobacillus 25·2, (3) starter plus micrococcus L_1, and (4) starter plus lactobacillus 25·2 and micrococcus L_1. Each group also included 2 control cheeses made in the 'aseptic' vat, but with the lid removed and without any aseptic precautions. One such cheese ('bulk' control) was made from normal bulk, flash-pasteurized milk (155 °F/5 sec); the other ('aseptic' control) was made using milk drawn aseptically from selected cows and heat treated in the vat as described above.

(*b*) *Without starter.* Attempts were also made to manufacture Cheddar cheeses similar to those described above but without the use of starter. The method was based on that of Mabbitt, Chapman & Berridge (1955) and was essentially as follows:

100 lb milk was pasteurized in the vat as described above and cooled to 95 °F. Hydrochloric acid (1000 ml 0·2 N) and gluconic acid lactone (250 g) were added and the vat rennetted in the normal way. The curd was cut after 10–15 min and the temperature raised to 100 °F. After a further 40 min the temperature was raised to 110 °F. The vat was held at this temperature for 5 min and then run. After drying the curd was cheddared for about 60 min; it was then milled and salted immediately. Twenty min after salting additional gluconic acid lactone (125 g) was scattered over the curd and thoroughly mixed in. The curd was then turned at intervals for about 60 min and hooped.

All cheeses were analysed and examined when 14 days old; a few, which were of abnormal composition, pH or body were rejected and replacement cheeses made as soon as possible.

Bacteriological examination of milk and curd

Milk samples were taken before and after pasteurization, and immediately before rennetting. Curd samples were taken at hooping. At 1, 2, 4, 8, 12, and 26 weeks cheese samples were drawn with a sterile trier, the surface of the cheese being sterilized with alcohol before sampling. Milk was homogenized for 2 min using an M.S.E. Ato-mix blender. 10 g proportions of cheese, taken from that part of the core farthest from the rind, were similarly homogenized in 90 ml Ringer solution. The milk or cheese homogenate was allowed to stand for 10 min before decimal dilutions were made in Ringer solution. Using 1-ml portions of suitable dilutions as inocula, 2 series of plates were prepared using lactose–yeast–phosphate–agar (Hunter, 1946) as growth medium. One series was treated in the usual way to give counts of total populations. Counts of non-starter populations were found from the second series by the method of Robertson (1960). To determine the proportion of the various types of

organisms present, 50 adjacent colonies were picked into yeast–litmus–milk from a suitable plate of each series. Tubes were incubated at 30 °C, inspected for acid production after 24 h and cultures examined microscopically after 3 days. In all the 'aseptic' cheeses containing starter the milk, curd and whey were also examined for the presence of phage.

Flavour assessment and chemical analysis

At the same time as the samples were taken for bacteriological examination, samples were also taken for flavour assessment. These were examined by 2 members of a flavour panel. As soon as Cheddar flavour was detected in a sample, plugs were drawn from all cheeses in the same group and flavours were compared by a 6-membered panel. Each person examined the cheeses independently and without knowledge of the nature of the experiment. Chemical analysis of the cheeses, together with assessments of their body and texture, were also made.

Table 1. *Details of cheeses manufactured*

Additions	Starter					Starter-free (gluconic acid lactone)
	HP	C_{13}	KH	ML_3	ML_8	
'Aseptic' make						
None	2	2	2	2	2	1
Lactobacillus 25·2	1	1	1	1	1	1
Micrococcus L_1	1	1	1	1	1	—
Lactobacillus 25·2 + Micrococcus L_1	1	1	1	1	1	1
Non-aseptic make (control)						
'Aseptic' milk	2	1	1	1	1	3
'Bulk' milk	2	2	1	2	1	1

RESULTS AND DISCUSSION

Cheese manufactured

After preliminary experiments to establish the technique 54 cheeses were made in the aseptic vat. Of these, 2 were rejected because of unsatisfactory composition or pH and 6 because milk or curd samples were found to be contaminated with adventitious organisms. The remaining 46 cheeses, made up of groups as shown in Table 1, were examined at intervals over a period of 26 weeks.

Manufacturing and compositional data for the 46 cheeses are given in Tables 2–5. The 'aseptic' conditions were found to impose severe limitations on the cheese-maker's traditional controls over the composition of the cheeses. Working through rubber gloves, the feel of the curd gave little indication of its moisture content and hence of the dry stirring required and the percentage of salt needed. This applied particularly to the manufacture of the 'non-starter' cheeses, since on these occasions the curd also had an unusual appearance. A further feature of the 'aseptic' conditions was the slower rate at which the curd lost moisture when the vat cover was in place, making it difficult to obtain as low a moisture in the solids-not-fat as is normal in New Zealand cheese. Despite these limitations, however, it was possible to produce cheeses (including those made without starter) which were of reasonably normal composition and pH, and of relatively good body and texture when examined at 14 days. A com-

parison of Tables 3 and 5 will show how closely the composition of the gluconic acid lactone cheeses approached that of the cheeses made in the normal manner using starter.

Table 2. *Manufacturing data for* 39 *cheeses made with starter ('aseptic' and non-aseptic make)*

	Average	Range
Acidity % at:		
Rennetting	0·16	0·14–0·17
Cutting	0·11	0·10–0·12
Running	0·14	0·13–0·15
Drying	0·17	0·16–0·18
Milling	0·35	0·32–0·37
Salting	0·52	0·49–0·55
Time, rennetting to drying, h:min	2:10	2:00–2:35
Time, drying to salting, h:min	1:30	1:00–1:55
Total making-time, h:min	3:40	3:05–4:10

Table 3. *Analyses at* 14 *days of* 39 *cheeses made with starter ('aseptic' and non-aseptic make)*

	Average	Range
Moisture, %	36·2	33·6–38·1
Fat, %	34·4	33·0–36·0
Fat/total solids, %	54·3	51·0–55·0
Moisture in solids-not-fat, %	55·2	52·5–57·0
Salt, %	1·5	1·4–1·8
Salt/moisture, %	4·1	3·7–4·9
pH	5·0	5·0–5·1

Table 4. *Manufacturing data for* 7 *'starter-free' cheeses ('aseptic' and non-aseptic make)*

	Average	Range
Acidity % at:		
Rennetting	0·24	0·22–0·27
Cutting	0·21	0·20–0·23
Running	0·25	0·25–0·26
Drying	0·27	0·27–0·28
Milling	0·31	0·31–0·34
Salting	0·32	0·30–0·35
Time, rennetting to drying, h:min	1:10	1:05–1:15
Time, drying to salting, h:min	1:05	1:00–1:10
Total making-time, h:min	2:10	2:05–2:15

Table 5. *Analyses at* 14 *days of* 7 *'starter-free' cheeses ('aseptic' and non-aseptic make)*

	Average	Range
Moisture, %	35·8	33·2–38·0
Fat, %	33·0	31·0–34·0
Fat/total solids, %	51·5	50·0–54·5
Moisture in solids-not-fat, %	53·4	50·0–55·0
Salt, %	1·7	1·6–1·9
Salt/moisture, %	4·8	4·5–5·4
pH	5·0	4·9–5·1

Bacteriological examinations

In the 'aseptic' cheeses no organisms other than those added to the cheese milk were detected in 0·2 g aliquot of milk, curd or cheese at any examination. The threshold for detection of adventitious organisms in curd and cheese was of the order of $1:5 \times 10^6$ starter count in those cheeses made from milk inoculated with starter only and about 1:100 non-starter count in cheeses made from milk inoculated with organisms other than starter. Within any group of experiments the changes in numbers during ripening of starter, lactobacilli and micrococci relative to each other were similar to those based on average counts shown in Fig. 2.

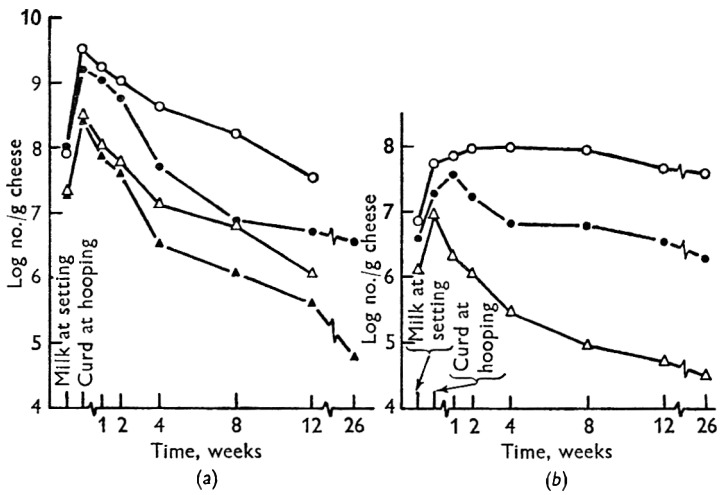

Fig. 2*a*. Starter counts in cheeses made under aseptic and under non-aseptic conditions. Average counts of *Str. lactis* (○—○) and of *Str. cremoris* (△—△) in control cheeses made under non-aseptic conditions from 'bulk' and from 'aseptic' milk. Average counts of *Str. lactis* (●—●) and of *Str. cremoris* (▲—▲) in cheeses made under aseptic conditions from milk inoculated with starter only.

Fig. 2*b*. Average counts of '*L. plantarum-casei*' strain 25·2, in cheeses made under 'aseptic' conditions from milk inoculated with this organism using starter (○—○) or gluconic acid lactone (●—●); and average counts of micrococcus L_1 in 'starter' cheeses made under 'aseptic' conditions from milk inoculated with this organism (△—△).

Starter counts in the control cheeses (non-aseptic make) were similar to those reported by Perry (1961) for normal cheeses. Strains of *Str. lactis* were again found to survive longer in cheeses than *Str. cremoris* strains.

In the 'aseptic' cheeses made from milk inoculated with the lactobacillus or the micrococcus, starter counts did not differ significantly from those in the corresponding control cheeses made under non-aseptic conditions. In 'aseptic' cheeses made without the addition of organisms other than starter (Fig. 2*a*), there was a more rapid decrease in starter numbers of both *Str. cremoris* and *Str. lactis* during the first few weeks of storage, but a less rapid decrease between the 12th and 26th week of storage (at 26 weeks the mean log no./g cheese for *Str. lactis* and *Str. cremoris* were 3·65 and 2·21, respectively). It is possible that starter growth in the young cheese is stimulated by the presence of other organisms, but that, in the absence of other organisms, starter bacteria can survive longer through lack of competition for nutrients.

At hooping the average count of non-starter organisms in the curd from bulk milk was $1{\cdot}5 \times 10^6$/g (range $0{\cdot}75 \times 10^6$–$2{\cdot}4 \times 10^6$ g). These counts increased about 20-fold during the first 4 weeks of storage and decreased slightly thereafter. Non-starter counts in the control cheeses made from 'aseptic' milk were about one-tenth to one-fifth of those in the control cheese made from 'bulk' milk.

The survival of the micrococcus strain L_1 in the 'aseptic' cheeses was similar to that reported for normal cheeses (Robertson & Perry, 1961) and appeared to be unaffected by the presence or absence of lactobacilli.

The survival of '*L. plantarum-casei*' strain 25·2 was similar in the 'aseptic' starter cheeses made with strains of *Str. cremoris* and *Str. lactis* and appeared to be unaffected by the presence of adventitious organisms or of the micrococcus strain L_1. However, in those cheeses in which gluconic acid lactone replaced the starter, the count of the lactobacillus decreased markedly between the 1st and 4th weeks of storage. This decrease occurred in the 2 'aseptic' gluconic acid lactone cheeses made from milk inoculated with the lactobacillus as well as in 2 similarly made cheeses which were subsequently rejected because of contamination. It is possible that gluconic acid lactone, or its breakdown products, inhibit bacterial growth. This is not, however, a general effect on all organisms, since non-starter counts were similar in the control cheeses made normally with starter and in those made with gluconic acid lactone. A symbiotic relationship between organisms such as '*L. plantarum-casei*' strain 25·2 and the strains of *Str. cremoris* and *Str. lactis* used as starters would offer an alternative explanation.

Flavour assessments

The control cheeses made from bulk and 'aseptic' milk developed the range of flavours characteristic of Cheddar. Definite Cheddar flavour was usually first detected at about 8 weeks and was more pronounced in the controls made from 'bulk' milk than in the controls made from 'aseptic' milk. These latter were slightly bitter at maturity.

The flavours of the 'aseptic' cheeses varied considerably. No definite Cheddar flavour was detected up to the 26-week examination in any of the 'aseptic' cheeses made with starter only or with the addition of '*L. plantarum-casei*' 25·2. The flavours of the young cheeses were generally slightly 'sharp' or 'acid' but this was not unpleasant and decreased somewhat with maturity, the cheeses having little or no flavour when older.

The addition of the micrococcus L_1 resulted in the development of a slight Cheddar flavour. This was not detected until the 26-week examination and was described as 'very mild'. At earlier examinations the flavours of these cheeses were described as 'clean and bland' and no flavour defects were noted.

In each group of 'aseptic' cheeses the best flavour resulted from the addition of both '*L. plantarum-casei*' 25·2 and the micrococcus L_1. As in the control cheeses, Cheddar flavour was first detected at about 8 weeks but it did not develop as strongly and at maturity was still 'mild'. These cheeses were also slightly 'sharp' or 'acid' in flavour.

'Lactis' flavour (Perry, 1961) was present in most of the cheeses made with strains of *Str. lactis* (ML_3 and ML_8) and was always more pronounced with ML_3 as starter than

with ML_8. In all control cheeses made with these starters the 'lactis' flavour developed after 2–4 weeks and became very pronounced at maturity. The flavour was much less pronounced in the cheeses made under 'aseptic' conditions and was not detected until the 26-week examination. At this examination the 'aseptic' cheeses made with ML_3 as starter had a slight but not unpleasant 'lactis' flavour. A similar flavour was present in the cheese made with ML_8 alone, but was not detected in the ML_8 cheeses made from milk inoculated with micrococcus L_1 and/or '*L. plantarum-casei*' 25·2. It appears therefore, that the production of 'lactis' flavour is associated with strains of *Str. lactis* and that it is intensified by the presence of non-starter organisms other than those added to the 'aseptic' milk in this investigation.

The flavours of the gluconic acid lactone cheeses, both controls and 'aseptic', were abnormal. They were generally unpleasant, being 'sharp' or 'bitter' with no indication of any Cheddar flavour. This failure to develop Cheddar flavour, even in the presence of '*L. plantarum-casei*' 25·2 and the micrococcus L_1, supports the conclusion of Mabbitt *et al.* (1955) that the products of starter are important in the production of good Cheddar flavour.

It is significant, however, that the starter-only 'aseptic' cheeses did not develop Cheddar flavour in the normal time. This could be associated with the lower starter populations in these cheeses or it could suggest that flavour does not depend on starter organisms alone. The beneficial effect on flavour of the micrococcus L_1, especially in combination with '*L. plantarum-casei*' 25·2, and the development of stronger Cheddar flavour in the control cheeses could thus be associated with the survival of starter organisms in cheeses containing other organisms, although the work of Robertson & Perry (1961) suggests that the micrococcus L_1 makes a more direct contribution to cheese flavour.

Body and texture

Whatever may be the effect of adventitious organisms on Cheddar flavour, they appear to play no major part in protein breakdown, since the body and texture of the 'starter-only' cheeses compared well with those of the 'bulk' controls at both 14 days and maturity. Further work involving a detailed analysis of the chemical composition of these cheeses is the subject of a separate publication (Lawrence, to be published).

The efficiency of the technique described is emphasized by the fact that, although the 'aseptic' vat was used in the main cheese-making room at the Institute factory where cheese manufacture on a semi-commercial scale was being carried out simultaneously (often using the same starter), phage was never found in samples taken from the 'aseptic' vat, yet was always detectable in samples taken during non-aseptic manufacture.

It would appear that the manufacture of cheeses under controlled bacteriological conditions offers a promising approach to the problem of cheese flavour and that for this work a sufficient degree of asepsis can be obtained using the relatively simple method described in this paper.

The authors gratefully acknowledge the helpful advice of Dr H. R. Whitehead; the advice and assistance in cheese-making of Messrs W. L. Harkness and J. Gilles; and the technical assistance of Mrs P. J. Kilgarriff.

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Printed in Great Britain

J. Dairy Res. (1964), **31**, 167

A meter for measuring pulsation ratio in milking machines

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(*Received* 10 *December* 1963)

Summary. A portable apparatus is described for indicating continuously as a measure of pulsation ratio the proportion of the cycle during which a liner is more than half open. With the machine set for milking, a probe with an electric switch which opens as the liner collapses is inserted in one liner, the others being closed with rubber bungs. The pulses of current from the switch are fed to a damping circuit so that the proportion of the cycle time during which current flows can be read on a meter. The problem of interpreting the various measures of pulsation ratio are discussed.

Pulsation ratio in the milking machine is usually measured by taking a continuous record of vacuum (extent to which the pressure is below the prevailing atmospheric pressure) in the pulsation chamber of a teat cup assembly over a few cycles, and then at some arbitrary value of vacuum, noting the proportion of the total cycle time for which the vacuum in the chamber is higher than this value. The Ruakura vacuum recorder (Whittleston, 1942) has been widely used, the pulsation ratio being measured at positions on the graph corresponding to atmospheric pressure, a vacuum of 7·5 inHg and more recently a vacuum of 5 inHg (Whittlestone & Olney, 1962). Pulsation ratio has also been measured by a cinematographic technique, the liner in a glass teat cup being filmed from a position at right angles to the direction of collapse, and the ratio defined as the proportion of each cycle for which the liner is more open than the mean position (Ardran, Kemp, Clough & Dodd, 1958).

The ratiometer described in this paper depends on liner wall movement. It is suitable for use in the field and has the advantage of allowing the ratio to be observed over longer continuous periods than is usually convenient with recording methods. The measuring circuit was suggested to us by our colleague, Mr H. Burton, to whom we are also indebted for an analysis showing that the response was expected to be linear.

