# JOURNAL OF DAIRY RESEARCH

VOLUME 36, 1969

CAMBRIDGE UNIVERSITY PRESS

# The rennet hysteresis of heated milk

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SUMMARY. The phenomenon of rennet hysteresis in heated milk is essentially due to reversible heat-induced changes of calcium phosphate equilibrium which affect the second or calcium ion stage of the rennet coagulation. The phenomenon is shown not only by heated milk but also by all heated caseinate systems provided they contain sufficient calcium to permit of coagulation with rennet and enough phosphate to ensure some degree of colloidal phosphate precipitation during the heating process; its occurrence does not require the presence of micellar casein or of $\beta$-lactoglobulin, or the initial presence of colloidal phosphate. Nevertheless, rennet hysteresis is greatly increased in these heated systems if $\beta$-lactoglobulin is present owing to the formation of a calcium caseinate/$\beta$-lactoglobulin complex which in its renneted condition is much less sensitive to calcium ions than is calcium para-caseinate. The resulting relative prolongation of the second stage of the rennet coagulation renders this phenomenon more apparent by increasing the proportion of the total time occupied by the hysteresis effect.

Rennet hysteresis, i.e. the occurrence of a time-lag before the increase in the rennet clotting time of milk brought about by heating attains its maximum, was first observed by Mattick & Hallett (1929), and has been attributed to a slow partial reversal of a heat-induced transfer of calcium phosphate from the serum to the caseinate micelles (Pyne, 1945). This change, it was held, affects only the second or calcium coagulation stage of the rennet coagulation. In a more recent study by Kannan & Jenness (1961) attention was drawn to the previously unrecognized effect of $\beta$-lactoglobulin on the phenomenon. While acknowledging that heat-induced phosphatic displacements participated in the phenomenon, these authors attributed both the immediate prolongation of the rennet clotting time which follows relatively severe heating of milk, and the rennet hysteresis of such heated milk, primarily to a heat-induced interaction between casein and $\beta$-lactoglobulin. Heat-induced interaction between $\beta$-lactoglobulin and $\kappa$-caseinate has since been definitely shown to occur (Sawyer, Coulter & Jenness, 1963).

A preliminary note by Morrissey & Pyne (1966) reports evidence which supports the views of Kannan & Jenness (1961) concerning the influence of $\beta$-lactoglobulin on the rennet hysteresis of milk. It confirms, however, the earlier view that the soluble salts in milk, and in particular calcium phosphate, exert a determining influence on the phenomenon and that this is quite independent of the intervention of $\beta$-lactoglobulin. The present paper provides further information on the roles and relative

importance of the various factors involved in rennet hysteresis and attempts to provide a more detailed and precise picture of the nature of the phenomenon.

<div style="text-align: center;">EXPERIMENTAL MATERIALS AND METHODS</div>

Fresh mixed milks and individual cow's milks, mainly from Friesian animals, were obtained from the University College herds, or from farms supplying milk to the University Experimental Creamery.

*Colloidal phosphate-free milks (CPF milks).* These were prepared by bringing skim-milk at 0 °C to pH 4·9 by addition, with mechanical stirring, of about 0·4 % by volume of concentrated HCl. The milks were stirred for 1 h after the addition of the acid and then dialysed for a total of 72 h at 0–5 °C against 40 volumes of the original skim-milk, changed at 24-h intervals.

*Synthetic milks.* These were prepared as follows:

(A) Colloidal calcium phosphate was formed in a neutral calcium caseinate solution, as described by McGann & Pyne (1960).

(B) Colloidal calcium phosphate was formed by raising the pH value of undialysed CPF milk from pH 4·9 to 7·1 by addition of sodium barbiturate solution of pH 8·5.

(C) Colloidal calcium phosphate was introduced into a strongly alkaline calcium caseinate solution by adjusting to pH 6·6 by addition of potassium phosphate solution of pH 5·0.

All these solutions were finally equilibrated against bulk milk by dialysis.

*Serum protein-depleted milks* were prepared by dispersing in appropriate amounts of milk dialysate the calcium caseinate-phosphate gel which separates on the bowl of the laboratory Sharples super-centrifuge. After overnight stirring at 0–2 °C the casein concentration in these preparations was adjusted to 2·5 % by suitable further additions of milk dialysate.

*Synthetic milk sera* were prepared by the method of Pyne & McHenry (1955).

*Phosphate-free milk sera* were prepared by replacing 0·1 M-sodium phosphate by 0·1 M-sodium maleate in preparing the sera just described.

*Serum protein* was prepared by the method of Kannan & Jenness (1961) and was used in these experiments as the source of $\beta$-lactoglobulin.

*Analytical methods.* Inorganic phosphate was determined by the colorimetric method of Fiske & Subbarow (1925), calcium plus magnesium by the EDTA titration method of Davies & White (1962), citrate by the colorimetric method of Marier & Boulet (1958) as modified by White & Davies (1963), pH by glass electrode and protein content by the amido-black method of Schober & Hetzel (1957). Colorimetric estimations were made with a Unicam SP 500 spectrophotometer.

Unless otherwise stated the heat treatment of milk used was 85 °C for 30 min.

<div style="text-align: center;">RESULTS</div>

<div style="text-align: center;">*Rennet hysteresis in milk systems devoid of $\beta$-lactoglobulin*</div>

The results in Table 1 show that rennet hysteresis is displayed by serum protein-free synthetic milk (preparation C) and by serum protein-free calcium caseinate systems which had been equilibrated against either milk or artificial milk sera and raised to a sufficiently high calcium ion concentration (about 5 m-mole/l $Ca^{2+}$) to

ensure rennet coagulation. Development of hysteresis was more obvious in the serum protein-depleted milks when they were renneted at somewhat lower temperatures than 35 °C, e.g. at 25 and 30 °C, owing to the relative prolongation of the second stage of the rennet coagulation (results not shown). Synthetic milks prepared by methods A and B did not in general show as marked a hysteresis as did those prepared by method C. An explanation of this behaviour is proposed in the Discussion.

When soluble phosphate was replaced in a CPF milk by the equivalent amount of maleate, by equilibration against an appropriate phosphate-free serum, rennet hysteresis was no longer observed (Table 1). Presence of phosphate in solution (in addition, of course, to dissolved calcium) was thus essential for the development of rennet hysteresis.

Table 1. *A comparison of the rennet hysteresis of various milk systems*

| | Rennet clotting time at 35 °C, sec | |
| --- | --- | --- |
| Material | Measured immediately | Measured 180 min later |
| Synthetic milk C | 212 | 265 |
| CPF milk | 77 | 178 |
| CPF milk (maleate replaces phosphate) | 140 | 125 |
| CPF milk (no $\beta$-lactoglobulin) | 67 | 102 |
| CPF milk (with $\beta$-lactoglobulin) | 195 | 348 |
| Milk | 162 | 336 |
| Milk + 3 mM/l-NEM* | 92 | 122 |

* N-ethylmaleimide.

### Influence of colloidal calcium phosphate on hysteresis

Observations of rennet hysteresis have hitherto been limited to those systems which, like milk or artificial milks, contained colloidal calcium phosphate. Nevertheless, the phenomenon can occur independently of the presence of this constituent, which merely serves, in fact, to increase the sensitivity of renneted caseinate systems to calcium ions (Pyne & McGann, 1962), and thus allows coagulation to occur at such relatively low calcium ion concentrations as those in milk.

### Effect of $\beta$-lactoglobulin on rennet hysteresis

The data in Table 1 also show the influence of $\beta$-lactoglobulin on hysteresis, and confirm the finding of Kannan & Jenness (1961) that presence of this protein during the heating of milk or other caseinate system not only markedly increases the degree of hysteresis, but also causes a permanent increase in their renneting times. Thus, subsamples of serum protein-reduced milk, brought to various levels of serum protein and heated before renneting, showed both these effects (results not tabulated). These changes were probably due, as has been suggested by Kannan & Jenness, to a heat-induced casein–$\beta$-lactoglobulin interaction which not only alters the sensitivity of the casein to attack by rennin but perhaps also makes the sensitivity to calcium of the rennet altered casein 'more critically dependent on its calcium phosphate content' as these authors express it. Heat-induced interaction between casein and $\beta$-lacto-globulin is inhibited by the presence of sulphydryl blocking agents during heating (Sawyer *et al.* 1963); correspondingly, both the increase in rennet clotting time of heated milk and the degree of hysteresis displayed were found to be diminished

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greatly when 3·0 mM/l N-ethylmaleimide (NEM) were present at the time of heating (Table 1).

Although rennet hysteresis is held to be a phenomenon which arises essentially from changes in the duration of the second or calcium precipitation stage of renneting (Pyne, 1945; Kannan & Jenness, 1961) it is in fact measured by comparing the total renneting time of the milk immediately after heating and some hours later. Total renneting time is largely determined in normal conditions by the duration of the first stage, which is usually fairly long compared with the second when measured at around 35 °C. If now casein–$\beta$-lactoglobulin interaction were to bring about a retardation of the second stage which was relatively greater than that of the first—perhaps in consequence of the rennet-altered protein complex so formed possessing a lower sensitivity to calcium ions than rennet-treated calcium caseinate—this interaction could, on account of the increased proportion of the total clotting time taken up by the second stage, similarly increase the degree of hysteresis as a percentage of the total clotting time of the milk. In view of these considerations it was decided to investigate the influence of casein–$\beta$-lactoglobulin interaction on the duration of each of the 2 stages of the rennet coagulation.

Table 2. *Comparison of the relative rates of release of NPN soluble in 12 % TCA from unheated and heated casein–$\beta$-lactoglobulin mixtures when treated with rennet under identical conditions*

| Time of rennet action, min | Optical density at 750 nm | | Increase in NPN, % | |
| --- | --- | --- | --- | --- |
| | Unheated, mean of duplicates | Heated, mean of duplicates | Unheated | Heated |
| 0 | 0·1464 | 0·166 | — | — |
| 5 | 0·2577 | 0·2432 | 76 | 46·5 |
| 10 | 0·296 | 0·2696 | 102·1 | 62·4 |
| 20 | 0·314 | 0·299 | 114·4 | 80·1 |
| 30 | 0·330 | 0·322 | 125·4 | 93·9 |

*Effect of casein–$\beta$-lactoglobulin interaction on the enzymic stage and on the calcium precipitation stage of coagulation with rennet*

The influence of heat on the enzymic stage of the rennet coagulation of milk is best examined under conditions which allow it to be isolated from the succeeding calcium precipitation stage. Measurement of the rate of release of NPN soluble in 12 % TCA appeared to provide a method of doing this. The NPN values shown in Table 2 and expressed in terms of optical density and of the percentage increase in NPN were obtained by the method of McGann & Pyne (1960), which employs the colorimetric procedure of Lowry, Rosebrough, Farr & Randall (1951). The results confirm a further finding of Kannan & Jenness (1961), namely that the presence of serum protein during the heating of milk significantly retards the rate of rennin attack on the caseinate. The results of further somewhat similar experiments (not included here) in which $\kappa$-casein was heated in presence and absence of serum protein also confirmed this finding.

The effect of heat-induced casein–$\beta$-lactoglobulin interaction on the second stage of rennet coagulation was determined by the method of Pyne & McHenry (1955).

A 2·5 % solution of calcium caseinate buffered with 10 mM/l sodium maleate was brought to the calcium ion concentration needed to give the unheated caseinate system a second stage coagulation time of between 60 and 120 sec at 29 °C. (The duration of the second stage was taken as being the approximately constant value which is eventually attained for the coagulation time at 29 °C following completion of the first stage at 0–2 °C. This first stage was usually complete in 45–60 min at 0–2 °C after addition of 2 % of commercial rennet solution.) When the caseinate–$\beta$-lactoglobulin solution was not heated the presence of the serum protein appeared to have at most only a very slight influence on the duration of the second stage (Table 3). But when the solution was heated the second stage of the coagulation was greatly retarded and, moreover, retarded to a relatively greater degree than the first stage, no doubt owing to the reduced sensitivity to calcium ions of the 'para-caseinate' when present in the form of a complex with $\beta$-lactoglobulin. This disproportionate retardation of the second stage of the coagulation—the stage implicated in the development of rennet hysteresis—can obviously account, in part at least, for the increased hysteresis shown by heated caseinate–$\beta$-lactoglobulin systems—including of course milk.

Table 3. *Effect of heat treatment in presence and absence of serum protein on the second stage rennin coagulation of calcium caseinate systems*

| Second-stage coagulation time at 29 °C, sec | | | |
|---|---|---|---|
| Unheated | | Heated | |
| No serum protein | 0·5 % serum protein | No serum protein | 0·5 % serum protein |
| 60 | 76 | 46 | 190 |
| 108 | 107 | 87 | 224 |
| 60 | 70 | 50 | 175 |
| 110 | 102 | 80 | 185 |

### Time order of caseinate–$\beta$-lactoglobulin interaction and displacement of calcium phosphate equilibrium

It is uncertain whether casein–$\beta$-lactoglobulin interaction mainly precedes or mainly follows the heat-induced displacement of calcium phosphate equilibrium to which the hysteresis phenomenon is essentially due, and indeed whether the order in which these reactions occur has any significance in relation to the degree of hysteresis which develops.

The following experiment, in which advantage was taken of the ability of NEM to interrupt the casein–$\beta$-lactoglobulin interaction, appears to indicate that the casein–$\beta$-lactoglobulin interaction largely precedes deposition of calcium phosphate on the caseinate. Samples of milk were withdrawn at varying time intervals after initiation of the heating process (at 85 °C) and cooled rapidly in ice to room temperature. They were then treated by addition of 3 mM/l NEM, stirred for 20 min, again brought rapidly to 85 °C and held at that temperature for the remainder of the 30 min.

The results are shown in Table 4 and indicated that for marked inhibition of hysteresis to occur the NEM had to be added within 2 min of the commencement of heating at 85 °C. The greater part of the interaction between casein and $\beta$-lactoglobulin would thus appear to be complete in about 2–3 min at 85 °C and accordingly

to precede the greater part of the changes of phosphate equilibrium which characterize the development of hysteresis. Once induced, the casein–$\beta$-lactoglobulin reaction appears to be relatively irreversible under normal conditions. Thus, rennet hysteresis can be fully re-developed in the same heated milk even in presence of subsequently added NEM by reheating it to 85 °C.

Table 4. *Rapidity of $\kappa$-casein–$\beta$-lactoglobulin interaction at 85 °C*

| Heating procedure | Rennet clotting time 35 °C, sec | |
|---|---|---|
| | Measured immediately | Measured 180 min later |
| (1) Skim-milk: heat at 85 °C for 30 min; no NEM | 162 | 286 |
| (2) Skim-milk: bring to 85 °C, immediately cool, add NEM and continue heating | 78 | 100 |
| (3) Skim-milk: heat at 85 °C for 1 min, immediately cool, add NEM and continue heating | 105 | 142 |
| (4) Skim-milk: heat at 85 °C for 2 min, immediately cool, add NEM and continue heating | 136 | 195 |
| (5) Skim-milk: heat at 85 °C for 3 min, immediately cool, add NEM and continue heating | 183 | 261 |
| (6) Skim-milk: heat at 85 °C for 4 min, immediately cool, add NEM and continue heating | 186 | 297 |
| (7) Skim-milk + NEM; heat at 85 °C for 30 min | 78 | 86 |

Table 5. *Effect of serum salt variations on the rennet coagulability of skim-milk*

| Serum salt (mM) | | | Rennet clotting time at 35 °C, sec | | |
|---|---|---|---|---|---|
| | | | Raw | Heated | |
| Ca + Mg | Inorganic P | Citrate | | Measured immediately | Measured 180 min later |
| **10** | 11·5 | 7·5 | 130 | 192 | 473 |
| 11·2 | 11·5 | 7·5 | 70 | 95 | 143 |
| **13** | 11·5 | 7·5 | 35 | 46 | 61 |
| **14** | 11·5 | 7·5 | 27 | 32 | 35 |
| 11·2 | **10** | 7·5 | 66 | 95 | 132 |
| 11·2 | **12·5** | 7·5 | 65 | 97 | 137 |
| 11·2 | **13·5** | 7·5 | 59 | 100 | 136 |
| 11·2 | 11·5 | **6** | 63 | 56 | 65 |
| 11·2 | 11·5 | **8·5** | 95 | 130 | 424 |
| 11·2 | 11·5 | **9·5** | 124 | 310 | 829 |

Figures in bold type represent experimental variations in concentration around the reference values established for a milk exhibiting only slight hysteresis (see below).

### Soluble milk salts in relation to hysteresis

Morrissey & Pyne (1966) noted that individual milks differed greatly in their capacity to display rennet hysteresis. These differences appeared in some cases to be due to differences in the serum salt conditions rather than in the $\beta$-lactoglobulin content of the milks, since milks showing marked hysteresis were frequently found to lose this characteristic when dialysed against milks exhibiting only slight hysteresis. These latter showed, conversely, an increase in rennet hysteresis.

In an attempt to isolate the precise salt compositional conditions which favour development of rennet hysteresis, the dialysate of a milk exhibiting only slight

hysteresis was analysed for the major soluble constituents thought likely to be involved, namely soluble calcium plus magnesium, phosphate and citrate. The milk was then modified in its soluble salt composition by dialysing it against various synthetic salt solutions adjusted over a range of compositions in respect of each of these 3 components.

Typical sets of results are shown in Table 5. They indicate that lowering the calcium ion concentration of the milk, whether by decreasing the level of calcium plus magnesium or by increasing the level of citrate, favoured the development of hysteresis. The effect of varying the phosphate concentration, however, appeared to be slight. These results, taken in conjunction with those relating to the influence of $\beta$-lactoglobulin noted earlier, would appear to indicate that hysteresis is favoured both by a relatively high $\beta$-lactoglobulin content and by a relatively low calcium ion concentration. Perhaps the same reason applies in both cases, namely that the presence of $\beta$-lactoglobulin or of low calcium concentration caused the second or calcium coagulation stage to be retarded relative to the first. However, certain exceptions to this generalization can occur. Thus, some low hysteresis milks were found to respond to increase in soluble calcium with an increased display of hysteresis. This matter will be dealt with in the Discussion.

## DISCUSSION

The present investigation confirms in essentials the views originally put forward by Pyne (1945), namely that the rennet hysteresis of milk is due to the relatively slow reversal of a heat-induced transfer of calcium phosphate from serum to casein micelles, a transfer which sensitizes them in their rennet-altered condition to precipitation by calcium ions. The presence of soluble phosphate is essential for the formation of this calcium phosphate and is thus a *sine qua non* for the development of rennet hysteresis. Hysteresis can occur on the other hand in the complete absence of $\beta$-lactoglobulin, but the degree of its manifestation unquestionably increases progressively with rise in $\beta$-lactoglobulin content. The effect of $\beta$-lactoglobulin appears to be to create, as a result of heat-induced interaction with caseinate, a caseinate/$\beta$-lactoglobulin complex which not only reacts more slowly with rennet, but which, in its rennet-altered form, displays also a lower sensitivity to calcium ions than does calcium para-caseinate. A retardation of both stages of renneting is thus produced, but it appears to be relatively greater in the second stage than in the first. The resultant relatively greater prolongation of the second stage thus increases the proportion of the total renneting time taken up by variations in the duration of the second stage, i.e. by those variations which are at the basis of the hysteresis phenomenon proper.

The explanation of rennet hysteresis put forward by Kannan & Jenness (1961), namely that "the interaction of $\beta$-lactoglobulin with the caseinate micelles makes the coagulability more critically dependent on the concentration of colloidal phosphate" cannot, however, be ruled out as being at least a contributory factor. The order in which the 2 reactions involved take place, namely casein–$\beta$-lactoglobulin interaction and heat-induced formation of colloidal calcium phosphate in solution, suggests that much of the calcium phosphate formed during heating will in fact be deposited on an already constituted caseinate/$\beta$-lactoglobulin complex. And it is indeed possible that

the calcium sensitivity of this complex when renneted may be more greatly in-fluenced by its calcium phosphate content than is calcium para-caseinate which has not interacted with $\beta$-lactoglobulin.

The differing hysteresis behaviours of the various 'artificial milk' preparations A, B and C, of which C alone displayed the phenomenon to any marked extent, is perhaps attributable to the differing methods employed in preparing them. McGann & Pyne (1960) have suggested that the micelles in milk may possess something of the nature of a double calcium salt of phosphate and caseinate. It is not altogether sur-prising, therefore, that the method of preparing the complex which best displays rennet hysteresis, namely neutralization of an alkaline calcium caseinate by a primary phosphate, is that which is most likely to give precisely this type of association. The alternative methods of preparation employed in which the calcium phosphate is formed under somewhat acid and in neutral conditions, respectively, may on the contrary lead to formation of phosphate precipitates which are merely physically protected by the caseinate.

As regards the influence of the salt composition of milk on hysteresis, it may seem strange that lowering the soluble calcium content led to increased rennet hysteresis in most milks which had been equilibrated against artificial sera, while raising it in-creased (at least in a limited number of samples) the hysteresis shown by certain low hysteresis milks. In seeking an explanation it must be borne in mind that hysteresis originates solely in those changes in the duration of the second stage of renneting which arise from reversible heat-induced changes of phosphate equilibrium. However, the *degree* of hysteresis which is exhibited in any particular case is determined by 2 factors, (1) the extent of this change of phosphate equilibrium—which depends chiefly on the salt composition of the milk serum—and (2) the $\beta$-lactoglobulin content of the milk. Somewhat low soluble calcium tends in general to cause a retardation of the second stage of the rennet coagulation relative to the first and thereby to accen-tuate the hysteresis, and this is probably the more general case, especially where the $\beta$-lactoglobulin content of the milk is normal or fairly high. On the other hand, raising the calcium concentration, and thus increasing the extent of the heat-induced changes of calcium phosphate equilibrium, may be the only way in which a milk low in $\beta$-lactoglobulin can be made to show a marked degree of hysteresis. Admittedly this treatment may result in an all-round shortening of the second stage of the coagu-lation relative to the first. Nevertheless, the greater degree of calcium phosphate precipitation involved may more than compensate for this and may allow the hys-teresis observed to become more evident by bringing about an increase in the differ-ence in the second-stage coagulation times of the milk measured immediately follow-ing heating and some hours later.

As to the mode of action of $\beta$-lactoglobulin in retarding renneting and in bringing about an increased development of rennet hysteresis, any conclusions must neces-sarily be somewhat speculative. The retardation of the enzymic stage of renneting following interaction of casein with $\beta$-lactoglobulin may be due to a reduced accessi-bility of the casein to rennet during the first stage of renneting. Retardation of the second or calcium precipitation stage may be due to a parallel blocking or rendering less accessible to calcium those groups of the rennet-altered casein which, when cross-linked by calcium, lead to coagulation. Such a reduced propensity of the para-caseinate

for calcium cross-linking could perhaps also render the system more critically dependent on the content of sensitizing colloidal phosphate which the micelles had acquired during the heating.

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# The heat stability of milk as affected by variations in pH and milk salts

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SUMMARY. The maximum and minimum heat stability exhibited by most milks over a relatively narrow range of pH values is shown also by synthetic colloidal calcium caseinate–calcium phosphate systems and even by simple caseinate systems, provided all possess adequate contents of $\beta$-lactoglobulin, soluble calcium and phosphate. The phenomenon is not, however, dependent on the presence of the characteristic micellar structure of the casein of milk. The minimum stability observed, usually around pH 6·9, is the most characteristic feature of the phenomenon and arises from heat induced deposition of calcium phosphate on a caseinate/$\beta$-lactoglobulin complex. This reaction, which tends to occur to a marked degree at relatively high pH values and calcium ion concentrations, sensitizes the complex to precipitation by calcium ions. The precise pH values at which the maximum and minimum stabilities occur can vary depending on the salt composition of the serum, since the latter can influence the solubility of calcium phosphate.

Despite numerous investigations, recent reviews (Rose, 1963, 1965) suggest that factors concerned in the heat coagulation of milk and the coagulation mechanism are still not completely understood. Nevertheless, since the pioneer work of Sommer & Hart (1919, 1922, 1926) the salt composition—or perhaps soluble-salt composition (Pyne & McHenry, 1955; Pyne, 1958, 1959)—has been generally accepted as exercising an important influence on the heat stability of milk. The heat stability of most individual milks can also be greatly altered by quite small changes of pH (Rose, 1961*a*). When a maximum stability was observed it was usually found by this author to occur between pH 6·5 and 6·7 and the accompanying minimum stability at around pH 6·9. Such milks—the majority—were later classified by Tessier & Rose (1964) as type A, while milks showing an approximately linear relationship between pH and heat stability over the pH range 6·3–7·5 were classified as type B. These variations in stability appeared to be related in the first instance to the $\beta$-lactoglobulin content of the milk (Rose, 1961*b*), but a role in the phenomenon was later assigned also to $\kappa$-casein by Tessier & Rose (1964). According to these latter authors it is the $\kappa$-casein/$\beta$-lactoglobulin ratio of the milk rather than the $\beta$-lactoglobulin content which primarily determines the shape of the pH/heat-stability curve. Rose (1962) also concluded that if the major inorganic salts influenced the heat stability of milk they did so only in the presence of $\beta$-lactoglobulin. This conclusion seemed to be at variance with certain observations on the role of the milk salts made earlier

in this laboratory. It was therefore decided to re-examine the influence of the milk salts, both colloidal and dissolved, on the heat stability of milk in the light of the studies of Rose and his colleague.

## EXPERIMENTAL MATERIALS AND METHODS

Experimental materials and methods in general, unless otherwise stated, were those already described (Morrissey, 1969).

*pH-adjusted milks.* The pH of milk was altered by slow addition, with constant mechanical stirring, of either 0·1 N-HCl or NaOH to furnish approximate pH values in the range 6·4–7·5. All adjusted samples were held for 3 h before determination of pH and heat stability.

*Heat-coagulation time.* This was determined by the method of Pyne & McHenry (1955).

## RESULTS

### Phosphate as a factor in the pH/heat-stability relation

Rose (1962) noted that removal of colloidal phosphate from milk eliminated the minimum usually observed in the heat stability curve around pH 6·9. Preliminary experiments on colloidal phosphate-free milks (CPF milks) confirmed the results of Rose in this regard. Removal of colloidal phosphate greatly increased heat stability at all pH values, and did in fact bring about a loss of the characteristic maximum and minimum values in the pH/heat-stability curve. However, the coagulation times of the CPF milks were considerably longer than those of the corresponding whole milks and may have been so long as to obscure the operation of the factors primarily implicated in the production of the pH/heat-stability relation observed by Rose in normal milk. The experiment was therefore repeated on a CPF milk, the soluble calcium content of which had been raised by dialysis against calcium-enriched milk (milk to which 10–12 ml 0·1 M-CaCl$_2$/l had been added, followed by readjustment of the pH to its original value). The CPF milk so treated was now found to have taken on some of the characteristics of type A milk. The stability curve was displaced towards the alkaline side compared with that for normal milk, minimum stability being generally observed to occur in the pH range 7·2–7·5. The results indicated, however, that soluble calcium content plays a role in determining the characteristic shape of the pH/heat-stability curve observed with CPF milk, and that the initial presence of colloidal calcium phosphate (as in milk) is not essential for the development of this feature.

Marked reduction in the soluble phosphate content of a milk (a condition conveniently attained by the phosphate-reducing dialysis technique of McGann & Pyne, 1960) was also found to be accompanied by changes in its pH/heat-stability pattern. Thus, reduction in phosphate content (mainly in soluble phosphate) from 57 to 28·6 mg P/100 ml caused a progressive shift in the position of the maximum and minimum stabilities towards the alkaline side. Figure 1 shows the curves obtained with subsamples of a milk examined after 4 and 16 h dialysis. The calcium content of the artificial serum against which the dialysis was conducted was raised slightly to compensate for some slight losses of colloidal phosphate during dialysis of the milk.