Description of the ratiometer

A circuit diagram with appropriate values of components is given in Fig. 1. The special switch *LS* which is placed in the milking liner closes as soon as the liner is half-open, remains closed throughout the open phase of the liner, and opens again as the liner passes through the half-collapsed position. For the part of the pulsation cycle during which *LS* is closed, capacitor *C* charges from battery *B* through resistor R_1. However, current will flow continuously in the part of the circuit containing resistors R_2 and R_3 and the microammeter *A*. After about 10 pulsations steady condi-

tions will be established in which the increment of charge accumulating in the capacitor during each cycle equals the increment of discharge during the part of the cycle when the switch is open. The meter pointer will then oscillate about a mean position. the amplitude of oscillation depending to some extent on the mechanical characteristics of the meter used. With the 2 meters tried the amplitude of the swing was between 1 and 2% of full scale at a pulsation rate of 66 c/min and about 5% at 33 c/min. Even at the low rate there was no difficulty in reading the mean position to 1%. The values of R_2 and R_3 are chosen so that the meter, scaled linearly 0–100, reads full scale when LS is held closed. The meter in use then gives a direct reading of the percentage of the total cycle time for which the liner is open (% liner open time).

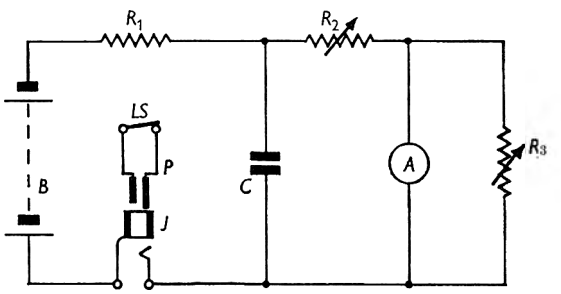

Fig. 1. Circuit diagram of the ratiometer. B, 9 V layer cell type battery; J and P, jack and plug; LS, special liner switch (see Fig. 2); R_1, 0·25 W, 150 kΩ; C, 1000 μF, 15 V, electrolytic; R_2, R_3, 25 kΩ, variable, 0·5 W; A, 50 μA, 1·5 kΩ, scaled 0–100 with legend '% liner open time'.

Some details of the special liner switch are given in Fig. 2. It was the most successful of several designs tried, and the dimensions given are appropriate for detecting the half-collapsed positions of many British liners in current use. Because the spring leaves of the switch are curved outwards during manufacture so that on assembly the contacts are under pressure, the switch will open only if both leaves are pressed. Therefore the assembly does not need to be exactly centred in the liner. For making measurements in wet liners it was found necessary to insulate the leaves of the switch and also the junctions of the leads. The switch is connected into the circuit by a plug and jack. Withdrawing the jack disconnects the battery.

ADJUSTING THE METER AND METHOD OF USE

Each time the instrument is to be used the plug of the lead is inserted into the jack and, if necessary, R_3 is adjusted to bring the needle of the meter to full scale deflexion. The liner switch is then held open to check that the needle comes to rest at the zero mark. The instrument is then ready for use. (If during use the insulation of the leaves of the switch becomes faulty, or if the switch becomes completely wetted with electrolyte, e.g. weak detergent solution, a positive reading will be obtained when the switch is held open.)

To take readings of pulsation ratio the milking machine is made ready for milking with all the mouths of the liners closed with, for example, rubber bungs and the clusters hung in such a way that the liners pulsate normally. The plane of collapse of the selected liner is then found with a finger and the special switch inserted so that the

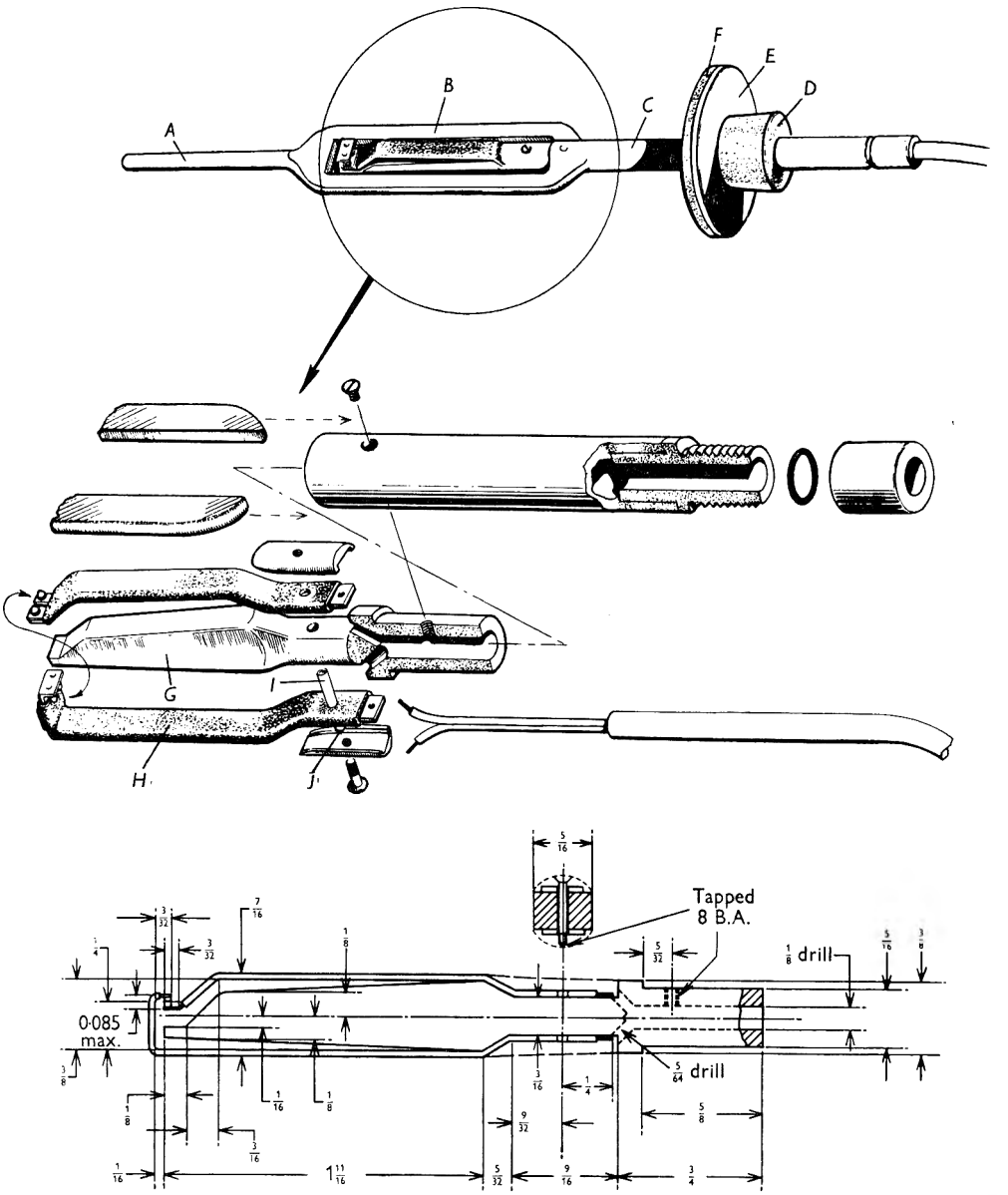

Fig. 2. Constructional details of the special liner switch. Dimensions in inches. *A*, rod $2\frac{1}{4}$ long $\times \frac{3}{16}$ diam. which enters the short milk tube, welded or silver-soldered to plate *B*, $3\frac{5}{8}$ long $\times \frac{7}{8} \times \frac{1}{10}$ with a slot $2\frac{1}{16}$ long $\times \frac{3}{8}$ wide, welded or silver-soldered for a distance of $\frac{5}{8}$ to *C*, a tube $3\frac{3}{16}$ long $\times$ $\frac{3}{8}$ O.D. $\times \frac{1}{4}$ I.D. with a gland to suit the cable used; *D*, rubber bung carrying a $\frac{1}{8}$ thick metal washer *E* and a $\frac{1}{8}$ thick rubber washer *F*, both $2\frac{1}{2}$ O.D.; *G*, switch support machined from fabric-reinforced thermoplastic bar; *H*, switch leaves built up from post office relay components and insulated with thin-walled P.V.C. tubing: *I*, insulating sleeve to prevent the screw contacting *H*: *J*, cover plates of fabric-reinforced plastic. The wires are threaded through the holes in *G* and soldered to the switch leaves *H*. The switch components are then assembled with epoxy resin adhesive (Araldite, AV 100 or 2 tube pack, CIBA (A.R.L.) Ltd., Duxford, Cambridge) on all mating surfaces and *G* is glued into tube *C* (plate *B* is bent aside and later re-straightened).

collapsing liner will press squarely on the leaves of the switch. By adjusting the collar on the stem, the contacts of the switch are placed about half-way along that part of the liner which collapses completely.

When the instrument is first used it is necessary to adjust the time constant of the circuit, i.e. the time for the pointer to fall to $1/e$ of the value indicated at the moment of switching off. The needle is adjusted to full scale deflexion (100), the jack is withdrawn, and the time for the needle to fall to a scale reading of 37 is measured. If the time is over 3 sec, R_2 is decreased, R_3 is readjusted to give full-scale deflexion, and the timing repeated.

A time constant of 3 sec allows the needle to take up a steady mean position in 10–15 sec at 60 c/min, with a needle swing of 1–2 scale divisions. If needle swing is a nuisance when lower pulsation rates are involved, a longer time constant may be found to be an advantage, although the instrument will, of course, respond more slowly. The following figures illustrate the point.

Pulsation rate, c/min	Time constant, sec	Time to steady reading, sec	Needle swing, % full-scale deflexion
30	3	14	6
	5	18	4
	8	40	3
60	3	14	2
	5	19	1
	8	43	1

PRECISION OF MEASUREMENT

Linearity of the measuring circuit was checked by replacing the liner switch with a cam-driven microswitch. At frequencies from 30 to 160 c/min, with the switch closed for proportions of each cycle varying from 25 to 75 %, the maximum deviation of the meter reading from the expected value was about 1 %, i.e. within the limits of accuracy of the microammeter.

Precision of the complete instrument was tested using the cinematographic technique of Ardran *et al.* (1958) at 100 frames/s. Four makes of liner were set up in glass teat cups and connected to an electric pulsator either as a single teat cup assembly or in a complete cluster with a single pulse tube 30 in long. The pulsator was driven by means of a cam-operated switch at frequencies of 30–90/min with the switch closed for 33, 50, 67 and 80 % of each cycle. A pulsator with a restricted opening was chosen so that the rate of change of pressure in the pulsation chamber of the teat cup was slow, thus giving fairly severe test conditions (Fig. 3*b*) for the switch which senses mechanically the position of the liner wall. Data at a pulsation rate of 66 c/min with electrical pulses persisting for 67 and 33 % of each cycle are given in Table 1. The error of the cinematographic technique due to indeterminacy of time measurement was minimized by averaging the results over at least 3 consecutive time cycles. From the data in Table 1 and similar results at other rates and ratios it was concluded that the precision of the ratiometer readings was about 2–3 % of full scale.

Table 1. *Comparison of pulsation ratios as percentage liner open time obtained cinematographically and by ratiometer with 4 makes of liner*

The glass cup with liner was either connected direct to an electric pulsator or formed part of a full milking cluster. Vacuum 15 inHg; pulsation rate, 66 c/min.

Make of liner		Cine		Ratiometer		Ratiometer error
	Current on to pulsator for 67 % of cycle					
A, extruded	Single cup	61·5		63·0		+1·5
	Full cluster		54·4		53·5	−0·9
B, moulded	Single cup	61·2		63·5		+2·3
	Full cluster		51·8		53·5	+1·7
C, moulded	Single cup	61·2		63·0		+1·8
	Full cluster		51·3		51·5	+0·2
D, moulded	Single cup	62·6		62·6		0
	Full cluster		53·5		53·5	+0·2
	Current on to pulsator for 33 % of cycle					
A, extruded	Single cup	30·2		31·5		+1·3
	Full cluster		21·0		20·8	−0·2
B, moulded	Single cup	30·9		32·0		+1·1
	Full cluster		21·9		23·2	+1·3
C, moulded	Single cup	29·3		31·2		+1·9
	Full cluster		16·5		19·7	+3·2
D, moulded	Single cup	30·0		30·5		+0·5
	Full cluster		18·6		18·5	−0·1

Table 2. *Pulsation ratios (as percentage liner open time) measured by ratiometer and by the Ruakura vacuum recorder of 10 milking machines on farms*

From the vacuum recorder charts ratios were calculated half-way between the maximum vacuum recorded and atmospheric pressure, and also at a distance from the maximum vacuum corresponding to 3 inHg.

			Unit 1			Unit 2			Unit 3		
			By recorder			By recorder			By recorder		
Make of milking machine	Pulsation rate, c/min	Milking vacuum, inHg below atmospheric pressure	Ratiometer	½-way line	3 inHg below max. vac.	Ratiometer	½-way line	3 inHg below max. vac.	Ratiometer	½-way line	3 inHg below max. vac.
A	42	13·6	55	72	55	54	69	5–	54	70	52
A	42	15·2	34	48	35	51	66	49	39	51	35
A	52	15·0	62	74	59	62	75	60	62	73	59
A	50	13·0	62	74	59	63	74	60	65	74	61
A*	78	12·6	45	69	48	40	65	43	26	61	30
B	60	14·9	43	71	58	45	71	49	46	73	52
B	46	14·2	50	74	57	53	73	5–	56	71	56
B	64	15·3	52	78	52	57	81	57	56	79	56
C	50	15·6	44	65	49	40	61	50	38	58	41
C+	58	15·0	36	69	48	45	69	46	44	68	49

* Fig. 3*d* refers to this farm.
+ Fig. 3*c* refers to this farm.

COMPARISON OF RATIOMETER READINGS WITH PULSATION RATIOS DETERMINED FROM VACUUM RECORDER CHARTS

Simultaneous records were made by a Ruakura vacuum recorder and a ratiometer with 3 makes of machine on 10 commercial farms. Ratios were calculated from the recorder charts at a position half-way between the maximum vacuum recorded and

atmospheric pressure. It can be seen from Table 2 that these ratios were a good deal higher in terms of percentage liner open time than the ratiometer readings. The differences would have been even greater if the measurements had been made on the chart at the position corresponding to a vacuum of 5 inHg and greater still at atmospheric pressure. As shown in Table 2, agreement was better if ratios were measured on the charts at a position corresponding to only 3 inHg from the maximum vacuum recorded. Two examples from the 10 farms of vacuum recorder traces showing low rates of change of pressure are reproduced in Fig. 3*c*, *d*.

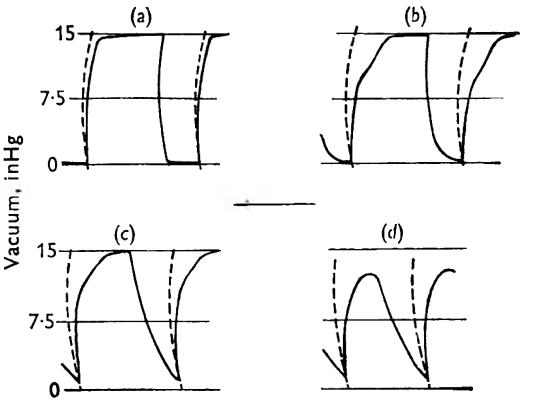

Fig. 3. Vacuum recorder traces with (*a*) a single teat cup assembly and (*b*) a whole cluster attached to the pulsator used in laboratory measurements. (*c*) and (*d*) are traces taken at 2 of the 10 commercial farms (see Table 2). Arrow indicates direction of movement of the paper relative to the pen.

DISCUSSION

The instrument described is capable of giving a reasonably precise indication of pulsation ratio defined by liner wall movement. However, as with ratios found from charts of the Ruakura vacuum recorder, there is some uncertainty as to the usefulness of the results obtained. With rates of change of pressure as high as those shown in Fig. 3*a*, it is of no great consequence what datum is used. On the other hand, for the conditions indicated in Fig. 3*b*, values for pulsation ratio (release:squeeze) are 90:10 measured at atmospheric pressure; 70:30 at a vacuum of 5 inHg; 63:37 half-way between atmospheric pressure and the maximum vacuum recorded; and 52:48 by the ratiometer described here. The difference between values obtained by the various methods would be even greater for the conditions shown in Fig. 3*c*, *d*. It is possible that the pulsation characteristics imposed on the liner by the pressure changes shown in Fig. 3*b*, *c*, *d* are all unsatisfactory for milking, but in fact no direct evidence to suggest this has as yet been published. Whittlestone & Olney (1962) proposed the 5 inHg datum on the basis that milk ceases to flow when the pressure in the teat cup chamber has risen to this value. While it may be true that effective pulsation preventing damage in the broadest sense to the cow coincides with sufficient force to stop milk flow, the assumption remains unproved. Our own data show that some force at least is exerted by the liner on the teat when the pressure difference across the liner is as low as 3 inHg.

The milking equipment used in Great Britain has for many years given charac-

teristically slow rates of flow of air from the pulsation chamber during evacuation. More recently the rate of entry of air has also in many instances been deliberately restricted to widen the ratio and to reduce the violence of the squeeze. It might well be that in striking a balance between fast milking and freedom from ill effects to the cow slow rates of change of pressure are to be preferred to fast rates. However, as the effect of pressure conditions as different as those shown in Fig. 3*a–d* is not known (except perhaps in terms of milking rate) it is not possible to attach any great significance to a single parameter such as an arbitrary pulsation ratio. Thus it would seem that the measurement of pulsation ratio can only be of limited usefulness, such as for detecting differences between units of the one installation, or irregular performance of one unit.

We acknowledge with gratitude the help of Mr H. Burton, Mr P. A. Clough and Dr F. H. Dodd at all stages of this work.

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Printed in Great Britain

J. Dairy Res. (1964), **31**, 175

The phosphoamidase action of rennin on casein

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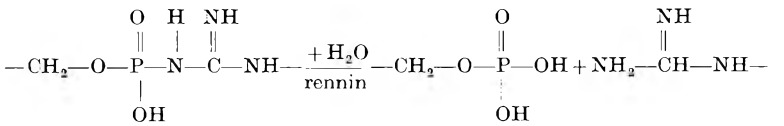
(*Received* 13 *December* 1963)

Summary. It is proposed that —N—P—N— bonds exist in casein and that these bonds can be hydrolysed probably at different rates by the action of rennin. It is proposed that the phosphorus is linked with the guanido group of arginine.