If, however, soluble phosphate is removed completely—this can be done most con-

veniently with CPF milk—the pH/stability pattern entirely changes. Thus, equilibrium dialysis of CPF milk against an artificial milk-type serum in which phosphate had been replaced by the equivalent amount of maleate produced a system in which the characteristic type A properties of the CPF milk were replaced by those of type B. These results suggest that for the development of the characteristic maximum and minimum in the pH/heat-stability curve of milk not only is the presence of $\beta$-lacto-globulin essential as Rose (1961 b) had shown, but so also is the presence of soluble phosphate.
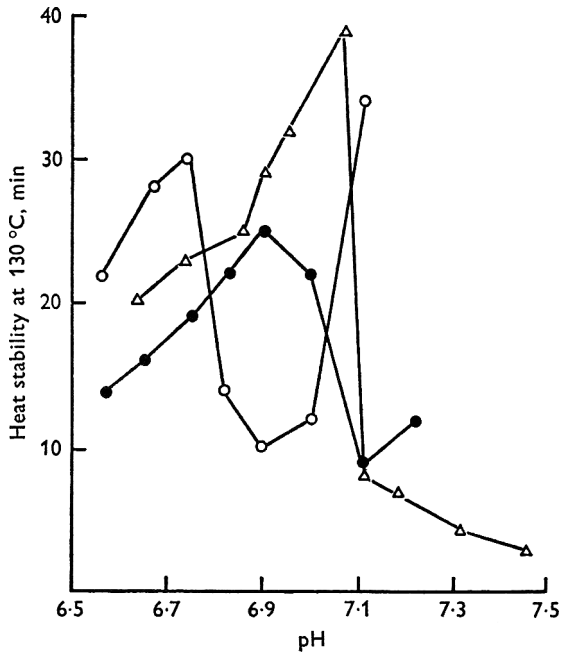


Fig. 1. Effect of soluble phosphate depletion on the pH/heat-stability characteristics of milk. ○, Control; ●, 4-h phosphate-reducing dialysis; △, 16-h phosphate-reducing dialysis.

### Influence of pH on the heat stability of synthetic milk

The synthetic milk whose preparation is described by Morrissey (1969) was found on examination to exhibit type A stability, i.e. to develop on slight changes of pH a maximum and minimum stability which closely resembled those of normal type A milk (Fig. 2). From this finding and that obtained with the calcium-enriched CPF milk already described, it is clear that the occurrence of type A features in milk is not dependent on the presence of the characteristic micellar structure of its calcium caseinate–calcium phosphate.

The contrary opinion expressed by Rose (1963), namely that removal of colloidal calcium phosphate from milk disrupts a micellar caseinate structure that he thought essential for the development of the normal pH/heat-stability features, was based on the observation (Rose, 1962) that synthetic milk, prepared by reprecipitation of colloidal calcium phosphate by alkali neutralization of an acidified type A milk, failed to display the type A characteristics of the parent milk. But the properties of a synthetic milk depend greatly on the method of preparation used (Morrissey, 1969), and it is

possible that the product prepared by Rose (1962) possessed too high a heat stability to display type A features under the experimental conditions employed. It will be remembered that CPF milk failed to display these features until its high stability had been appreciably reduced by addition of calcium salts.
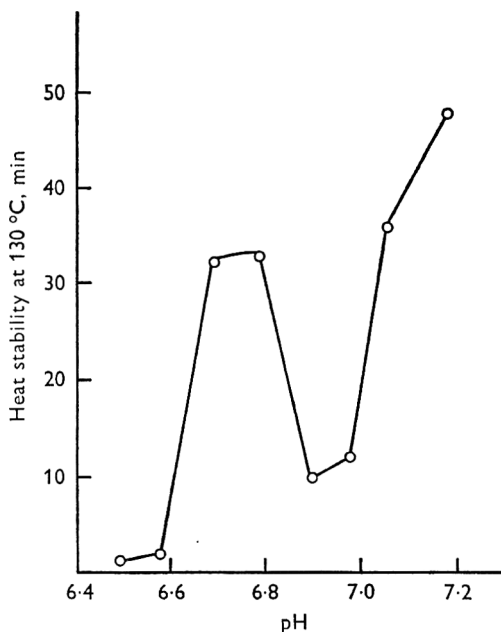


Fig. 2. The relation between pH and heat stability of a synthetic caseinate-phosphate milk.

### Effect of interchanging milk sera on the pH/heat-stability curves of various milks

Interchanging milk sera by dialysis of a small volume of one milk against large volumes of another tends as a rule to shift the pH/stability pattern of the dialysed milk towards that of the milk against which it has been dialysed. Thus, a type A milk dialysed against another type A milk tends to exhibit the pH values for maximum and minimum stability characteristic of the latter (Fig. 3). A type A milk dialysed against a type B tends to lose its characteristic type A minimum stability. A type B dialysed against a type A tends to acquire a minimum stability around pH 6·9 (Fig. 4). The results suggest, in agreement with the findings of Rose (1961b), that important factors controlling the pH/heat-stability pattern of milk are present in solution.

However, a certain number of milks fail to respond in this fashion. Clearly not all stability differences between milks can be ascribed to differences in milk serum composition alone; differences in $\beta$-lactoglobulin content or rather in $\beta$-lactoglobulin/$\kappa$-casein ratio must also operate as Tessier & Rose (1964) have noted. Bearing in mind that type A behaviour is favoured both by high $\beta$-lactoglobulin content (Rose, 1961b) and by high soluble calcium (see below) the following explanation could possibly account for the behaviour observed when the sera of these milks were interchanged. Thus, dialysis of type A milk high in $\beta$-lactoglobulin and somewhat low in soluble

calcium against a type B milk low in $\beta$-lactoglobulin and high in soluble calcium might well reinforce the type A characteristic of the former, and dialysis of the same type B milk against the same type A milk reinforce those of the type B milk.

### *Influence of divalent cations on the pH/heat-stability curve*

In order to determine more precisely the extent to which salt factors, and in particular variations in divalent cation concentration, may influence the pH/heat-stability characteristics of normal milk, a number of type A milks were modified in their soluble salt systems by dialysis against salt solutions in which the phosphate and citrate levels were maintained at their approximately average milk values of 10 and 9 mM/l, respectively, while soluble calcium and magnesium taken together, ([Mg]/[Ca] = 0·35) were made to vary from 10·5 to 13·5 mM/l.
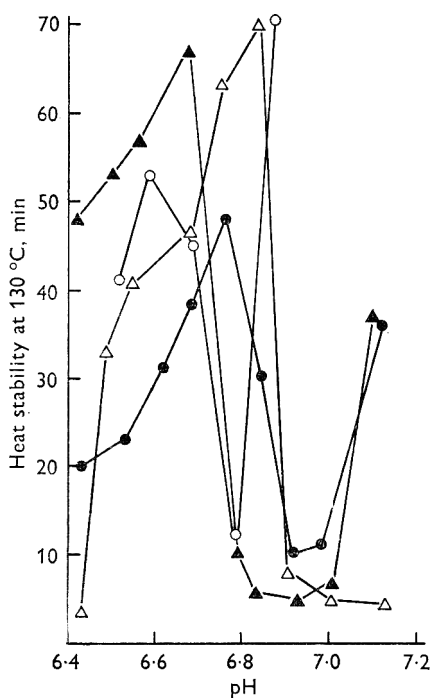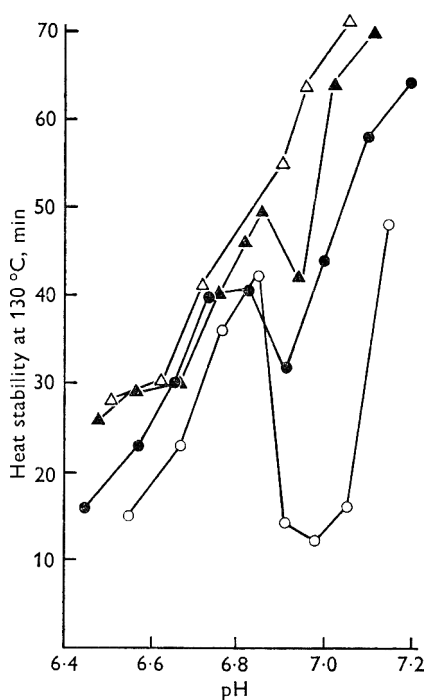


Fig. 3

Fig. 4

Fig. 3. Effect of interchanging (by dialysis) milk sera from type A milks on the pH/heat-stability curve. ○, Milk no. 1; ●, milk no. 1 dialysed against milk no. 2; △, milk no. 2; ▲, milk no. 2 dialysed against milk no. 1.

Fig. 4. Effect of interchanging (by dialysis) milk sera from Type A and Type B milks on the pH/heat-stability curve. ○, Type A milk; ●, type A milk dialysed against type B milk; △, type B milk; ▲, type B milk dialysed against type A milk.

The results for one such milk are shown in Fig. 5. They indicate that a moderately high concentration of soluble calcium in combination with soluble phosphate and $\beta$-lactoglobulin is essential for the development of the characteristic pH-sensitive variations in heat stability, with its marked minimum at pH 6·9 or higher. Changes in the level of divalent cations determined mostly the type of classification (A or B) and

gave rise to marked changes in the coagulation time at the pH of minimum stability. They did not greatly affect the coagulation time at the pH of maximum stability. These results demonstrate the importance of high soluble calcium and magnesium—a condition normally associated with low soluble phosphate—for the development of a minimum heat stability at pH 6·9 or higher, and are thus consistent with earlier findings of Rose (1961 a) that the maximum heat stability was significantly correlated negatively both to the ratio of soluble calcium to soluble inorganic phosphate and the ratio of ionic calcium to soluble inorganic phosphate. Here again, a few milks were found which, while responding in the above manner to slight reductions in the level of calcium and magnesium used, behaved quite differently when these reductions were more marked, developing a new minimum which was, however, definitely displaced towards the alkaline side.
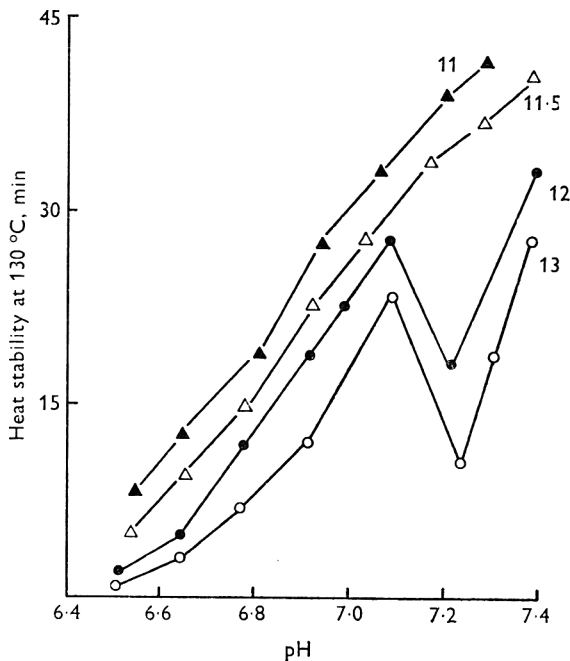
Fig. 5. Effect of altering the divalent cation content on the pH/heat-stability characteristics of milk. Figures beside curves indicate concentrations, in mM/l, of calcium + magnesium.

*Influence of other factors*

(a) *Conditions under which the complex of casein and β-lactoglobulin is formed.* Much the same pH/heat-stability patterns were obtained whether the casein/β-lactoglobulin complex was formed by heating a solution of the 2 proteins at pH 7·0 prior to adjusting the pH to the various values of the range studied, or in the usual way by heating the mixture at the various pH values. This finding does not appear to offer any support for the suggestion of Rose (1963), by way of explanation of the influence of pH on heat stability, that the extent to which the caseinate/β-lactoglobulin complex is formed in heated milk may be pH-dependent.

Thus, when solutions of sodium (or calcium) caseinate and β-lactoglobulin were

adjusted to pH 7·0, heated at 90 °C for 10 min, cooled, converted into a calcium caseinate–calcium phosphate 'milk' as described elsewhere (Morrissey, 1969), and equilibrated against bulk-milk by dialysis, this synthetic milk then displayed much the same type of maximum and minimum heat stability curve at the various pH values 6·6–7·5 as was observed in systems which were not preheated. The sole difference of note was that the maximum and minimum points were found to be shifted somewhat further towards the alkaline side in the systems which had been preheated (Fig. 6).
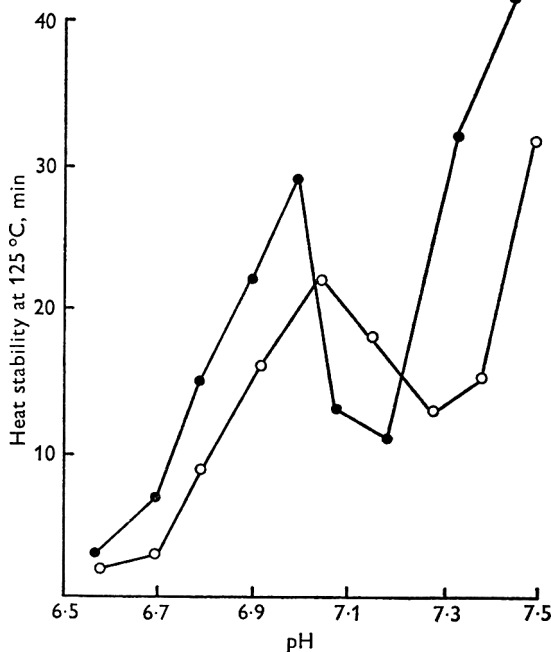


Fig. 6. The pH/heat-stability characteristics of a calcium caseinate–phosphate 'milk' prepared from a sodium caseinate–$\beta$-lactoglobulin solution which had been preheated at 90 °C for 10 min. ○, Preheated solution; ●, control (not preheated).

(b) *Influence of salts of the alkali metals.* Additions of relatively small concentrations of potassium chloride and sodium chloride (10 and 20 mM/l, respectively), to milks were found to stabilize slightly some individual milks and destabilize others. These differences appeared to arise from marked changes in the shape of the pH/heat-stability curves, which in general suffered a displacement of their maximum and minimum stabilities towards more alkaline values. Very definite destabilization above pH 7·0 was observed especially when the concentrations of potassium chloride and sodium chloride exceeded 20 and 30 mM/l, respectively. Potassium chloride was in general more effective than sodium chloride in altering the stability pattern.

DISCUSSION

The results obtained in the present work suggest that the minimum stability usually shown by milk at around pH 6·9 is due to precipitation of calcium phosphate on a caseinate/$\beta$-lactoglobulin complex which is thereby rendered more sensitive to the action of cations—calcium in particular.

Contrary to suggestions in the literature, neither the initial colloidal phosphate content of the milk nor the characteristic micellar structure of caseinate as it occurs in milk seems to play a role of any importance in this phenomenon; nor does the pH at which the formation of the caseinate/$\beta$-lactoglobulin complex occurs. Thus, CPF milk which has been raised to a sufficiently high calcium ion concentration to confer on it an average coagulation time typical of normal milk, and synthetic milks containing $\beta$-lactoglobulin, exhibit the same pH/stability pattern as normal milk. The heat-induced casein–$\beta$-lactoglobulin interaction which lies at the basis of (and under suitable serum salt conditions gives rise to) this typical pH/stability pattern with a type A minimum in the pH 7·1–7·3 region occurs independently of the pH at which the heating is conducted, and of the presence of milk salts. This is clear from the experiment described above in which an approximately neutral sodium caseinate–$\beta$-lactoglobulin solution, appropriately heated and subsequently converted into a synthetic milk by addition of the necessary salts, introduction of colloidal phosphate and other compounds, was found to display the typical pH/stability pattern of normal milk. This pattern, with its marked maximum and minimum, cannot therefore be attributed (*pace* Rose, 1963) to pH-dependent differences in the degree of interaction of casein and $\beta$-lactoglobulin. This leaves then as the factors involved in the development of the typical pH/stability of normal milks only the $\beta$-lactoglobulin content of the milk and the milk serum salts.

As to the role of these salts, the following points have been established. The typical pH/stability (Type A) relation of milk is lost if the phosphate of the serum is entirely replaced by another anion (e.g. by maleate); the pH values at which the minimum and maximum stabilities occur are displaced towards the alkaline side if the soluble phosphate content is reduced; the minimum stability is either brought into existence or, if already present, is accentuated if the soluble calcium content of the milk is increased. These findings are all consistent with the view that the minimum heat stability of milk encountered about pH 6·9 is due to heat-induced precipitation of calcium phosphate on a caseinate/$\beta$-lactoglobulin complex (likewise formed as a result of the heating), which is thereby sensitized to the coagulating action of calcium salts.

The curves shown in Fig. 5 make it clear that the characteristic portion of the typical pH/heat stability curve of milk is the marked minimum which occurs at pH 6·9 or higher. The maximum stability observed at pH values greater than 7·0 has no independent existence but arises from the presence of the neighbouring minimum. In the absence of the complicating deposition of calcium phosphate, heat stability would increase steadily with increase in pH. The minimum stability may be regarded as arising from the marked tendency of the serum calcium phosphate, at pH 6·9 or higher, to form a precipitate of colloidal calcium phosphate which is deposited on the caseinate/$\beta$-lactoglobulin complex and greatly increases its sensitivity to precipi-

tation by calcium ions. It is the superimposition of this destabilizing effect of a calcium phosphate precipitation at about pH 6·9 on the stabilizing effect of increasing pH which gives rise to the maximum which immediately precedes the minimum stability.

The effect of added sodium chloride and potassium chloride in shifting the minimum stability of a milk to a somewhat higher pH value than 6·9 is perhaps ascribable to the increased solubility of calcium phosphate in the presence of these salts, a phenomenon noted by Rose & Tessier (1959) in another connexion. In these conditions, a somewhat higher degree of alkalinity, i.e. a higher pH value, may be required to ensure the degree of precipitation of calcium phosphate needed to minimize the stability. The somewhat greater effectiveness of potassium salts as compared with sodium salts in altering the stability of milk is perhaps to be accounted for by its greater cation exchange efficiency, a point also made by Rose & Tessier (1959).

The phenomenon observed here may also account for the results obtained with a few milks which, while responding to slight reductions in the levels of calcium and magnesium by showing type B features, developed a new minimum when these reductions were more marked. The reductions in the concentrations of these divalent cations could likewise increase the $[Na + K]/[Ca + Mg]$ ratio.

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# Simple methods for the purification of crude κ-casein and β-casein by treatment with calcium phosphate gel

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SUMMARY. Batch methods applicable on a large scale are described for the purification of crude κ- and β-casein. κ-Casein, dissolved in urea-containing buffer, was freed from $\alpha_s$- and β-caseins by treatment with calcium phosphate gel and recovered in about 60 % yield. β-Casein was freed from most impurities by adsorption on to calcium phosphate gel at pH 7·8 in the presence of urea and elution with 6 M-urea–N-$NH_4OH$ at 4 °C. The recovery was about 50 %.

As expected from the solubilities of their calcium salts (Waugh, 1961), κ-casein is not adsorbed on calcium phosphate gel at room temperature and neutral pH, $\alpha_s$-casein is strongly adsorbed and not readily eluted and β-casein is adsorbed but can be eluted at low temperature and high pH. This observation forms the basis of the methods described in the present paper for removal of the main casein contaminants from crude preparations of κ- and β-caseins. Thus, treatment of crude κ-casein with calcium phosphate gel removes $\alpha_s$- and β-caseins, and β-casein can be freed from κ-casein by adsorption onto the gel and from $\alpha_s$-casein by subsequent elution. It is necessary to carry out the complete procedures in the presence of urea in order to prevent the formation of casein complexes.

Previously N. J. Berridge (personal communication) had shown that κ-casein could be purified by chromatography in a hydroxyapatite column. The present work is a development of this, calcium phosphate gel being used in preference to hydroxyapatite because of its greater convenience; hydroxyapatite is relatively difficult to prepare, does not retain its activity on storage and does not adsorb $\alpha_s$- and β-caseins sufficiently strongly for batchwise application.

## EXPERIMENTAL

### Materials

*Buffer.* Unless otherwise indicated, the buffer solution used contained 0·05 M potassium phosphate buffer at pH 6·8, 0·005 M-KCl, 4·5 M-urea and 0·013 M-2-mercaptoethanol.

*Calcium phosphate gel.* Calcium phosphate gel was prepared as described by Colowick (1955). It was suspended in water and the concentration of the suspension determined from the weight of a known volume dried at 100 °C. Just before use, the required amount of gel, calculated on a dry-weight basis, was recovered by centrifugation and resuspended in buffer.

*Crude casein fractions.* Casein fractions were prepared from the milks of individual genetically typed Friesian cows from the Institute herd. For each fraction the milk from a cow producing only the required genetic variant was chosen. Crude $\kappa$-casein A was prepared by the method of Zittle & Custer (1963) except that the ethanol precipitation was omitted. Crude $\beta$-casein A was prepared by the method of Aschaffenburg (1963). $\alpha_s$-Casein B was prepared as described by Zittle, Cerbulis, Pepper & Della Monica (1959) except that the final precipitation step was carried out 3 times instead of twice.

### Methods for purification of crude $\kappa$- and $\beta$-casein

*$\kappa$-Casein.* Crude $\kappa$-casein was dissolved in buffer to a concentration of about 2% and dialysed against the same buffer. To this solution was added calcium phosphate gel suspended in buffer (1 g gel/g protein) and the mixture was stirred at room temperature for 30 min. The gel was removed by centrifugation and the supernatant liquid treated with a further 3 batches of gel in the same manner. The 4 batches of gel were then combined and washed twice with buffer (17·5 ml/g gel) by stirring for 15 min followed by centrifugation. The washes and the supernatant liquid recovered after the final gel treatment were combined, freed from urea by dialysis against water and the $\kappa$-casein recovered by precipitation at pH 4·6.

*$\beta$-Casein.* A 2% solution of crude $\beta$-casein in buffer was treated with 4 batches of calcium phosphate gel (1·5 g gel/g protein) as described for $\kappa$-casein. The 4 batches of gel were then combined and washed 3 times with buffer (12 ml/g gel) by stirring for 10 min followed by centrifugation. The washes and the supernatant liquid remaining after the final gel treatment were either discarded or, if required for analysis, dialysed against water to remove urea and the casein recovered by precipitation at pH 4·6. $\beta$-Casein was eluted from the gel with 6 M-urea–N-NH$_4$OH at 4 °C. The gel was suspended in the eluting solution (15 ml/g gel) and the mixture cooled to 4 °C and stirred for 30 min at this temperature. The gel was recovered by centrifugation and eluted with a further 2 lots of 6 M-urea–N-NH$_4$OH in the same way. The eluents were combined, freed from urea by dialysis against water and the $\beta$-casein precipitated at pH 4·9. The product was freed from small amounts of calcium phosphate by solution in water at pH 7·8 and reprecipitation at pH 4·9.

### Analytical methods

*Determination of protein.* The protein content of casein solutions was determined spectrophotometrically assuming $E_{280}^{1\%} = 10\cdot0$ for $\alpha_s$-casein B (Zittle, 1961), $E_{280}^{1\%} = 12\cdot2$ for crude and purified $\kappa$-casein A (Zittle & Custer, 1963) and $E_{280}^{1\%} = 4\cdot8$ for crude and purified $\beta$-casein A (Zittle & Walter, 1963). Recoveries of protein after carrying out purification procedures were based on spectrophotometric determination.

*Starch-gel electrophoresis.* Analytical electrophoresis was done with starch gels at pH 8·6 containing urea and dithiothreitol, as described by Cheeseman (1968).

*Calcium precipitation and stabilization tests.* The extent of precipitation of $\beta$-casein by various levels of CaCl$_2$ was determined as described by Zittle & Walter (1963). The degrees of stabilization of $\alpha_s$- and $\beta$-casein by $\kappa$-casein were determined by the methods of Zittle (1961) and Zittle & Walter (1963), respectively.

*Renneting of $\kappa$-casein.* A 4% solution of purified $\kappa$-casein in water at pH 6·5 was

incubated at 30 °C with Chr. Hansen cheese-making rennet at 1:350 dilution. After 100 min the enzyme was destroyed by addition of N-NaOH to raise the pH value to above 9. For electrophoresis the protein was solubilized by addition of urea to the mixture.

## RESULTS

### *Purification of κ-casein*

The starch-gel electrophoretic patterns of crude and purified κ-casein are shown in Plate 1(a). This indicates that all of the $\alpha_s$-casein and most of the β-casein were removed during the purification procedure. However, minor casein components of low mobility were not entirely removed. These conclusions are confirmed by the electrophoretic pattern obtained after treatment of purified κ-casein with rennet.

The recoveries of protein after purification in 3 separate experiments are shown in Table 1.

### Table 1. *Recoveries of κ-casein after purification*

| Expt no. | Initial amount of crude κ-casein, g | Amount of purified κ-casein recovered, g | Recovery, % |
|---|---|---|---|
| 1 | 7·7 | 6·0 | 78 |
| 2 | 6·0 | 3·6 | 60 |
| 3 | 8·0 | 4·0 | 50 |

### Table 2. *Recoveries of β-casein after purification*

| Expt. no. | Initial amount of crude β-casein, g | Amount of purified β-casein recovered, g | Recovery, % |
|---|---|---|---|
| 1 | 10·3 | 4·0 | 39 |
| 2 | 9·0 | 4·5 | 50 |
| 3 | 4·0 | 1·6 | 40 |
| 4 | 2·5 | 1·6 | 64 |

### *Purification of β-casein*

The starch-gel electrophoretic patterns of the supernatant liquid remaining after gel treatment of crude β-casein, the buffer washes and the gel eluents are shown in Plates 2(a) and (b). The electrophoretic patterns of crude and purified β-casein are compared in Plate 1(b).

The recoveries of protein from the purification procedure are shown in Table 2 for 4 separate experiments.

### *Properties of purified casein fractions*

Purified κ-casein stabilized $\alpha_s$-casein against precipitation by 10 mM-CaCl$_2$; at a ratio of κ-casein/$\alpha_s$-casein of 0·12, 90 % of the $\alpha_s$-casein remained soluble.

With purified β-casein the extent of precipitation by 10 mM-CaCl$_2$ and the extent of stabilization by purified κ-casein were determined. The results obtained with 2 different preparations of purified β-casein are compared with those of Zittle & Walter

(1963) in Table 3. Starch-gel electrophoresis indicated that preparation 2 was slightly less pure than preparation 1 in that it contained a little more material with the mobility expected for temperature-sensitive casein.

Table 3. *Precipitation of purified β-casein by $CaCl_2$ and stabilization by purified κ-casein*

|  | Preparation 1 | Preparation 2 | Results of Zittle & Walter (1963) |
|---|---|---|---|
| Extent of precipitation (%) by 10 mM-$CaCl_2$ | 86 | 81 | 78 |
| Ratio of κ-casein/β-casein required for complete stabilization | 0·25 | 0·20 | 0·15 |
| Extent of stabilization (%) at κ-casein/β-casein = 0·15 | 73 | 87 | 99 |

### DISCUSSION

The methods described above allowed the removal of the major casein contaminants from crude preparations of κ- and β-casein, but neither of the products was completely pure. The purified κ-casein contained minor casein components of low electrophoretic mobility, probably temperature-sensitive and γ-caseins, which were not adsorbed by calcium phosphate gel. The purified β-casein contained unidentified material forming about 4 electrophoretic bands close to that of β-casein as well as very small amounts of material with a mobility in the region expected for temperature-sensitive casein (Groves, McMeekin, Hipp & Gordon, 1962).

The purified κ-casein stabilized both $α_s$- and β-caseins against $CaCl_2$ precipitation. With $α_s$-casein the results agreed quantitatively with those obtained by Zittle (1961). Different batches of β-casein, however, differed both among themselves and from the β-casein used by Zittle & Walter (1963) in the ratio of κ-casein required for stabilization and the extent of precipitation by 10 mM-$CaCl_2$. It is not clear whether these variations arose from slight changes in structure resulting from the relatively drastic procedure used in elution of β-casein from the gel or from differences in purity between samples.

Most of the published methods for the purification of crude κ- and β-caseins involve either chromatography or gel filtration (for κ-casein see Hill, 1963; Yaguchi, Davies & Kim, 1968; Cheeseman, 1968; for β-casein see Groves *et al.* 1962; Garnier, Ribadeau-Dumas & Mocquot, 1964; Gehrke, Freeark, Oh & Chun, 1964; Thompson, 1966) and, although they may often yield highly purified products, they are usually time-consuming and applicable to only relatively small amounts of material. The advantages of the methods now described are that they are applicable on a large scale (the main limiting factor being the size of centrifuge available), and that they use only simple apparatus and techniques.
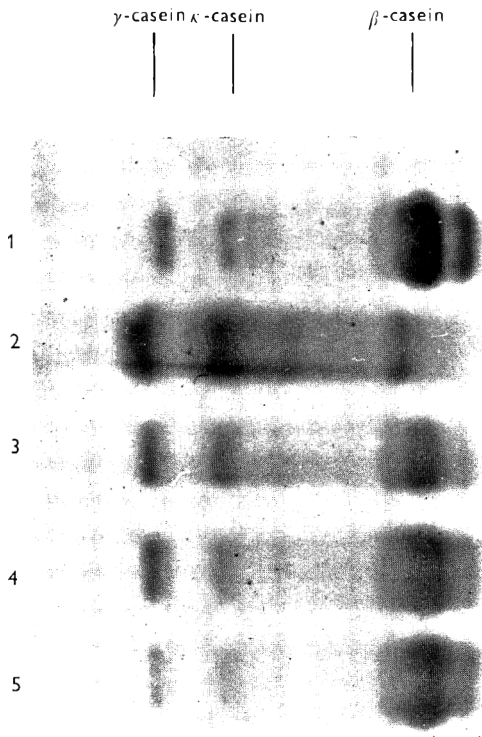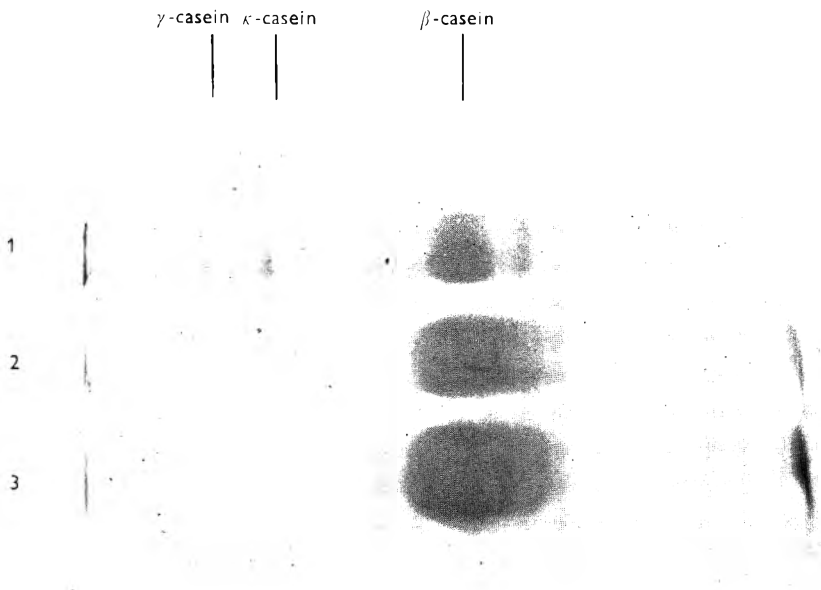
(a)

(b)

(a)



(b)

MARGARET L. GREEN
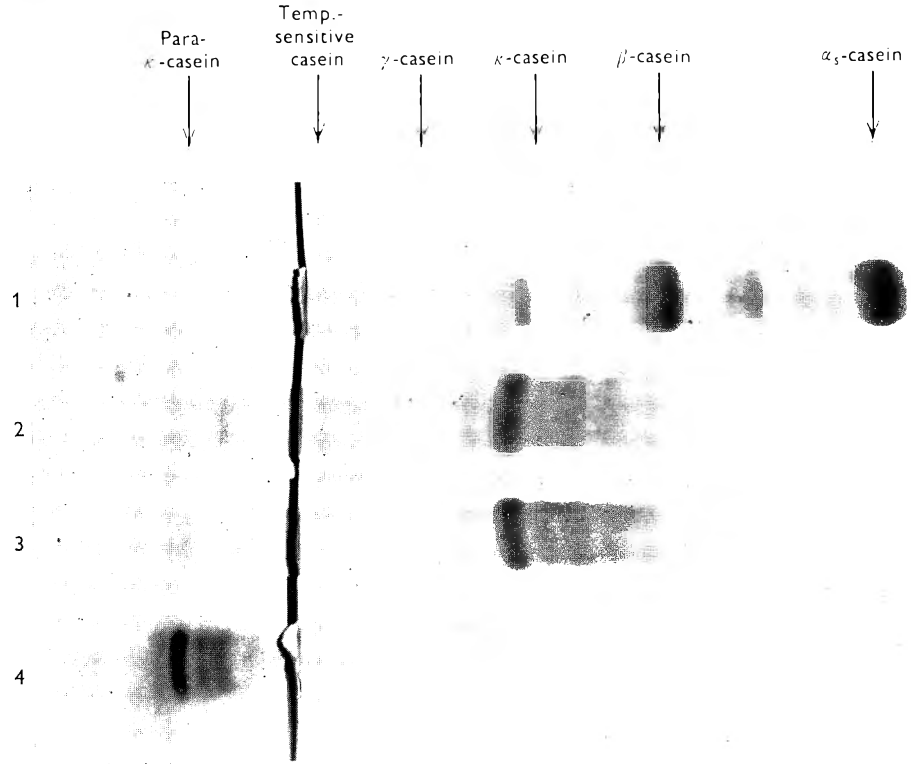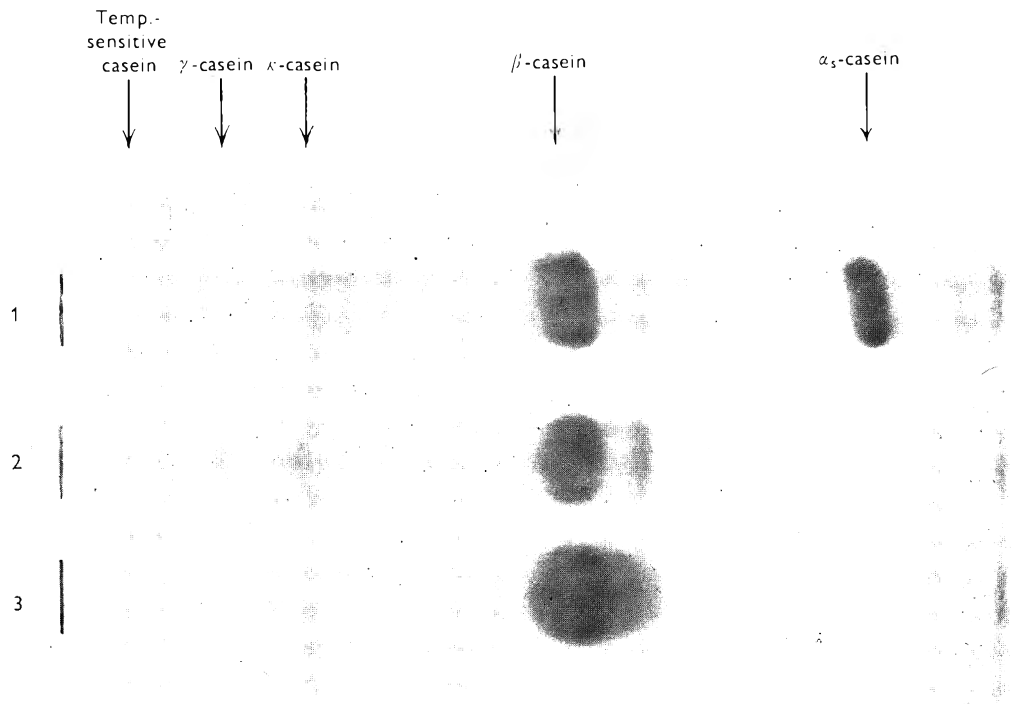
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## EXPLANATION OF PLATES

### PLATE 1

Starch-gel electrophoretic patterns. (a) The purification of κ-casein A by calcium phosphate gel treatment. Samples: 1, milk; 2, crude κ-casein; 3, purified κ-casein; 4, purified κ-casein after rennet treatment. (b) The purification of β-casein A by calcium phosphate gel treatment. Samples: 1, milk; 2, crude β-casein: 3, purified β-casein.

### PLATE 2

Starch-gel electrophoretic patterns of the various stages of β-casein A purification by calcium phosphate gel treatment. (a) Samples: 1, crude β-casein; 2, supernatant remaining after treatment with calcium phosphate gel; 3, 4, 5, first, second and third gel washes. (b) Samples: 1, crude β-casein; 2, first gel eluent; 3, second gel eluent.

# Studies on the mechanism of gel formation in the California mastitis test reaction*

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SUMMARY. The effects of various factors on gel formation in the California Mastitis Test (CMT) were studied to obtain information on the mechanism of the reaction. Death of the leucocytes in milk, produced either by storing the milk at 5 °C or by freezing and thawing, markedly reduced the viscosity of the gel formed by the milk with CMT reagent. Samples of skim-milk to which leucocytes were added gave increasingly viscous gels with CMT reagent as the pH was increased to 10·0. Soluble casein increased the viscosity of the gel formed by leucocytes with CMT reagent. The viscosity of the gel was decreased by the addition of $CaCl_2$ and NaCl. On microscopic examination the gel formed in the CMT appeared as an irregularly arranged fibrillar network containing DNA. Deoxyribonuclease I rapidly eliminated the gel-forming property of leucocyte nuclei with CMT reagent, and EDTA inhibited this enzyme activity. Crude protease, trypsin, papain and bacterial protease decreased the gel-forming property to different degrees. The presence of EDTA with these enzymes resulted in the formation of a viscous, ropy gel before the addition of CMT reagent. It was concluded that at least a part of the protein of the DNA-protein complex of leucocyte nuclei, in addition to the native polymer of DNA, was necessary for gel formation in the CMT reaction.

The California Mastitis Test (CMT) was developed by Schalm & Noorlander (1957) for the detection of mastitic milk. The test was based on the reaction between anionic surfactants, such as alkyl aryl sulphonates at 3–5 % concentration, with milk of high cell content. The reaction varied from the formation of a slight precipitate of amorphous material to the development of a viscid gel. Several workers have studied the mechanism of gel formation, but their results are somewhat conflicting. Carroll & Schalm (1962) found that nucleated cells produced a typical CMT reaction when

added to normal milk whereas non-nucleated cells did not. These workers also reported that the formation of gel in the CMT was prevented by treatment of the milk with deoxyribonuclease I, but not by treatment with ribonuclease and trypsin. Christ (1962) contended that proteins reacted with the detergent, becoming either precipitated, denatured or bound into a protein–detergent complex. It has also been demonstrated (Dounce & Monty, 1955) that a small amount of deoxyribonuclease I can cleave much of the DNA from the residual protein in gellable nuclei without causing extensive depolymerization of DNA but destroying the gel-forming power of the nuclei. Bernstein (1956) believed that the gel structure of DNA-protein gels was determined by intermolecular association bonds in which proteins were the primary participants. Scruggs & Ross (1964) showed that the viscosity of DNA gels is affected by the presence of proteins and univalent and divalent cations.

The primary object of the work reported in this paper was to determine more precisely the mechanism of gel formation in the CMT on milk. This has been done by studying the influence of various factors on the viscosity of the gel formed in the CMT reaction. An attempt was also made to determine the microscopic appearance of the gel.

### MATERIALS AND METHODS

*Determination of viscosity*

*Fisher electroviscometer* (Fisher Scientific Co., New York). The samples to be tested were warmed to $25 \pm 2$ °C. Approximately 50 ml of milk sample and 50 ml of CMT reagent ('Mastest solution', Norden Laboratories, Lincoln, Nebraska) were mixed in a sample cup and attached to the viscometer with the X-1 bobbin in place. The reaction mixture was equilibrated in the water bath for about 5 min, and then the viscosity was noted 20 sec after starting the viscometer motor, at which time the viscosity reading reached a peak.

*Brookfield viscometer* (Brookfield Engineering Laboratories, Stoughton, Massachusetts). Depending upon the viscosity of the sample, either spindle 1 or 2 was used, with a speed of 60 rev/min. Equal quantities of milk and CMT reagent were mixed in a round, 250 ml capacity $14 \cdot 5 \times 5$ cm glass jar. The highest viscosity reading was noted for each sample.

*California mastitis test*

About $1 \cdot 5$ ml of sample was swirled with an equal volume of CMT reagent in each cup of a paddle ('CMT Testep', Norden Laboratories, Lincoln, Nebraska). The CMT score was recorded as recommended by Schalm & Noorlander (1957). In a negative reaction the mixture forms a liquid with no evidence of precipitation. A score of 1 indicates precipitation but no gel formation; scores of 2 and 3 represent slight and distinct gel formation; T indicates a trace of precipitate, respectively. Catalase tests were conducted by an inverted tube method described by Nageswararao, Blobel & Derbyshire (1965). Leucocyte counts and tests for leucocyte viability by trypan blue staining were performed as previously described (Nageswararao *et al.* 1965).

*Effect of storing milk at 5 °C on the viscosity of the gel formed with CMT reagent*

Eight milk samples with leucocyte counts ranging from $1 \times 10^5$ to $6 \times 10^6$ cells/ml were obtained from individual cows. The viscosity of each sample with CMT reagent was determined with the Fisher electroviscometer within 2 h after milking. The samples were then held at 5 ° C for 5 days and retested daily. At each time interval, catalase activity and the proportion of viable leucocytes were also estimated.

*Effect of freezing and thawing of milk on the viscosity of the gel formed with CMT reagent*

Five milk samples with leucocyte counts ranging from $1 \times 10^6$ to $4 \times 10^6$ cells/ml were obtained from individual cows. Each sample was divided into 2 equal portions. One portion was used as a control and the other was rapidly frozen in a solid $CO_2$ and alcohol mixture, and then rapidly thawed in a 25 °C water bath. The freezing and thawing procedure was repeated once, and 100 ml were retained after each cycle of freezing and thawing. Total leucocyte counts, the proportion of live leucocytes and the CMT score were estimated on each control and treated sample. The viscosities after treatment with CMT reagent were determined with the Fisher electroviscometer.

*Isolation of leucocytes*

Milk was collected from cows with mastitis selected from the University of Wisconsin dairy herds. This milk was centrifuged in 50 ml-size conical glass centrifuge tubes at $400 \times g$ for 15 min at 0–2 °C. The sediment was suspended in chilled 0·15 M-NaCl solution and washed once and then the cell clumps were dispersed by homogenizing in tissue grinders (Corning Glass Works, Corning, New York) of 150 $\mu$ clearance. The leucocyte suspension was then washed once more in 0·15 M-NaCl solution.

*Effect of pH on the viscosity of the gel formed with CMT reagent*

Raw skim-milk obtained from a dairy plant was centrifuged at $2000 \times g$ for 20 min at 5 °C. The fat and sediment layers were discarded, the resulting skim-milk was warmed to $25 \pm 2$ °C and divided into several portions of 200 ml each. To different portions of skim-milk either citric acid or NaOH was added, and the pH was adjusted to 6·0, 6·4, 6·6, 6·8, 7·0, 8·0, 9·0, 10·0, 11·0 or 12·0. Equal numbers of leucocytes were added to each sample, and then CMT reagent was added and viscosity was estimated with the Brookfield viscometer. Three experiments were conducted, in which low, medium and high viscosity gels were obtained by adjusting the numbers of leucocytes added to the samples. Viscosity was also determined on control skim-milk samples to which no leucocytes had been added, and the viscosity readings on these were used to correct the values obtained for the samples containing leucocytes.

*Effect of casein, $CaCl_2$, NaCl and EDTA on the viscosity of the gel formed by leucocyte suspensions with CMT reagent*

Skim-milk was prepared as described above. Casein solution was prepared by adding 2·5 g of casein and 1·0 ml N-NaOH to 95 ml of water, with vigorous stirring. When the casein had dissolved, the pH was adjusted to 6·6 with 1 N-HCl, and the

solution was divided into several portions. To some of these portions of casein solution lactose was added to a concentration of 5 %. To different portions of casein plus lactose, of water and of skim-milk, 0·3 or 0·6 % of $CaCl_2$ or 0·3 or 0·6 % of NaCl were added. To one portion of skim-milk EDTA was added to a concentration of 2 %. The pH of all the above preparations was adjusted to 6·6. Each was divided into 2 portions to one of which leucocytes were added. The viscosity of each preparation with and without leucocytes was determined with the Brookfield viscometer after the addition of CMT reagent. These experiments were conducted with samples which gave gels of low, medium and high viscosity depending upon the numbers of leucocytes which were added.

*Microscopic appearance of the gel formed with CMT reagent*

Milk samples containing several millions of leucocytes, and leucocytes obtained from milk and suspended in 0·15 M-NaCl, were prepared for microscopic examination by the following method. Each sample was mixed with an equal volume of CMT reagent, and the gel which formed was fixed with 10 % formalin, embedded in paraffin and cut into sections of 7 or 15 $\mu$ thickness. Another preparation of the gel was rapidly frozen and cut into 5, 10 or 15 $\mu$ sections which were fixed with acetone. The sections were stained by the haematoxylin-eosin and Feulgen methods (McManus and Mowry, 1960).

*Isolation of nuclei*

Leucocyte nuclei were prepared by a procedure, based on that described by Dounce (1955), which has been described in detail elsewhere (Nageswararao & Derbyshire, 1969).

*Effect of enzymes on the gel-forming property of nuclei preparations*

Nuclei suspensions in water of pH 6·6 were prepared to give a CMT score of 3, and equal volumes of suspension were distributed in 50 ml Erlenmayer flasks. To one group of flasks, 40 % EDTA of pH 6·6 was added to give concentrations of 0·2, 1·0 or 2·0 % and the volume in the other group was similarly adjusted with water. The following enzymes were added to different groups of EDTA-treated and untreated nuclei suspensions: 10 mg crude protease (Nutritional Biochemicals Corp., Cleveland, Ohio); 5, 10 or 20 mg of 2 × crystallized trypsin (Nutritional Biochemicals Corp., Cleveland, Ohio); 10 or 20 mg of 2 × crystallized papain (Nutritional Biochemicals Corp., Cleveland, Ohio); 10 or 20 mg of bacterial protease (Bacterial Protease, Crystallized B Grade, Calbiochem, Los Angeles, California) or 1 mg of 2 × crystallized deoxyribonuclease 1 (Nutritional Biochemicals Corp., Cleveland, Ohio) for each 10 ml of nuclei suspension. Nuclei suspensions without added enzymes were included as controls. All the suspensions were incubated in a water bath at 37 °C. Samples were taken at 15, 30, 60 and 90 min and the CMT score was determined.

## RESULTS

*Effect of storing milk on the viscosity of the gel formed with CMT reagent*

The results are presented in Table 1. The viscosity of the gel formed by the samples with CMT reagent decreased day by day and the proportion of live leucocytes decreased from 81 % immediately after milking to 26 % by the fifth day. The $O_2$ liberated in the catalase test did not decrease significantly.

*Effect of freezing and thawing of milk on the viscosity of the gel formed with CMT reagent*

Two cycles of rapid freezing and thawing did not destabilize milk. It will be seen from Table 2 that after freezing and thawing once, the total leucocyte count decreased to almost half that of the control milk, and the proportion of live leucocytes decreased from more than 50 % to 10 or 15 %. The CMT score decreased from 3 to $T$ and the viscosity reading also decreased after one freezing and thawing. The total leucocyte count and the viscosity of the gel formed with CMT reagent were further decreased after a second freezing and thawing.

Table 1. *Effect of storing milk at 5 °C on the viscosity of the gel formed with CMT reagent, and on the proportion of live leucocytes and catalase activity of the milk (mean values of 8 samples)*

| | No. of days at 5 °C | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 |
| Relative viscosity* | 18·9 | 16·5 | 15·4 | 12·8 | 11·4 | 10·7 |
| Proportion of live leucocytes, % | 81 | 70 | 51 | 35 | 33 | 26 |
| Catalase activity ($O_2$ produced, %) | 48·6 | 51·0 | 48·0 | 47·0 | 49·6 | 48·6 |

\* Determined by Fisher electroviscometer.

Table 2. *Effect of freezing and thawing of milk on the viscosity of the gel formed with CMT reagent*

| Experiment no. | Treatment | CMT score | Relative viscosity* | Total leucocytes × 10⁴ | Live leucocytes, % |
|---|---|---|---|---|---|
| 1 | Control | 3 | 21·0 | 568 | 50 |
| | After first freezing and thawing | T | 14·0 | 304 | 10 |
| | After second freezing and thawing | T | 13·0 | 285 | 10 |
| 2 | Control | 3 | 28·0 | 1007 | 52 |
| | After first freezing and thawing | T | 12·5 | 498 | 15 |
| | After second freezing and thawing | T | 9·5 | 425 | 12 |

\* Determined by Fisher electroviscometer.

*Effect of pH on the viscosity of the gel formed with CMT reagent*

The viscosity obtained by the addition of CMT reagent to skim-milk with added leucocytes was much lower at pH 6·0 and 6·4 than at pH 6·6. Between pH 8·0 and 10·0 the viscosity increased markedly with pH. At pH 11·0 and 12·0 the viscosity decreased significantly. These results are summarized in Table 3.

*Effect of casein, CaCl₂, NaCl and EDTA on the viscosity of the gel formed by leucocyte suspensions with CMT reagent*

The results are summarized in Table 4. Casein solution gave a more viscous gel with CMT reagent than did either skim-milk or water containing the same number of leucocytes. Addition of lactose to the casein solution did not increase the viscosity of the gel. The viscosity of the gel formed by the skim-milk suspensions was about the same as that formed by the aqueous suspensions when the leucocyte counts were medium or high. With low leucocyte counts the viscosity of the gel formed by the skim-milk suspension was approximately twice that of the gel formed by the aqueous suspension. Skim-milk containing 2 % EDTA gave a more viscous gel than that

Table 3. *Effect of pH of milk on the viscosity of the gel formed with CMT reagent*

(NT = not tested.)

| pH of milk | pH of reaction mixture | Viscosity* | | |
|---|---|---|---|---|
| | | Experiment 1† | Experiment 2‡ | Experiment 3‡ |
| 6·0 | 6·25 | 49 | 26·8 | 10·3 |
| 6·4 | 6·7 | 57 | 34·5 | 13·5 |
| 6·6 | 6·8 | 67 | 38·4 | 13·1 |
| 6·8 | 6·8 | NT | 35·7 | 14·2 |
| 7·0 | 7·0 | 63 | 31·8 | 14·6 |
| 8·0 | 8·0 | 83 | 35·1 | 18·5 |
| 9·0 | 8·8 | 90 | 47·6 | 18·1 |
| 10·0 | 10·0 | 110 | 55·7 | 20·0 |
| 11·0 | 11·0 | 64 | 57·6 | 14·6 |
| 12·0 | 11·5 | 50 | NT | NT |

* Determined by Brookfield viscometer. Values are corrected for viscosity of control skim-milk with CMT reagent.
† Spindle 2, 60 rev/min.
‡ Spindle 1, 60 rev/min.

Table 4. *Effect of casein, CaCl₂ and EDTA on the viscosity of the gel formed by leucocytes with CMT reagent**

(NT = not tested.)

| Suspending medium | Experiment | | | |
|---|---|---|---|---|
| | 1† | 2† | 3† | 4‡ |
| Water | 9 | 25 | 56 | 137 |
| Water + 0·3 % CaCl₂ | 12 | 37 | 60 | 98 |
| Water + 0·6 % CaCl₂ | NT | NT | 4 | 2 |
| Skim-milk | 16 | 30 | 51 | 140 |
| Skim-milk + 0·3 % CaCl₂ | NT | NT | 41 | NT |
| Skim-milk + 0·6 % CaCl₂ | 9 | NT | 17 | 40 |
| Skim-milk + 2 % EDTA | NT | 52 | 78 | 161 |
| Casein 2·5 % + 5 % lactose | 25 | NT | 75 | 178 |

* Determined by Brookfield viscometer. The values are corrected for the viscosity of control samples.
† Spindle 1, 60 rev/min.
‡ Spindle 2, 60 rev/min.

formed by water or skim-milk containing equal numbers of leucocytes. The viscosity of the gel formed in the casein solution closely corresponded with that formed by skim-milk containing 2 % EDTA, in the presence of equal numbers of leucocytes.

The presence of 0·3 or 0·6 % NaCl in water, skim-milk or casein solution slightly decreased the viscosity of the gel formed with CMT reagent. When 0·3 % $CaCl_2$ was present in water, the viscosity of the gel formed was slightly higher in the presence of small numbers of leucocytes but considerably decreased when large numbers of leucocytes were present. In the presence of 0·6 % $CaCl_2$ both skim-milk and water, containing leucocytes, formed white broken clots and precipitate after the addition of CMT reagent.

## Microscopic appearance of the gel formed with CMT reagent

The gel appeared as an irregularly arranged fibrillar network (Plates 1, 2) and Feulgen-positive material could be seen when the leucocyte count of the samples was very high. If the cell count in the milk or leucocyte suspension was less than $2 \times 10^7$ cells/ml, no distinct fibrillar network could be seen in formalin fixed sections, and in frozen sections the gel could be detected only with difficulty by the Feulgen stain.

Table 5. *Effect of deoxyribonuclease I and proteases on the gel-forming property of nuclei suspensions with CMT reagent (viscosity expressed as CMT score)*

| | Time of incubation at 37 °C, min | | | |
|---|---|---|---|---|
| Treatment | 15 | 30 | 60 | 90 |
| Control | 3 | 3 | 2 | 2 |
| Control + 1 % EDTA | 3 | 3 | 3 | 3 |
| Control + 0·2 % EDTA | 3 | 3 | 3 | 3 |
| Deoxyribonuclease I 1 mg/10 ml | 0 | 0 | 0 | 0 |
| Deoxyribonuclease I 1 mg/10 ml + 1 % EDTA | 3 | 3 | 3 | 3 |
| Protease (crude) 10 mg/10 ml | T | T | 0 | 0 |
| Protease (crude) 10 mg/10 ml + 1 % EDTA | 1* | T | 0 | 0 |
| Trypsin 20 mg/10 ml + 0·2 % EDTA | 2† | T* | 0* | 0 |
| Papain 20 mg/10 ml + 0·2 % EDTA | 2† | 1† | T† | 0* |
| Bacterial protease 20 mg/10 ml + 0·2 % EDTA | T* | 0 | 0 | 0 |

\* Viscous before the addition of CMT reagent.
† Ropy before the addition of CMT reagent.

## Effect of enzymes on gel formation by nuclei with CMT reagent

The results of this study are summarized in Table 5. The nuclei suspension without either EDTA or enzymes showed only a slight decrease in the gel-forming property with CMT reagent during incubation at 37 °C, as indicated by the decrease in CMT score from 3 to 2. When 2·0, 1·0 or 0·2 % EDTA was added, the gel-forming property was maintained even after incubation for 90 min. Deoxyribonuclease I completely

eliminated gel formation in less than 15 min, and the addition of 2·0 or 1·0 % EDTA completely prevented this activity of deoxyribonuclease I.