Introduction

Much interest has been shown in the action of rennin on casein and recently Lindquist (1963) reviewed the whole field. However, little interest has been shown in the phosphoamidase action of rennin.

Berridge (1945) inferred that rennin, in its action on casein, possessed such an activity. Holter & Li (1950) demonstrated that crystalline rennin had a hydrolytic action on a substituted amide of phosphoric acid and this prompted Nitschmann & Varin (1951) to suggest that such groups could exist in casein and could be attacked by rennin. Using electrometric titration data, Dyachenko (1959) showed that the isoelectric point of casein lay between pH 4·6 and 4·7, while that of paracasein (the product remaining after rennin action) was between pH 5·0 and 5·2. He also showed that the essential difference between the titration curves for the 2 caseins lay in the 'divergence in the strongly alkaline region starting from pH 10·2 when the ϵ-NH_2 groups of lysine and the guanidine groups of arginine exhibit their buffering effect'. Since lysine amino groups had been shown not to increase with rennin action (Inikhov, 1922; Koning & Stadhouders, 1962), the appearance of strongly alkaline groups in paracasein was attributed to arginine guanido groups. Dyachenko postulated that rennin splits a —P—N— linkage and proposed the following reaction.



Cheeseman, Rawitscher & Sturtevant (1963) have questioned the esterase activity proposed for rennin by Garnier, Mocquot & Brignon (1962).

It seemed desirable, therefore, to examine further the activity of rennin in the release of the bound phosphate in casein.

EXPERIMENTAL

Preparation of rennin

Ernstrom's method (1958) was followed, except that the last 3 steps were eliminated and the rennin from step 5 was dissolved in a minimum of distilled water, freeze-dried and then stored at −15 °C.

Starch-gel electrophoresis of the purified rennin indicated 2 major bands and 3 very minor bands (Aiyar, 1963). Ernstrom reported 4–5 electrophoretic peaks for his crystalline rennin using free-boundary electrophoresis. It is of interest to note that when the 2 major bands from the starch gel were eluted and re-examined by the same procedure they each gave 2 identical bands at positions corresponding to the sites of the major bands in the original run. The 2 bands thus appear to represent different states of aggregation of the enzyme, the extent of aggregation being controlled by an equilibrium condition.

Preparation of alkaline milk phosphomonoesterase

The method of Morton (1955) was followed up to and including the dialysis stage in step 4 after which the dialysed extract was freeze-dried and then stored at −15 °C.

Starch-gel electrophoresis of the purified enzyme indicated 2 major and 4–5 minor bands.

Preparation of freeze-dried acid casein

The method followed was that of Hipp, Groves, Custer & McMeekin (1959) and Wake & Baldwin (1961) which gave a casein containing 2·0% ash and 0·74% phosphorus.

Urea starch-gel electrophoresis indicated 15–17 components (Aiyar, 1963), which was comparable with the results of Wake & Baldwin (1961) and Neelin, Rose & Tessier (1962).

Preparation of casein substrate

A smooth paste was first prepared by mixing the weighed quantity of the freeze-dried acid casein with a small quantity of distilled water and this was then dispersed with more distilled water. To the stirred casein suspension, 0·1 N-NaOH was added dropwise to adjust the pH to 6·75. Nisin was added to give a final concentration of 100 ppm. and the casein solution was then made up to the required volume (Nisin did not interfere with the enzyme actions of the rennin or alkaline phosphomonoesterase).

Determination of phosphate liberation

(*a*) *With rennin*

20 ml of a 3·5% (w/v) casein solution (pH 6·7) was held at 38 °C in a temperature-controlled water bath and 5 ml of an aqueous solution of the enzyme was then added with mixing to give the desired enzyme/substrate ratio (w/w). After the desired time the reaction was stopped by pouring the mixture into a stoppered measuring cylinder containing 25 ml vanadium molybdate solution prepared according to Method Sheet no. 60, Unicam Instruments (1961). The vanadium molybdate precipitated the casein, inactivated the enzyme and reacted with liberated orthophosphate ions to give the soluble, orange coloured vanadium-phosphomolybdate complex. The tube was rinsed with distilled water and the combined reaction mixture and wash made up to 75 ml and filtered, through Whatman No. 42 paper on a Buchner funnel.

The filtered samples were set aside for at least 2 h to permit full colour development. Since there was a continuous slow aggregation of the reaction products it was found necessary to refilter the solutions before measuring transmissions. Repeated filtrations between 4 and 24 h after colour development did not significantly affect the final transmission reading.

Percentage transmission was measured on a Beckman DU Spectrophotometer at 420 mμm.

(*b*) *With phosphomonoesterase*

(i) At pH 6·7—method as for rennin;

(ii) at pH 9·5—to 20 ml of a 3·5% (w/v) casein solution made up in the double strength $Na_2CO_3/NaHCO_3$ buffer of Tramer & Wright (1950) at pH 9·5 was added 5 ml of enzyme solution in a similar buffer. The reaction was stopped and phosphate liberation measured as in (*a*).

Using the synthetic substrate disodium *p*-nitrophenyl phosphate it was found that use of double strength $Na_2CO_3/NaHCO_3$ buffer did not affect the activity of the phosphomonoesterase.

(*c*) *With rennin then phosphomonoesterase*

To 20 ml of a 3·5% (w/v) casein solution (pH 6·7) the desired quantity of rennin was added as a powder. At the appropriate time the phosphomonoesterase was added as a solution in 5 ml of double strength $Na_2CO_3/NaHCO_3$ buffer. This increased the pH to 9·5, inactivating the rennin, but giving optimum conditions for phosphomonoesterase activity. The reaction was stopped and phosphate liberation measured as in (*a*).

(*d*) *With phosphomonoesterase then rennin*

To 15 ml 4·66% (w/v) casein solution in double strength $Na_2CO_3/NaHCO_3$ buffer at pH 9·5 was added 5 ml of the phosphomonoesterase in a similar buffer. At the appropriate time 5 ml of approximately 0·2 N-HCl was added to reduce the pH to 6·65–6·70 and the rennin was added as a powder. The reaction was stopped and phosphate liberation measured as in (*a*).

The final casein concentration was the same for all series.

Determination of exposure of guanido groups

The Sakaguchi (1925) test was used to detect the exposed guanido groups, the test being performed on a duplicate sample treated in a manner identical with that used for measuring phosphate liberation. The 2 tests were performed simultaneously. Two ml of the reaction mixture were removed at the appropriate time and transferred to a colorimeter tube containing 0·5 ml 1 N-NaOH (this stopped rennin action immediately); 1 ml of distilled water was added, followed by 0·5 ml of 0·1% (w/v) naphthol in 70% ethanol and 1 ml of 5% (w/v) sodium hypochlorite solution. The colour development was carried out with the tubes immersed in a water bath at 38 °C. The colour was developed for 2 min after the addition of reagents, the tubes were removed, allowed to cool in the dark, and percentage transmission read at 420 mμm

(maximum absorption) after a minimum of $\frac{1}{2}$ h had elapsed to permit full colour development. The colour was stable for at least 2 h.

A standard arginine solution was prepared by dissolving 120·92 mg of arginine monochloride (L.R. grade) in distilled water, the pH was adjusted to 7·0 with N/20 NaOH and the volume made up to 100 ml. The arginine content of this solution was taken as 100 mg % (w/v).

To prepare the calibration curve for the guanido group of arginine, measured quantities of the standard arginine solution were added to 2 ml of a 2·8 % (w/v) casein solution (i.e. the same concentration of casein as in the test samples). Distilled water was added to equalize volumes and this was followed by the Sakaguchi test reagents. Total volume was in all cases 5 ml.

The casein solutions used to measure the colour after the Sakaguchi reaction were opaque but control experiments in which casein samples were incubated with rennin for periods of up to 24 h and then treated with 0·5 ml 1 N-NaOH, as in the Sakaguchi reaction, showed no change in opacity. The increased adsorption that occurred after such periods of incubation and with the addition of all the Sakaguchi reagents was considered, therefore, to be due to increased colour production as the result of rennin action.

Since arginine was not released into solution by rennin action, but remained attached to the para-casein it was not possible to remove the casein by precipitation and then measure the colour in the clear supernatant. A filtered sample gave no colour test for soluble arginine. Control tests without enzyme addition were run with each experiment.

The results given in Table 2 are each the mean of 6 separate determinations and are based on the assumption that both free and bound arginine (free guanido group) have similar colour values. The differences between each succeeding mean value are, respectively, significant at the following values $< 0{\cdot}025$, $< 0{\cdot}01$, $< 0{\cdot}005$.

RESULTS

(*a*) *Phosphatase activity of rennin and alkaline phosphomonoesterase on 2 phosphomonoesterase substrates*

(i) *Disodium* p-*nitrophenyl phosphate*

The modified Aschaffenburg–Mullen phosphate test (Tramer & Wright, 1950) was carried out using the 2 enzymes. Two buffer systems were used, the $Na_2CO_3/NaHCO_3$ buffer with reaction pH 9·6 and Na_2HPO_4/KH_2PO_4 buffer pH 6·2 (Vogel, 1959).

Observation at 2 and 18 h indicated no activity with rennin at either pH, whereas the milk phosphomonoesterase was very active at the alkaline pH and still retained considerable activity at the acid pH.

(ii) *Glucose*-1-*phosphoric acid* (*di-potassium salt*)

Phosphate liberation was measured after incubation at pH 9·6. Milk phosphomonoesterase released all the phosphorus within 1 h.

It is interesting to note that commercial rennet showed limited phosphoesterase activity with both these substrates.

(*b*) *Liberation of phosphate from casein by rennin and phosphomonoesterase used individually or together*

Results are given in Table 1. These results are the average of 2 entirely independent experiments. Transmission values of duplicate samples differed by a maximum of 2 units (0·026 mg P_2O_5). Total phosphorus content in 700 mg casein used for each test amounted to 5·2 mg P or 12 mg P_2O_5 equivalent.

The extended reaction time (30 h) for phosphomonoesterase was based on Perlman's (1955) observation that phosphomonoesterase acted only slowly on unfractionated casein.

Table 1 clearly indicates that alkaline milk phosphomonoesterase under the conditions of the experiment does not release phosphorus from casein, whereas rennin liberated 3·1–3·4 % of the total phosphorus in 1 h. Furthermore, alkaline phosphomonoesterase was not able to release any further phosphorus after the preliminary action of rennin on the substrate.

Table 1. *Liberation of phosphate from casein by rennin and alkaline phosphomonoesterase acting individually or together*

Sample	Reaction*	% transmission at 420 mμ	Phosphate released, mg P_2O_5	Phosphorus released, % total P
1	C 6·7/1 h + 5 ml D.S.B./30 h	105	Nil	Nil
2	C 9·5/30 h + 5 ml 0·2 N-HCl/1 h	105	Nil	Nil
3	R^N + P 9·5/30 h	105	Nil	Nil
4	C 6·7/1 h + P 9·5/30 h	105	Nil	Nil
5	C 6·7 + R^N/1 h + 5 ml D.S.B./30 h	74	0·40	3·4
6	C 6·7 + R^N/1 h + P 9·5/30 h	75	0·39	3·3
7	C 9·5 + P/30 h + R^N 6·7/1 h†	76	0·37	3·1

* C = 20 ml of 3·5 % casein solution; 6·7 and 9·5 refer to reaction pH; D.S.B. = double strength carbonate/bicarbonate buffer pH 9·5; R^N = rennin; P = alkaline phosphomonoesterase; 1 h and 30 h refer to time in hours; enzyme/substrate ratios used: rennin, 1:87·5; phosphomonoesterase 1:116·7.

† Rennin was added as powder after 5 ml 0·2 N-HCl had been added to bring reaction back to pH 6·7.

(*c*) *Phosphatase activity of different rennin concentrations on casein at a fixed reaction time and of fixed rennin concentration over extended reaction times*

Enzyme/substrate ratios of 1:2188, 1:1094, 1:728, 1:364, 1:182, 1:87·5 were used and the phosphate liberated after 1 h was measured. It was found that with the most dilute system (1:2188) no detectable phosphate was liberated in 1 h, although a subsequent experiment gave 3·1 % of phosphorus released after 52 h incubation. The quantities of phosphate released in 1 h with the remaining enzyme/substrate ratios were, respectively, 0·32, 0·50, 0·94, 1·9, 3·3 %. The latter value agrees with values for the same enzyme/substrate ratio given in Table 1.

Fig. 1 shows the effect of different enzyme/substrate ratios on the rate of release of phosphate and on its extent over 24 h. Using an enzyme/substrate ratio of 1:87·5 and incubating for 24 h, 9·5 % of the phosphorus was released, the slope of the curve indicated that further phosphate could be released given time.

Fig. 2 shows the effect of increasing the substrate concentration on the release of phosphorus over 24 h using the same concentration of enzyme. Since the plot of the

phosphorus released under these conditions shows no sign of flattening out it is assumed that saturation or limiting conditions of the enzyme–substrate relationship have not yet been reached, and in an endeavour to apply approximate quantitative relationships between the released phosphorus and the exposed guanido groups of

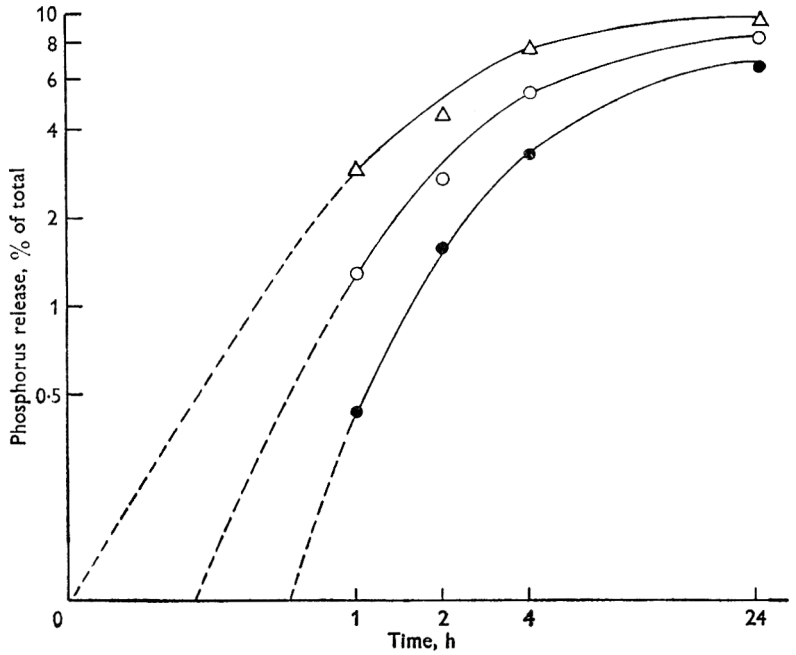


Fig. 1. The effect of various enzyme/substrate ratios on the release of phosphorus after different periods of incubation. Enzyme/substrate ratios: △, 1:87·5; ○, 1:182; ●, 1:364. Scales: ordinate, log (1 cycle); abscissa, log (3 cycles).

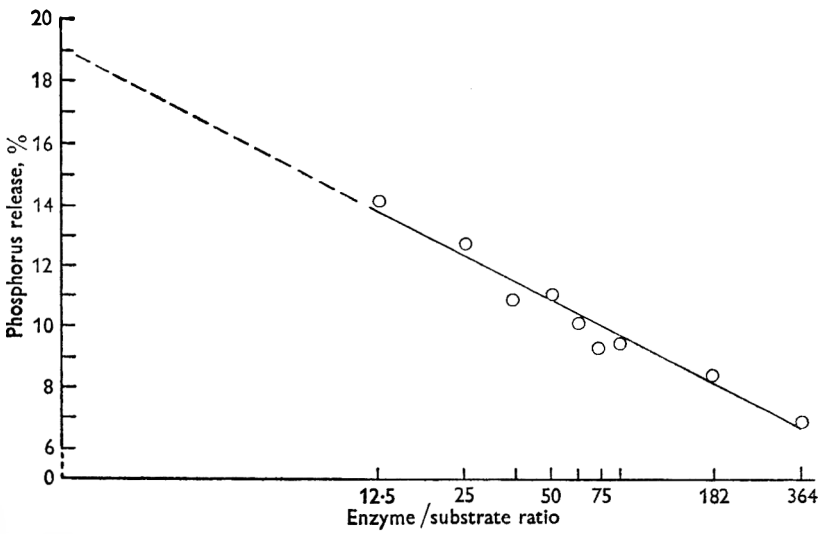


Fig. 2. The effect of various enzyme/substrate ratios on the total amount of phosphorus released during 24 h incubation. Scales: ordinate, linear; abscissa, log (1 cycle).

arginine the curve has been extrapolated to zero to obtain the probable maximum value for phosphorus released. By calculation the regression equation for the data depicted in Fig. 2 is

$$Y_c = 19{\cdot}14 - 4{\cdot}913x,$$

where x is the log value of the enzyme/substrate ratio, the variance is 0·24 and the standard error of the estimate is 0·49 % P. It would therefore appear that about 20 % of the total phosphorus of casein can be released by rennin.

The pH of the casein solutions during a 24 h experiment with added rennin generally dropped from 6·7 to 6·2–6·3 and during this time the solution became increasingly opaque and ultimately deposited a precipitate. The opaqueness was very obvious when compared with non-rennin treated controls after 4 h incubation. It is possible that as a result of rennin action on the dispersed casein, reactive groups are exposed which permit casein aggregates of greater than colloidal dimensions to develop or, alternatively, the casein molecule may, under the action of rennin, be unfolding to give a denatured product as proposed by Berridge (1942).

The drop in pH was found to have no measurable effect on rennin activity when a veronal–acetate buffer (Michaelis, 1931) at pH 6·7 was used to stabilize the reaction.

Table 2. *Exposure of guanido groups and liberation of phosphate from casein by rennin*

Sample	Reaction*	Phosphorus released		Arginine exposed		Moles arginine exposed / Moles phosphorus released
		mg/100 g casein	moles × 10^5/100 g casein	mg/100 g casein	moles × 10^5/100 g casein	
1–4	C 6·7+5 ml distilled water	Nil	Nil	Nil	Nil	
5	C 6·7+RN/1 h	20	65	730	420	6·5:1
6	C 6·7+RN/2 h	41	132	860	500	3·8:1
7	C 6·7+RN/4 h	56	180	980	560	3·1:1
8	C 6·7+RN/24 h	71	230	1210	700	3·0:1
9	C 6·7+RN/48 h	—	—	1160	670	—
10	C 6·7+6·25 ml N-NaOH+RN/24 h	—	—	Nil	Nil	—

* For explanation of abbreviations see footnote to Table 1; enzyme/substrate ratio, 1:87·5.