The addition of crude protease made the nuclei suspension slightly viscous in 15 min without the addition of CMT reagent, and the gel-forming property with CMT reagent was eliminated after treatment with the protease for 30 min. Similar results were obtained with the crude protease in the presence of EDTA except that the viscosity of the suspension before the addition of CMT reagent was slightly higher. Trypsin decreased the gel-forming property of the nuclei suspension but did not eliminate it. During the treatment with trypsin the nuclei suspension showed various degrees of viscosity before the addition of CMT reagent. Papain eliminated the gel-forming property of nuclei in less than 60 min. In the presence of 1·0 or 2·0 % EDTA, trypsin and papain caused the formation of a very viscous, ropy or stringy gel by the nuclei suspension before the addition of CMT reagent. This gel was digested slowly, but did not disappear even after 90 min of incubation. The action of trypsin or papain on the nuclei suspension in the presence of 0·2 % EDTA also produced a somewhat ropy gel, but the gel-forming property of the nuclei with CMT reagent was eliminated in less than 60 min by trypsin, and in less than 90 min by papain. Bacterial protease eliminated the gel-forming property of nuclei with CMT reagent in the absence of EDTA, and in the presence of 0·2 % EDTA, in 30 min. The reaction mixture showed various degrees of viscosity during the initial stages of bacterial protease action. In the presence of 1·0 % EDTA the reaction mixture formed a thick gel without the addition of CMT reagent, and the gel-forming property with CMT reagent was not eliminated even after 90 min of incubation.

## DISCUSSION

Our studies on the effect of holding milk at 5 °C, and freezing and thawing of milk indicated a close correlation between the presence of live leucocytes and the ability of milk to form a gel with the CMT reagent. This finding is of practical significance in the use of the CMT in the detection of mastitis by the examination of bulk milk samples. Holding of milk at 5 °C results in gradual death of the leucocytes, which is accompanied by a decrease in the viscosity of the gel formed with CMT reagent. Thus, if bulk milk were held for several days before testing, the CMT score would not be expected to represent the total leucocyte count accurately. This is in general agreement with the findings of Postle & Blobel (1965), who compared CMT scores with leucocyte counts on bulk milk. We also confirmed in this study that holding milk for several days did not significantly decrease $O_2$ liberation in the catalase test.

It was clear from our studies that the presence of soluble casein considerably increased the viscosity of the gel formed by leucocyte suspensions with CMT reagent. Leucocytes suspended in milk at the normal pH range and leucocytes suspended in water produced gels of similar viscosity with CMT reagent. At the normal pH of milk, little of the casein in milk is in solution, but at higher pH values, at which more viscous gels are produced, solubility of the casein would be increased. The addition of EDTA to milk containing leucocytes increased the viscosity of the gel formed, probably due to chelation of calcium by EDTA resulting in solubilization of casein.

The microscopic examination of stained preparations of CMT gels yielded relatively

little information other than an indication of the presence of DNA in the fibrillar net-work. When the leucocyte count was less than $2 \times 10^7$ cells/ml the gel structure was not maintained on formalin fixation, and insufficient DNA was present to detect by staining and microscopic examination.

Our studies with enzymes indicated that either deoxyribonuclease I or proteolytic enzymes could eliminate the gel-forming property of nuclei with CMT reagent. The formation of gel during the course of action of proteolytic enzymes may indicate that the gel is formed spontaneously after removal or exposure of some parts of the protein of the nuclei. The increase in the viscosity of the nuclei suspension in the presence of EDTA during the action of proteolytic enzymes indicates that possibly $Na^+$, present in EDTA or a chelating agent like EDTA, either enhances gel formation or enters into combination with polymers of DNA-protein complexes to form a thick gel. Deoxy-ribonuclease I eliminated the gel formation of nuclei with CMT reagent in a short time because breaking a few bonds in the DNA polymer is enough to prevent gel formation (Dounce & Monty, 1955), whereas proteolytic enzymes may have to digest most of the proteins to small peptides to accomplish the same result. It could be possible that the location of the protein that enters into gel formation is such that it could not be readily attacked by proteolytic enzymes which have specificity for particular amino acids. We conclude that the native polymer of DNA and a protein com-ponent of the DNA-protein complex of nuclei are necessary for gel formation in the CMT. We postulate the mechanism of gel formation by milk containing leucocytes with CMT reagent to consist of the liberation of DNA-protein complexes from the leucocyte nuclei by the surfactant, followed by spontaneous gel formation by the DNA-protein complexes. Cations and chelating agents may have the role of linking polymers of DNA-protein complexes in the gel formation.

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## EXPLANATION OF PLATES

### PLATE 1

Section of formalin-fixed gel 7 $\mu$ thick formed by milk containing $5 \times 10^7$ leucocytes/ml. Stained by Feulgen method and recorded by phase-contrast illumination. $\times 500$.

### PLATE 2

Section of frozen gel 10 $\mu$ thick formed by leucocyte suspension of $2 \times 10^7$ cells/ml. Stained by Feulgen method and recorded by phase-contrast illumination. $\times 500$.

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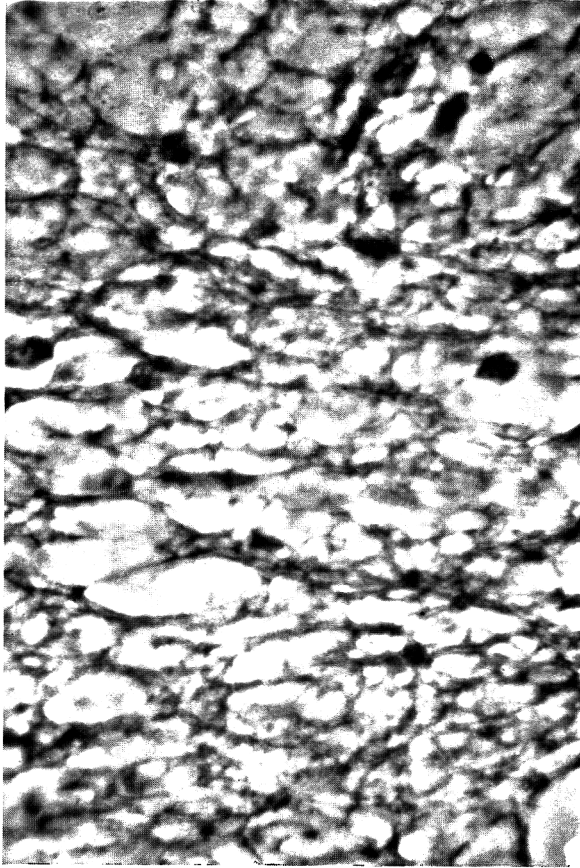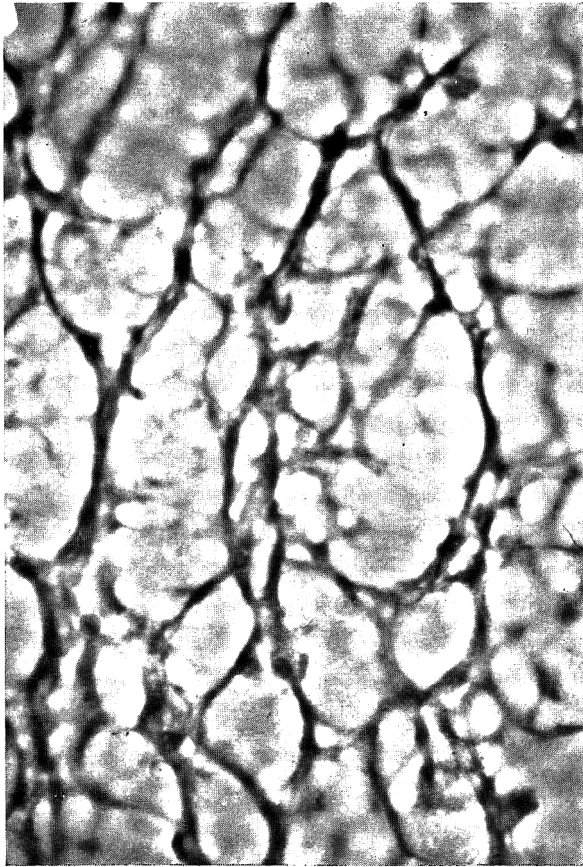
# The effects of dietary palmitic and stearic acids on milk yield and composition in the cow

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SUMMARY. The effects of the isocaloric replacement of starch in the concentrate by either 5 or 10 % 'stearic acid' (85 % pure) or by 10 % 'palmitic acid' (85 % pure) on the yield and composition of milk were investigated in a feeding experiment with 12 cows in mid-lactation. The concentrate mixtures were given with a high-roughage diet that supplied 4·4 kg of hay and 2·7 kg of sugar-beet pulp/day.

The inclusion of 5 % 'stearic acid' in the concentrate mixture resulted in increases in the yields of milk, milk fat, solids-not-fat (SNF) and lactose and a reduction in the content of protein in the milk, whereas, when the level of 'stearic acid' in the concentrate was increased to 10 %, there was an increase in only the yield of milk and a reduction in the contents but not in the yields of SNF and protein in the milk. The inclusion of 10 % 'palmitic acid' in the concentrate mixture also reduced the contents of SNF and protein in the milk but produced increases in the yields of milk, milk fat and lactose and also in the content of fat in the milk.

None of the dietary treatments resulted in any changes in the concentration of blood glucose.

When stearic or palmitic acid was added to the diet of the cow there were large increases in the output of milk fat (Steele & Moore, 1968*b*), in spite of the fact that these acids were included in a diet that already contained enough fat to supply the mammary gland with the fatty acids which are known to be derived directly from the blood (Barry, Bartley, Linzell & Robinson, 1963). Therefore, it was decided to investigate whether the increase in milk fat output would be greater when these fatty acids were given with a very low-fat diet. In addition, an attempt was made to determine whether these increases could be maintained over a longer period and, if so, whether the longer periods would allow any changes in milk output to be shown more clearly.

## EXPERIMENTAL

### Experimental animals

Twelve Ayrshire cows, which had calved on average 61 days before the beginning of the experiment, were used. Eight of them were in their first lactation and the other 4 had completed more than 1 lactation. During the experiment the cows were housed in a byre and yoked in stalls equipped for individual feeding. The cows were exercised in a concrete yard for 2 h each day. They were milked at intervals of 15 and 9 h. Their daily rations were given in 3 portions each day and water was given *ad lib.*

*Experimental diets*

The cows were given 4·4 kg of a mature hay and 2·7 kg of sugar-beet pulp/day. The compositions (percentages of the dry matter) of the hay and sugar-beet pulp, respectively, were as follows: crude protein, 6·8, 11·9; crude fibre, 34·0, 16·6; ether extract, 1·8, 1·4; ash, 6·6, 6·1; nitrogen-free extractives, 50·8, 64·0. Four different concentrate mixtures were given to the cows. Although the actual amounts for each cow varied with milk yield (see below), the mean daily intakes (kg) of each of the constituents of the basal (low-fat) concentrate mixtures were: barley, 2·72; starch, 1·54; decorticated extracted groundnut meal, 1·23; blood meal, 0·23; molasses, 0·23; minerals, 0·23. The other 3 concentrate mixtures supplied the same intakes of each of the constituents as the low-fat concentrate except that part of the starch was replaced by an isocaloric amount of fatty acids. In 2 of the concentrate mixtures, 'stearic acid' made up 5 and 10 % of the weight of the concentrates and in the third, 'palmitic acid' made up 10 % of the weight. Thus, the mean daily intakes (kg) of the 3 types of concentrate mixtures were 6·18 for the low-fat concentrate mixture, 5·72 for the concentrate mixture containing 5 % added fatty acids and 5·25 for the concentrate mixture containing 10 % added fatty acids. The fatty acids were obtained from Prices (Bromborough) Ltd, Wirral, Cheshire, and were 85 % pure. The total fatty acid contents (g/100 g dry matter) of the hay, sugar-beet pulp and the low-fat concentrate were 0·54, 0·47 and 0·93 %, respectively.

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

*Experimental design*

The experimental design was three 4 × 4 Latin squares which were balanced for carry-over effects. The duration of each experimental period was 35 days and the change-overs between treatments were made gradually over 4 days. The 4 adult cows were randomly assigned to the treatment sequences of one of the squares and the 8 first-lactation cows were randomly assigned to the treatment sequences of the other 2 squares.

*Sampling and methods of analysis*

A 2-day composite sample of milk was taken on days 16 and 18 and another 2-day composite sample on days 23 and 25; one 4-day composite sample was taken on days 30–33 of each period. Fat and SNF contents were determined on these samples as described previously (Steele & Moore, 1968a). The contents of protein (N × 6·38) and lactose were also determined on these samples by the micro-Kjeldahl and Wahba (1965) methods, respectively.

Two samples of blood were taken from the subcutaneous abdominal vein on the 26th day of each period and the concentrations of glucose in the samples of plasma were determined by the method of Bergmeyer & Brent (1963). The first blood sample

was taken at 06.00 h, i.e. before the animal had been given the first daily feed, and the second sample was taken at 14.00 h, i.e. 2 h after the second portion of the daily ration had been given.

### Statistical treatment of results

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

### RESULTS

The different experimental treatments had no significant effects on the weights of the cows during the experiment: the mean weights (kg) of the cows when they were given the low-fat concentrate mixture, the concentrate mixture containing 5 % 'stearic acid', that containing 10 % 'stearic acid' and that containing 10 % 'palmitic acid' were, respectively, 440, 438, 441 and 439; S.E.M., ±2·3.

Table 1. *Mean daily yields and the composition of the milk produced by the cows on days 30–33 of each period*

| | Dietary treatments | | | | |
| | Diet with low-fat concentrate mixture | Diet with concentrate mixture containing | | | |
| | | 5 % 'stearic acid' | 10 % 'stearic acid' | 10 % 'palmitic acid' | S.E.M. |
|---|---|---|---|---|---|
| Milk yield, kg | 13·6 | 14·8*** | 14·2*** | 14·6*** | ±0·11 |
| Milk fat yield, g | 546 | 585* | 557 | 661*** | ±17·7 |
| Milk fat content, % | 4·01 | 3·95 | 3·92 | 4·53*** | ±0·097 |
| SNF yield, kg | 1·19 | 1·29** | 1·23 | 1·22 | ±0·034 |
| SNF content, % | 8·75 | 8·72 | 8·66* | 8·36*** | ±0·039 |
| Lactose yield, g | 614 | 683*** | 650 | 667** | ±18·9 |
| Lactose content, % | 4·51 | 4·61 | 4·58 | 4·57 | ±0·057 |
| Protein yield, g | 448 | 464 | 442 | 450 | ±13·3 |
| Protein content, % | 3·29 | 3·14*** | 3·11*** | 3·08*** | ±0·045 |

*, **, ***. Significantly different ($P < 0.05$, $P < 0.01$, $P < 0.001$, respectively) from the values obtained with the unsupplemented ration.

Table 2. *Mean concentrations of glucose in the plasma of the cows, mg/100 ml*

| | Dietary treatments | | | | |
| | Diet with low-fat concentrate mixture | Diet with concentrate mixture containing | | | |
| | | 5 % 'stearic acid' | 10 % 'stearic acid' | 10 % 'palmitic acid' | S.E.M. |
|---|---|---|---|---|---|
| Sample taken at 06.00 h | 52·8 | 52·6 | 54·0 | 52·7 | ±2·04 |
| Sample taken at 14.00 h | 53·8 | 54·7 | 54·6 | 53·1 | ±2·32 |

Analysis of the two 2-day composite samples taken on days 16 and 18 and on days 23 and 25 showed that treatment effects on the yield of milk fat were evident in the early part of each experimental period and were maintained until the end of each

period. However, treatment effects on the yields of milk and SNF developed slowly during each experimental period and did not reach maximum values until the end of the fourth week. Therefore, the results given in Table 1 for milk yield and composition are mean values for the 4-day composite samples taken on days 30–33 of each period.

The inclusion of 5 % 'stearic acid' in the concentrate mixture resulted in increases in the yields of milk, milk fat, SNF and lactose and a reduction in the content of protein in the milk, whereas when the level of 'stearic acid' in the concentrate mixture was increased to 10 %, there was an increase in only the milk yield and a reduction in the contents, but not in the yield, of both SNF and protein in the milk. The inclusion of 10 % 'palmitic acid' in the diet also reduced the contents of SNF and protein in the milk, but produced increases in the yields of milk, milk fat and lactose and also in the content of fat in the milk.

The results in Table 2 show that the concentration of plasma glucose was unaltered by dietary treatment and that there were no differences in plasma glucose concentration between the samples taken at 06.00 h and at 14.00 h.

## DISCUSSION

Blood glucose is known to be the precursor of milk lactose (Reiss & Barry, 1953) and, although the diets that contained added fatty acids resulted in an increased output of milk lactose, none of them altered the concentration of blood glucose. It has been shown in previous experiments (Steele & Moore, 1968b) that an increased uptake by the udder of long-chain fatty acids from the blood produced a marked reduction in the yields of fatty acids in the milk fat that are derived by de novo synthesis from acetate and $\beta$-hydroxybutyrate. Thus, a proportion of the acetate taken up by the mammary gland could be utilized for the synthesis of adenosine triphosphate which in turn could be utilized in the synthesis of milk lactose (Storry & Rook, 1961). Since milk is isotonic with blood (Rook, Storry & Wheelock, 1965) the increase in milk lactose would thus enable a corresponding increase in milk output to occur.

In spite of the fact that the control concentrate mixture used in the present experiment were of low-fat content (cf. Steel & Moore, 1968b), the increases in the milk fat output which were observed when 'stearic acid' constituted 5 % of the concentrate or when 'palmitic acid' constituted 10 % (Table 1) were of the same magnitude as those previously observed (Steele & Moore, 1968b). The difference in the magnitudes of the responses obtained with the 2 acids, which cannot be explained by the different levels of intake, can be accounted for by the difference in the digestibilities of these 2 acids (Steele & Moore, 1968c), palmitic acid being more readily absorbed than stearic acid. However, the absence of a significant increase in milk fat output when 'stearic acid' constituted 10 % of the concentrate mixture does not agree with previous findings (Steele & Moore, 1968b). No explanation for this difference can be offered at present, but the main differences between the present and previous experiments (Steele & Moore, 1968b) are that different breeds of cows were used and that the control (low-fat) ration used in the present experiment contained very much less fat than that used previously.

The author thanks Dr M. E. Castle and Dr J. H. Moore for their interest and encouragement and Mr J. N. Watson, Dr R. C. Noble and Mr N. H. Strachan for their assistance.

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# The effects of dietary palmitic and stearic acids on milk fat composition in the cow

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SUMMARY. The effects of the isocaloric replacement of starch in a low-fat concentrate mixture by either 5 or 10 % 'stearic acid' (85 % pure) or 10 % 'palmitic acid' (85 % pure) on the yield and composition of milk fat were investigated in a feeding experiment with 12 cows in mid-lactation. The concentrate mixtures were given with a high-roughage diet that supplied daily 4·4 kg of hay and 2·7 kg of sugar-beet pulp.

The inclusion of 5 or 10 % 'stearic acid' in the concentrate mixture decreased the concentrations and yields of 10:0, 12:0, 14:0, 14:1, 16:0 and 16:1 in the milk fat but increased the concentrations and yields of 18:0 and 18:1. When the concentrate mixture contained 10 % 'stearic acid' the decreases in the yields of the shorter chain acids and the increases in the yields of 18:0 and 18:1 in the milk fat were more pronounced than when the concentrate mixture contained 5 % 'stearic acid'. The inclusion of 10 % 'stearic acid' in the concentrate mixture also decreased the concentrations of 6:0 and 18:2 in the milk fat. The inclusion of 10 % 'palmitic acid' in the concentrate mixture decreased the concentrations of 6:0, 8:0, 10:0, 12:0, 14:0, 14:1, 18:0 and 18:2 in the milk fat but increased the concentrations of 16:0 and 16:1. This dietary treatment also reduced the yields of 6:0, 10:0, 12:0, 14:0 and 18:2 but increased the yields of 4:0, 16:0 and 16:1 in the milk fat. The inclusion of 5 % 'stearic acid' or 10 % 'palmitic acid' in the concentrate mixture increased the yield of total milk fat.

The inclusion of 10 % 'palmitic acid' or 10 % 'stearic acid' in the concentrate mixture increased the relative proportions of propionic and *iso*-butyric acids in the rumen liquor but decreased the proportions of *n*-butyric and valeric acids. The inclusion of 'stearic acid' at the level of 5 % in the concentrate mixture increased the relative proportions of *iso*-butyric and *iso*-valeric acids but decreased the proportion of butyric acid in the rumen liquor.

In a previous investigation (Steele & Moore, 1968c) it was shown that when the diet of the cow was supplemented with 600 g/day of palmitic or stearic acids there were large increases in the yields of palmitic and palmitoleic acids or of stearic and oleic acids in the milk fat. The increased yields of $C_{16}$ and $C_{18}$ fatty acids were accompanied by decreased secretions of $C_{10}$, $C_{12}$ and $C_{14}$ fatty acids in the milk fat. These changes occurred in spite of the fact that the palmitic or stearic acid was added to a basal diet that already supplied 450 g of total fatty acids (mainly $C_{16}$ and $C_{18}$ acids) per day; the yield of total fatty acids in the milk of the cows during the experiment was also

about 450 g/day. Thus, only about 57 % of the total fatty acids in the supplemented diets was derived from the added stearic or palmitic acids. To elucidate further these changes, an investigation has been carried out in which the fatty acids were incorporated into a basal diet of low-fat content. The basal diet supplied 96 g of total fatty acids/day, and in the high-fat diets about 85 % of the total fatty acids was derived from the added stearic or palmitic acids.

### EXPERIMENTAL

#### Experimental animals

The experimental animals were 12 Ayrshire cows that had calved on average 61 days before the beginning of the experiment. Details of the housing and management of the animals have been given elsewhere (Steele, 1969).

#### Experimental diets

Throughout the experiment each cow was given 4·4 kg of a mature hay and 2·7 kg of sugar-beet pulp/day. Four different concentrate mixtures were given. The control (low-fat) concentrate mixture was formulated so that when the cows were given this ration their daily intake of total fatty acids was less than 100 g. The other 3 concentrate mixtures were made up by isocalorically replacing starch in the low-fat concentrate mixture by either 5 or 10 % 'stearic acid' or by 10 % 'palmitic acid'. The fatty acids were obtained from Price's (Bromborough) Ltd, Wirral, Cheshire.

The fatty acid contents and compositions of the various dietary components and added fatty acids are given in Table 1. Further details of the diets have been given elsewhere (Steele, 1969). As described by Steele (1969), the amounts of concentrate mixtures given to each cow during the experiment were adjusted for milk yield.

Table 1. *Total fatty acid contents and fatty acid compositions of the dietary constituents*

| Fatty acid composition (wt percentages of the total) | Hay | Sugar-beet pulp | Control concentrate mixture | 'Stearic acid' | 'Palmitic acid' |
|---|---|---|---|---|---|
| 16:0 | 30·3 | 26·3 | 23·4 | 7·7 | 85·4 |
| 17:0 | 9·9 | 4·0 | 6·1 | 0·4 | 0·9 |
| 18:0 | 5·9 | 14·4 | 8·3 | 85·4 | 10·5 |
| 18:1 | 8·8 | 13·1 | 33·0 | 2·5 | 1·2 |
| 18:2 | 23·7 | 31·9 | 27·5 | 3·1 | 1·7 |
| 18:3 | 20·5 | 4·6 | 1·5 | 0·5 | 0·1 |
| Total fatty acid content (g/100 g dry matter) | 0·54 | 0·47 | 0·93 | 99·1 | 99·4 |

#### Experimental design

The experimental design was three 4 × 4 Latin squares which were balanced for carry-over effects. The duration of each experimental period was 35 days and the change-overs between treatments were made gradually over 4 days.

## Sampling and methods of analysis

One 4-day composite sample was obtained from the milk produced by each cow on the last 4 days of each period. The concentrations of total fat in these samples were determined by the method described previously (Steele & More, 1968a). A sample of the fat that had been extracted from the milk with chloroform-methanol (2:1, v/v) was hydrolysed by the method of Hutton & Seeley (1966). The proportions of the various fatty acids were then determined by gas-liquid chromatography with an instrument fitted with dual flame ionization detectors and a temperature programmer (Pye-Unicam Ltd, Cambridge, England). The separations were carried out in glass columns ($0.64 \times 214$ cm) packed with a stationary phase of Carbowax 20 M terephthalic acid on a support of Chromosorb G (Perkin–Elmer Ltd, Beaconsfield, England).

On the last day of each period, samples of rumen liquor were taken from each cow by stomach tube (Steele & Moore, 1968b). The proportions of the individual volatile fatty acids in the samples of rumen liquor were then determined according to the method of Cottyn & Boucque (1968).

## Statistical treatment of results

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

### RESULTS

The mean weight percentages and yields of the major fatty acids in the milk fat produced in the last 4 days of each experimental period are given in Tables 2 and 3. When 'stearic acid' constituted either 5 or 10 % of the concentrate mixture there were significant decreases in the concentrations and yields of 10:0, 12:0, 14:0, 14:1, 16:0 and 16:1 in the milk fat; there were large increases in the concentrations and yields of 18:0 and 18:1. These effects were more pronounced when 'stearic acid' was included at the higher level in the concentrate mixture. The inclusion of 10 % 'stearic acid' in the concentrate mixture decreased the concentrations of 6:0 and 18:2 in the milk fat. When the cows were given the concentrate mixture containing 10 % 'palmitic acid' there were significant decreases in the concentrations of 6:0, 8:0, 10:0, 12:0, 14:0, 14:1, 18:0 and 18:2 and significant increases in the concentrations of 16:0 and 16:1 in the milk fat. The inclusion of 10 % 'palmitic acid' in the concentrate mixture also decreased the yields of 6:0, 10:0, 12:0, 14:0 and 18:2 but increased the yields of 4:0, 16:0 and 16:1. When the cows were given the concentrate mixtures containing either 5 % 'stearic acid' or 10 % 'palmitic acid' the yields of total milk fatty acids were significantly increased.

The concentrations of the total steam-volatile fatty acids and the relative proportions of the individual acids in the rumen liquor of the cows during the last day of each experimental period are given in Table 4. The effects of the various dietary treatments were small. The inclusion of either 10 % 'palmitic acid' or 10 % 'stearic acid' in the concentrate mixture resulted in increases in the concentrations of propionic, *iso*-butyric and *iso*-valeric acids and decreases in the concentrations of *n*-butyric and *n*-valeric acids. The inclusion of 5 % 'stearic acid' in the concentrate mixture re-

sulted in increases in the concentrations of *iso*-butyric and *iso*-valeric acids and a decrease in the concentration of *n*-butyric acid. None of the dietary treatments had any effect on the concentrations of total volatile fatty acids in the rumen liquor.

Table 2. *Mean weight percentages of the major fatty acids in the milk fat produced during the last 4 days of each period*

| | | Dietary treatments | | | |
|---|---|---|---|---|---|
| | Diet with low-fat concentrate mixture | Diet with concentrate mixture containing | | | |
| Fatty acids | | 5 % 'Stearic acid' | 10 % 'Stearic acid' | 10 % 'Palmitic acid' | S.E.M. |
| 4:0 | 3·0 | 3·0 | 3·2 | 2·9 | ± 0·10 |
| 6:0 | 1·8 | 1·6 | 1·5* | 1·3*** | ± 0·07 |
| 8:0 | 1·4 | 1·3 | 1·2 | 1·0*** | ± 0·11 |
| 10:0 | 2·8 | 2·1*** | 1·8*** | 1·4*** | ± 0·10 |
| 12:0 | 3·3 | 2·4*** | 2·1*** | 1·6*** | ± 0·10 |
| 14:0 | 13·2 | 10·8*** | 9·6*** | 8·1*** | ± 0·37 |
| 14:1 | 2·0 | 1·4*** | 1·2*** | 1·5*** | ± 0·12 |
| 16:0 | 36·4 | 26·9*** | 23·9*** | 49·8*** | ± 0·92 |
| 16:1 | 3·1 | 2·1*** | 2·1*** | 5·2*** | ± 0·25 |
| 18:0 | 4·2 | 11·1*** | 12·5*** | 3·3* | ± 0·38 |
| 18:1 | 16·2 | 26·5*** | 28·7*** | 14·9 | ± 0·88 |
| 18:2 | 2·0 | 1·8 | 1·6* | 1·3*** | ± 0·16 |

*, ***, Significantly different ($P < 0.05$, $P < 0.001$, respectively) from the value obtained with the unsupplemented ration.