(*d*) *Release of phosphorus and exposure of guanido groups of arginine as a result of rennin action on casein*

Table 2 indicates that exposure of guanido groups as the result of rennin action on casein occurred much more quickly than the release of phosphorus. The release of phosphorus was comparable with that of earlier experiments. Thus, the ratio of moles of phosphorus released to moles of arginine exposed was 1:6·5 at the end of the first hour, but was only 1:3·0 at the end of 24 h as phosphate release continued and arginine exposure levelled off. No further guanido groups were detectable on incubating for a further 24 h. Results discussed earlier (Fig. 2) indicate that ultimately around 20 % of the total phosphorus appears to be capable of release by rennin. Twenty per cent of the total phosphorus in the casein used is equivalent to 148 mg P/100 g casein or 480×10^{-5} moles P/100 g casein. Thus, the ultimate ratio of moles of arginine exposed to moles phosphorus released approximates 1·5:1.

DISCUSSION

The present experiments have indicated that about 20 % of the phosphorus in casein may be released through the action of rennin, but no release of phosphorus was evident through phosphomonoesterase action. Purified rennin did not have phosphatase action on the synthetic substrate, di-sodium *p*-nitrophenyl phosphate, whereas the alkaline phosphomonoesterase was extremely active. Commercial rennet had some activity, which was not unexpected, since it is possible for contaminating enzymes from the calf mucosa to get into the rennet during its commercial extraction.

Kalan & Telka (1959) observed an 85 % dephosphorylation of whole casein, using calf intestinal mucosa acid phosphomonoesterase, which was in general agreement with Zittle & Bingham's finding (1959) based on the use of alkaline milk phosphomonoesterase which, however, had shown greatest activity on casein at neutral pH.

Sampathkumar, Sundararajan & Sarma (1957) reported 55 % dephosphorylation of whole casein using an acid pyrophosphatase from soyabean, and Sundararajan & Sarma (1957) dephosphorylated whole casein to the extent of 77 % using a phosphoprotein phosphatase (acid) from rat tissues. In general, results from the action of phosphatases on casein appear confusing. However, the results obtained by all the above workers indicate that about 15–20 % of the total 'phosphorus' in casein was not released by their enzyme preparations.

In this present study it has been deduced that rennin may be capable of liberating about 20 % of the total phosphorus in casein and in view of the quoted results of other workers with various phosphatases it is suggested that this phosphorus could arise from phosphate linkages which appear to resist the action of phosphatases in general.

Perlman (1955) indicated that 40 % of the total phosphorus in α-casein was bound through an —N—P—O— link and Dyachenko (1959) postulated a phosphoamidase action of rennin on casein, and suggested that the phosphoamidase action was on phosphorus linked to the guanido group of arginine.

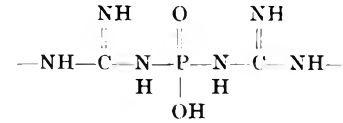
Perlman (1955) considered that the remaining phosphate linkages in casein were of the ester or pyrophosphate type. No consideration was given to a linkage of the type —N—P—N—.

If 40 % of the phosphate linkages in α-casein were of the —N—P—O— type, then it should be possible to liberate orthophosphate ions by the combined action of rennin and alkaline phosphomonoesterase. Table 1 (samples 4, 6 and 7) showed that rennin plus the alkaline phosphomonoesterase prepared for these studies released no more orthophosphate than was released by rennin alone. Since free orthophosphate ions only are capable of forming the phosphomolybdate complex (Durrant & Durrant, 1962; Pauling, 1952), rennin must be capable of completely hydrolysing some of the phosphate linkages present, and since the rennin used in this study did not possess measurable phosphoesterase activity, it is reasonable to conclude that some phosphorus in casein is linked —N—P—N—.

With regard to the exposure of guanido groups of the arginine residues in acid casein by rennin action the results indicate that ultimately for every mole of phosphorus released, more than 1 mole of arginine appeared to be exposed.

This exposure of arginine appeared to go at a very much faster rate initially than the release of phosphorus. Thus, the ratio of phosphorus released to arginine exposed

(at the guanido groups) was of the order of 1:6–1:7 after 1 h. The ultimate 1:1·5 ratio of phosphorus released to arginine exposed suggests an —N—P—N— link in the casein molecule, i.e. 2 arginine residues linked by their guanido groups through phosphorus as follows:



Half of the rennin-sensitive phosphorus is probably attached to only 1 guanido group.

A reasonable explanation for the observed changing ratio of phosphorus released to guanido groups exposed can be derived if one bears in mind that only phosphorus completely released as orthophosphate ions is measurable, whereas the guanido group exposed through the hydrolysis of only one bond of the —N—P—N— linkage becomes detectable. Since a 1:1 ratio of —N—P—N— linkages and —N—P linkages has been proposed, let us consider for mathematical convenience the hydrolysis of 66 of each of these linkages in casein by rennin. It is assumed that one phosphorus bond in —N—P—N— is more readily hydrolysed than either the remaining bond or the simple N—P bond and that this difference in hydrolysis rate is of the order 11:1. Table 3 indicates the gradual change in the ratio of guanido groups exposed to phosphorus released which would be expected with the passing of time.

Table 3. *Calculated change in the ratio moles arginine exposed: moles phosphorus released*

Time interval, h ...	t_0	t_1-x	t_1	t_2	t_4	t_{24}	t_∞
No. of original –N–P–N– linkages remaining	66	55	0	—	—	—	—
No. of original –N–P linkages remaining	66	65	60	—	—	—	—
Total no. of –N–P linkages remaining	66	75	120	110·4	101·2	94·0	0
No. of 'P' released from all –N–P	—	2	12	21·6	30·8	38	132
No. of 'N' exposed from all –N–P	—	2	12	21·6	30·8	38	132
No. of 'N' exposed from all –N–P–N–	—	11	66	66	66	66	66
Total no. of 'N' exposed	—	13	78	87·6	96·8	104	198
Ratio 'N':'P' $\left(\frac{\text{moles arginine exposed}}{\text{moles phosphorus released}}\right)$	—	6·5	6·5	4·05	3·1	2·7	1·5
Observed ratio 'N':'P'	—	—	6·5	3·8	3·1	3·0	—

To have an equivalent time basis for comparison with the observed change in 'N':'P' ratio, the number of moles of 'P' released for the various time intervals used in the calculation is the same as that observed for the release of phosphate with an enzyme/substrate ratio of 1:87·5 and since this is the same condition as used in determining the observed 'N':'P' ratio the calculated and observed figures are comparable. The rates are found to agree very well.

No new α-amino end group has been demonstrated on the para-casein or para-κ-casein molecules formed during the initial fast reaction of rennin on casein. This would not be expected if, as suggested in this study, the phosphorus were attached to guanido groups at either end. Cleavage of either P—N bonds would only expose guanido groups and not produce a new α-amino end group.

The work of Dyachenko (1959) indicated that the basic groups exposed by rennin action on casein stem mainly from arginine, and results from this study support this view.

In conclusion, it appears that an —N—P—N— link is present in casein; the phosphorus links 2 arginine residues through their guanido groups; rennin is capable of hydrolysing N—P bonds and may hydrolyse the bonds of —N—P—N— at different rates.

Acknowledgement is made to the N.Z. Rennet Co. Ltd. for contributing the commercial rennet from which the purified rennin was prepared; to Aplin & Barret Ltd., for the sample of Nisin; to The Dairy Research Institute (N.Z.) for the milk used in preparing the acid casein and for the buttermilk used in preparing the alkaline milk phosphatase.

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Printed in Great Britain

Steam distillation of taints from cream

IX. Vapour/liquid equilibrium relationships for indole and skatole in water and cream

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(*Received* 30 *December* 1963)

SUMMARY. The vapour/liquid equilibrium coefficients for the steam distillation of indole and skatole from water solution over the range of concentrations 0·1 to 20–30 ppm. were found to be 2·4 and 4·7, respectively. The coefficient for indole in a cream containing 30% fat was found to be 0·31, and for skatole, in a cream containing 40% fat, 0·14. It is concluded that complete removal of indole and skatole contaminations from cream by use of steam distillation equipment is not commercially practicable.

Indole and skatole have been shown to be present in appreciable quantities in some milks and creams and to be carried forward into the resultant butter. Clarke *et al.* (1937) found that high indole content of cream and butter may be due to breakdown of proteins by bacteria. Hussong & Quam (1943) reported that feeding of 'peppergrass' (*Lepidium virginicum*) to cows caused the milk and butter to have a strong off-flavour, similar to the odour of indole, and that butter from cows fed on peppergrass had indole contents as high as 22 ppm. in the fat compared with the normal value of 0·2–0·3 ppm. Conochie (1950) found that on feeding of 'peppercress' (*L. hyssopifolium*) for several days there was a rapid rise in the indole content from 0·06 ppm. in the butterfat to a content of 6·8 ppm., with the simultaneous appearance of a strong peppercress taint in the milk. Similar increases in indole content were caused by the feeding of *L. bonariense* (Conochie, 1953). Conochie found that indole and skatole are normally present in the fat of the milks of the cow and goat in concentrations ranging from 0·05 to 0·3 ppm., but in the fats from milks of 2 herds of cows feeding on *Lepidium* under field conditions he found 10·4 and 4·4 ppm. of indole and 10·3 and 5·1 ppm. of skatole, and in the fats from milks of 4 goats feeding on a diet containing *Lepidium* he found up to 11·3 ppm. of indole and up to 18·7 ppm. of skatole. Conochie showed also that butter from *Lepidium*-tainted cream contained abnormal proportions of both indole and skatole, and he stated (1950) that 0·4 ppm. of indole affects the flavour of butter.

There has not previously been any quantitative investigation of methods for removing indole and skatole from cream. The usual method of cream treatment for removal of off-flavours is steam distillation in equipment such as the Murray Vacreator (concurrent flow of steam and cream) or the A.P.V. Cream Treatment Unit (counter-

current flow of steam and cream). Indole is reported to be 'readily volatile in steam' (Thorpe, 1928), and many of the methods of estimation of indole in biological fluids utilize steam distillation as the first step in the analysis (Clarke *et al.*, 1937; Hussong & Quam, 1943). There are, however, no quantitative data on the steam volatility of either indole or skatole.

Indole and skatole are only slightly soluble in cold water but they are soluble in hot water, from which they can be crystallized. They are more soluble in fat than in water, and in the methods of analysis used by Clarke *et al.* (1937) and by Hussong & Quam (1943) for indole, the assumption is made that all the indole is present in the fat phase. From the results given in previous papers in this series (McDowall, 1959*a*, *b*, *c*) it can be expected that the extent of removal of indole and skatole from cream by steam distillation will be dependent both on the vapour/liquid equilibrium coefficients for these substances in water solution and on their solubility partition between fat and skim-milk at the temperature of steam distillation.

METHODS

The vapour/liquid equilibrium relationships were measured in the continuous vaporization equilibrium still described by McDowall (1955). The contents of indole and skatole in the vapour condensate and in the liquid at time of partition were estimated by direct measurements of the absorption in a Beckman Spectrophotometer. Indole was found to give maximum absorption at 218 mμm and skatole at 222 mμm. The measurements were made at these wave-lengths and the amounts present estimated from a standard curve prepared using indole and skatole from the same source.

For the measurements in cream the contents of the substances in the residual cream were calculated by difference as already described for the higher diketones (McDowall, 1959*b*).

RESULTS

Distillation from water

The results for indole, Fig. 1, show that over the range of concentrations 0·1 to about 30 ppm. in the liquid at time of partition the relationship between concentration in the vapour (C_V) and concentration in the liquid (C_L) was linear, $C_V/C_L = 2{\cdot}4$. The results for skatole, also in Fig. 1, show that the relationship was linear over the range of concentrations 0·1–20 ppm. in the liquid at time of partition, $C_V/C_L = 4{\cdot}7$. Skatole in water solution is thus shown to be more readily volatile than is indole.

Distillation from cream

The results are given in Fig. 2. The cream from which the indole was distilled contained approximately 30 % of fat. The C_V/C_L relationship was linear up to the maximum concentration investigated, about 16 ppm. in the liquid at the time of partition, $C_V/C_L = 0{\cdot}31$. The cream from which the skatole was distilled contained 40 % of fat. The C_V/C_L relationship was linear over a similar range of concentrations, $C_V/C_L = 0{\cdot}14$.

The presence of fat thus greatly reduced the vapour/liquid equilibrium coefficients

for both indole and skatole (compare the results obtained in similar experiments with diacetyl and other diketones, McDowall, 1959*b*). Because of the higher fat content of the cream from which skatole was distilled, the coefficients found for indole and skatole in cream are not strictly comparable. The results indicate very clearly, however, that although indole and skatole can be separated from aqueous solutions by

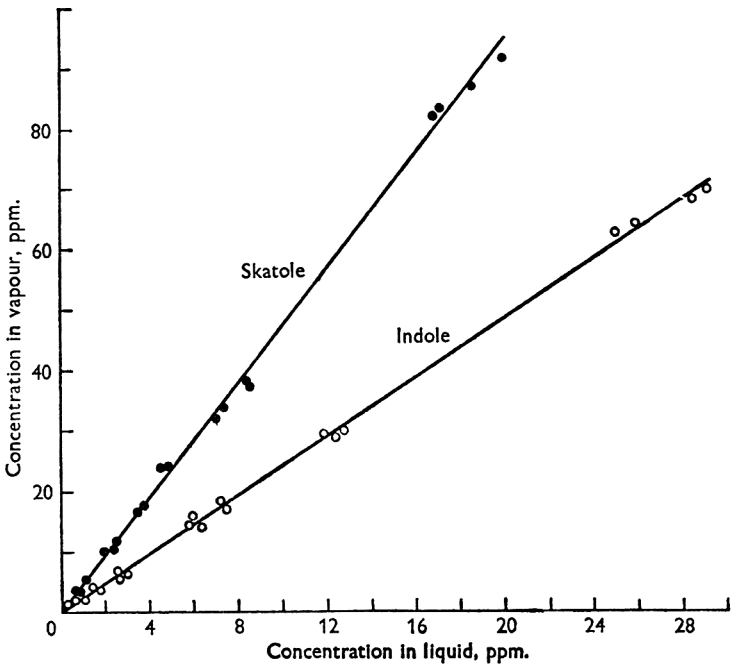

Fig. 1. Vapour/liquid equilibrium relationships for steam distillation of indole and skatole from aqueous solutions.

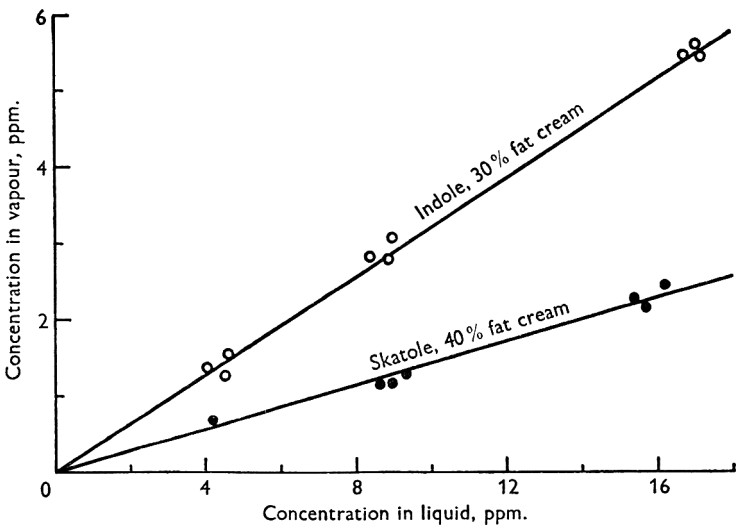

Fig. 2. Vapour/liquid equilibrium relationships for steam distillation of indole and skatole from cream.

steam distillation their separation from a fat-containing liquid such as cream could be expected to take place only with the expenditure of relatively large amounts of steam. Complete removal of these substances from cream in the equipment now available would be commercially impracticable.

The author is indebted to Mr A. K. R. McDowell and Mr E. R. Elley for assistance with the analytical work.

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Printed in Great Britain

J. Dairy Res. (1964), **31**, 189
With 1 plate

Teneur en caséine κ de la caséine de colostrum de vache

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(*Received* 21 *January* 1964)

RÉSUMÉ. La caséine de colostrum de vache étudiée renferme 0·85 % d'acide *N*-acétyl-neuraminique, et libère par action de la présure 2·95 % d'azote soluble dans l'acide trichloracétique à 12 % (NPN). Elle semble donc contenir une proportion élevée de caséine κ que l'on peut évaluer à 35 % environ.

Les micelles de caséine du colostrum renferment une quantité d'acide *N*-acétyl-neuraminique d'autant plus importante que leur taille est plus faible. La caséine soluble, qui correspond à 25 % de la caséine du colostrum, en renferme 1·52 % et donne 6·24 % de NPN par action de la présure.

La caséine de colostrum et surtout la caséine soluble de colostrum semblent être des sources intéressantes de caséine κ et de caséino-glycopeptide.

SUMMARY. Casein derived from cow's colostrum contains 0·85 % *N*-acetyl neuraminic acid and liberates 2·95 % nitrogen soluble in 12 % trichloroacetic acid (NPN) under the action of rennin. It therefore appears to contain a large proportion of κ-casein (calculated to be approximately 35 %).

The smaller the micelles of casein in colostrum the larger is the amount of *N*-acetyl neuraminic acid they contain. Soluble casein, i.e. 25 % of the casein in colostrum, contains 1·52 % of this acid and gives 6·24 % NPN under the action of rennin.

Casein and particularly the soluble casein of colostrum appear to be important sources of κ-casein and caseino-glycopeptide.

INTRODUCTION

Le colostrum de vache a une teneur en acide *N*-acétyl-neuraminique beaucoup plus élevée que le lait normal (Clark, Jackson et Pallansch, 1962). Il nous a paru intéressant, dans le cadre de travaux poursuivis sur la caséine κ et sur la structure des micelles du lait, de voir si la caséine de colostrum présentait la même caractéristique, et si, dans ce cas, à une teneur élevée en acide *N*-acétyl-neuraminique correspondait une teneur élevée en caséine κ.

Un travail poursuivi simultanément sur la composition des micelles du lait normal en relation avec leur taille nous a conduit en outre à étudier ces mêmes facteurs (teneur en acide *N*-acétyl neuraminique et en caséine κ) sur différentes fractions obtenues du colostrum par centrifugation.

MATÉRIEL ET MÉTHODES

Colostrum

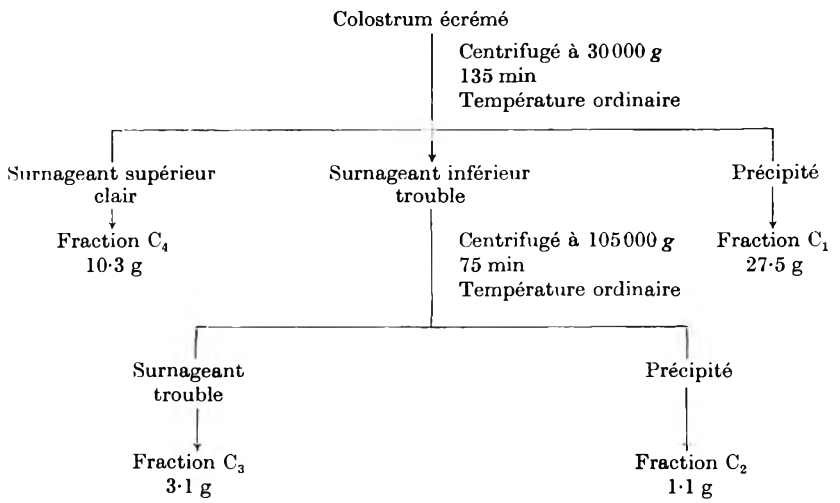
Le colostrum utilisé provenait de vaches individuelles de race Française-Frisonne Pie Noire appartenant au troupeau du Centre National de Recherches Zootechniques de Jouy-en-Josas; il était prélevé dans les 24 h qui suivaient le vêlage de chaque vache.

Caséine isoélectrique

La caséine a été préparée à partir de colostrum écrémé, dilué avec 3 fois son volume d'eau, par 2 précipitations successives (à pH 4·5 et 4·6, respectivement), lavages à l'eau, redissolution à pH 7 et lyophilisation.

Deux préparations ont été ainsi faites. On a obtenu environ 50 g de caséine par litre de colostrum.

Tableau 1. *Schéma de fractionnement des micelles de colostrum*



Fractions obtenues par centrifugation

Par centrifugation fractionnée, on a obtenu 4 fractions: C_1, C_2, C_3 et C_4, correspondant à des tailles de micelles décroissantes. Les fractions centrifugées ont été remises en suspension dans l'eau, précipitées 2 fois à pH 4·6, lavées à l'eau, redissoutes à pH 7 et lyophilisées.

La caséine soluble (fraction C_4), non centrifugeable, a été préparée de la même manière par précipitation du surnageant. Le schéma de fractionnement est donné dans le Tableau 1.

Courbes de 'NPN'

L'action de la présure sur des substrats contenant de la caséine κ se traduit par la libération du caséino-glycopeptide soluble dans l'acide trichloracétique à 12 % (Garnier, 1957). L'étude de l'augmentation de la quantité d'azote (NPN) dans la

fraction soluble dans l'acide trichloracétique à 12 % en fonction du temps fournit un renseignement sur la teneur en caséine κ du substrat.

Les courbes de NPN en fonction du temps ont été réalisées suivant la technique de Garnier (1963), variante de la méthode utilisée par Alais, Mocquot, Nitschmann et Zahler (1953).

Présure

On a utilisé de la présure cristallisée Hansen.

Dosages d'acide N-acétyl-neuraminique

Ils ont été effectués par la méthode de Warren (1959).

Electrophorèse en gel d'amidon en milieu urée

La technique de Wake et Baldwin (1961) a été employée: tampon discontinu de Poulik, urée 7 M, 7 V/cm, 4° C, 16 h.

RESULTATS—DISCUSSION

Le Tableau 2 indique les teneurs, en acide *N*-acétyl-neuraminique et en NPN libéré par action de la présure, de la caséine de colostrum et des différentes fractions obtenues par centrifugation.

Les courbes de libération de NPN par la présure en fonction du temps sont représentées dans la Fig. 1.

Tableau 2. *Teneurs en acide N-acétyl-neuraminique, et NPN libéré par action de la présure, de la caséine de colostrum et des différentes fractions obtenues par centrifugation*

	NANA*, %	NPN†, % de l'azote total
Caséine de lait normal	0·36	1·4
Caséine de colostrum	0·85	2·95
Fraction C_1	0·67	—
Fraction C_2	0·88	—
Fraction C_3	1·17	—
Fraction C_4	1·52	6·24

* NANA, acide *N*-acétyl-neuraminique.

† NPN, $(NPN_T - NPN_0)/N_T \times 100$: NPN_T, azote soluble dans l'acide trichloracétique à 12 % après action de la présure; NPN_0, azote soluble dans l'acide trichloracétique au temps 0; N_T, azote total avant action de la présure.

On voit que la caséine de colostrum a une teneur beaucoup plus élevée en acide *N*-acétyl-neuraminique que la caséine de lait normal, et que cette même teneur dans les fractions de centrifugation augmente lorsque la taille des micelles diminue, pour atteindre une valeur de 1·5 % dans la caséine soluble. Ces résultats confirment ceux de Sullivan, Fitzpatrick et Stanton (1959) obtenus à partir du lait normal.

De plus la quantité de NPN libérée par action de la présure paraît être en rapport avec la teneur en acide *N*-acétyl-neuraminique des différentes fractions. Il semble donc bien que la caséine de colostrum renferme une quantité élevée de caséine κ. Si l'on admet que la teneur en acide *N*-acétyl-neuraminique de celle-ci est égale à 2·3 %, la caséine de colostrum et la fraction C_4 renfermeraient, respectivement 37 et 66 % de

caséine κ. On obtient des valeurs assez similaires (30 et 62 %) en admettant que la caséine κ libère 10 % de NPN. Il est à noter d'ailleurs que la fraction C_4 se trouble par action de la présure en absence de calcium, comme on l'observe pour les fractions très enrichies en caséine κ.

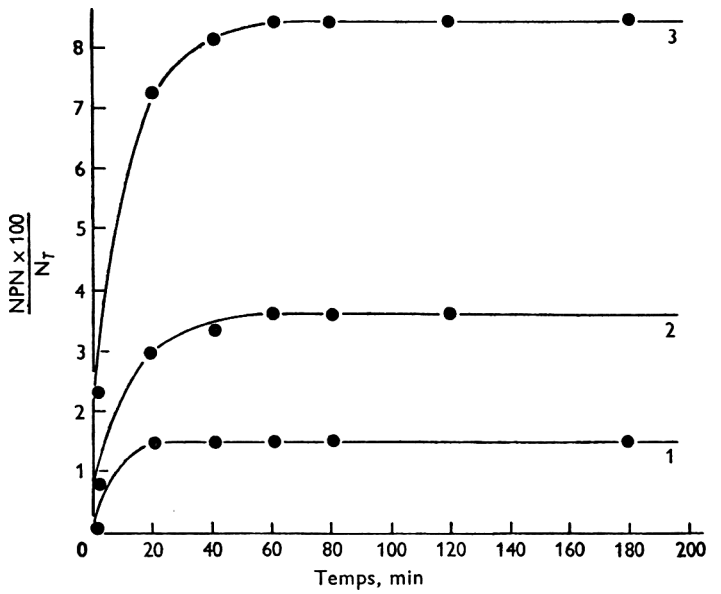
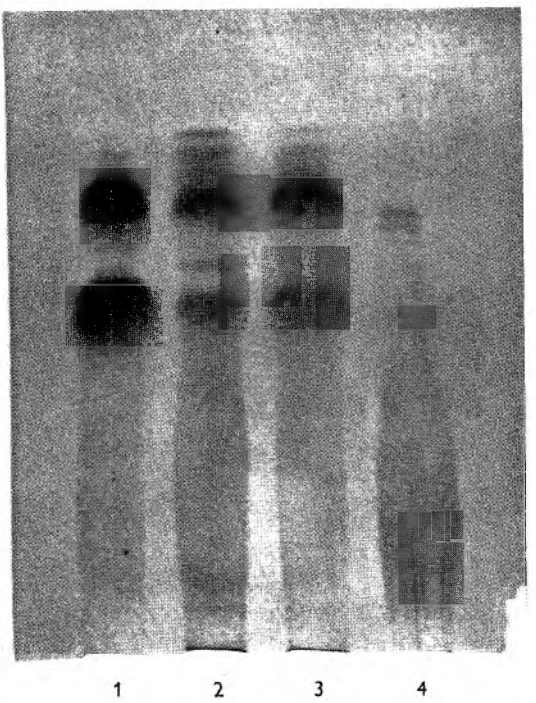


Fig. 1. Courbes de libération d'azote soluble dans l'acide trichloracétique à 12 % en fonction du temps. 1, Caséine de lait normal; 2, caséine de colostrum; 3, caséine soluble de colostrum (fraction C_4). Concentration en protéine, 3·2 %; concentration en présure, 0·91 μg/ml ($7{\cdot}6 \times 10^{-2}$ U.P./ml); température, 37 °C.

Le Tableau 1 indique que la caséine soluble du colostrum (fraction C_4) correspond à 25 % environ de la caséine totale. C'est donc que la taille moyenne des micelles est beaucoup plus faible dans le colostrum que dans le lait normal pour lequel la caséine soluble représente 3 à 7 % de la caséine totale (Bohren et Wenner, 1961). Ceci est en relation avec la teneur élevée en caséine κ de la caséine de colostrum, car le rapport des concentrations des deux autres constituants principaux de cette caséine (caséines α_s et β) semble être analogue à celui que l'on trouve dans la caséine de lait normal, comme on peut le voir sur les électrophorèses en gel d'amidon présentées dans la Planche 1. Cependant certains constituants mineurs semblent appartenir en propre à la caséine de colostrum, ou s'y trouver en des proportions nettement différentes de celles que l'on observe dans la caséine de lait normal. Notons d'ailleurs que ces électrophorèses montrent pour la caséine de colostrum et pour la fraction C_4 une traînée importante, caractéristique de la caséine κ.

La caséine de colostrum (ou la fraction C_4 que l'on obtient facilement) paraît être une source intéressante de caséine κ et pourrait en particulier être utilisée dans l'étude du caséino-glycopeptide que l'on obtient en quantité importante, particulièrement à partir de la fraction C_4.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Nous remercions Monsieur G. Mocquot, Directeur de la Station Centrale de Microbiologie et Recherches Laitières, et Monsieur J. Garnier, pour l'intérêt qu'ils ont porté à ce travail et les conseils qu'ils nous ont prodigués.

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PLANCHE 1

Electrophorèse des caséines en gel d'amidon: urée 7 M, pH 8·6; tampon discontinu de Poulik; concentration en protéine, 0·8%; tension, 7 V/cm; durée, 16 h; température, 4 °C. 1. Caséine de lait normal (préparée par précipitation isoélectrique). 2. Caséine soluble de colostrum (fraction C_4). 3. Caséine de colostrum (préparée par précipitation isoélectrique). 4. Caséine κ préparée selon McKenzie et Wake (1961).

Printed in Great Britain

J. Dairy Res. (1964), **31**, 195

Factors affecting the fat globule sizes during the homogenization of milk and cream

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(*Received* 23 *January* 1964)

Summary. The homogenization of milk and cream with a piston-type homogenizer has been studied. An examination has been made of the influences upon the mean fat globule diameters of changes in milk fat content, flow rate and homogenization temperature. The results are discussed in terms of the variations in the parameters q and P_0 of the empirical equation $d = (P_0/P)^q$ relating the globule diameter d to applied pressure P.

INTRODUCTION

Several hypotheses have been proposed to explain the mechanism by which the fat globules in milk are reduced in size on passing through a homogenizer (Trout, 1950). Since there are very few experimental data to justify any of these hypotheses, a study has been made of some of the factors which affect the sizes of the fat globules during this processing. Such an investigation became practicable following the development by Goulden & Phipps (1960) of a rapid spectroturbidimetric method for determining the mean volume-surface diameter ($d = \Sigma nd^3/\Sigma nd^2$) of the fat globules in a homogenized emulsion. The present studies are an extension of preliminary work on milk homogenization using this method (Goulden & Phipps, 1962).

Ashworth (1949), Deackoff & Rees (1957), and Leviton & Pallansch (1959) have previously used turbidimetric methods for limited investigations on homogenized milk, but these studies did not lead to values for fat globule diameters and were restricted to milk of normal fat content. Although turbidimetric methods employing a single wavelength provide an exact mean globule diameter only when applied to an emulsion in which the globules are all the same size, effects due to the spread of globule sizes in homogenized milk and cream are small enough to be neglected in the present study.

Preliminary work on milk showed that the performance of a piston-type homogenizer can be described by the empirical equation $d = (P_0/P)^q$, where d is the mean fat globule diameter obtained at the pressure P. The parameter q is the slope of the graph of $\log d$ against $\log P$, while P_0 is the pressure corresponding to unit globule diameter. The main object of the present investigation was to determine the effects of changes in fat content, temperature, and flow rate upon the values of P_0 and q, the parameters used here to characterize homogenization efficiency.

EXPERIMENTAL

A Rannie homogenizer incorporating the standard 'liquid whirl' type homogenizing valve (Wittig, 1950) was used in the experiments. For studying the effects of changes in flow rate, the normal rate of 22 gal/h was increased to 40 and 52 gal/h by changing the motor and pulleys.

Creams of different fat contents were prepared by mixing thick cream (approximately 35 % fat) with the appropriate volumes of whole or separated milk. After heating to the required temperature by standing the cream container in a hot water bath, each cream was passed through the pre-warmed homogenizer. Samples were collected at a series of homogenization pressures, allowing 2 pints to flow between samples to ensure that the preceding sample was washed out.

Homogenized creams were diluted with separated milk to a fat content of about 3·5 % enabling the standard Gerber method to be used for the determination of fat content. For turbidity measurements, 1 ml of each diluted homogenized cream was made up to 250 ml (or 500 ml depending on the globule diameter) with water containing 2 ml N-sodium hydroxide solution and 2 ml fat-globule declumping solution. The declumping solution was equivalent to that of Haugaard & Pettinati (1959) and consisted of 13 g EDTA (Na salt) and 5 g Tween 20 as emulsifier made up to 1 l with distilled water after adjusting the pH to 10 by the addition of alkali. Turbidities were measured at 40 °C using a modified Unicam SP 500 spectrophotometer (Goulden, 1958; Goulden & Phipps, 1962), operating at wavelengths of 0·6 and 0·9 μm. Most of the recorded d values were calculated from turbidities measured at 0·6 μm (Goulden & Phipps, 1960) since the rate of change of turbidity with d is greater at this wavelength. For poorly homogenized samples containing relatively large fat globules, readings obtained at the 0·9 μm wavelength were used to confirm that the first scattering maximum for 0·6 μm had not been passed.

The efficacy of the fat-globule declumping solution was checked as far as possible by examination of diluted samples under the microscope. Even thick, highly clumped homogenized creams appeared wholly unclumped following dilution and addition of the solution. The repeatability of the $\log d/\log P$ relationship on any one cream sample provided supporting evidence for the effectiveness of the declumping procedure.

RESULTS

Fat content

Fig. 1 illustrates some of the results obtained from homogenization experiments with creams of different fat contents. At any particular pressure it can be seen that an increase in fat content leads to a larger mean fat globule diameter, i.e. homogenization is less effective. The $\log d/\log P$ relationships do not deviate far from linearity except at high pressures, and approximate values of the parameters P_0 and q can therefore be obtained by drawing straight lines through the points at lower pressures. It may be seen in Fig. 2*a* that both the exponent q and P_0^q, the numerator in $d = (P_0/P)^q$, decrease with increasing fat content; it follows that to account for the larger globule diameters as the fat content increases (Fig. 1), the effect of q in reducing the denominator of the equation must predominate.

Temperature

The results illustrated in Fig. 3 show that for milk there is practically no decrease in d values as the temperature is increased, i.e. homogenization is not appreciably improved. For cream with 12% fat, however, homogenization is measurably more effective the higher the temperature; for even greater fat contents, the temperature dependence becomes quite marked. These effects can also be seen from the logarithmic plots shown in Fig. 4*a*; the variations of P_0 and q with temperature are shown in Fig. 2(*b*).

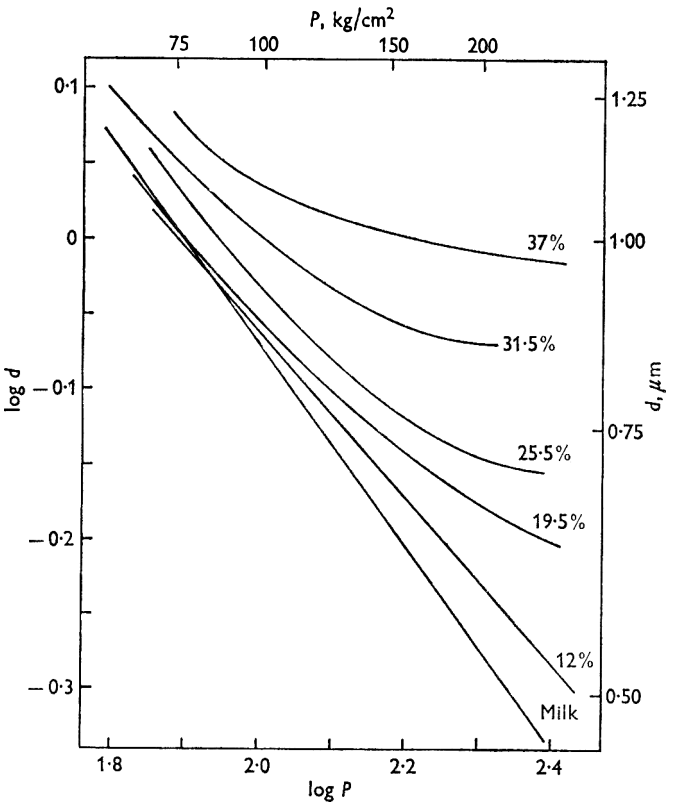

Fig. 1. Effects of fat content (%) on the relationship between the mean fat globule diameter (d) and the homogenization pressure (P). Temperature 70 °C, flow rate 22 gal/h.