Table 3. *Mean yields (g/day) of total and individual fatty acids in the milk fat produced during the last 4 days of each period*

| | | Dietary treatments | | | |
|---|---|---|---|---|---|
| | Diet with low-fat concentrate mixture | Diet with concentrate mixture containing | | | |
| Fatty acids | | 5 % 'Stearic acid' | 10 % 'Stearic acid' | 10 % 'Palmitic acid' | S.E.M. |
| 4:0 | 15·5 | 16·7 | 16·9 | 18·0** | ± 0·84 |
| 6:0 | 9·1 | 9·1 | 8·3 | 7·9** | ± 0·44 |
| 8:0 | 7·3 | 7·0 | 6·5 | 6·3 | ± 0·73 |
| 10:0 | 14·4 | 11·4*** | 9·8*** | 8·9*** | ± 0·69 |
| 12:0 | 17·3 | 13·2*** | 10·9*** | 10·0*** | ± 0·75 |
| 14:0 | 68·8 | 59·7*** | 50·8*** | 50·0*** | ± 2·59 |
| 14:1 | 10·5 | 8·0** | 6·5*** | 9·2 | ± 0·75 |
| 16:0 | 189·5 | 148·5*** | 126·8*** | 306·6*** | ± 7·29 |
| 16:1 | 16·3 | 11·8** | 11·1** | 31·8*** | ± 1·62 |
| 18:0 | 22·1 | 61·5*** | 66·5*** | 20·4 | ± 2·75 |
| 18:1 | 84·2 | 146·6*** | 152·5*** | 91·8 | ± 6·25 |
| 18:2 | 10·4 | 10·0 | 8·6 | 7·8** | ± 0·93 |
| Total | 516 | 554* | 528 | 625*** | 16·8 |

*, **, ***, Significantly different ($P < 0.05$, $P < 0.01$, $P < 0.001$, respectively) from the value obtained with the unsupplemented ration.

Table 4. *Mean concentrations (m-equiv./100 ml) of total volatile fatty acids and the mean relative proportions (molar percentages of the total) of the individual volatile fatty acids in the rumen liquor of the cows during the last day of each period*

| | Dietary treatments | | | | |
|---|---|---|---|---|---|
| | Diet with low-fat concentrate mixture | Diet with concentrate mixture containing | | | |
| | | 5 % 'Stearic acid' | 10 % 'Stearic acid' | 10 % 'Palmitic acid' | S.E.M. |
| Acetic | 69·4 | 70·1 | 69·9 | 69·9 | ± 0·46 |
| Propionic | 15·2 | 15·6 | 16·2** | 17·0*** | ± 0·39 |
| *iso*-Butyric | 0·50 | 0·58* | 0·61** | 0·62** | ± 0·037 |
| *n*-Butyric | 13·8 | 12·8* | 12·2*** | 11·3*** | ± 0·44 |
| *iso*-Valeric | 0·23 | 0·33** | 0·35** | 0·33** | ± 0·037 |
| *n*-Valeric | 0·86 | 0·80 | 0·77* | 0·75** | ± 0·040 |
| Total | 9·6 | 10·2 | 10·3 | 9·6 | ± 0·39 |

*, **, ***, Significantly different ($P < 0.05$, $P < 0.01$, $P < 001$, respectively) from the values obtained with the unsupplemented ration.

## DISCUSSION

The finding that the yields of the milk fatty acids of medium chain length ($C_{10}$, $C_{12}$ and $C_{14}$ fatty acids) were reduced when the diet of the cows was supplemented with either palmitic or stearic acids (Table 3) confirms the results of earlier experiments (Steele & Moore, 1968c). Also in agreement with the results of these earlier experiments, the decreased yields of the milk fatty acids of medium chain length were not related to a reduced synthesis of acetate and butyrate by the rumen micro-organisms (Table 4). Moore & Steele (1968) have suggested that an increased uptake by the mammary gland of long-chain fatty acids from the blood triglycerides leads to increased concentrations of long-chain fatty acids or fatty acyl CoA derivatives in the mammary gland. Increased concentrations of fatty acids or fatty acyl CoA derivatives inhibit acetyl CoA carboxylase (Howanitz & Levy, 1965; Smith & Dils, 1966; Hibbitt, 1966) which catalyses the rate limiting step in fatty acid synthesis in the bovine mammary gland (Ganguly, 1960). An increased uptake by the mammary gland of 16:0 or 18:0 from the blood triglycerides would therefore reduce the secretion of those fatty acids that are synthesized from acetate in the mammary gland by the malonyl CoA pathway (Moore & Steele, 1968). However, there was no indication that the addition of stearic acid to the diet of the cow decreased the yield of butyric acid in the milk fat (Table 3). Moreover, the inclusion of 10 % 'palmitic acid' in the concentrate mixture resulted in a small but significant increase in the yield of butyric acid in the milk fat (Table 3). Similar findings have been reported by Storry, Rook & Hall (1967). A possible explanation of the different effects of dietary long-chain fatty acids on the secretion of butyric acid and on the secretion of the other fatty acids that are synthesized from acetate in the mammary gland of the cow may be found in the work of Kumar, Singh & Keren-Paz (1965). In experiments with the supernatant fractions of the mammary glands of lactating rabbits, Kumar *et al.* (1965) showed that avidin markedly inhibited the synthesis of the $C_6$ to $C_{16}$ fatty acids when acetate, acetoacetate, butyrate or $\beta$-hydroxybutyrate

were used as substrates. On the other hand, avidin had little effect on the synthesis of butyrate when acetate, acetoacetate or $\beta$-hydroxybutyrate were used as substrates. Thus, it appeared that in the mammary gland of the rabbit the synthesis of butyrate from acetate does not involve the carboxylation of acetyl CoA. If the same mechanism exists in the mammary gland of the cow, then the inhibition of acetyl CoA carboxylase by increased concentrations of long-chain fatty acids or fatty acyl CoA derivatives should not reduce the synthesis of butyrate from acetate. Since the inhibition of acetyl CoA carboxylase does not decrease the rate of synthesis of butyrate from acetate but decreases the rate of synthesis of the $C_6$ to $C_{16}$ fatty acids from butyrate (Kumar et al. 1965) then under those conditions there should be a tendency for butyrate to accumulate in the mammary gland. This may account for the fact that the secretion of butyric acid in the milk fat was actually increased when the cows were given the concentrate mixture containing 10 % 'palmitic acid'.

When the cows were given the concentrate mixture containing 10 % 'stearic acid', the total yield of the major $C_{18}$ fatty acids (18:0 plus 18:1) in the milk fat was increased by 113 g/day, and 60 % of this increase was accounted for by the increased yield of 18:1. On the other hand, when the cows were given the concentrate mixture containing 10 % 'palmitic acid', the total yield of $C_{16}$ fatty acids (16:0 plus 16:1) in the milk fat was increased by 133 g/day, but only 12 % of this increase was accounted for by the increased yield of 16:1. These findings are consistent with those of Bickerstaffe & Annison (1968), who incubated 18:0 or 16:0 with the microsomal fraction obtained from mammary tissue of lactating goats; at similar substrate concentrations, the extent of desaturation of 16:0 was only about 20 % of that of 18:0.

The results for the effect of dietary palmitic acid on the yields of 18:0 and 18:1 in the milk fat (Table 3) do not agree with the results of the previous experiment (Steele & Moore, 1968c) in which it was shown that the inclusion of 10 % of 'palmitic acid' in the concentrate mixture markedly decreased the yields of 18:0 and 18:1 in the milk fat. These differences are probably due to the fact that the fatty acid content of the basal diet used in the previous investigation (Steele & Moore, 1968c) was considerably greater than that of the basal diet used in the present investigation. The addition of palmitic acid to the diet that already contained considerable amounts of fatty acids would not be expected to affect the concentration of triglycerides in the plasma to any great extent. Under these conditions, the increase in the concentration of 16:0 in the plasma triglycerides would be accompanied by decreases in the concentrations of 18:0 and 18:1 in this plasma lipid fraction. Thus, the total concentrations of 18:0 and 18:1 circulating in the plasma as triglycerides would decrease and the amounts of these two $C_{18}$ fatty acids taken up by the mammary gland would also decrease. The addition of palmitic acid to the low-fat diet would be expected to increase the concentration of plasma triglycerides, and although they would contain increased concentrations of 16:0 and decreased concentrations of 18:0 and 18:1, the total concentrations of 18:0 and 18:1 circulating in the plasma as triglycerides would not necessarily be decreased. The amounts of 18:0 and 18:1 taken up by the mammary gland from the plasma triglycerides would thus be unaltered.

In experiments with sheep, Steele & Moore (1968d) obtained digestibility coefficients of 82 and 53 % for 16:0 and 18:0, respectively. When these values were applied to the results of the previous experiments with cows (Steele & Moore, 1968c) it was

found that when 10 % of 'palmitic acid' was included in the concentrate mixture, the increased yield of 16:0 plus 16:1 in the milk fat was equivalent to 27 % of the supplementary 16:0 in the diet that had been absorbed. When 10 % of 'stearic acid' was included in the concentrate mixture, the increased yield of 18:0 plus 18:1 in the milk fat was equivalent to 28 % of the supplementary 18:0 in the diet that had been absorbed. From the results of the present experiment (Table 3) it can be calculated that the increased yield of 16:0 plus 16:1 in the milk fat was equivalent to 39 % of the supplementary 16:0 in the diet that had been absorbed, and that the increased yield of 18:0 plus 18:1 in the milk fat was equivalent to 36 % of the supplementary 18:0 in the diet that had been absorbed. The fact that these values are greater than the corresponding values obtained from the previous experiment (Steele & Moore, 1968c) is, no doubt, a reflexion of the lower fatty acid content of the basal diet used in the present experiment.

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# The relationships between dietary fatty acids, plasma lipid composition and milk fat secretion in the cow

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SUMMARY. The effects of the isocaloric replacement of starch in a low-fat concentrate mixture by either 5 or 10% 'stearic acid' (85% pure) or by 10% 'palmitic acid' (85% pure) on the composition of the plasma lipids were investigated in a feeding experiment with 12 cows in mid-lactation. The concentrate mixtures were given with a high roughage diet that supplied daily 4·4 kg of hay and 2·7 kg of sugar-beet pulp. A study was made of the relationships between the compositions of the plasma and milk lipids.

The inclusion of 10% 'stearic acid' or 10% 'palmitic acid' in the concentrate mixture increased the concentration of total plasma fatty acids. Irrespective of dietary treatment, about 40% of the total plasma fatty acids occurred in the cholesteryl ester fraction, 54% in the phospholipid fraction, 3% in the triglyceride fraction and 3% in the unesterified fatty acid fraction. There was a positive curvilinear relationship between the concentration of unesterified fatty acids in the plasma and the yield of total milk fatty acids.

In the plasma triglycerides, the concentrations of 16:0 and 16:1 were decreased and the concentration of 18:0 was increased when the concentrate mixture contained 'stearic acid'; the concentration of 16:0 was increased and the concentrations of 18:0, 18:1 and 18:2 were decreased when the concentrate mixture contained 'palmitic acid'. Similar changes were observed in the compositions of the plasma unesterified fatty acids when the cows were given the different diets.

In the plasma cholesteryl esters, the concentration of 16:0 was decreased and the concentrations of 18:3 and 20:4 were increased when the concentrate mixture contained stearic acid; the concentrations of 16:1, 18:3 and 20:4 were increased and the concentration of 18:2 was decreased when the diet was supplemented with palmitic acid. The addition of stearic acid to the diet increased the concentration of 18:0, 18:1 and 18:3 in the plasma phospholipids but decreased the concentrations of 16:0, 18:2, 20:3 and 20:4. When the diet contained palmitic acid the concentrations of 16:0, 16:1, 18:1 and 18:3 in the plasma phospholipids were increased but the concentrations of 18:0, 18:2 and 20:3 were decreased.

The major fatty acid circulating in the plasma of the cows was 18:2, which accounted for about 45% of the total plasma fatty acids. Only about 0·7% of the total plasma 18:2 occurred in the plasma triglycerides.

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

In the previous paper Noble, Steele & Moore, (1969) described an experiment in which a study was made of the effects of dietary palmitic and stearic acids on milk fat composition in the cow. In this experiment blood samples were taken from the cows on the various dietary treatments. The plasma lipids have now been analysed and the results of these analyses are reported together with a discussion of the relationships between the compositions of the plasma and milk lipids.

## EXPERIMENTAL

Full details of the cows, experimental diets and procedures have been given by Steele (1969). Briefly, 12 Ayrshire cows were each given 4·4 kg of hay and 2·7 kg of sugar-beet pulp/day. Four different concentrate mixtures were given; one of these was of low-fat content, and when the diet containing this concentrate mixture was given to the cows their daily intake of total fatty acids was less than 100 g. The other 3 concentrate mixtures were made up by isocalorically replacing starch in the low-fat concentrate mixture by either 5 or 10 % 'stearic acid' (85 % pure) or by 10 % 'palmitic acid' (also 85 % pure). The fatty acid contents and compositions of the various dietary components and added fatty acids have been given by Noble et al. (1969). The amounts of concentrate mixture given to each cow were adjusted for milk yield. The experimental design was three 4 × 4 Latin squares which were balanced for carry-over effects. The duration of each experimental period was 35 days and the change-overs between treatments were made gradually over 4 days. One 4-day composite sample was obtained from the milk produced by each cow on the last 4 days of each period. Blood samples were taken from the subcutaneous abdominal vein on the 26th day of each period. The milk fat was extracted and analysed as described by Noble et al. (1969). The lipids were extracted from the samples of plasma by the method of Nelson & Freeman (1959) and the plasma lipids were analysed by the methods described by Moore, Noble & Steele (1968). The results were analysed statistically by the methods outlined by Cochran & Cox (1957).

## RESULTS

The concentrations of the total plasma fatty acids and of the fatty acids contained in the various lipid fractions in the plasma of the cows given the different diets are presented in Table 1. The concentrations of the total fatty acids in the plasma of the cows when they were given the concentrate mixture containing 10 % 'stearic acid' or 10 % 'palmitic acid' were significantly higher than the concentrations observed when they were given the unsupplemented diet. Although the inclusion of 10 % 'stearic acid' or 10 % 'palmitic acid' in the concentrate mixture tended to increase the concentration of triglyceride fatty acids in the plasma, none of the treatment differences in plasma triglyceride concentration reached the 5 % level of significance. There were no significant differences in the concentrations of unesterified fatty acids in the plasma of the cows when they were given the various experimental diets, but again there was a tendency for the diets supplemented with fatty acids to result in slightly high concentrations of plasma unesterified fatty acids. Similarly, the inclusion of stearic acid or palmitic acid in the concentrate mixture tended to

increase the concentration of phospholipid and cholesteryl esters in the plasma but only when the concentrate mixture contained 10 % 'palmitic acid' did the increases reach the 5 % level of significance. There were no marked changes in the composition of the plasma lipids and, irrespective of dietary treatment, about 40 % of the total plasma fatty acids occurred in the cholesteryl ester fraction, 54 % in the phospholipid fraction, 3 % in the triglyceride fraction and 3 % in the unesterified fatty acid fraction. Nevertheless, as the concentration of total fatty acids in the plasma increased, there were slight decreases in the proportion of the total plasma fatty acids that occurred in the unesterified form.

Table 1. *Mean concentrations (mg/100 ml) of total plasma fatty acids and of the fatty acids present in the individual lipid fractions of the plasma of the cows on each dietary treatment*

| | Diet with low-fat concentrate mixture | Diet with concentrate mixture containing | | | |
| | | 5 % 'Stearic acid' | 10 % 'Stearic acid' | 10 % 'Palmitic acid' | S.E.M. |
| --- | --- | --- | --- | --- | --- |
| Total fatty acids | 92·8 | 127 | 148* | 176** | ± 24·4 |
| Triglyceride fatty acids | 3·26 | 3·29 | 4·59 | 5·76 | ± 1·18 |
| Unesterified fatty acids | 3·86 | 4·47 | 4·26 | 4·73 | ± 1·02 |
| Phospholipid fatty acids | 47·4 | 72·7 | 78·7 | 93·0* | ± 14·9 |
| Cholesteryl ester fatty acids | 38·3 | 47·0 | 60·5 | 73·1* | ± 12·8 |

*, **, Significantly different ($P < 0.05$, $P < 0.01$, respectively) from the value obtained with the unsupplemented diet.

Table 2. *Mean weight percentages of the major fatty acids in the plasma triglycerides of the cows on each dietary treatment*

| | | Dietary treatments | | | |
| Fatty acids | Diet with low-fat concentrate mixture | Diet with concentrate mixture containing | | | |
| | | 5 % 'Stearic acid' | 10 % 'Stearic acid' | 10 % 'Palmitic acid' | S.E.M. |
| --- | --- | --- | --- | --- | --- |
| 16:0 | 30·0 | 25·8* | 22·4*** | 47·6*** | ± 2·05 |
| 16:1 | 5·6 | 4·0** | 4·1* | 5·9 | ± 0·56 |
| 18:0 | 21·5 | 33·7*** | 36·8*** | 16·5** | ± 1·86 |
| 18:1 | 20·6 | 20·3 | 18·9* | 15·5*** | ± 0·84 |
| 18:2 | 12·7 | 8·6* | 10·5 | 8·2** | ± 1·33 |

*, **, ***, Significantly different ($P < 0.05$, $P < 0.01$, $P < 0.001$, respectively) from the value obtained with the unsupplemented diet.

The fatty acid compositions of the plasma triglycerides of the cows on the different dietary treatments are given in Table 2. The inclusion of 5 or 10 % 'stearic acid' in the concentrate mixture significantly decreased the concentrations of 16:0 and 16:1 and significantly increased the concentration of 18:0. Perhaps rather surprisingly, the concentration of 18:1 in the plasma triglycerides was not increased when the cows were given the concentrate mixture containing 5 % 'stearic acid'. Moreover, when

the concentrate mixture containing 10 % 'stearic acid' was given, there was a small but significant decrease in the concentration of 18:1 in the plasma triglycerides. The addition of 10 % 'palmitic acid' to the diet resulted in a marked increase in the concentration of 16:0 in the plasma triglycerides and significant decreases in the concentrations of 18:0, 18:1 and 18:2; there were no significant changes in the concentration of 16:1.

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

| | | Dietary treatments | | | |
| | Diet with low-fat concentrate mixture | Diet with concentrate mixture containing | | | |
| Fatty acids | | 5 % 'Stearic acid' | 10 % 'Stearic acid' | 10 % 'Palmitic acid' | S.E.M. |
|---|---|---|---|---|---|
| 16:0 | 27·9 | 24·2** | 22·3*** | 42·9*** | ± 1·08 |
| 16:1 | 3·51 | 2·9 | 2·8 | 3·6 | ± 0·42 |
| 18:0 | 25·9 | 37·0*** | 40·4*** | 22·8* | ± 1·15 |
| 18:1 | 30·5 | 26·7** | 23·2*** | 22·1*** | ± 1·42 |
| 18:2 | 7·4 | 5·2** | 5·7* | 4·6** | ± 0·71 |

*, **, ***, Significantly different ($P < 0.05$, $P < 0.01$, $P < 0.001$, respectively) from the value obtained with the unsupplemented diet.

The compositions of the unesterified fatty acids in the plasma of the cows are given in Table 3. When the cows were given the concentrate mixture containing 5 or 10 % 'stearic acid' the concentration of 18:0 in the unesterified fatty acids was markedly increased but the concentrations of 16:0, 18:1 and 18:2 were decreased. The addition of 10 % 'palmitic acid' to the concentrate mixture resulted in a pronounced increase in the concentration of 16:0 in the unesterified fatty acids and significant decreases in the concentrations of 18:0, 18:1 and 18:2. Comparison of the results in Tables 2 and 3 shows that irrespective of dietary treatment there were consistent differences in the compositions of the fatty acids in the plasma triglycerides and unesterified fatty acids. The plasma triglycerides contained higher concentrations of 16:0, 16:1 and 18:2 but lower concentrations of 18:0 and 18:1 than the plasma unesterified fatty acids. In the plasma triglycerides, the 18:0/18:1 ratios were consistently higher and the 16:0/16:1 ratios were considerably lower than the corresponding ratios in the plasma unesterified fatty acids. Nevertheless, the effects of diet on the fatty acid compositions of the plasma triglycerides and unesterified fatty acids showed close similarities. Thus, there appeared to be rectilinear relationships between the concentrations of 18:0, 16:0, 16:1 and 18:2 in the plasma triglycerides and the corresponding concentrations of these fatty acids in the plasma unesterified fatty acids (Figs. 1 and 2).

The fatty acid compositions of the plasma cholesteryl esters are given in Table 4. Although the concentration of 16:0 in the plasma cholesteryl esters was significantly decreased by the inclusion of 5 or 10 % of 'stearic acid' in the concentrate mixture, the concentration of 16:0 in this plasma lipid fraction was not altered significantly when the concentrate mixture contained 10 % of 'palmitic acid'. On the other hand,

when the cows were given the concentrate mixture containing palmitic acid, there was a pronounced and highly significant increase in the concentration of 16:1 in the plasma cholesteryl esters. The concentrations of 18:0, 18:1 and 20:3 in the plasma cholesteryl esters were unaffected by dietary treatment but the concentrations of
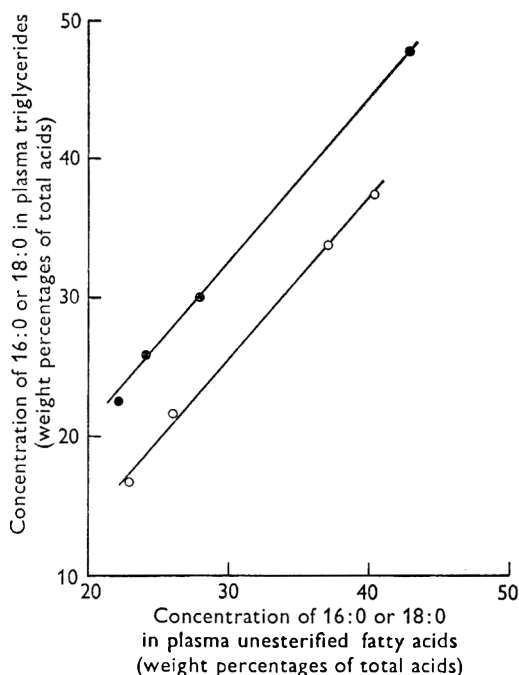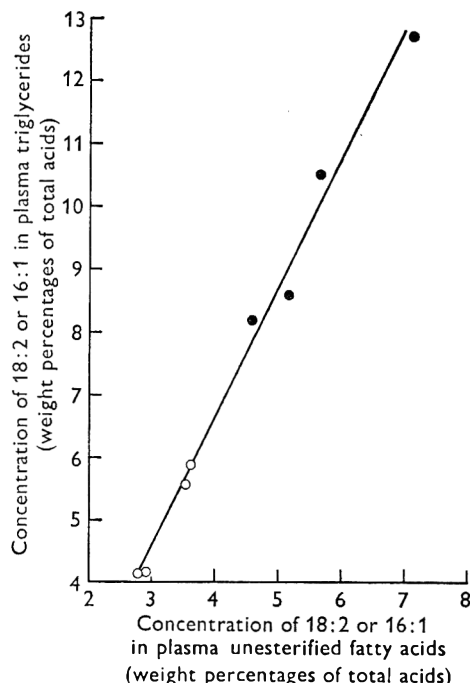


Fig. 1. Relationship between the concentrations of 16:0 (●) and 18:0 (○) in the triglycerides and unesterified fatty acids in the plasma of cows given different diets.

Fig. 2. Relationships between the concentrations of 18:2 (●) and 16:1 (○) in the triglycerides and unesterified fatty acids in the plasma of cows given different diets.

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

| | | Dietary treatments | | | |
| | Diet with low-fat concentrate mixture | Diet with concentrate mixture containing | | | |
| Fatty acids | | 5 % 'Stearic acid' | 10 % 'Stearic acid' | 10 % 'Palmitic acid' | S.E.M. |
|---|---|---|---|---|---|
| 16:0 | 6·2 | 5·0*** | 4·2*** | 5·8 | ± 0·30 |
| 16:1 | 3·8 | 4·6 | 4·4 | 13·2*** | ± 0·55 |
| 18:0 | 0·9 | 0·9 | 0·9 | 0·9 | ± 0·27 |
| 18:1 | 5·6 | 6·2 | 6·4 | 5·7 | ± 0·35 |
| 18:2 | 69·2 | 66·8 | 68·4 | 56·8*** | ± 1·39 |
| 18:3 | 4·4 | 6·5*** | 6·6*** | 6·7*** | ± 0·36 |
| 20:3 | 1·0 | 0·9 | 1·0 | 0·9 | ± 0·11 |
| 20:4 | 2·2 | 2·8** | 2·9** | 2·6* | ± 0·22 |

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

18:3 and 20:4 were significantly increased when the cows were given the concentrate mixtures with added fatty acids. The 18:2 content of the cholesteryl esters was significantly decreased when the cows were given the diet supplemented with palmitic acid.

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

| | | Dietary treatments | | | |
|---|---|---|---|---|---|
| | Diet with low-fat concentrate mixture | Diet with concentrate mixture containing | | | |
| Fatty acids | | 5 % 'Stearic acid' | 10 % 'Stearic acid' | 10 % 'Palmitic acid' | S.E.M. |
| 16:0 | 15·9 | 14·7** | 14·4*** | 23·1*** | ±0·37 |
| 16:1 | 1·0 | 0·8 | 0·9 | 2·0*** | ±0·11 |
| 18:0 | 24·9 | 28·3*** | 27·7*** | 21·0*** | ±0·39 |
| 18:1 | 13·1 | 15·8*** | 16·2*** | 16·9*** | ±0·56 |
| 18:2 | 30·2 | 27·7** | 27·9** | 25·3*** | ±0·77 |
| 18:3 | 1·1 | 1·3* | 1·4* | 1·4* | ±0·11 |
| 20:3 | 4·6 | 3·8*** | 3·4*** | 3·6*** | ±0·18 |
| 20:4 | 3·1 | 2·7* | 2·6** | 2·8 | ±0·18 |
| 22:5 | 1·1 | 1·1 | 1·0* | 0·9** | ±0·07 |
| 22:6 | 0·2 | 0·3 | 0·2 | 0·2 | ±0·03 |

*, **, ***, Significantly different ($P < 0.05$, $P < 0.01$, $P < 0.001$, respectively) from the value obtained with the unsupplemented diet.

Table 6. *Mean total concentrations (mg/100 ml) of individual fatty acids in the plasma of the cows on each dietary treatment*

| | | Dietary treatments | | |
|---|---|---|---|---|
| | Diet with low-fat concentrate mixture | Diet with concentrate mixture containing | | |
| Fatty acids | | 5 % 'Stearic acid' | 10 % 'Stearic acid' | 10 % 'Palmitic acid' |
| 16:0 | 12·0 | 15·0 | 15·9 | 30·5 |
| 16:1 | 2·2 | 3·0 | 3·6 | 12·0 |
| 18:0 | 13·9 | 23·7 | 25·8 | 22·2 |
| 18:1 | 10·2 | 16·3 | 18·5 | 21·8 |
| 18:2 | 41·5 | 52·1 | 64·1 | 65·7 |
| 18:3 | 2·2 | 4·0 | 5·1 | 6·2 |
| 20:3 | 2·5 | 3·2 | 3·2 | 4·0 |
| 20:4 | 2·3 | 3·3 | 3·8 | 4·5 |
| 22:5 | 0·5 | 0·8 | 0·8 | 0·9 |
| 22:6 | 0·1 | 0·2 | 0·1 | 0·2 |

The fatty acid compositions of the plasma phospholipids are given in Table 5. In the plasma phospholipids, the 16:0, 18:2, 20:3 and 20:4 contents were significantly decreased and the 18:0, 18:1 and 18:3 contents were significantly increased when the cows were given the diets supplemented with stearic acid. When the diet was supplemented with palmitic acid, the concentrations of 16:0, 16:1, 18:1 and 18:3 in the plasma phospholipids were significantly increased and the concentrations of

18:2, 20:3 and 22:5 were significantly decreased. Although the different dietary treatments brought about considerable changes in the concentrations of 16:0 and 18:0 these 2 saturated fatty acids together accounted for about 44 % of the major fatty acids in the plasma phospholipids of the cows on each diet.

The total concentrations of the individual fatty acids in the plasma of the cows are given in Table 6, from which it may be seen that 18:2 was the major fatty acid circulating in the plasma of the cows on each dietary treatment. This essential fatty acid accounted for about 45 % of the total plasma fatty acids, but it should be noted that only about 0·7 % of the total plasma 18:2 occurred in the plasma triglycerides.

## DISCUSSION

Allowing for the differences in experimental conditions, the concentrations and fatty acid compositions of the various lipid fractions in the plasma of the experimental cows were similar to those reported by other investigators (e.g. McClymont & Vallance, 1962; Duncan & Garton, 1963; Storry & Rook, 1965).

Comparison of the yields of total milk fatty acids by the cows on the various dietary treatments (Noble *et al.* 1969) with the results given in Table 1 showed that the yield of total milk fatty acids was not related to the concentration of total plasma fatty acids or the concentrations of fatty acids circulating in the plasma as triglycerides, phospholipids and cholesteryl esters. The fatty acids in the plasma phospholipids and cholesteryl esters cannot be taken up by the mammary gland but those in the plasma triglycerides can be utilized for milk fat synthesis (Barry, Bartley, Linzell & Robinson, 1963; Annison, Linzell, Fazakerley & Nicholls, 1967). Although a relationship between the concentration of plasma triglycerides and the secretion of fatty acids in the milk might thus have been expected, it must be remembered that only a proportion of the total milk fatty acids is derived from the plasma triglycerides (e.g. Annison *et al.* 1967) and that only a proportion of the total plasma triglycerides (those present in the chylomicrons and low-density lipoproteins) can donate fatty acids to the mammary gland for milk fat synthesis (Barry *et al.* 1963). However, there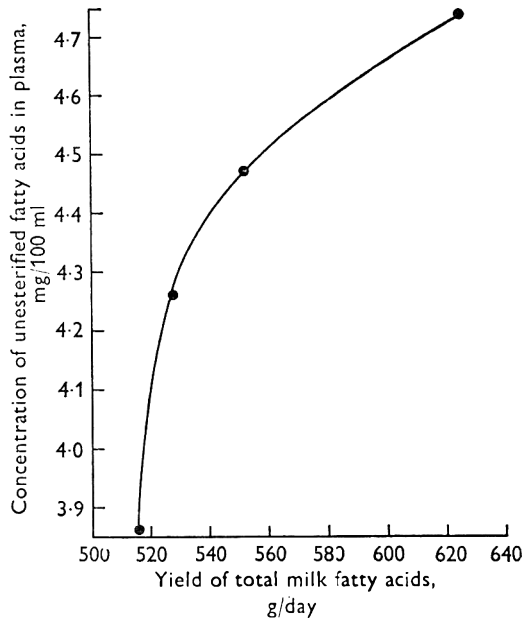 appeared to be a positive curvilinear relationship between the yield of total milk fatty acids and the concentration of unesterified fatty acids in the plasma of the cows given the different diets (Fig. 3). This relationship is not easy to explain for, although it has been established that the plasma unesterified fatty acids are in equilibrium in the mammary gland with a pool of unesterified fatty acids derived from the plasma triglycerides (West, Annison & Linzell, 1967), there is no evidence for a net uptake of unesterified fatty acids from the plasma by the mammary gland (McClymont & Vallance, 1962; Barry *et al.* 1963; Hartmann & Lascelles, 1964; Kronfeld, 1965; Annison *et al.* 1967). In non-lactating animals, some plasma unesterified fatty acids may arise from the hydrolysis of lipoprotein triglycerides by lipoprotein lipase in the blood (Havel, 1958) but the greater part is derived from adipose tissue triglycerides (Annison, 1964). However, it is unlikely that the changes in the concentrations of unesterified fatty acids in the plasma of the cows in the present experiment are due to the effects of the different dietary treatments on the release of unesterified fatty acids from the adipose tissue triglycerides. In non-lactating ruminants, increased concentrations of plasma unesterified fatty acids due

to increased release from adipose tissue triglycerides are associated with decreased concentrations of glucose and acetate in the blood (Annison, 1960; Lindsay, 1961). The concentration of glucose in the plasma of the experimental cows was unaltered by dietary treatment (Steele, 1969) and it is most unlikely that there were any alterations in the concentrations of acetate in the blood (Noble et al. 1969). Moreover, the weights of the cows remained constant during the experiment (Steele, 1969). The increased yield of total milk fatty acids was associated with an increased uptake of blood triglycerides by the mammary gland (Noble et al. 1969), and, due to the action of lipoprotein lipase in the mammary tissues (McBride & Korn, 1963; Robinson,



Fig. 3. Relationship between the yield of total milk fatty acids and the concentration of unesterified fatty acids in the plasma of cows given different diets.

1963; Barry et al. 1963; Schoefl & French, 1968), this would result in increased concentrations of unesterified fatty acids in the mammary gland (Moore & Steele, 1968). Since the plasma unesterified fatty acids are in equilibrium with the unesterified fatty acids in the mammary gland (West et al. 1967) the increased concentration of unesterified fatty acids in the plasma (Table 1, Fig. 3) may simply be a reflexion of the increased pool size of unesterified fatty acids in the mammary gland.

When the diet of the cow is supplemented with 18:0 or fats containing high proportions of 18:0, the concentration and yield of 18:1 in the milk fat is increased (Steele & Moore, 1968a, b, c; Noble et al. 1969). Although the mammary gland contains an enzyme system that desaturates 18:0 to 18:1 (e.g. Lauryssens et al. 1960; Annison et al. 1967; Bickerstaffe & Annison, 1968a) it is known that similar enzyme systems occur in other tissues such as the liver (e.g. Raju & Reiser, 1967) and small intestine (Bickerstaffe & Annison, 1968b). The desaturation of 18:0 to 18:1 in the liver or small intestine could also contribute to the increased concentration and yield of 18:1 in the milk fat when cows are given supplementary 18:0 in the diet. How-

ever, in the plasma triglycerides of the cows given the concentrate mixture containing 5 or 10 % 'stearic acid', the concentration of 18:1 was unaltered or even slightly decreased (Table 2) and the 18:0/18:1 ratio was markedly increased (Table 7). It can be seen from Table 7 that, irrespective of dietary treatment, the 18:0/18:1 ratio in the milk fat (mean 0·34) was considerably less than the corresponding ratio in the plasma triglycerides (mean 1·43). Since increased desaturation of 18:0 to 18:1

Table 7. *Comparison of the ratios of certain fatty acids in the plasma triglycerides and milk fat of the cows on each dietary treatment*

| | Dietary treatments | | | |
| | Diet with low-fat concentrate mixture | Diet with concentrate mixture containing | | |
| Ratio | | 5 % 'Stearic acid' | 10 % 'Stearic acid' | 10 % 'Palmitic acid' |
| --- | --- | --- | --- | --- |
| 18:0/18:1 | | | | |
| Milk fat | 0·26 | 0·42 | 0·44 | 0·22 |
| Plasma triglycerides | 1·04 | 1·66 | 1·95 | 1·06 |
| 16:0/16:1 | | | | |
| Milk fat | 11·7 | 12·8 | 11·4 | 9·46 |
| Plasma triglycerides | 5·40 | 6·42 | 5·41 | 8·07 |
| (18:0+18:1)/18:2 | | | | |
| Milk fat | 10·2 | 20·9 | 25·8 | 14·0 |
| Plasma triglycerides | 3·31 | 6·28 | 5·30 | 3·90 |

in the liver or small intestine would have been expected to result in increased concentrations of 18:1 in the plasma triglycerides, it would appear that the increased concentrations and yields of 18:1 in the milk fat of the cows given supplementary stearic acid (Noble *et al.* 1969) were almost entirely due to the desaturation in the mammary gland of the increased amounts of 18:0 taken up from the plasma triglycerides. In contrast, the 16:0/16:1 ratio (Table 7) in the milk fat (mean 11·4) was considerably greater than the corresponding ratio in the plasma triglycerides (mean 6·33). This is presumably a reflexion of the fact that the desaturating enzyme in the mammary gland has a relatively low specificity for 16:0 (Bickerstaffe & Annison, 1968*a*). Nevertheless, it is clear from the results in Table 4 that there is an enzyme system in certain tissues of the cow, possibly the liver, that actively desaturates 16:0 and that the resulting 16:1 is preferentially incorporated into the plasma cholesteryl esters. It may be calculated that when the cows were given the control diet the concentration of 16:1 circulating in the plasma as cholesteryl esters was 1·5 mg/100 ml, whereas when the cows were given the diet supplemented with palmitic acid the corresponding concentration of 16:1 was 9·7 mg/100 ml.

It is often stated that the low concentrations of essential fatty acids in the milk fat of the cow are due to the efficient hydrogenation of dietary 18:2 in the rumen (e.g. Tove & Mochrie, 1963). Although extensive hydrogenation of 18:2 in the rumen undoubtedly occurs, the results in Table 6 emphasize that there is no lack of 18:2 circulating in the plasma of the lactating cow. However, about 99 % of this 18:2 occurs in those plasma lipid fractions that cannot donate fatty acids to the mammary gland for milk fat synthesis. The specific distribution of 18:2 amongst the lipid

fractions in the plasma of the cow must therefore be the major factor that determines the low concentration of 18:2 in the milk fat. However, the results in Table 7 suggest that other factors may be involved. Since it is now known that all of the $C_{18}$ fatty acids in the milk fat are derived from the blood triglycerides (e.g. Annison et al. 1967), it might have been expected that the $(18:0 + 18:1)/18:2$ ratio in the blood triglycerides would be similar to the corresponding ratio in the milk fat. Unexpectedly, the $(18:0 + 18:1)/18:2$ ratio in the blood triglycerides (mean, 4·8) was markedly less than the corresponding ratio in the milk fat (mean, 17·7). This suggests that there is also specificity in the distribution of 18:2 amongst the triglycerides of the different plasma lipoproteins and that the 18:2 content of the triglycerides in the chylomicrons and low-density lipoproteins that are taken up by the mammary gland is less than the 18:2 content of the total plasma triglycerides.

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# Dietary fatty acids and the plasma phospholipids of the lactating cow

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SUMMARY. Cows were given either a low-fat concentrate mixture or a concentrate mixture that contained 5 or 10 % 'stearic acid' (85 % pure) or 10 % 'palmitic acid' (85 % pure). The concentrate mixtures were given with a high roughage diet that supplied 4·4 kg of hay and 2·7 kg of sugar-beet pulp/day. Blood samples were taken from the cows on each dietary treatment and the plasma phospholipids were analysed.

Phosphatidyl choline accounted for 70 % of the plasma phospholipids when the cows were given the low-fat diet and about 86 % of the plasma phospholipids when the cows were given the diets supplemented with the fatty acids. The inclusion of the fatty acids in the concentrate mixtures decreased the relative proportions of the plasma phosphatidyl ethanolamine, sphingomyelin and lysophosphatidyl choline but the relative proportion of the plasma phosphatidyl serine remained unchanged.

When the diet was supplemented with stearic acid the concentrations of 18:0 and 16:0 in the phosphatidyl choline were unaltered but the concentration of 18:1 was increased and the concentration of 18:2 was decreased. When the diet was supplemented with palmitic acid the concentrations of 16:0 and 18:1 in the phosphatidyl choline were increased and the concentrations of 18:0 and 18:2 were decreased. In contrast, the inclusion of stearic acid in the diet increased the concentration of 18:0 in the phosphatidyl serine and decreased the concentration of 16:0; the concentrations of 18:1 and 18:2 were unchanged. The fatty acid composition of the plasma phosphatidyl ethanolamine was unaffected by dietary treatment. The effects of diet on the fatty acid compositions of the phosphatidyl choline and lysophosphatidyl choline were similar. Supplementation of the diet with stearic acid increased the concentrations of 18:0 and 18:1 in the plasma sphingomyelin and decreased the concentrations of 16:0 and 18:2. The addition of palmitic acid to the diet increased the concentration of 16:0 in the sphingomyelin but it decreased the concentrations of the other constituent fatty acids.

In a previous paper (Moore, Steele & Noble, 1969) it was reported that when cows were given a low-fat concentrate mixture or concentrate mixtures supplemented with either stearic or palmitic acid, there were changes in the concentration of total phospholipids in the plasma and in the concentrations of the $C_{16}$ and $C_{18}$ fatty acids in the total plasma phospholipids. It was of interest, therefore, to determine whether these changes were due to the effects of diet on the relative proportions of the individual phospholipids in the plasma or whether they were due to the effects of diet on the

concentrations of $C_{16}$ and $C_{18}$ fatty acids in the individual plasma phospholipids. Accordingly, pooled samples of the total plasma phospholipids obtained from the cows on each dietary treatment were fractionated and analysed and the results are now reported.

### EXPERIMENTAL

Detailed descriptions of the experimental cows, diets and procedures have been given by Steele (1969), Noble, Steele & Moore (1969) and Moore et al. (1969). Briefly, 12 cows in mid-lactation were each given 4·4 kg of hay and 2·7 kg of sugar-beet pulp/day. Four different concentrate mixtures were given; 1 of these was of low-fat content and the other 3 concentrate mixtures were made up by isocalorically replacing starch in the low-fat concentrate mixture by either 5 or 10 % 'stearic acid' (85 % pure) or by 10 % 'palmitic acid' (85 % pure). The experimental design was three 4 × 4 Latin squares which were balanced for carry-over effects and the duration of each experimental period was 35 days. Blood samples were taken from the subcutaneous abdominal vein on the 26th day of each period. The lipids were extracted from the samples of plasma by the method of Nelson & Freeman (1959) and after the analyses reported in the previous paper (Moore et al. 1969) had been carried out, the samples of total plasma phospholipids obtained from the cows on each dietary treatment were pooled. The pooled plasma phospholipids were then fractionated and analysed by the methods described in detail by Moore & Williams (1963, 1964), Noble & Moore (1965) and Moore & Noble (1969).

### RESULTS AND DISCUSSION

The compositions of the pooled plasma phospholipids of the cows on the different diets are given in Table 1 from which it may be seen that phosphatidyl choline was the major phospholipid in the plasma of the experimental cows. The results for the cows on the low-fat diet are in fair agreement with those of Nelson (1967), who reported that phosphatidyl choline accounted for 74·5 % of the plasma phospholipids

Table 1. *Compositions of the pooled plasma phospholipids of the cows on each dietary treatment. Concentrations of individual phospholipids are expressed as percentages of the total plasma phospholipids*

| | Dietary treatments | | | |
| | Diet with low-fat concentrate mixture | Diet with concentrate mixture containing | | |
| | | 5 % 'Stearic acid' | 10 % 'Stearic acid' | 10 % 'Palmitic acid' |
| --- | --- | --- | --- | --- |
| Phosphatidyl choline | 70·4 | 83·4 | 88·2 | 87·2 |
| Lysophosphatidyl choline | 5·5 | 5·2 | 1·3 | 1·7 |
| Phosphatidyl ethanolamine | 1·9 | 1·3 | 0·9 | 0·9 |
| Phosphatidyl serine | 2·4 | 2·3 | 2·4 | 2·4 |
| Sphingomyelin | 19·8 | 8·0 | 7·2 | 7·9 |

of the cow, and those of Dawson, Hemington & Lindsay (1960), who found that phosphatidyl choline and lysophosphatidyl choline together accounted for 82 %. Phosphatidyl choline is the major phospholipid in the plasma of man (Phillips, 1958;

Marinetti, Albrecht, Ford & Stotz, 1959; Dawson *et al.* 1960; Nelson & Freeman, 1960; Nelson, 1962; Phillips & Dodge, 1967) and many other species (Dawson *et al.* 1960; Nelson, 1967; Moore & Noble, 1969). The other 2 diacyl phospholipids, phosphatidyl ethanolamine and phosphatidyl serine, were relatively minor components and together comprised only 4·3 % of the total plasma phospholipids of the cows when they were given the low-fat diet; normal values for man are 5·6–6·4 % of phosphatidyl ethanolamine plus phosphatidyl serine (Gjone, Berry & Turner, 1959; Nelson & Freeman, 1960; Nelson, 1962). The percentages of lysophosphatidyl choline and sphingomyelin in the plasma phospholipids of the cows given the low-fat diet (5·5 and 19·8, respectively), are of the same order as the corresponding values (8·4 and 14·9, respectively), reported by Nelson (1967) for cow plasma. When the concentrate mixture given to the cows was supplemented with 5 or 10 % 'stearic acid' or 10 % 'palmitic acid' the proportion of phosphatidyl choline in the total plasma phospholipids was increased to about 86 % and the proportions of phosphatidyl ethanolamine and sphingomyelin were decreased. The proportion of lysophosphatidyl choline was decreased only when the cows were given the concentrate mixtures containing 10 % 'stearic acid' or 10 % 'palmitic acid'. The proportion of phosphatidyl serine in the plasma phospholipids was unaltered by dietary treatment.

Table 2. *Weight percentages of the major $C_{16}$ and $C_{18}$ fatty acids in the diacyl phospholipids in the plasma of the cows on each dietary treatment*

| | | Dietary treatments | | | |
| | | | Diet with concentrate mixture containing | | |
| Phospholipid | Fatty acids | Diet with low-fat concentrate mixture | 5 % 'Stearic acid' | 10 % 'Stearic acid' | 10 % 'Palmitic acid' |
|---|---|---|---|---|---|
| Phosphatidyl choline | 16:0 | 15·9 | 15·2 | 15·1 | 24·4 |
| | 16:1 | 0·6 | 0·7 | 1·0 | 1·0 |
| | 18:0 | 28·0 | 28·1 | 28·4 | 20·0 |
| | 18:1 | 15·0 | 18·8 | 21·6 | 20·7 |
| | 18:2 | 34·7 | 31·1 | 28·5 | 28·2 |
| | 18:3 | 1·2 | 1·4 | 1·2 | 1·3 |
| Phosphatidyl ethanolamine | 16:0 | 20·2 | 19·6 | 21·6 | 21·7 |
| | 16:1 | 4·6 | 4·8 | 4·3 | 4·3 |
| | 18:0 | 23·8 | 24·2 | 23·4 | 23·2 |
| | 18:1 | 25·3 | 26·3 | 25·0 | 25·6 |
| | 18:2 | 18·9 | 18·0 | 18·2 | 17·9 |
| | 18:3 | 1·4 | 1·8 | 2·3 | 1·8 |
| Phosphatidyl serine | 16:0 | 15·0 | 11·8 | 8·3 | 19·8 |
| | 16:1 | 2·4 | 2·0 | 1·0 | 2·6 |
| | 18:0 | 44·9 | 48·5 | 52·2 | 40·7 |
| | 18:1 | 23·8 | 23·6 | 23·6 | 23·0 |
| | 18:2 | 10·0 | 9·9 | 10·9 | 9·9 |

The concentrations of the $C_{16}$ and $C_{18}$ fatty acids in the 3 plasma diacyl phospholipids of the cows are given in Table 2. In spite of the changes in dietary treatment, 16:0 and 18:0 together accounted for about 44 % of the total fatty acids in the phosphatidyl choline and phosphatidyl ethanolamine and about 60 % of the total fatty acids in the phosphatidyl serine. Of the 3 diacyl phospholipids in the plasma of

the cow, the phosphatidyl serine contained the highest concentration of 18:0 and the lowest concentration of 18:2; the phosphatidyl choline contained the highest concentration of 18:2. A similar pattern of distribution of fatty acids has been reported for the diacyl phospholipids in the plasma of man (Phillips & Dodge, 1967) and in the plasma of the rabbit (Moore & Noble, 1969). When the concentrate mixture was supplemented with either 5 or 10% 'stearic acid', the concentrations of 18:0 and 16:0 in the phosphatidyl choline were unaltered but the concentration of 18:1 was increased and the concentration of 18:2 decreased. Presumably some of the additional 18:0 derived from the diet was desaturated in the tissues (e.g. Raju & Reiser, 1967; Bickerstaffe & Annison, 1968) and the resulting 18:1 tended to replace a proportion of the 18:2 in the $\beta$-position of the phosphatidyl choline (Moore, Williams & Westgarth, 1965). When the concentrate mixture given to the cows contained 10% 'palmitic acid', the concentration of 16:0 in the plasma phosphatidyl choline was increased and the concentration of 18:0 was decreased; again, the concentration of 18:1 was increased and the concentration of 18:2 was decreased. In this instance, it seems reasonable to suppose that the additional 18:1 was derived from the chain elongation in the tissues of the dietary 16:0 (Wakil, 1964), and the desaturation of the resulting 18:0. The different dietary treatments had little or no effect on the fatty acid composition of the plasma phosphatidyl ethanolamine. In contrast to the

Table 3. *Weight percentages of the major $C_{16}$ and $C_{18}$ fatty acids in the lysophosphatidyl choline and sphingomyelin in the plasma of the cows on each dietary treatment*

| | | | Dietary treatments | | |
| | | Diet with low-fat concentrate mixture | Diet with concentrate mixture containing | | |
| Phospholipid | Fatty acid | | 5% 'Stearic acid' | 10% 'Stearic acid' | 10% 'Palmitic acid' |
|---|---|---|---|---|---|
| Lysophosphatidyl | 16:0 | 31·6 | 30·9 | 32·8 | 38·6 |
| choline | 16:1 | 2·6 | 2·3 | 2·1 | 2·5 |
| | 18:0 | 22·5 | 23·2 | 21·4 | 15·4 |
| | 18:1 | 20·2 | 25·2 | 28·3 | 26·5 |
| | 18:2 | 17·5 | 12·5 | 10·0 | 11·2 |
| Sphingomyelin | 16:0 | 56·0 | 47·1 | 40·0 | 64·4 |
| | 18:0 | 16·9 | 23·2 | 28·3 | 14·4 |
| | 18:1 | 11·3 | 18·7 | 22·7 | 8·3 |
| | 18:2 | 10·3 | 6·4 | 4·3 | 8·5 |

phosphatidyl choline, the inclusion of 5 or 10% of 'stearic acid' in the concentrate mixture resulted in an increase in the concentration of 18:0 and a decrease in the concentration of 16:0 in the phosphatidyl serine; the concentrations of 18:1 and 18:2 were unchanged. When the concentrate mixture contained 10% 'palmitic acid', the 16:0 content of the phosphatidyl serine was increased and the concentration of 18:0 was decreased; again, the concentrations of 18:1 and 18:2 were unchanged.

The concentrations of the $C_{16}$ and $C_{18}$ fatty acids in the lysophosphatidyl choline and sphingomyelin in the plasma of the cows are given in Table 3. In the lysophosphatidyl choline, 16:0 and 18:0 together accounted for about 54% of the total

fatty acids. It seems reasonable to conclude, therefore, that cow plasma contained similar proportions of $\alpha$- and $\beta$-lysophosphatidyl choline (cf. Tattrie & Cyr, 1963). The lysophosphatidyl choline in the plasma of the cow would thus appear to be similar to that in the plasma of man (Williams, Kuchmak & Witter, 1966; Phillips & Dodge, 1967) and of the rabbit (Moore & Noble, 1969). The effects of diet on the fatty acid composition of the plasma lysophosphatidyl choline were very similar to the effects on the fatty acid composition of the plasma phosphatidyl choline (Table 2). This similarity is presumably a reflexion of the metabolic relationship that exists between these 2 plasma phospholipids. Both are derived mainly from the liver, homogenates of which have been shown to catalyse the hydrolysis of phosphatidyl choline with the formation of the 2 structural isomers of lysophosphatidyl choline (Van Den Bosch & Van Deenen, 1965; Waite & Van Deenen, 1967). As with the sphingomyelin in the plasma of man (Hanahan, Watts & Pappajohn, 1960; Nelson, 1962; Williams *et al.* 1966; Phillips & Dodge, 1967) and of the rabbit (Moore & Noble, 1969), palmitic acid was the major saturated fatty acid in the sphingomyelin of cow plasma. Supplementation of the diet of the cows with stearic acid increased the concentration of 18:0 and 18:1 in the plasma sphingomyelin and decreased the concentration of 16:0 and 18:2. Supplementation of this diet with palmitic acid increased the concentration of 16·0 in the plasma sphingomyelin but it decreased the concentrations of the other constituent fatty acids.

The results of this investigation showed that the addition of stearic acid or palmitic acid to the diet of the cow influenced both the proportions of the individual components of the total plasma phospholipids and the fatty acid compositions of the individual plasma phospholipids. The patterns of change in fatty acid composition were quite characteristic for certain of the individual plasma phospholipids. This was undoubtedly due to the fatty acid specificities of the enzymes that are concerned with the synthesis of the various phospholipids in the liver.

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# The winter decline in the solids-not-fat content of herd bulk-milk supplies

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SUMMARY. (1) A survey of the composition of the milk of individual cows in 4 commercial herds in the West Riding of Yorkshire was undertaken from October 1967 to May 1968.

(2) In 3 of the 4 herds there was a decline in solids-not-fat (SNF) content throughout the winter feeding period. Milk protein content was at a minimum in January–February but in all 3 herds lactose content also declined and in 2 of the herds this was the major cause of the fall in SNF content.

(3) The decline in lactose content was the result partly of the characteristic effect of an advance in lactation, partly of an increase in the incidence of mastitis throughout the winter period. In all herds there was a significant inverse relationship between lactose content and cell count in the bulked milk of individual cows.

(4) In 3 of the herds autumn-calving (October–December) animals showed a pronounced fall in milk protein content in the early months of lactation which was not observed in summer-calving (July–September) animals. There was no response in SNF or protein content to supplementary concentrates offered towards the end of the winter-feeding period, whereas with the commencement of grazing in the spring there was an immediate increase in both protein and SNF contents.

Rowland (1942–4) observed in some parts of the country during the early years of the second world war a decline during the winter period in the solids-not-fat (SNF) content of herd bulk milks. Pre-war experience suggested that the SNF content at that time should have been high. He attributed the change to the inexperience of farmers in the production and feeding of arable crops on which increasing reliance was then being placed. A winter decline is still a characteristic feature of the seasonal curve for the SNF content of herd bulk milk (Anon, 1967) and from the results of a survey Edwards (1958) concluded that this decline may have its origin in inadequate energy nutrition. However, recent unpublished observations by members of the National Agricultural Advisory Service and advisory personnel of commercial firms have suggested that there is little or no improvement in SNF content in response to

supplementary concentrate feeding at the time of the most marked depression in SNF content. Either energy undernutrition under the conditions which prevail in commercial herds throughout the winter period causes irreversible changes in milk composition which have not been recognized in experimental studies, or there are factors other than energy undernutrition contributing to the decline in SNF content.

Energy undernutrition is known to affect primarily the protein content of milk (see Rook, 1961). A survey of changes in compositional quality of the milk of cows in commercial herds throughout the winter period has therefore been undertaken to determine the contribution of deficiencies in protein and lactose contents to the winter decline in SNF content, and the response in SNF content to the feeding of supplementary concentrates towards the end of the winter-feeding period.

## EXPERIMENTAL

### Experimental procedure

The survey was undertaken over the period October 1967–May 1968. Four Friesian herds, 3 within a 20-mile radius of Leeds and a fourth on the Yorkshire–Lancashire border, were selected. The creamery records for all 4 herds in the 3 previous years showed a pronounced fall in SNF content of the bulk milk from autumn to late winter. Details are given in Table 1.