For the homogenization of milk, Deackoff & Rees (1957) reported a significant increase in light transmission at higher homogenization temperatures, an effect which corresponds to a small reduction in d values. In the present investigation, it was found that reproducible results were obtained only when the heat treatment prior to homogenization was sufficient to ensure complete melting of the fat. This was readily achieved with the batch heating method used here, but in the flow-through type of heat interchanger used by Deackoff & Rees, it might well have been that the fat was incompletely melted (Deackoff, personal communication) and resulted in lower homogenization efficiency.

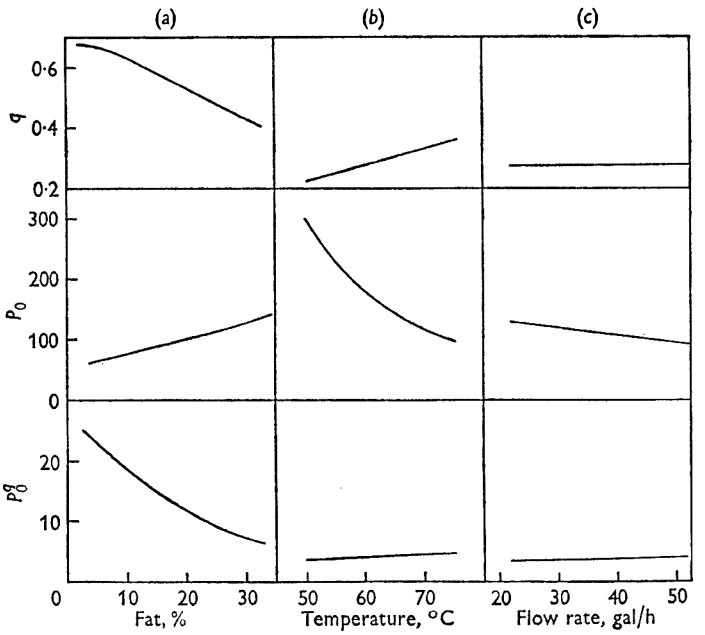

Fig. 2. Changes in the parameters q, P_0 and P_0^q with (*a*) fat content; temperature 70 °C, flow rate 22 gal/h; (*b*) temperature; fat content 30 %, flow rate 22 gal/h; (*c*) flow rate; fat content 30 %, temperature 70 °C.

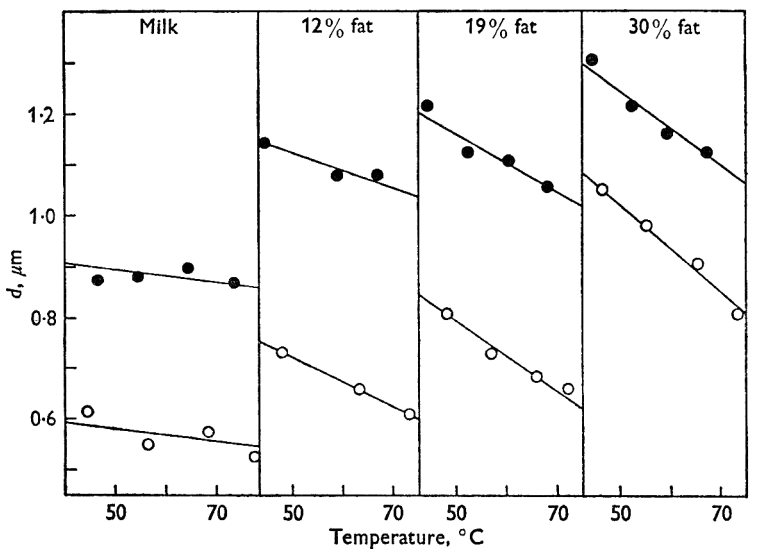

Fig. 3. Effects of temperature of homogenization of milk and cream on the mean fat globule diameter (d) at pressures of 70 kg/cm² (●) and 210 kg/cm² (○).

Flow rate

As shown by the results illustrated in Fig. 4(*b*), the influence of change in flow rate is apparent only with the higher fat content creams. A small reduction in d values occurs at higher flow rates. This effect is associated with a small change in P_0, since the value of q remains unchanged (Fig. 2*c*). Flow rate effects are imperceptible when the fat content is below about 12 %.

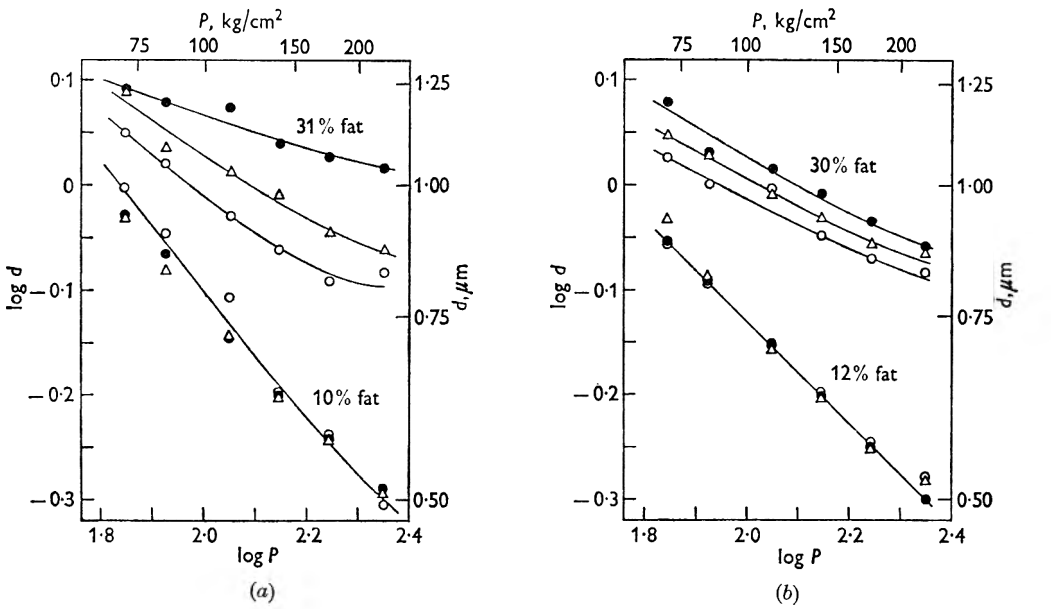

Fig. 4. Effects of temperature and flow rate on the relationship between $\log d$ and $\log P$. (*a*) Flow rate 22 gal/h. Temperatures: 50 °C (●), 65 °C (△) and 75 °C (○). (*b*) Temperature 70 °C. Flow rates: 22 gal/h (●), 40 gal/h (△) and 52 gal/h (○).

DISCUSSION

Definite patterns of behaviour have emerged in the variation of fat globule diameter with changes in the conditions of homogenization. Homogenization efficiency depends on the valve design (Goulden & Phipps, 1962) since a valve with flat faces is inferior to one with corrugated faces, e.g. the 'liquid-whirl' type valve (Wittig, 1950). Many experiments carried out over more than a year also show that the $\log d/\log P$ relations are sensitive to the condition of the homogenizing valve.

In general, as the results here show, it is to be expected that $\log d/\log P$ lines will be curvilinear when the fat content is greater than about 10 %. On some occasions, however, straight lines have been obtained for creams of even 30 % fat. Repeated experiments show that while the degree of curvature is about the same for cream samples taken over a short period of a few weeks, changes in the curvature occur at different times of the year. These changes might well be connected with variations in the stability of the emulsion. It would appear possible that the line curvature itself is due to an opposing coalescence process occurring in the homogenizing valve immediately following the break-up of the fat globules.

The equation $d = (P_0/P)^q$ is wholly empirical but it is to be hoped that the results obtained here will give a lead to the development of a comprehensive theory capable of explaining all the phenomena observed in the homogenization of the milk emulsion system.

The authors are indebted to Mrs M. Fowler and Miss A. J. W. Harrison for assistance in the preparation and homogenization of the creams and to Mr A. G. Walters, Mrs J. S. M. Conway and Mrs B. Harris for assistance in the globule size measurements.

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Printed in Great Britain

J. Dairy Res. (1964), **31**, 201

Reviews of the progress of dairy science

Section D. Nutritive value of milk and milk products

Part I. Nutritive value of milk proteins

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(*Received* 18 *February* 1964)

Part II. Nutritive value of milk fat

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(*Received* 29 *December* 1963)

CONTENTS

In the last Section D review it was stated that in future the subject would be discussed under seven topic headings and that three or four of these topics would be considered in each biennial review. After further consideration it has been decided that it would be more appropriate to include discussion of the nutritive value of milk and milk products for man and animals within the other topics. Accordingly it is now proposed to divide the subject as follows:

(1) Nutritive value of milk proteins.
(2) Nutritive value of milk fat.
(3) Nutritive value of carbohydrates and minerals of milk.
(4) Fat soluble vitamins in milk.
(5) Water soluble vitamins in milk and milk products.

Normally two or three of these topics will be covered in each biennial review.

PART I. NUTRITIVE VALUE OF MILK PROTEINS

INTRODUCTION

This section of the review deals with the nutritive value of milk proteins for man and for animals. It is based in the main on a selection of papers published in the 4 years since this subject was last reviewed (70), though as the nutritive value of milk for man was considered by McGillivray (69) in 1962 the review of this aspect of the topic covers only a 2-year period.

The long-term upward trend in milk production in main dairying countries was maintained in 1961 and total output showed a further slight rise in 1962. This continuing rise has not been accompanied by a similar increase in liquid milk consumption, and the greater part of the additional supplies has been used for manufacturing purposes. This has resulted in small increases in the production of cheese and condensed milk and larger increases in the production of butter and dried skim-milk. Casein production has also expanded in recent years (23, 24, 33-35).

The increasing appreciation of the nutritive significance of the proteins of milk, indicated by the introduction of a quality payment scheme for protein in the Netherlands in 1957 (97), is further emphasized in the adoption by the U.K. Milk Marketing Board (5) of the recommendation by the Cook Committee (51) of differential payment schemes for solids-not-fat. Dietary and other factors influencing the composition of milk, and hence its nutritive value, are comprehensively reviewed by Rook (104) and Kiddy (55).

The nutritive value of dairy products was the specific concern of the 7th International Dairy Federation Seminar (88), held in Sweden in 1961; among the papers read were reviews by Hartog of the nutritional role of milk and dairy products in the diet of West European countries, with special reference to the nutrition of young children (cf. (42), and by Kon on the amino acid composition of the milk proteins in relation to their nutritive value.

The 16th International Dairy Congress, held at Copenhagen in 1962, attracted few original contributions dealing with the nutritive value of milk. However, one of the Congress lectures was given by Kon (59) who gave an excellent account of recent studies of the nutritive value of milk, with particular reference to milk fat, milk protein and lactose, and to the effects of processing.

Other recent reviews include those by de Groot (40) of the effects of heat treatment on the nutritive value of milk and by Kon (58) of the nutritional effects on milk of chemical additives and processing.

General problems of protein nutrition are discussed in 2 conference reports published by the National Academy of Sciences—National Research Council (85, 86). In the first, some of the basic principles of the nutritional evaluation of proteins and amino acids are reviewed and a series of papers is presented on the use of milk and other food proteins in diets for malnourished people in many different countries. The second is devoted to a critical appraisal of methods of evaluation of protein quality and includes a glossary in which an attempt is made to define and thence standardize the terminology used. These are noteworthy publications; the contributors include many of the foremost workers in protein nutrition, both in the laboratory and in the

field, and their conclusions and suggestions for further research should be read by all interested in these problems.

Several microbiological methods for assessing the nutritive value of proteins have been developed, but until recently none has found wide acceptance. This has been largely because the methods using bacteria required lengthy preliminary enzymic hydrolyses of the test protein, and that using the protozoan *Tetrahymena pyriformis* needed unfamiliar techniques. These difficulties are avoided in the method developed by Ford (30) which makes use of a powerfully proteolytic strain of *Streptococcus zymogenes*. This organism grows quickly with an adequate intact protein as the main source of nitrogen. The method is rapid and ratings of nutritive value for a variety of food proteins, including samples of dried skim-milk, correlated well with values obtained in biological tests with rats.

BIOLOGICAL VALUE

Amino acid composition

The nutritive value of a dietary protein depends on the pattern and quantity of essential amino acids that it presents to the body after digestion and absorption from the intestine, and it measures the extent to which these amino acids fulfil the body's requirements for amino acids. Man's requirements are still not definitely settled, but on the basis of existing knowledge F.A.O. (28) postulated a provisional reference pattern of amino acids. Measured against this pattern total milk proteins provide a sufficiency of all amino acids except total sulphur amino acids (cystine and methionine). Recent work (cf. (85)) indicates that the suggested allowance of tryptophan is certainly, and that of sulphur amino acids probably, too high.

A precise knowledge of the amino acid composition of a protein is valuable in setting an upper limit to its nutritive value, but often it cannot be more than a guide to the biological value of the protein, since not all the constituent amino acids may be available and be liberated during digestion. The proteins of raw milk are generally well digested and the biological value of these proteins correlates well with their amino acid composition. However, with heated milk or milk products the correlation is less satisfactory (see p. 207).

Recent developments in column chromatography have enormously increased the accuracy and reproducibility of determinations of the amino acid composition of foodstuffs. Unfortunately the diversity of methods and analytical procedures used in the past has resulted in a wide scatter of values for the content of any one amino acid in milk proteins, and a study of any set of tables of amino acid composition of proteins will show values for many amino acids varying by a factor of two or more. As it seems unlikely that the amino acid content of these proteins really shows such variation, there is still a need for careful determinations of the composition of both total and individual milk proteins, with appropriate corrections for losses during hydrolysis. Furthermore, a more critical appraisal of their results by individual workers could eliminate the occasional publication of further unsatisfactory data resulting from unreplicated assays or technical error.

Two groups of workers have reported carefully conducted determinations of the amino acid composition of the A and B forms of β-lactoglobulin (37, 92); both groups

are agreed that the A form contains more aspartic acid and valine but less glycine and alanine than the B form. Amino acid analyses of the α-, α_1-, α_2-, and α_3-caseins have been reported (46); α_1-casein contained more glycine, methionine and histidine and less alanine, cystine and tyrosine than α_3-casein, whereas α_2-casein contained more aspartic acid, threonine, valine and methionine but less serine, tyrosine, phenylalanine, lysine, arginine and tryptophan than α_1- or α_3-casein.

The results of an extensive series of determinations of the amino acid content of individual samples of milk from Dutch and Jersey cows, and of cattle feeds, rumen contents and beef, are given in two monographs by Bigwood (9, 10); mean values show relatively little variation for the content in milk per 16 g nitrogen of any amino acid except methionine, and there was no significant effect due to stage of lactation or diet. The wide range of values found for methionine (0·6–3·7 g/16 g nitrogen) almost certainly stemmed from analytical difficulties with this amino acid. In a subsequent article Bigwood (11) reports values for the amino acid content of cow's colostrum and of human colostrum and milk, compares his results with literature values and discusses the differences in nutritive value of human and cow's milk proteins in the light of their amino acid make-up.

The surprisingly low values reported for the cystine (trace) and methionine (1·35 g/16 g nitrogen) contents of casein prepared from bulked evaporated goat's milk and the high value for histidine (3·65 g/16 g nitrogen) (66) may be due to faulty analyses.

Ion-exchange chromatographic and microbiological assays of the amino acid composition of milk proteins, and the effects thereon of pasteurization, sterilization (20 sec at 130 °C followed by in-bottle heating for 14 min at 118 °C), and spray-drying, are reported by Pol & Groot (96). They found a 19 % loss of cystine after spray-drying and losses of 13 % of cystine and 6 % of lysine after sterilizing. The only consistently significant reduction in performance in rat growth tests was with sterilized milk. Similar losses of lysine, and possibly of cystine, were reported for milk sterilized at 122–124 °C for 20 min (89).

Determination in animal tests

Further studies of the effect of different methods of preservation or processing on the biological value of milk proteins are reported.

Sydow (117) has carried out an extensive series of tests with rats to investigate various factors that might influence the results of determinations of nitrogen retention. Little effect due to source, breed, or management was found during these experiments and results were obtained for the biological value of differently processed milks that accord well with those of earlier workers.

The addition of nisin to certain canned foods to reduce spoilage due to spore-forming organisms is now legalized in Britain. Such addition enables milder heat treatment to be used without impairing the keeping quality of the product and extensive tests with rats given dose levels equivalent to 14 times the possible human dose showed no untoward effects (36). Gregory, Henry & Kon (38) have compared the nutritive value of normally evaporated milk with that of evaporated milk prepared from the same batch of raw milk, but to which 100 units of nisin per g of evaporated

milk were added and which was sterilized by less drastic heating. The biological value and true digestibility of the milk proteins were: raw milk, 92 and 96; normal evaporated milk, 88 and 93; evaporated milk containing nisin and sterilized at 105 °C for 15 min, 90 and 95; evaporated milk containing nisin and sterilized at 113 °C for 3 min, 89 and 96. These values were essentially unchanged after storage for 1 year. The biological values of the first and last evaporated milks were significantly lower than that of the raw milk. It would seem, however, that the beneficial effect of heating at the lower temperature is too small to be nutritionally important.

The effect of hydrogen peroxide as a preservative for raw milk on the nutritive value of milk has been further studied. Gregory, *et al.* (39) investigated the effect of 0·05 % (w/v) of H_2O_2 on the nutritive value of the proteins, vitamin content and bacterial flora of samples of bulked milk incubated for 8 h at 24 °C. Two subsamples of milk were studied, one with a low count and the other deliberately contaminated and with a high bacterial count. The nutritive value of the proteins of the samples was measured in rat tests and microbiologically with *Streptococcus zymogenes*. Compared with those of the untreated clean milk, both the biological value and true digestibility of the proteins of the H_2O_2-treated samples were slightly, but significantly reduced. This loss was confirmed by the microbiological tests which indicated that the impairment was due to a reduction of both the total and available methionine contents of the proteins. In a similar study Roushdy (105) was unable to detect any effect on the nutritive value of the proteins of H_2O_2-treated milk as measured by rat growth tests. Such a method, however, is less sensitive to small differences than the balance-sheet method used by Gregory *et al.*, and the finding that some loss of methionine occurs during peroxide treatment was not unexpected since this amino acid is known to be unstable in the presence of H_2O_2.