Calving dates and lactation numbers were recorded. Herds were subjected to their usual management routine. Farm foods on offer during the winter-feeding period and until the commencement of grazing in the spring were as follows:

| For maintenance | For production |
|---|---|
| Herd A—hay, chopped swedes or mangolds. | Rolled, home-grown barley plus balancer meal (4 lb/gal milk produced). |
| Herd B—hay, or hay plus brewers' grains. | Sugar beet pulp (3 lb) plus maize germ meal (1 lb), or purchased barley mixture (4 lb) for the first gal, and dairy concentrates (4 lb for each additional gal milk produced). |
| Herd C—hay, or hay plus kale, silage or mangolds. | Crushed home-grown oats (4 lb) for the first gal, and dairy concentrates (3 lb for each additional gal milk produced). |
| Herd D—hay. | Rolled, home-grown barley plus balancer meal (4 lb) for the first gal, dairy concentrates (4 lb for each additional gal milk produced). |

An assessment of the actual level of feeding was considered impracticable and chemical analyses of the feedstuffs offered were not undertaken.

From the second week of March each herd was divided into 2 groups, balanced as closely as possible for milk yield and composition, lactation stage and number. The animals in one group were given supplementary proprietary dairy concentrates (starch equivalent, 63; protein equivalent, 10·5) at a rate of 6 lb per head per day for a period of 6 weeks, or until cows were turned out to grass in the spring.

Farms were visited at one evening's and the following morning's milking once a fortnight throughout the period of the survey. The milk from each cow was weighed and a representative sample taken for chemical analysis. At the morning milking an additional sample was taken into a sterile bottle for bacteriological examination.

## *Methods of analysis*

A weighted composite sample of morning and evening milk was prepared for each cow at each sampling occasion. It was analysed for fat content by the Gerber method (British Standards Institution, 1955), for total solids gravimetrically (British Standards Institution, 1951), for nitrogen by the Kjeldahl technique and for lactose by the chloramine-T method (British Standards Institution, 1963). SNF content was calculated by difference.

Cell count was determined by a standard method (Anon, 1968) except that duplicate milk films were examined. Bacterial content was determined by plating a standard loopful of fresh milk on 5 % sheep blood agar and also on Edward's medium. The plates were incubated at 37 °C and examined after 24- and 48-h incubation.

Table 1. *Details of the herds under investigation*

Maximum and minimum values for the SNF content ( %)
of herd bulk milks during the winter-feeding period

| Farm | Average herd size | 1964–5 | | 1965–6 | | 1966–7 | | Average lactation no. 1967–8 |
| | | Max. | Min. | Max. | Min. | Max. | Min. | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| A | 15 | 8·67 (Oct.) | 8·39 (Feb.) | 8·71 (Oct.) | 8·33 (May) | 8·88 (Oct.) | 8·47 (Jan.) | 3·4 |
| B | 36 | 8·76 (Oct.) | 8·37 (Apr.) | 8·73 (Oct.) | 8·49 (Feb.) | 8·54 (Oct.) | 8·15 (Jan.) | 1·8 |
| C | 23 | 8·65 (Oct.) | 8·39 (Feb.) | 8·58 (Dec.) | 8·31 (Mar.) | 8·64 (Nov.) | 8·12 (Mar.) | 3·0 |
| D | 24 | 8·55 (Oct.) | 8·31 (Feb.) | 8·51 (Nov.) | 8·30 (Feb.) | 8·57 (Oct.) | 8·43 (Mar.) | 2·9 |

### RESULTS

### *Herd bulk values*

Herd size and herd average values for yield, and SNF, protein and lactose contents are given in Table 2. Shortly after the start of the survey, several animals in herd A were sold after calving down, and several others did not calve until the end of the winter period. The herd bulk values did not show the expected trend in the SNF content of their milk. The other 3 herds were predominantly late-summer and autumn calving and there were few changes in the composition of the herds. In all 3 herds the average values for SNF content showed an erratic but progressive fall from October to February. The extreme changes in average SNF contents in the 3 herds were from 8·68 (November 4) to 8·10 (January 12) in herd B; 8·78 (November 7) to 8·31 (January 26) in herd C and 8·68 (November 28) to 8·30 (February 9) in herd D. In all 3 herds, milk protein content was at a minimum in January–February but in herds B and C the main cause of the drop in SNF content was a fall in the content of lactose. There was no exact correspondence between short-term changes in SNF contents and the sum of changes in lactose and protein contents but the trends in SNF content reflected those in lactose and protein contents.

*Lactation trends in milk composition*

Average values for milk composition for the months of November, January and March for animals in each herd which continued in lactation from the beginning of November to the end of March are reported in Table 3. For animals in herds A and C. the characteristic increase in protein content with advance in lactation was pronounced, whereas animals in herds B and D showed only erratic changes. All herds

Table 2. *Herd average values for milk yield and weighted herd average values for SNF, protein (total $N \times 6\cdot38$) and lactose contents, together with herd numbers*

| Farm | | Oct.* | Nov. | Dec. | Jan. | Feb. | Mar. | Apr. | May† |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Month of sampling | | | | |
| A | No. animals sampled | 15 | 11 | 11 | 11 | 16 | 13 | 12 | 14 |
| | Herd changes‡ | | +2−6 | +1−1 | +4−4 | +7−2 | +2−5 | −1 | +2 |
| | Daily milk yield, kg | 10·5 | 8·4 | 7·7 | 9·9 | 12·1 | 14·1 | 12·5 | 14·9 |
| | SNF, % | 8·58 | 8·41 | 8·61 | 8·83 | 8·76 | 8·76 | 8·72 | 9·03 |
| | Protein, % | 3·19 | 3·17 | 3·19 | 3·45 | 3·34 | 3·23 | 3·19 | 3·51 |
| | Lactose, % | 4·40 | 4·40 | 4·46 | 4·49 | 4·42 | 4·51 | 4·38 | 4·53 |
| B | No. of animals sampled | 32 | 35 | 34 | 35 | 43 | 43 | 35 | 26 |
| | Herd changes‡ | | +3 | −1 | +2−1 | +4−2 | +7−1 | +2−10 | −9 |
| | Daily milk yield. kg | 15·2 | 15·0 | 13·0 | 11·9 | 11·7 | 12·3 | 12·0 | 16·6 |
| | SNF, % | 8·43 | 8·52 | 8·43 | 8·20 | 8·33 | 8·42 | 8·42 | 8·64 |
| | Protein, % | 3·00 | 2·95 | 2·93 | 2·88 | 2·97 | 3·04 | 2·96 | 3·20 |
| | Lactose,% | 4·55 | 4·58 | 4·48 | 4·37 | 4·39 | 4·43 | 4·48 | 4·57 |
| C | No. of animals sampled | 24 | 23 | 21 | 22 | 23 | 20 | 20 | 22 |
| | Herd changes‡ | | +2−3 | +2−4 | +4−3 | +1 | −3 | +1−1 | +4−2 |
| | Daily milk yield, kg | 9·3 | 8·7 | 8·6 | 9·6 | 9·1 | 8·8 | 8·2 | 8·9 |
| | SNF, % | 8·68 | 8·60 | 8·50 | 8·50 | 8·43 | 8·39 | 8·37 | 8·71 |
| | Protein, % | 3·25 | 3·18 | 3·25 | 3·20 | 3·16 | 3·21 | 3·27 | 3·42 |
| | Lactose, % | 4·41 | 4·34 | 4·23 | 4·29 | 4·25 | 4·24 | 4·18 | 4·31 |
| D | No. of animals sampled | 19 | 21 | 20 | 23 | 24 | 23 | 23 | 23 |
| | Herd changes‡ | | +3−1 | +3−4 | +3 | +1 | +1−2 | | |
| | Daily milk yield, kg | 14·4 | 13·7 | 15·7 | 15·5 | 14·7 | 14·0 | 14·2 | 16·1 |
| | SNF, % | 8·57 | 8·58 | 8·63 | 8·51 | 8·33 | 8·40 | 8·44 | 8·69 |
| | Protein, % | 3·02 | 3·07 | 3·0~ | 3·01 | 2·95 | 3·04 | 3·01 | 3·34 |
| | Lactose, % | 4·50 | 4·53 | 4·55 | 4·52 | 4·50 | 4·47 | 4·41 | 4·42 |

\* The herds were sampled once only during Oct. and May and twice monthly from Nov. to Apr.

† Animals at pasture.

‡ Animals added to or removed from milking herd since last sampling.

showed the characteristic lactational decline in lactose, and in herds B, C and D there was an accompanying increase in the weighted mean cell count. The most marked decline in lactose content was in herd C in which there was the highest average cell count and a pronounced increase in count with advance in lactation. In all 4 herds an increase in cell count was associated with a decrease in milk lactose content (Table 4). The predominant pathogenic bacteria isolated from the herds were: herd A, haemolytic staphylococci and *Streptococcus uberis*; herd B, haemolytic staphylococci and *Str. agalactiae*; herd C, *Str. agalactiae* and *Str. uberis*; herd D, *Str. agalactiae*.

The average values for milk lactose content in November and March for animals from all 4 herds grouped according to lactation number are given in Table 5. The

Table 3. *Average values for milk yield and SNF, protein and lactose contents, together with a weighted mean cell count for those cows in each herd which lactated throughout the winter period*

| Farm | | Month | | |
|------|------|------|------|------|
| | | Nov. | Jan. | Mar. |
| A | No. of animals | 6 | 6 | 6 |
| | Daily milk yield, kg | 19·9 | 16·9 | 13·4 |
| | SNF, % | 8·26 ± 0·244 | 8·38 ± 0·175 | 8·61 ± 0·171 |
| | Protein, % | 3·07 ± 0·109 | 3·25 ± 0·152 | 3·47 ± 0·106 |
| | Lactose, % | 4·33 ± 0·178 | 4·39 ± 0·160 | 4·19 ± 0·189 |
| | Weighted mean cell count, 000s | 834 | 665 | 391 |
| B | No. of animals | 28 | 28 | 28 |
| | Daily milk yield, kg | 15·4 | 11·6 | 11·1 |
| | SNF, % | 8·58 ± 0·063 | 8·16 ± 0·088 | 8·31 ± 0·058 |
| | Protein, % | 2·97 ± 0·046 | 2·92 ± 0·048 | 3·08 ± 0·017 |
| | Lactose, % | 4·62 ± 0·033 | 4·36 ± 0·044 | 4·40 ± 0·086 |
| | Weighted mean cell count, 000s | 169 | 511 | 907 |
| C | No. of animals | 13 | 13 | 13 |
| | Daily milk yield, kg | 12·6 | 9·1 | 5·9 |
| | SNF, % | 8·73 ± 0·082 | 8·66 ± 0·084 | 8·75 ± 0·130 |
| | Protein, % | 3·20 ± 0·063 | 3·39 ± 0·057 | 3·70 ± 0·120 |
| | Lactose, % | 4·49 ± 0·064 | 4·23 ± 0·078 | 4·09 ± 0·089 |
| | Weighted mean cell count, 000s | 723 | 895 | 1845 |
| D | No. of animals | 17 | 17 | 17 |
| | Daily milk yield, kg | 14·5 | 13·0 | 11·5 |
| | SNF, % | 8·79 ± 0·033 | 8·53 ± 0·027 | 8·44 ± 0·024 |
| | Protein, % | 3·13 ± 0·117 | 3·09 ± 0·072 | 3·16 ± 0·056 |
| | Lactose, % | 4·53 ± 0·064 | 4·53 ± 0·051 | 4·44 ± 0·054 |
| | Weighted mean cell count, 000s | 234 | 334 | 714 |

Table 4. *Relationship between lactose content (%) and cell count of the milk of individual cows*

| Farm | No. of observations | Correlation coefficient | Regression of lactose on cell count (lactose = $a + b$ log. cell count × $10^{-3}$) | |
|------|------|------|------|------|
| | | | $a$ | $b$ |
| A | 108 | 0·352 | 4·81 ± 0·08 | − 0·152 ± 0·039*** |
| B | 457 | 0·168 | 4·60 ± 0·05 | − 0·087 ± 0·024*** |
| C | 222 | 0·465 | 4·74 ± 0·06 | − 0·191 ± 0·025*** |
| D | 223 | 0·376 | 4·78 ± 0·04 | − 0·121 ± 0·020*** |

*** $P < 0·001$.

Table 5. *The decline in lactose content (%) throughout the winter period in cows of different lactation number*

(Values are a mean, with standard error)

| Lactation no. | No. of cows | Sampling date | |
|------|------|------|------|
| | | Nov. | Mar. |
| 1 | 28 | 4·61 ± 0·04 | 4·40 ± 0·04 |
| 2 | 14 | 4·52 ± 0·05 | 4·24 ± 0·07 |
| 3 | 9 | 4·54 ± 0·09 | 4·33 ± 0·10 |
| 4 | 3 | 4·41 ± 0·10 | 4·29 ± 0·12 |
| 5 | 5 | 4·40 ± 0·07 | 4·18 ± 0·10 |
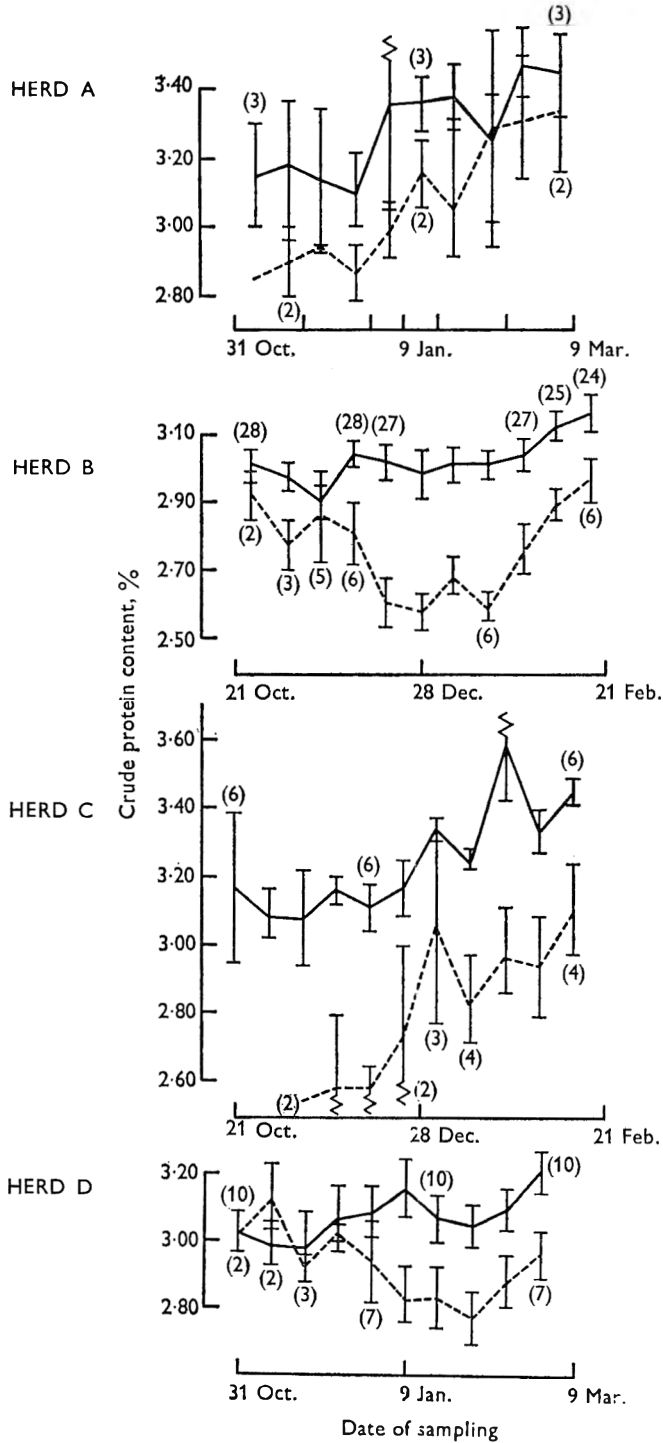
Fig. 1. Changes throughout the period of winter feeding in the protein content of the milk of: summer-calving animals (July–September), ——; autumn-calving animals (October–December), ----. Figures in parentheses are the number of animals contributing to the mean.

average lactose content decreased with increase in lactation number but there was no evidence of a more marked depression with advance in lactation in aged than in first lactation cows. The distribution of animals from the individual herds between lactation number groups was not, however, even. Two-thirds of the animals in their first lactation were in herd B.

### Effect of calving date on the trend in protein content

Though herd mean values for milk protein content did not show a pronounced fall throughout the winter period, important differences in trends were observed between summer and autumn calving animals (Fig. 1). In all 4 herds, animals calving in July–September showed a slow, progressive increase in the protein content of their milk, similar to previously observed trends with lactation from about the third month after calving (Rook & Campling, 1965). In herds B and D, however, animals calving October–December showed a pronounced downward trend in milk protein content until mid-February, only a part of which could be attributed to the normal decreases in protein content in early lactation. Moreover, for these 2 herds and for herd C, throughout December, January and February the average protein content for the milk of autumn-calving animals was consistently lower than that for summer-calving animals at a corresponding stage of lactation. There was no distinction between the calving groups in the trends in lactose content.

Table 6. *The increase in the yield and protein content of milk in response to the feeding of an additional 6 lb of concentrates per head per day*

(Values reported are the mean difference, with standard error, between values for cows which received the supplement and a comparable group of control animals. The figures in parentheses denote the numbers of pairs of animals.)

| | Fourth week of supplementary feeding | | Sixth week of supplementary feeding | |
| | Yield, kg/day | Protein, % units | Yield, kg/day | Protein, % units |
| Farm | | | | |
|---|---|---|---|---|
| A | $+0\cdot8\pm0\cdot8$ | $+0\cdot04\pm0\cdot10$ (6) | $+1\cdot3\pm0\cdot7$ | $-0\cdot12\pm0\cdot14$ (6) |
| B | $+1\cdot6\pm0\cdot3$*** | $+0\cdot07\pm0\cdot04$ (18) | $+1\cdot9\pm0\cdot5$** | $+0\cdot07\pm0\cdot08$ (16) |
| C | $-0\cdot3\pm0\cdot6$ | $+0\cdot05\pm0\cdot09$ (7) | $0\cdot0\pm1\cdot0$ | $+0\cdot01\pm0\cdot08$ (7) |
| D | $+1\cdot1\pm0\cdot7$ | $-0\cdot01\pm0\cdot10$ (11) | $+0\cdot8\pm0\cdot8$ | $0\cdot00\pm0\cdot05$ (11) |

** $P < 0\cdot01$.     *** $P < 0\cdot001$.

### Responses to supplementary feeding

The increases in milk yield and protein content in response to the feeding of additional concentrates in March–April are given in Table 6. With the exception of herd C, supplementary feeding produced small increases in milk yield and in herd B the effect was significant at the end of the fourth and sixth weeks of supplementary feeding. The responses in milk protein content were small and variable, ranging from $-0\cdot12$ percentage units at the end of the sixth week of supplementary feeding in herd A to $+0\cdot07$ percentage units at the end of the fourth and sixth weeks in herd B: in no instance was the effect significant ($P > 0\cdot05$). Supplementary feeding was not associated with any change in lactose content.

Three of the 4 herds showed the expected fall in SNF content in the period October–February. There was a corresponding slight fall in protein content but there was also a fall in the content of lactose, and in 2 of the herds this was the main cause of the decline in SNF content. In each of the 3 herds a high proportion of the cows in milk at the beginning of the survey continued in lactation until the end of March and part of the decline in lactose content would arise from the normal lactational changes. However, the presence of known pathogens coupled with an increase throughout the winter period in the proportion of animals with a high cell count in their milk indicates that an increase in the incidence of mastitis may also have contributed to the decline in lactose content. Waite & Blackburn (1957) drew attention to the high incidence in commercial herds of animals with sub-clinical mastitis as indicated by an unusually high cell count in their milk, but concluded that stage of lactation had little effect on the numbers of cells. Recently, however, Waite (1968) has reported in older cows an increase from the 58th to the 163rd day of lactation in the number of quarters infected and in which the milk lactose content was depressed. He also observed in older cows a drop in milk lactose content from about the 80th to the 90th days in lactation which was unrelated to udder infection. This may reflect a change with age in the lactational curve in lactose content. Such factors may, especially in predominantly autumn-calving herds, be a more common cause of a winter decline in SNF content than has previously been recognized.

Though the decline throughout the winter period in the herd average values for protein content was much less marked than had been expected, there was evidence that in autumn-calving animals (October–December) in herds B, C and D milk protein content was depressed in the first months of lactation: the values for autumn-calving animals during January and February were consistently lower than those for summer-calving animals (July–September) during October and November. If it is assumed that the most likely cause of this depression was energy undernutrition, 2 possibilities arise. There may be, through an overestimation of the nutritive value of autumn grazing, a tendency in some commercial herds to under-feed autumn-calving animals during the usual 'steaming-up' period and throughout the first weeks of lactation, which may depress milk protein content in the first months of lactation (Foot, Line & Rowland, 1963). Alternatively, overestimation of the quality of hay or silage on offer could result in a continuing undernutrition throughout the winter period, and the effect on milk protein content may be more pronounced in animals in early lactation, when milk yield may be maintained at the expense of body reserves, than in animals at a later stage of lactation in which the peak in yield has been passed.

By mid-February there was in all 4 herds a sharp upward trend in milk protein content in autumn-calving animals, probably the result of a lactational trend. This preceded the introduction of supplementary feeding which in all herds produced little or no change in milk protein content, confirming the reports of advisory workers in the field. This lack of response is puzzling in view of an immediate and marked increase in protein content observed with the commencement of grazing, an effect thought to be related to an improvement in energy nutrition (Rook & Rowland, 1959).

Increases in milk protein content in response to additional feed energy are, however, known to be dependent on a specific increase in the uptake of propionate from the rumen (Rook & Balch, 1961). In cattle receiving a diet of spring grass, the mixture of acids in the rumen may contain 25 % or more (on a molar basis) of propionic acid, a value rarely achieved with diets of hay and proprietary or farm concentrate mixtures (Rook, 1964). The addition of supplementary concentrates to a basal diet containing a high proportion of poor quality roughage could give little or no increase in the production of propionic acid in the rumen and under those circumstances an increase in milk protein and SNF contents would not be expected.

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# Synthetic peptide and ester substrates for rennin

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SUMMARY. Rennin hydrolysed the phe-met bond in the peptide H-ser-leu-phe-met-ala-OMe (i.e. methyl ester), the amino acid sequence of which is similar to that around the phe-met bond attacked by rennin in $\kappa$-casein. Rennin did not attack other peptides from this sequence not containing serine, and it is suggested that, in both $\kappa$-casein and the pentapeptide, the enzymic attack is accelerated by the nearby serine side chain. Rennin also hydrolysed sulphite esters such as phenyl sulphite ester and some N-substituted imidazole compounds such as benzoyl imidazole. Phenyl sulphite esters may be suitable substrate for assaying the activity of preparations of rennin.

One of the striking features of the behaviour of rennin (E.C.3.4.4.3) is the difference between its relatively weak activity in general proteolysis and the rapidity of its 'specific' attack upon the rennin-sensitive bond in casein. As a proteolytic enzyme it has an optimum at about pH 4 (*vide* Fish, 1957), whereas the specific action on casein remains rapid at rather higher pH values. For example, at pH 6·5 and 30 °C, and with an enzyme-substrate ratio of 1–20000, the attack on casein in milk can be completed in 1 min. The bond split in this attack has been identified as being between phenylalanine and methionine residues in $\kappa$-casein (Delfour, Jollès, Alais & Jollès, 1965). In the present work it was established initially that the dipeptide phe-met, whether protected or not, was not attacked by rennin. In order to study the reason for this difference, a number of peptides simulating parts of the amino acid sequence around the phe-met bond in casein were prepared. From work by Jollès, Alais & Jollès (1962) and in our own laboratory (R. Beeby, personal communication) this sequence is ser-leu-phe-met-ala. A preliminary report on the action of rennin on peptides from this sequence has been published (Hill, 1968). This paper contains a more complete account of this work, as well as the results of tests on other synthetic substrates such as phenyl sulphite esters and N-substituted imidazole compounds.

## MATERIALS AND METHODS

All materials were of analytical reagent grade, and amino acids were chromatographically pure L-amino acids. Pronase was the pure grade, obtained from the Kaken Chemical Co., Tokyo, and carboxypeptidase A (E.C.3.4.2.1), leucine amino peptidase (E.C.3.4.1.1.), and pepsin (E.C.3.4.4.1.) were the products of the Mann Laboratories, New York. Rennin used in these experiments was purified from commercial rennet by salt precipitation and chromatography on DEAE cellulose as

described in Hill & Laing (1967). It was stored as a stock solution of 0·5 % in M NaCl at pH 5·4 and 3 °C.

Peptides were prepared by coupling $N$-$o$-nitrophenylsulphenyl (NPS) amino acid dicyclohexylamine (DCHA) salts, using dicyclohexylcarbodiimide (DCCI) as coupling agent, with hydrochloride amino acid or peptide esters according to the method of Zervas, Borovas & Gazis (1963), except in the case of the peptide containing serine. For this, removal of the NPS-protecting group by the recommended treatment with methanolic HCl led to considerable loss of product, presumably by reaction of the liberated $o$-NPS Cl with the serine hydroxyl. This loss was avoided by treating the NPS-protected peptide with thioglycollic acid in pyridine (Fontana, Marchiori, Moroder & Scoffone, 1966) which liberated the protecting group as an unreactive, readily separable disulphide. In these circumstances the serine peptide could be prepared without protecting the serine hydroxyl group. The peptide esters phe-met-, phe-met-ala-, leu-phe-met-ala-, and H-ser-leu-phe-met-ala-OMe (i.e. methyl ester) were prepared in this way. The purity of the peptides was checked by thin layer chromatography (TLC) on silica gel, and the absence of racemization by digesting them with pronase and aminopeptidase. These peptides were digested completely by both the above enzymes, indicating that racemization had not occurred during preparation of the peptides. They chromatographed as single spots in TLC, except for the final penta-peptide which contained a trace of serine. Phenyl sulphite ester, and $p$-chlorophenyl, $o$-bromophenyl and $p$-bromophenyl sulphite esters were synthesized by the method of Carré & Libermann (1932). Phenyl sulphite ester was stored as a 20 % solution in dry isopropanol at 3 °C. Acetyl imidazole was prepared by coupling imidazole (2 mM) with acetic acid (glacial, 2 mM in 10 ml dry methylene chloride using DCCI (2·2 mM) as coupling agent. This was found to be a more convenient method than that of Boyer (1952) and was also used for the synthesis of benzoyl, salicyloyl and cinnamoyl imidazole. Reaction was rapid, usually being complete in less than an hour, when the product was readily separated by filtration from the precipitated dicyclohexyl urea. After removal of solvent *in vacuo*, the product was stored in a desiccator over $P_2O_5$.

For the tests of the action of rennin on the peptides, solutions of approximately 2 mM in peptide concentration and 0·01 $N$ in sodium acetate were adjusted to the desired pH with acetic acid. They were treated with rennin, approximately $10^{-6}$ M, at 30 °C. The extent of reaction was estimated by assaying the liberated amine groups using trinitrobenzenesulphonic acid (TNBS) according to the method of Harmeyer, Sallmann & Ayoub (1968) except that the absorbance of the TNBS products was measured at 430 nm, not 360 nm. The progress of the reaction was also followed by TLC of the reaction mixture on silica gel in chloroform-methanol (7:3) butanol-acetic acid-water (4:1:1), phenol-water (75:25) and propanol-ammonia (7:3). Controls were similarly assayed and chromatographed.

The reaction between rennin and the phenyl sulphite esters or the substituted imidazole compounds was followed either in a pH stat (Radiometer Titrator TTT 1 C and Titrigraph SBR 21 c) at pH 3·5–4 and 30 °C or in the case of the phenyl sulphite ester by observing the increase in the absorbance at 270 nm and at 25 °C as phenol was released by the rennin. Rennin concentrations were about $10^{-6}$ M. Because of the limited solubility (about $10^{-4}$ M) of these substrates, the solutions in the pH stat experiments were unbuffered in order to obtain maximum sensitivity

in detecting the hydrolysis. When the reaction was followed spectrophotometrically, the solutions were buffered with glycine-HCl rather than with acetate buffers, as it was found that the hydrolysis of phenyl sulphite ester was catalysed by acetate ion. In calculating rates of enzymic hydrolysis, allowance was made for the non-enzymic hydrolysis determined in control solutions.