No significant differences were found in the biological value or utilization of the proteins of sterilized milk stored in clear glass bottles for 6, 10 or 26 weeks in the dark at 4, 20 or 38 °C, in diffuse sunlight at 20 °C or in direct sunlight and artificial light at 38 °C (19, 48).

Rather surprisingly, in short-term growth experiments with rats, the proteins of homogenized milk appeared to be utilized somewhat more efficiently than those of raw milk (91).

Pion & Rerat (94) compared the nutritive value of skim-milk powders prepared from the same batch of milk by spray-drying or by roller-drying, either with the milk sprayed on to the roller or by the process in which the milk is picked up by a roller from a trough. Analysis of the amino acid content of these products showed no appreciable differences except that about 20 % of the lysine of the second roller-dried milk had been destroyed. This finding was borne out in rat growth tests which showed no difference between the milks when they were fed as the sole source of protein, but a marked inferiority of the low-lysine roller-dried milk when they were fed as supplements to a lysine-deficient diet.

A steady deterioration was found (101) in the biological value for rats, but little change in digestibility, of samples of roller-dried and spray-dried skim-milk and of a concentrate containing 50 % of skim-milk analysed every 2 months during storage in paper sacks.

Biological values for rats are reported (49) for the proteins of skim-milk, acid casein,

and lactic caseins prepared with *Streptococcus cremoris* and with *Lactobacillus bulgaricus* and *Str. thermophilus* of 76, 73, 74 and 76, respectively.

Commercially available samples of dried whey can differ widely in nutritive value and the type of processing appeared not to be the determining factor when 9 samples of spray- or roller-dried whey were fed to rats as the sole protein source in diets containing 6 % protein and 37 % lactose, though in all cases the rate of growth and efficiency of nitrogen utilization was markedly inferior to that of rats given lactalbumin. However, when the whey was fed as a source of energy and comprised 60 % of a diet containing casein and methionine, the samples of roller-dried whey were significantly superior to the spray-dried products (6).

AVAILABILITY OF AMINO ACIDS FROM MILK PROTEINS

The proteins of raw milk are generally well digested and the amino acids of casein are as readily available to the rat as are free amino acids (103). Likewise, the growth and efficiency of nitrogen utilization of mice fed a diet containing crystalline β-lactoglobulin were the same as those of mice fed an amino acid mixture which corresponded exactly to the native protein (12). Tryptophan was shown to be wholly available in 2 samples of casein that contained 1·5 and 1·2 g of the amino acid per 16 g nitrogen (57).

However, ionizing radiations, heating or unsatisfactory storage of milk products may reduce the availability of amino acids, particularly of lysine, and thus reduce the nutritive value of the proteins.

The deleterious effects of radiation on milk proteins have been further studied and, although gamma irradiation of casein with up to 5 Mrad was without effect on its proteolysis by papain, pepsin or trypsin (87), irradiation of β-lactoglobulin with 4·7 Mrad, but not with 0·5 or 1 Mrad, caused a 40 % decrease in the amino nitrogen liberated during digestion with trypsin; this fall was associated with a 7 % loss of lysine, which also occurred after irradiation with 1·3 Mrad (75).

Mauron (86) has summarized his studies of the availability of lysine from milk and processed milks, as measured by rat growth tests, *in vitro* digestion studies and chemical methods using fluorodinitrobenzene procedures. It is apparent from these results that the preparation of spray-dried milk powder and sweetened condensed milk does not alter the availability of lysine, whereas the manufacture of evaporated milk and roller-dried milk powder causes a 20–40 % reduction in the amount of available lysine and even greater losses may occur if the powder be scorched during drying. The results by the different methods are in reasonably good agreement when the chemical method used is that developed by Carpenter (17). Values obtained by the simpler fluorodinitrobenzene procedure proposed by Schober & Prinz (107) were appreciably higher than those obtained in any of the other tests, and it seems that this method is not satisfactory for milk products. Pion (93), however, preferred Schober & Prinz's method to the earlier version of Carpenter's procedure (18) in his study of the total and available lysine contents of a series of spray- and roller-dried skim-milk powders. He found 90–97 % of the lysine available in all the samples except in one discoloured roller-dried powder in which it was 75 % available. Generally similar conclusions to the foregoing were reached by Tsugo, Yamauchi &

Yoshino (120) who determined the change in available lysine caused by heating milk products or milk proteins with lactose, and by Pol & Groot (96) who confirmed the loss of available lysine during heat sterilization and found a small loss during pasteurization.

Mauron (86) found no loss of available tryptophan or tyrosine in any sample, as measured by *in vitro* digestion, but a 10–20 % inactivation of methionine occurred during roller-drying. Similar findings were reported by Ford (31) who has developed a method for the microbiological assay of available amino acids using the proteolytic microorganism, *Str. zymogenes*. This organism was used to measure available methionine, leucine, isoleucine, arginine, histidine, tryptophan and valine in dried skim-milk powder before and after heating in steam at 120 °C for up to 18 h. These conditions were extremely severe and the content of available lysine, which was determined by the fluorodinitrobenzene method, fell sharply, and after 30 min heating only 14 % remained; the contents of available arginine and histidine both fell to about 42 % of the initial values during this period. The availability of methionine, valine, leucine, isoleucine and tryptophan also decreased during the heating, but at a slower rate than that of the 3 basic amino acids. In a subsequent paper Ford & Porter (32) reported the changes found in the availability of amino acids in dried skim-milk powder, whale meat meal and fish meal after dry heating at 105 °C for 0·5, 1, 2 or 5 h. Heating reduced the contents of available methionine, tryptophan and lysine in all the materials, but it was noteworthy that the loss of available lysine was relatively much greater in the heated skim-milk powder. Similar conclusions were reached by Carpenter and his colleagues (100) who showed in rat feeding tests that the effect of heating a mixture of buffalo casein and glucose of high moisture content was to reduce the availability of lysine more than that of methionine or of total sulphur amino acids.

Milk proteins are such a good source of lysine that even a severely heated roller-dried milk, in which 50 % of the lysine was no longer available, proved a satisfactory protein source for 5 infants (8). However, such losses of lysine are far more serious when milk proteins are being used in a supplementary role to compensate for the deficiencies of vegetable proteins, because the high lysine content of milk proteins is responsible for much of their well-known value as supplements. Accordingly, when the availability of lysine is impaired as a result of processing, the supplementary value of the proteins is reduced. Thus the efficiency of dried skim-milk as a supplement to bread in supporting rat growth was proportional to its content of available lysine (27, 50), and growth and efficiency of protein utilization by rats given a white wheat flour diet supplemented with sweetened condensed milk (lysine 97 % available) was greater than that when the flour was supplemented with evaporated milk (lysine 78 % available) (77).

Conflicting views continue to be held about the cariogenic properties of heat-damaged dried milk powders. The earlier work on this topic by McClure and his colleagues is reviewed by Jansen (52) in his comprehensive account of the role of lysine in nutrition. Briefly, McClure & Folk (68) found that lysine supplementation of diets containing such dried milks improved growth and afforded a striking decrease in the susceptibility of rats to caries. Subsequently Edmonds, Madsen & Smith (26) showed that a supplement of lysine caused a reduced incidence of caries even when added to a diet that contained autoclaved dried skim-milk and that allowed normal

growth. However, Mauron and his colleagues (22, 76) found no difference in the incidence or severity of carious lesions in rats given diets containing freeze-dried or fresh milk, or spray-dried or roller-dried skim-milk with contents of available lysine ranging from 28 to 100 %. Several factors, including the strain of rat used and the type of carious lesion produced may explain these discrepancies, but it seems probable that the cariogenic properties of heat-damaged dried skim-milk are not to be attributed solely to a reduction in their content of available lysine.

MILK PROTEINS IN INFANT NUTRITION

Problems of infant feeding have been discussed in several reviews. Thus, Aitken & Hytten (1) have prepared a careful and critical review covering all aspects of this confused and somewhat contentious field. Jelliffe (53) discusses the decline in breast feeding caused by increasing urbanization in tropical regions and considers ways in which the frequently resulting malnutrition can be combated with reference to the particular problems of the various regions. A series of reports published on infant feeding in various countries includes discussion of breast feeding in the culture of the United States (3), feeding Chinese, Indian and Malay infants in Singapore (78), infant feeding practices in West Germany (7), Uganda (25), Afghanistan (102), South Africa (29) and the Philippines (83). The report of a symposium on infant feeding held in New York included contributions on the amino acid requirements of infants (47) and the differences between human and cow's milk (122). Problems associated with cow's milk allergy have been reviewed by Stanfield (116) and by Collins-Williams (20).

The nature and amount of digestive enzymes secreted by the human baby have been little studied in the past. Recently, however, Japanese workers (54, 84) have examined the proteolytic activity of gastric juice from infant stomachs and have shown by pH-activity curves, and paper electrophoretic studies that such activity was due solely to pepsin; no rennin or cathepsin was present. The development of the secretion of proteolytic activity in the stomachs of groups of infants and children aged from 0–6 months to 7–14 years was followed by Tolckmitt & Hundt (118) who determined the tryptophan content of gastric contents after a drink of skim-milk and found statistically significant differences in proteolytic activity between different age groups up to 4 years.

The relative nutritive merit of the total proteins of human and cow's milk is still the subject of investigation. Because of their higher total content of sulphur amino acids, whey proteins have a biological value close to 100, whereas casein has a value of around 70, and since human milk contains slightly more whey protein than casein, whereas in cow's milk only about one-fifth of the total protein is contributed by whey protein, it is to be expected that isonitrogenous diets containing human milk proteins would show a higher biological value than those containing cow's milk protein. Earlier attempts to establish the superiority of human milk proteins were unsuccessful (cf. (70)), but in a recent preliminary communication (95) values for NPU_{st}, as measured with rats accustomed to a high intake of lactose, are reported for human milk of 100 and for cow's milk of 85.

Tomarelli & Bernhart (119) have continued their studies by measuring the biological value by several rat assay procedures of the proteins of cow's milk and whey

and of a mixture of the two in amounts simulating the casein–whey protein ratio of human milk; they obtained values of 83, 94 and 92, respectively. The whey proteins used in these experiments had been demineralized by electrodialysis (4); similar products are being used in infant foods as they allow the preparation of diets with both mineral and protein compositions closer to those of human milk (71). Gel filtration through Sephadex can also be used for the removal of soluble milk salts (60).

Although it is well established that the proteins of both human and cow's milk are well digested by the infant, from a nutritional standpoint a possible source of difference between these milks may lie in the rate at which individual amino acids are liberated and absorbed. Miyao & Ishihara (79, 80) have compared the rate of liberation of amino nitrogen from raw human and pasteurized cow's milk by pepsin and by human gastric juice at pH 5 and by trypsin and by human duodenal juice at pH 7. They found that only traces of amino nitrogen were produced from either milk during digestion at pH 5, but that at pH 7 human milk was digested more rapidly and gave a more rapid liberation of lysine and arginine than cow's milk. Subsequently, the absorption by the isolated rat intestine of the enzymic and acid hydrolysis products of the proteins of these milks was studied by Miyao, Tsuneishi & Hosogi, who found that the rate of absorption of amino nitrogen was greater for human than for cow's milk (81). However, when the milk had been heated to 65 °C for 30 min or 100 °C for 10 min before digestion, the rate of absorption for heated human milk was less than for unheated, but the reverse was true for cow's milk (82).

MILK PROTEINS IN DIETS FOR YOUNG PIGS AND FOR PRE-RUMINANT ANIMALS

Elucidation of the special value of milk proteins in the nutrition of the young pig and the young calf still awaits a clearer understanding of the factors governing protein digestion and utilization in these animals. Perhaps the 3 most important factors likely to exert an influence are the amino acid composition and digestibility of the protein, the levels of proteolytic enzyme activity in the gut, and the clotting properties of the milk. However, the significance of any of these factors seems rather confounded by the results reported in the interesting series of papers by Roy & Shillam and their collaborators (108-115) on the effect of heat treatment on the nutritive value of milk for the young calf. These workers have carried out a thorough investigation of the performance during the first 3 weeks of life of calves given colostrum followed by raw, pasteurized or UHT-treated milk, or by a milk substitute diet prepared from skim-milk powder, fat and fat soluble vitamins. Their principal finding was that any heat treatment of milk that results in the denaturation of about half of the whey proteins causes a reduction in its nutritive value. Thus calves given roller-dried skim-milk, UHT-treated milk and spray-dried skim-milk that had been submitted to a severe (74 °C for about 30 min) preheating treatment grew significantly less well than calves given raw or pasteurized milk or spray-dried skim-milk that had had a mild (77 °C for 15 sec) pre-treatment. The incidence of scouring did not differ between calves fed the different diets and similar results were obtained both when the general incidence of scouring was high and when it was low. Supplementing the diets with Ca, to improve the clotting properties (111), with chlortetracycline (110) or with selenium (112) did not influence the results. The mean apparent digestibility

of the nitrogen in mild pre-treated spray-dried milk was greater than in severe pre-treated milk, but there were no differences between treatments in the utilization of the apparently digested nitrogen or in the biological value of the proteins for the calves. There is as yet no satisfactory explanation for the apparent dependence of the young calf on a good supply of undenatured whey proteins but, as Shillam & Roy (110) suggest, it seems that whenever possible milk substitute diets for young calves should contain dried milk in which little or no denaturation of the whey proteins has occurred.

The protein requirements of the calf have been further investigated (13, 14-16, 63). Contrary to an earlier report, formalin-treated skim-milk was found to be unpalatable to 2-week old calves and to cause severe scouring (98). Dried skim-milk that had been stored for about a year was inferior to the fresh product when reconstituted for calves (62).

The nutrition of the young pig has been comprehensively reviewed by Lucas & Lodge (67).

Further confirmation has been supplied of the superiority of milk proteins to soya-bean proteins in baby pig diets (43, 72, 73, 90). Kratzer & Porter (61) investigated the effect of pH on the digestion of soya protein and casein *in vitro* by pepsin and found that raising the pH of digestion from the optimum at pH 2 caused a more rapid fall-off in the rate of digestion of soya protein than of milk protein. This may imply that milk or synthetic-milk diets based on milk proteins may be more rapidly digested by pepsin at the pH of the stomach contents of young pigs than similar diets based on soya proteins. Higher plasma amino acid levels were found in 2-week-old pigs weaned on to milk protein than on to soya protein, though the differences had disappeared by the time the animals were 6–8 weeks old (99). The effect of age at weaning and of the nature of the diet on the rate of development of the blood serum protein pattern of piglets has been studied by Lecce & Matrone (64, 65). They found that weaning at 1 day on to diets containing cow's milk caused some delay in reaching the mature pattern, but that weaning on to amino acid diets simulating milk proteins did not promote normal changes; piglets weaned after 4 days showed no delay in serum protein development.

The secretion of proteolytic enzymes has been studied in calves (2, 44), lambs (2, 121), goats (56) and pigs (41). The levels secreted appear to be adequate in the pig, for no improvement in performance was found when proteolytic enzymes were added to diets (21, 72, 74). A response was obtained, however, when a bacterial protease was added to a calf starter diet (106). Little difference was found in the extent of proteolysis of milk and of synthetic milk diets containing casein or isolated soyabean protein in the abomasum of the young calf (45).

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PART II. NUTRITIVE VALUE OF MILK FAT

INTRODUCTION

The last occasion when the nutritive value of milk fat was discussed in Section D was when the topic was included in a general review of the nutritive value of milk and milk products by McGillivray (1960), though in a more recent contribution to the Section (McGillivray, 1962) attention was directed to some papers and reviews which appeared during 1960 and 1961 in which the possible relationship between milk fat intake and the incidence of coronary disease was discussed. Now, for the first time, a part of Section D is devoted entirely to the nutritive value of milk fat.

In assembling material for this contribution, the reviewer's terms of reference have been to peruse as many as possible of the papers that have been published during the past 5 years or so dealing with effects resulting from the ingestion of milk fat by man or animals. It is hoped that the final selection of papers to be included represents a fair indication of progress in the assessment of the nutritive value of milk fat—a topic which has many facets, ranging from digestibility to nutritional pathology. Fundamental to the nutritive value of milk fat is its chemical composition, but since this is dealt with at intervals in this series of Reviews and has been discussed recently in detail elsewhere by the present reviewer (Garton, 1963), it is not considered further here except in relation to linoleic acid and its possible importance in infant nutrition.

NUTRITIVE VALUE OF MILK FAT FOR ANIMALS

After much controversy it was concluded some 20 years ago that, for the rat, milk fat probably did not possess nutritive properties superior to those of many other fats. Recently, this conclusion has been confirmed as applying to some other species, namely the pig, guinea-pig and dog by Crampton, Shaw, Mackay & Schad (1960). Groups of prematurely weaned young of each of these 3 species were fed for 4 or 6 weeks on a suitable basal diet to which was added 20 % of butterfat or one of several fats which included soyabean oil, linseed oil, rape-seed oil, coconut oil, cottonseed oil, a fish oil, lard and tallow. No consistent significant differences in growth, voluntary feed intake, apparent digestibility or weight gain per unit of 'digested calories' were observed between any of the groups.

However, the substitution of milk fat by other fats (especially highly unsaturated vegetable oils) in 'filled' milks for calf rearing has been known for some time to affect adversely the growth and physical condition of the animals. The problem was studied in considerable detail in the U.S.A. by Adams, Gullickson, Gander & Sautter (1959) and Adams, Gander, Gullickson & Sautter (1959*a*, *b*). Filled milks containing maize oil, hydrogenated vegetable oil, lard or butter oil were prepared weekly and fed to groups of calves and the digestibility of these fats and their effects on the physical condition of the animals were compared with results obtained when skim-milk (0·1 % butterfat) or whole milk (3·5 % butterfat) was fed. Animals given the filled milk containing maize oil were very badly affected in many ways, the disorders including muscular weakness, diarrhoea, low resistance to infection, poor feed utilization and poor weight gains. These effects were not observed in calves given the other filled milks, but signs of muscular weakness were present in the groups given

lard or butter oil prepared from oxidized butter; this could be remedied by giving supplementary tocopherol which also prevented muscular weakness in the calves given maize oil, though it did not have any other markedly beneficial effect. The detrimental effects of maize oil were much less marked when the filled milk was made daily (instead of weekly), thus allowing less time for oxidation of the unsaturated fatty acids to occur; the daily weight gains were, however, still not improved. The apparent digestibility, adjusted for 'metabolic' faecal fat, of butter oil (97 %) was significantly greater than that of any of the other fats and the proportions of free fatty acids, soaps and neutral fat in the faecal lipids of the calves given butter oil were similar to those in the group given skim-milk; all the other fats gave rise to an enhanced excretion of soaps.

In this country Raven & Robinson (1958) reported that hydrogenated palm oil was very much inferior to butterfat as a source of energy for calves; this was due partly to its somewhat lower digestibility, but mainly to very poor utilization of the digested palm oil compared with that of butterfat, as judged by their respective protein-sparing effects. Subsequently it was found (Raven & Robinson, 1959) that when unhydrogenated palm oil was used a marked improvement in nitrogen retention resulted which was not improved further when, as a means of introducing shorter-chain fatty acids, part of the oil was replaced by unhydrogenated palm-kernel oil. Unhydrogenated palm-kernel oil by itself was inferior to unhydrogenated palm oil in its protein-sparing effect (Raven & Robinson, 1960). As in the American work, the diets containing vegetable oils gave rise to an enhanced faecal excretion of soaps, especially Ca soaps.

The addition of 10 % of butter, tallow, lard or hydrogenated cottonseed oil to a 'starter' ration for the early weaning of calves did not significantly affect weight gain or hay consumption over an 8-week period beginning when the animals were 6 days old (Miller, 1962).

The hydrolysis of milk fat by 'pre-gastric' esterase (of salivary origin) in the abomasum of the young calf was studied by Ramsey & Young (1961). The milk was given either orally or put by rubber tube directly into the abomasum to minimize the concomitant entry of pre-gastric esterase. Free short-chain fatty acids, in particular butyric acid, appeared quite rapidly in the abomasum, though higher (water-insoluble) fatty acids were released more slowly and remained trapped in the curd particles, whereas the water-soluble acids apparently passed from the abomasum fairly quickly. Considerably less hydrolysis was observed in the experiments in which the milk was put directly into the abomasum than when it was given orally, indicating that the pre-gastric enzyme (or enzymes) is of greater lipolytic significance for the calf than gastric lipase *per se*. It has sometimes been suggested that the calf may have a limited ability to digest milk fat. To investigate this possibility Grimes & Gardner (1959) gave groups of calves a daily fat intake which averaged 95, 128, 196 or 286 g using milk containing 3, 6 or 9 % of fat. In all the groups the digestibility was high, the values being 96·2, 95·9, 97·4 and 94·2 % corresponding, in that order, to the increasing amounts of milk fat given; the incidence of scouring was, however, high in calves given the milk containing 9 % of fat.

In a study of the effects of dietary fat on the carcass and liver lipids of the weanling rat, Bhalerao, Endres & Kummarow (1961) gave the animals a diet containing 20 %

of each of the following fats, namely, fresh milk fat, thermally oxidized milk fat, fresh maize oil and fresh lard; the oxidized milk fat was made by heating the fat at 200° C for periods of several hours. The composition of the tissue lipids examined showed that part of the major component fatty acids of the respective dietary fats had entered the tissue fats, and compared with animals given fresh milk fat, those given the oxidized fat showed no significant differences. An unexpected observation was the presence of a greater proportion of arachidonic acid in the liver lipids of the rats given milk fat compared with those given maize oil in spite of the low levels of linoleic acid and arachidonic acid in milk fat and the high proportion of linoleic acid (the precursor of arachidonic acid) in maize oil. Branched-chain fatty acids of milk fat appeared in the tissue lipids of the rats.

Later Bhalerao, Inoue & Kummarow (1963) examined the lipids of the intestinal lymph of rats given 1 ml of fresh or thermally oxidized samples of milk fat, coconut oil, maize oil or olive oil. Lymph was collected for 24 h following the administration of the fat. Compared with the fresh fats, about 10 % less of the oxidized fats was absorbed and this was related, as might be expected, to the unsaturated fatty acids, of which oxidation polymers apparently were not being absorbed. Little or no difference was observed between the relative proportions of saturated fatty acids in the lymph lipids following the feeding of a fresh or a thermally oxidized fat. In confirmation of earlier findings by other workers the short-chain saturated fatty acids in milk fat appeared in only very small proportions in the lymph lipids, having presumably been absorbed via the portal circulation. The proportions of C_{18} di-unsaturated acid in the lymph lipids were 9·4 and 2·5 %, respectively, in animals given fresh and oxidized milk fat, and the corresponding values for arachidonic acid were 2·0 and 0·9 %, indicating a reduced synthesis (presumably in the liver) of the latter acid.

In earlier papers (Johnson, Sakuragi & Kummarow, 1956; Bhalerao, Johnson & Kummarow, 1959) it had been shown that thermally treated butterfat, unlike similarly treated maize oil or hydrogenated soyabean oil, did not have a growth-depressant effect on rats and that the acetone-insoluble glycerides of butterfat are in some way able to counteract the effect of toxic products or prevent their formation during the oxidative treatment of the fat.

Stemming from the great interest shown in the past decade in the etiology of cardiovascular disease, many pathological studies have been conducted using experimental animals whose rations often included butterfat. It is not proposed to document these experiments here, except to mention a paper by Moore & Kon (1963) which seems of particular relevance to recent considerations regarding diet and heart disease in man (*vide infra*). These workers found that, contrary to the widespread assumption that saturated fat is the most important dietary factor in cardiovascular disease, atheromatous lesions similar in severity to those produced on a diet containing 20 % of butterfat can also arise in rabbits fed on a high carbohydrate diet devoid of fat.

Experiments concerned with nutritional pathology sometimes lead to a closer study of normal processes in the body. The observations of Thomas & Hartroft (1959) and O'Neal, Thomas & Hartroft (1959) that myocardial and renal infarcts produced in rats fed on a diet containing bile salts, cholesterol and butter did not result when maize oil replaced butter, led to the finding that the butter-fed animals showed a

longer 'clot-lysis' time (Scott, Alousi & Thomas, 1961) and had higher serum lipid values than those given maize oil (Scott, Hale, Hale, Goodale & Tejada, 1962). These results prompted a detailed study of absorption from the alimentary tract by Jones, Scott, Morrison, Kroms & Thomas (1963); following the feeding to rats of 2 ml of butter or maize oil an examination was made by phase microscopy and electron microscopy of the jejunal mucosa and the chyle, and the fatty acid composition of the lipids of the chyle, blood and liver was determined. There was a fairly close relationship between the component fatty acids of the chyle lipids (mostly triglycerides) and those of the respective dietary lipids and, to a lesser extent, this was true also of the serum lipids. However, there was a considerable difference between the chyle lipids in their microscopic appearance. Whereas in the rats fed on maize oil the chylomicra were 'smooth-bordered' and small (diameter 1 μm or less), those in the animals given butter were composed predominantly of a mixture of very small irregularly shaped particles and of 'giant' chylomicra measuring up to 30 μm in diameter. Though it was not possible to be certain that these 'giant' chylomicra did not represent the products of coalescence *in vitro* of small chylomicra, an apparent difference was indicated in the nature of the particulate lipid resulting from the assimilation of butterfat as compared with maize oil; it is to be hoped that future studies on these lines will include other fats and other animal species and that the protein as well as the lipid of the chylomicra will be examined.

NUTRITIVE VALUE OF MILK FAT FOR MAN

Milk fat in infant nutrition

On the basis of weight gains, length increments and general clinical assessment over a 6-month period Goalwin & Pomeranze (1961) found no differences between the nutritive value for babies of breast milk, cow's milk (diluted 1:1) and a 'prepared' milk containing unsaturated vegetable fat. The comparison was prompted by the fact that breast milk fatty acids have a much higher content of essential fatty acids (EFA) than the fatty acids of cow's milk; whereas the former contain about 8% [7% as linoleic acid (*cis-cis*-9,12-octadecadienoic acid) and 1% as arachidonic acid], the latter usually contain only 1–2% of octadecadienoic acid (consisting of linoleic acid together with non-active isomers) and 0·5% of arachidonic acid. The fatty acids of the 'prepared' milk contained much more linoleic acid (about 14%).

That EFA are required by infants was shown 3 years earlier by Hansen, Haggard, Boelsche, Adam & Wiese (1958). These workers found that as little as 1·3% of the dietary calories as linoleic acid cured the dermal signs of EFA deficiency (occasioned by feeding skim-milk) in 2–4 weeks. It has been known for some time that in EFA deficiency tri-unsaturated fatty acid (5,8,11-eicosatrienoic acid) is produced endogenously from oleic acid in an effort, albeit it seems in vain, on the part of the body to provide a physiologically acceptable substitute for EFA. Based on this response an objective criterion for deciding whether or not EFA deficiency exists has been suggested by Wiese, Hansen & Adam (1958). Minimal 'normal' serum values in babies for di-, tri- and tetra-unsaturated fatty acids were associated with the feeding of a diet which provided about 1% of the total calories as linoleic acid. Other studies by Adam, Hansen & Wiese (1958) and Combes, Pratt & Wiese (1962) indicated that the

optimum utilization of milk constituents for the growth of infants was related to their linoleic acid intake. The foregoing studies have recently been confirmed by the same group of workers (Hansen *et al.*, 1963) in a clinical and chemical study on many more infants fed on milk mixtures which varied in the kind and amount of fat which they contained.

However, as Combes *et al.* (1962) comment, 'many thousands of full-term and premature infants have grown well, without recognized clinical evidence of any nutritional deficiency, on diluted cow's milk and on half-skim-milk mixtures', and opinion on the comparative aspects of breast- and artificial feeding generally supports the view that mixtures of cow's milk, water and sugar are satisfactory substitutes for breast milk (see review by Aitken & Hytten, 1960) though they usually provide less linoleic acid than would account for 1 % of the total energy value. Breast milk provides about 4 % of its total energy value as linoleic acid, and it is an open question whether or not it might be desirable, for infant feeding, to supplement some samples or preparations of cow's milk with linoleic acid. The question can be answered only when the optimum dietary level (which may vary with age) has been clearly defined and this, in turn, depends on advances in our knowledge of the fundamental biochemical role of EFA. In the meantime it should be remembered that the amount of linoleic acid in cow's milk fat can vary considerably and that values ranging from 0·2 to 2·7 % of the total fatty acids have been reported (see review by Shorland & Hansen, 1957). This should be borne in mind when clinical and metabolic studies by different workers are being compared since different responses to cow's milk may depend on their relative EFA content. For example, it is possible that one cause of eczema in babies is lack of adequate amounts of EFA and the disagreement which exists (see Aitken & Hytten, 1960) regarding whether or not there is a greater incidence of the condition in infants given liquid diets based on cow's milk than in those given breast milk may be related to different amounts of EFA being provided by the cow's milk.

Droese & Stolley (1960) discussed problems which may arise when attempts are made to increase the fat content of diluted bovine milk for infant feeding and considered that it was probably better to supplement cow's milk with an unsaturated vegetable oil than to replace milk fat entirely with a higher proportion of another fat (cf. Jochims, 1959). However, the use of preparations containing cottonseed oil, as tried by Jochims (1961), could not be recommended yet for general use in the home, though diluted milk containing emulsified butter appeared to be satisfactory. The undesirability of using 'filled' milks prepared from coconut oil and non-fat milk solids was discussed by Rice (1960).

Prompted by the observation that fatty streaking of the intima of arteries may occur in the very early life of a baby and the possible association between high blood cholesterol values and development of atherosclerosis, several groups of workers have investigated the concentration of cholesterol in the serum of infants in relation to the fat in the diet. In adults it is known that the feeding of polyunsaturated fatty acids results in a decrease in plasma cholesterol values and so Pomeranze, Goalwin & Slobody (1958) suggested that babies given breast milk might show lower serum cholesterol values than those given cow's milk since, as discussed above, the linoleic acid content of human milk fat is greater than that of cow's milk fat. However, no

significant differences in the effects of these 2 milk fats were found by Fomon & Bartels (1960) and Goalwin & Pomeranze (1962); significantly lower values for serum cholesterol were observed in babies given 'synthetic' milks containing soyabean oil or maize oil, though when solid food was added to the diet the serum cholesterol rose to values similar to those observed in babies given breast milk or cow's milk. Similar results were reported by Lindquist & Malmcrona (1960) who, in addition to estimating serum cholesterol, showed that total serum lipid values were less in babies given a 'synthetic' milk containing maize oil than in infants which were breast-fed or given cream in a 'prepared' milk.

What appears to be an important, hitherto-undiscovered role for fat in the diet of infants is described and discussed by Gillman *et al.* (1961) and Gillman, Gilbert, Savage, Gillman & Scragg (1961). Incidental to studies on the etiology of kwashiorkor, the utilization of pyruvate, acetate and glucose for the synthesis of cholesterol and for the synthesis of fatty acids and glycerol of triglycerides in both normal and kwashiorkor-affected infants was investigated. It was concluded that up to the age of 2 years and possibly longer, an infant cannot readily synthesize cholesterol or the fatty acids of triglycerides from glucose or even pyruvate and is largely dependent on acetate (acetyl CoA) derived from the catabolism of milk fat; glycerol synthesis was more extensively promoted by acetate than by pyruvate. Observations apparently so fundamental as these clearly require confirmation and extended study in infants and in the young of other species.

Milk fat in the nutrition of adults, with special reference to cardiovascular disease

Though no direct cause-effect relation has been established between lipids in plasma and coronary heart disease it has been known for some time that a correlation exists between the incidence of the condition and higher than 'normal' values for plasma cholesterol; stemming from this correlation a plethora of papers has appeared during the past 10 years on the effect of ingested polyunsaturated fatty acids in bringing about a lowering of plasma cholesterol values. It has also been well established that the consumption of a diet containing a considerable proportion of its energy value as fat of a low degree of overall unsaturation is accompanied by an increase in plasma cholesterol values. Milk fat is particularly effective in this respect and thus there grew up a considerable campaign (especially in the U.S.A.) to discredit dairy products in general as predisposing the consumer to cardiovascular disease. It is not proposed here to discuss the already well-documented background to this topic but to direct attention to some recent publications which present a more balanced attitude to the problem.

An Interdepartmental Committee (1960) presented its report on 'Milk Composition in the United Kingdom'. The Medical Panel of the Committee heard a great deal of evidence on the question of a possible link between fat consumed in milk and butter and cardiovascular disease. The Committee as a whole accepted the Panel's view 'that the evidence, though suggestive, is not conclusive and that a great deal of research is urgently needed to clarify the relationship between atheroma and/or coronary thrombosis and lipids of different kinds'. The Panel also concluded that, in the future should a reduction in the consumption of fat be indicated, then 'milk

would be among the last of foods rich in fat of which less should be consumed' in view of its valuable contribution of other nutrients. The Panel did not, however, think that any benefit would be gained from the consumption of greater amounts of milk fat by any section of the community. In the meantime no reduction in the fat content of milk was called for by the Committee and 'indeed it may never be'.

At a seminar of the International Dairy Federation held in Sweden in 1961 Morton (1962) discussed the composition and nutritive value of food fat, with special reference to milk fat. With respect to atherosclerosis and coronary heart disease and diet he saw very little reason to criticize milk fat as a component of a mixed diet—a conclusion, he adds, 'which is not at variance with historical experience'. Kon (1962) endorsed this conclusion in his lecture to the 16th International Dairy Congress in Copenhagen and gave prominent consideration to the comprehensive epidemiological study by Yerushalmy & Hilliboe (1957) in which (i) positive correlations were observed between deaths due to cardiovascular disease and the total energy value of the diet consumed, the amount of animal (saturated) fat and the amount of animal protein in the diet; and (ii) negative correlations between mortality and intake of vegetable fat and vegetable protein. Whilst there is a considerable body of evidence from clinical and animal experiments to support the positive correlations in respect of intake of total calories and of saturated fat, Kon pointed out that in several countries there appeared to be no striking relationship between the consumption of butter and mortality due to cardiovascular disease (cf. Davis, 1961) and went on to suggest that the polyunsaturated fatty acid:energy ratio of the diet should perhaps be considered in relation to its possible pathogenic potential 'irrespective of whether the energy is supplied as carbohydrate or as non-polyunsaturated fat'.

Whether or not one needs to be 'ratio conscious' in this matter remains to be seen (cf. Kinsell, 1963), though the emphasis on the energy value of the diet is in accord with the current concept that this is of considerable importance in relation to plasma lipid levels and possibly to the development of pathological lesions. Albrink, Meigs & Man (1961) have shown that, in the post-absorptive state, high values for plasma triglycerides are positively correlated with coronary heart disease; prolonged alimentary lipaemia is also characteristic of the condition, indicating that the fundamental metabolic defect is a delayed removal from the plasma of triglycerides of endogenous or exogenous origin. Albrink (1962) emphasizes that, whilst plasma cholesterol values (in the post-absorptive state) are significantly affected by the amount and kind of dietary fat, the triglycerides are not, though marked changes in the plasma content of the latter are occasioned by alterations in the total energy value of the diet, especially in respect of carbohydrate. The evidence presented and discussed in great detail by Albrink (1962) strongly suggests a close link between excess calorie intake, increased plasma triglyceride values, impaired carbohydrate tolerance and coronary artery disease. It is apparently more than coincidental that in several parts of the world (especially in the so-called 'Western countries') a high calorie intake is associated with a high intake of animal fat of which the dairy products milk, butter and cheese form a considerable part. An all-round reduction in diet is probably a good thing for many people, particularly those in sedentary occupations, but again let it be stressed that there appears to be no good reason why, if this were done, it should be at the disproportionate expense of dairy products. It is

perhaps worth remembering in the face of the voluminous literature on this subject that it has been a truism for many years in life insurance offices that over-eating and heart disease are closely associated!

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Printed in Great Britain at the University Printing House, Cambridge
(*Brooke Crutchley, University Printer*)