<div style="text-align:center">RESULTS</div>

*Peptide substrates*

For the peptides phe-met-, phe-met-ala-, and H-leu-phe-met-ala-OMe, the rates of reaction with rennin were negligible, little or no reaction being detectable by TLC or by the TNBS assay after several days at pH 3·7 or 5·9 and 30 °C. In the case of the leucine tetrapeptide, the TNBS assay indicated that one bond was split after 40 days at room temperature, but it is difficult to assign much significance to this result. By contrast, the pentapeptide H-ser-leu-phe-met-ala-OMe was hydrolysed moderately rapidly at pH 3·7 and 4·7, the time for half reaction being approximately 4 h at pH 3·7 and 3 h at pH 4·7. The reaction was somewhat slower at pH 5·9 (half-time 12 h) and no reaction was detectable at pH 6·3. The reaction did not follow first order kinetics, but after an initial rapid reaction in the first 10–15 min, the rate of reaction dropped off rapidly suggesting a possible competitive inhibition of the enzyme by products of the reaction. Tests made on portions of the reaction mixture showed that the rennin did not suffer any permanent inhibition as it remained fully active in coagulating a standard skim-milk substrate (Berridge, 1952). When the rennin action was completed, the TNBS assay indicated that one bond only had been split by the rennin. This was confirmed by TLC, which showed that the reaction mixture contained two peptides (with $R_F$ values in 75:25 phenol-water of 0·80 and 0·30) in place of the original pentapeptide of $R_F$ 0·95. The new C-terminal amino acid formed as a result of the rennin action was released by treating the reaction mixture with carboxypeptidase A at pH 8. TLC of the products showed that the amino acid first released was phenylalanine. This was identified by comparison with standards chromatographed on silica gel in butanol-acetic-water (4:1:1), phenol-water (85:15) and propanol-ammonia (7:3) (*vide* Brenner, Niederwieser & Pataki, 1962) in order to distinguish between phenylalanine, leucine, and methionine. The initial release of phenylalanine showed that the bond split was the phe-met bond, and the action by rennin was limited to this. As previously stated, all the foregoing peptides were completely digested by pronase at pH 9, and by leucine aminopeptidase at pH 8 and 30 °C.

The value of $K_m$ for the action by rennin on the pentapeptide was estimated from a series of tests in which the rennin concentration was $10^{-6}$ M, the substrate concentration ranged from $1·3 \times 10^{-3}$ M to $1·7 \times 10^{-4}$ M, and the initial velocity was taken as the average velocity over the first 10 min. Under these conditions, $K_m$ was found from a Lineweaver-Burk plot (1934) to be $4 \pm 2$ mM (Fig. 1), the accuracy of the estimate being reduced by the limited solubility of the substrate.

*Substituted imidazole compounds*

The first substituted imidazole to be tested was acetyl imidazole, and this did show an enhanced rate of hydrolysis at pH 3·7 and 21 °C on the addition of rennin. Unfortunately, the rate of non-enzymic aqueous hydrolysis was so rapid that

quantitative work on the enzymic hydrolysis was difficult. Other acyl imidazoles such as benzoyl, salicyloyl, isobutyryl and cinnamoyl imidazole were prepared in an effort to obtain a material more stable towards aqueous hydrolysis and having a structure that might favour binding on the rennin. Like acetyl imidazole, the rate of hydrolysis
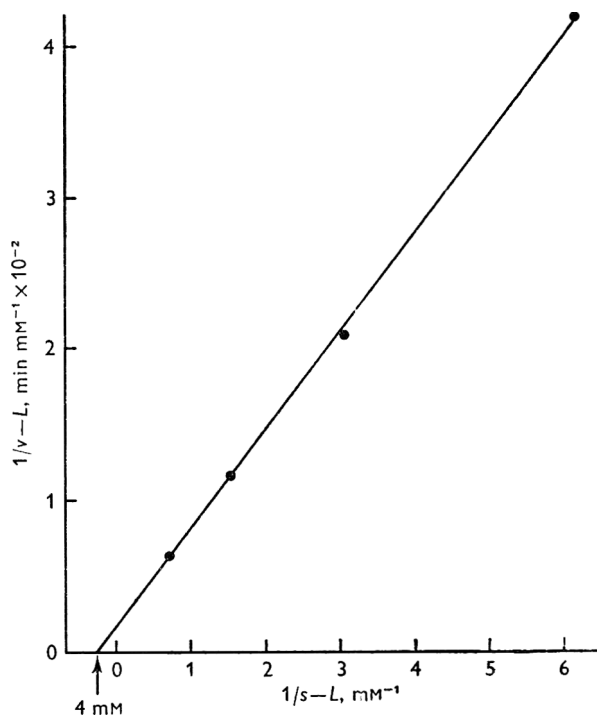


Fig. 1. Lineweaver-Burk plot for the action of rennin on ser-leu-phe-met-ala-OMe. Conditions—rennin $10^{-8}$ M, in 0·01 N sodium acetate at pH 4·7 and 30 °C.

of benzoyl was enhanced by the addition of rennin. For example, when 22 mg of benzoyl imidazole in 2 ml of chloroform was added to 10 ml of water maintained in the pH stat at pH 3·7 and 22 °C, and the aqueous phase stirred to maintain a saturated solution of benzoyl imidazole, the rate of proton uptake due to the aqueous hydrolysis alone was $7·4 \times 10^{-6}$ moles/h. On adding rennin (0·25 mg) the proton uptake was $20 \times 10^{-6}$ moles/h. The aqueous hydrolysis of the isobutyryl and salicyloyl imidazole was inconveniently rapid, while that of cinnamoyl imidazole was unaffected by rennin. At pH 3·7 the rennin was not inactivated by the cinnamoyl imidazole. Although there were definite effects by rennin on the hydrolysis of the acetyl and benzoyl imidazole, the relatively rapid aqueous hydrolysis caused problems of storage and difficulty in obtaining reproducible results.

*Phenyl sulphite esters*

Reid & Fahrney (1967) have shown that phenyl sulphite esters are good substrates for pepsin, and in view of the similarity in specificity between pepsin and rennin it seemed possible that rennin also might act upon them. This proved to be the case. Of those tested, *viz.* phenyl, *o*-chlorophenyl, *o*-bromophenyl and *p*-

bromophenyl sulphite esters, the *p*-bromophenyl ester formed the best substrate for rennin, as it was for pepsin (Reid & Fahrney, 1967).

However, the phenyl sulphite ester was a more convenient substrate than the *p*-bromophenyl sulphite, being more soluble and less prone to form micelles at concentrations near $10^{-4}$ M. Figure 2 shows the course of the reaction at pH 3·7 and 25 °C between rennin and the ester, together with curves showing the hydrolysis in control solutions. The rate of the non-enzymic hydrolysis, while not negligible, is small compared with that caused by the enzyme. The rate of the latter was found to be directly proportional to rennin concentration in the range $0.25-1 \times 10^{-6}$ M, and to substrate concentration in the range $0.2-1.2 \times 10^{-4}$ M. As with pepsin, the failure to observe Michaelis and Menten kinetics is no doubt due to the value of $K_m$ for the
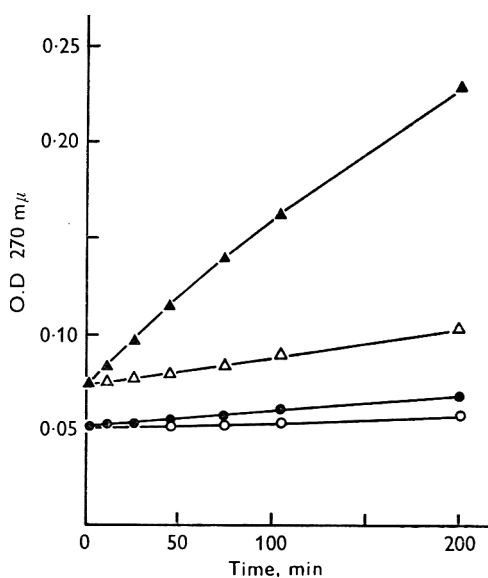


Fig. 2. Action of rennin on phenyl sulphite ester at pH 3·7 and 25 °C. ○, Control adjusted to pH 3·7 with HCl only; ●, control adjusted to pH 3·7 with 0·032 N glycine-HCl; △, as for ●, but containing $0.85 \times 10^{-6}$ M rennin inactivated by boiling 1 m; ▲, as for ●, but containing 0·85 $\times 10^{-6}$ M active rennin. Concentration of phneyl sulphite ester $1.3 \times 10^{-4}$ M.

reaction being considerably in excess of the limiting solubility (cf. Reid & Fahrney, 1967). At 25 °C, over-all rate constants for the reaction showed a broad optimum in the pH range 3·7–4, where optimum proteolytic activity also is shown (Fish, 1957). The over-all rate constant in these conditions is $35-40 \times 10^2$ l. mole$^{-1}$.min$^{-1}$ and is about one third of that found for pepsin (Reid & Fahrney, 1967). At pH 4·7 the value of the rate constant dropped considerably, to $8 \times 10^2$ l. mole$^{-1}$.min$^{-1}$. As stated earlier the hydrolysis was also catalysed by acetate ion, $K_{CH_3COO-}$ being 0·7 l. mole$^{-1}$.min$^{-1}$. An acetate ion concentration of about $10^{-2}$ M can therefore cause a rate of hydrolysis comparable to that caused by $10^{-6}$ M rennin.

DISCUSSION

The work described in this paper was mainly concerned with the nature of the rennin-sensitive bond in casein. There were, however, other points of interest, such as the desirability of providing a practical synthetic substrate for use in assays of rennin activity. At present, these assays are made using reconstituted skim-milk as a substrate (Berridge, 1952) so that there is no absolute standard for the measurement of activity. Considering the compounds on which rennin was found to act in the present study, the phenyl sulphite esters appear to offer the best prospect for developing such a standard substrate. Unlike the pentapeptide substrate, they are readily preparable, and although sensitive to moisture, can be stored in dry isopropanol, for example, at 3 °C for many months without deterioration. The solubility of these esters, however, is small, and relatively sophisticated techniques are therefore needed to measure the activity of the enzyme. In order to develop a simple assay for use in industry, it would appear desirable to modify the substrate so as to increase its solubility or increase the sensitivity of the test. One possible way of obtaining increased sensitivity might be to prepare a sulphite ester having as one component a fluorescent alcohol, similar to the substrates developed for other enzymes by Guilbault (vide Guilbault & Sadar, 1968).

Rennin is very similar to pepsin in its specificity (Foltmann, 1966) and the action by rennin on the sulphite esters is further evidence of this. Like pepsin, rennin has recently been shown to possess an active centre carboxyl group (Stepanov, Lobareva & Mal'-Tsev, 1968), while Reid & Fahrney (1967) have presented evidence that the action of pepsin on the sulphite esters might involve the formation of a mixed anhydride between an enzyme carboxyl and the sulphite ester. In this connexion it is worth noting that, although the rate constant for the enzymic hydrolysis is about 6000 times greater than that for the acetate-catalysed reaction, the hydrolysis of the sulphite ester is nevertheless appreciably catalysed by acetate ion.

The tests with the substituted imidazoles were undertaken because it was considered that they should form suitable substrates for investigating the role of the (possibly) catalytically active lysine (Hill & Laing, 1967) in the action of rennin. Although enzymic activity could be demonstrated, it appears that a different approach to this problem will be needed.

The fact that rennin either does not act, or acts very slowly upon the peptides phe-met-, phe-met-ala, and H-leu-phe-met-ala-OMe while reacting readily with the peptide H-ser-leu-phe-met-ala-OMe to split the phe-met bond, suggests rather strongly that the attack upon this bond is catalysed by the serine side chain. An alternative possibility is that the serine is concerned in the binding of the substrate to the enzyme. However, in its attack upon the B chain of insulin (Fish, 1957), rennin hydrolyses only those bonds adjacent to hydrophobic side chains; these chains and not serine are therefore the likely binding sites for the enzyme. Moreover, rennin does not appear to possess a catalytically active serine residue (Hill & Laing, 1967) and it is therefore possible that the serine in the substrate supplies a function lacking in the enzyme.

Although the pentapeptide simulates a part of the sequence in the $\kappa$-casein, the attack by rennin upon the phe-met bond in $\kappa$-casein is very much ($\simeq 10^3$) faster than

its attack upon the similar bond in the pentapeptide. The effect of the serine must therefore be regarded as a part only of the reason for the rapidity of the attack on the bond in casein. The value of $K_m$ obtained for the action on the pentapeptide substrate was 4 mM, while in casein itself the rate of reaction is linear with casein concentration up to 9 % (R. D. Hill, unpublished) or almost 1 mM, assuming 1 mole of $\kappa$-casein/$10^5$ g casein. The value of $K_m$ for the reaction with casein must therefore be somewhat in excess of 1 mM, so that the strength of the binding of the enzyme to the pentapeptide substrate must be similar to that of its binding to casein. Better binding between enzyme and substrate is therefore an inadequate explanation for the rapidity of the action on casein. In addition, it is necessary to account for the fact that the rate of the action on the pentapeptide substrate had dropped to zero at pH 6·3, whereas the action on casein is still rapid at pH 6·5 and above. In the preliminary report, evidence was presented that an additional catalytic effect may be provided by a histidine side chain, and from experiments on fibrinogen it was further suggested that similar structures might exist near the sensitive bonds in other proteins (Hill, 1968). Since this report was published, Jollès, Alais & Jollès (1968) have given the amino acid sequence of a tryptic peptide from $\kappa$-casein, containing the phe-met bond. They differ in the position ascribed to the serine residue, but report the presence of 2 histidines removed 4 and 7 residues, respectively, from the phe-met bond. This is in accord with the suggestion that histidine residues may have a particular role in rendering this bond sensitive to the action of rennin.

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# The interrelationship of the viscosity, fat content and temperature of cream between 40° and 80 °C

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SUMMARY. The dynamic viscosity coefficients $\eta$ of creams containing up to 50 % fat have been determined at temperatures of approximately 40, 50, 60, 70 and 80 °C. All the creams behaved as Newtonian liquids, the shear stress being proportional to rate of shear up to the maximum rate used of 100 sec$^{-1}$. At a given temperature a linear dependence of log $\eta$ on $(\phi + \phi^{5/3})$ was obtained for $\phi < 0.4$, where $\phi$ is the concentration (w/w) of fat. Interpolation formulae have been derived to enable $\eta$ to be calculated at any temperature between 40 and 80 °C and for any fat content up to 40 %. Formulae for the density $\rho$ of cream have also been deduced to permit kinematic viscosities $\eta/\rho$ to be computed. Nomograms have been constructed to enable $\eta$ and $\rho$ to be readily determined without the use of the interpolation formulae and when slight loss in accuracy is unimportant.

## INTRODUCTION

Recent consideration has been given to the physical properties of milk and cream at high temperatures, particularly to the flow characteristics and their relation to the degree of fat globule disruption which occurs when these fluids pass through a homogenizer. To identify laminar, transitional or turbulent flow requires a knowledge of a fluid's viscosity. With cream, the viscosity is highly dependent on the fat content and temperature but no definite relationships have been reported over the ranges of these variables appropriate to the present studies.

The form of the relation between the dynamic viscosity coefficient $\eta$ of an oil-in-water emulsion and the fraction $\phi$ of the dispersed oil phase is not immediately apparent, for although several equations have been proposed they have proved of limited application (Sherman, 1962). However, the experimental results of Leviton & Leighton (1936) on the viscous properties of emulsions containing milk fat are particularly relevant. An extension of an equation applicable to dilute emulsions and derived theoretically by Taylor (1932) was found to describe their results, viz:

$$\log_e \frac{\eta}{\eta_0} = 2 \cdot 5 \frac{\eta_f + 2\eta_0/5}{\eta_f + \eta_0} (\phi + \phi^{5/3} + \phi^{11/3}), \tag{1}$$

where $\eta_f$ and $\eta_0$ are the viscosities of the dispersed oil or fat phase and the continuous medium, respectively. The relation is essentially empirical and contains no terms to account for the deformation and interaction of fat globules (Schowalter, Chaffey &

Brenner, 1968). This equation was found to apply to milk-fat emulsions of various values of $\phi$ up to 0·365 (w/w) and with continuous media consisting of different concentrations of separated milk. Viscosity measurements were made at one temperature (64 °C) only, that is, with $\eta_f$ constant, but by varying the composition of the continuous phase, concomitant changes in $\eta_0$ were produced and confirmation was obtained of the validity of the factor $2\cdot5\,(\eta_f + 2\eta_0/5)/(\eta_f + \eta_0)$ deduced by Taylor.

Curvilinear relationships between the temperature and the viscosity of milk and cream may be deduced from the data in the literature but no single mathematical equation would appear to apply over wide ranges of temperature.

The present paper describes an investigation into the dependence of the viscosity and the density of cream upon its fat content over the range 0–50 % ($\phi = 0$–0·5) and upon temperature between 40 and 80 °C. These ranges were chosen so as to be comparable with those previously used in a study by Goulden & Phipps (1964) of factors affecting the homogenization process. The results are presented in the form of empirical equations from which viscosity and density values may be interpolated. In addition, the equations have been transformed into nomograms to enable rapid interpolations to be made.

### MATERIALS AND METHODS

*Cream samples.* Cream was separated from fresh, bulked milk from the Institute's farm and aliquots were immediately diluted and gently mixed with the separated milk in various proportions to give a series of cream samples of different fat contents ranging from 0 to about 50 %. These samples were analysed for fat content (w/w) by the Gerber method (British Standards Institution, 1955) and for total solids by a gravimetric method (British Standards Institution, 1963), the solids-not-fat (SNF) being obtained by difference. The SNF percentages of the various separated milks were rather higher than the average of 8·7 % for commercial supplies in England and Wales (Rook, 1961). However, these differences and the small influence of natural variations in SNF attributable to the season, breed of cow and other factors were ignored and no allowance was made in the equations that follow for variations in the composition of the serum component of the cream samples.

Immediately before determining its viscosity, each cream sample was de-gassed under reduced pressure at approximately the temperature selected for measurement; this treatment prevented gas bubbles from interfering with the measurements.

*Viscometer.* A Couette-type rotary viscometer was used (Fig. 1) consisting of 2 stainless steel coaxial cylinders, the outer one A (inside diam. 2·16 cm) capable of rotation and the inner one B (outside diam. 2·0 cm) suspended by a phosphor-bronze torsion wire C (40 swg); the annulus between the cylinders was filled with the fluid to be tested. Damping of the suspended cylinder could be controlled by adjusting the depth to which a thin, cylindrical Perspex 'skirt' D attached to the suspension was immersed in a static trough E of Silicone fluid; further control of damping was possible by altering the viscosity (about 10 cP) of the Silicone fluid. The 'skirt' also sealed off the interior of the viscometer and prevented continuous evaporation of the liquid under test. A pointer attached to the suspension and in register with a fixed circular scale F graduated in degrees indicated the angular deflexion of the inner cylinder upon rotation of the outer one.

The temperature of the viscometer was regulated by the thermostatically controlled, independently stirred, oil bath G and the adjustable air heater H. The latter was adjusted for each run to equalize the temperature of the air in the enclosed space and that of the liquid under test, thereby preventing condensation of liquid on the under surfaces of the Perspex disk J. Temperatures were measured with calibrated copper/constantan thermocouples of 40 swg wires and a vernier potentiometer (W. G. Pye Ltd., Cambridge). A thermocouple for measuring the temperature of the liquid was cemented with Araldite into the wall of the inner cylinder at about its midheight. The wire leads were led through a channel inside the cylinder, out of a hole in the stem and bent over to bring their bared ends close to the platform K.
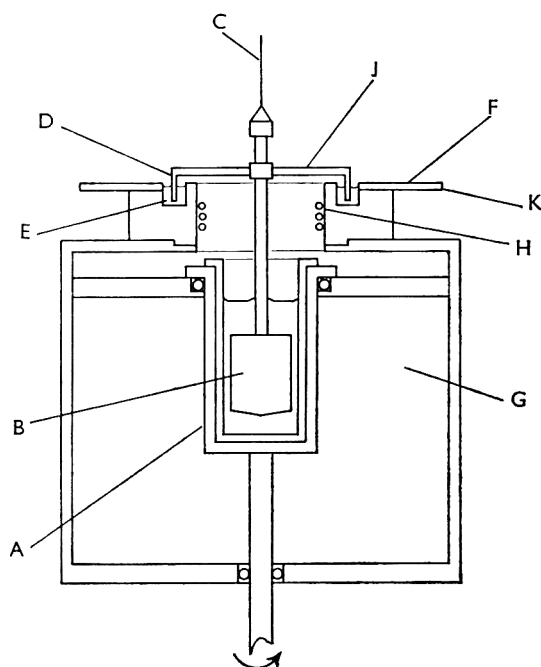


Fig. 1. Schematic diagram of viscometer. Not drawn to scale. A, Outer cylinder; B, inner cylinder; C, torsion wire; D, Perspex 'skirt'; E, trough; F, scale; G, oil-bath; H, air heater; J, Perspex disk; K, platform.

Temperatures could then be measured at any time that the inner cylinder was stationary, whether deflected or not, by connecting the wires to their appropriate extension leads from the potentiometer by special clips; the connexions produced no extraneous thermoelectric effects. The measurements of fluid temperature were considered accurate to $\pm 0.1$ °C.

Uniform speeds of rotation of the outer cylinder of 5–75 rev/min could be obtained giving a range of shear rates of approximately 7–100 sec$^{-1}$. Rotational speeds were given directly in rev/min on a TSA 3436 digital frequency meter (Venner Electronics, New Malden, Surrey) which was coupled to a photo-electric transducer device (Hird Brown Ltd., Bolton, Lancs) monitoring the speed of the drive-motor.

*Viscometer calibration and viscosity measurements.* The assembled apparatus was brought to thermal equilibrium at the temperature at which viscosities were to be

measured and calibrated by introducing de-gassed, pre-warmed, distilled water (8·5 ml) and measuring angular deflexions (degrees) of the suspension for several cylinder speeds; the experiment was repeated with a fresh sample of water. The temperature of the water in the viscometer was measured before and after each run; changes during a run with either water or cream were usually less than 0·25 °C.

For a deflexion $\alpha$ corresponding to a rotational speed $N$ the viscosity $\eta$ of the liquid under test was given by

$$\eta = K\alpha/N, \tag{2}$$

where $K$ is a constant embodying the torsional constant of the suspension wire and a factor which is a function of the geometry of the apparatus. With water, a Newtonian liquid, a plot of $\alpha$ versus $N$ at constant temperature was linear. $K$ was determined by substituting the measured slope (i.e. $\alpha/N$) of the best fitting straight line to these data into equation (2) together with the appropriate viscosity for water interpolated from tabulated values (Kaye & Laby, 1956). The average value of $K$ from the 2 experiments was taken to be the calibration constant. $K$ values were reproducible to within 1 % up to 60 °C and to 2 % at higher temperatures.

Calibration was immediately followed by measurements at the same temperature on each of the freshly separated, de-gassed creams. Since all cream samples showed Newtonian behaviour up to the maximum shear rate possible, a mean value of $\alpha/N$ was determined for each sample as before and the viscosity coefficients (in cP) determined from equation (2), using the known value of $K$. The mean of duplicate determinations of $\eta$ was obtained for each filling of the viscometer. Two sub-samples of each cream were tested and the 2 results for $\eta$ averaged to give the accepted value. The absolute accuracy of the measurements is difficult to assess but the variation between sub-samples of a cream was within ± 3·4 % with only 4 exceptions showing discrepancies up to 11 %. The viscosities of 7 or 8 cream samples were measured at each of the temperatures 40, 50, 60, 70 and 80 °C.

The customary precautions associated with this type of viscometry were taken to minimize errors.

*Density measurements.* The density of each de-gassed cream sample was determined, using pyknometers of 5 ml capacity, at the temperature at which the sample's viscosity was measured. Duplicate measurements were made and averaged.

<center>RESULTS AND DISCUSSION</center>

*Viscosity coefficients*

The combined effects of de-gassing and holding at high temperatures produced a slight 'oiling-off' of the fat in some cream samples but this seemed to have no influence on the results. By introducing pre-warmed cream into the thermally stabilized viscometer, the time for thermal equilibration after filling was kept short and effects due to the upward settling of fat were minimized. Since prolonged heating might change the cream's protein structure and affect its viscosity (Whitaker, Sherman & Sharp, 1927; Caffyn, 1951) measurements were made rapidly; if results were not obtained within about 10 min of assembling the apparatus, the experiment was abandoned.

Over the range of shear rates available all cream samples at all the temperatures

exhibited Newtonian behaviour, that is, the shear stress (proportional to deflexion $\alpha$) was directly proportional to the corresponding shear rate (proportional to rotational speed $N$). This contrasts with the non-Newtonian properties of cream of about 50 % fat content at 20 °C (Prentice, 1967). Experimental difficulties prevented viscosity measurements at the high rates of shear that can occur in a homogenizing valve but it would seem reasonable to assume Newtonian behaviour of cream in such circumstances, provided the temperature is greater than 40 °C and the fat thus in the molten state, and to regard the present viscosity values as appropriate at high shear rates, too.

Many trial fits, both graphical and numerical, were made of the data to equations considered likely to represent the interrelationship of $\eta$ and $\phi$ at constant temperature. The form of the empirical equation (1), omitting the term $\phi^{11/3}$ which gave an over-correction of curvature, was found to give an acceptable fit at all the temperatures provided $\phi < 0\cdot4$. The equation to the straight lines, therefore, may be written as

$$\log_{10}\eta = A(\phi+\phi^{5/3})+\log_{10}\eta_0, \tag{3}$$

where $\eta_0$ (cP) is the viscosity of the continuous medium, i.e. the separated milk. The slopes $A$ of the lines and the intercepts $\log_{10}\eta_0$ on the axis of $\log_{10}\eta$ ($\phi = 0$) are both
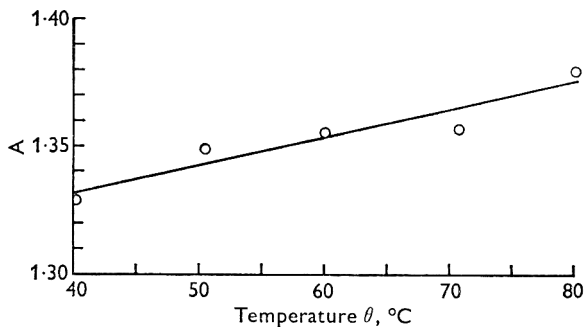


Fig. 2. Dependence of the coefficient $A$ in equation (3) on temperature.

functions of temperature. Regression analyses of the data obtained at each temperature showed $A$ to be linearly related to the temperature $\theta$ (Fig. 2), the equation to the regression line being given by

$$A = 1\cdot2876+11\cdot07\times10^{-4}\,\theta, \tag{4}$$

where $\theta$ is in deg C. The similarities of equations (1) and (3) suggest that their slopes might be related, but the magnitude and variations of $A$ in the present experiments appeared to be unrelated to those of the corresponding factor $2\cdot5\,(\eta_f+2\eta_0/5)/(\eta_f+\eta_0)$.

The values of log $\eta_0$ were found to be inversely proportional to temperature, and the regression line between 40 and 80 °C was calculated to be

$$\log_{10}\eta_0 = 0\cdot7687\,(10^3/T)-2\cdot4370, \tag{5}$$

where the temperature $T$ here is in °K. This equation represents the experimental data to rather better than $\pm0\cdot5\%$; it is also a very good representation of the

viscosity values for separated milk (8·96 % total solids) in the same temperature range given by Whitaker et al. (1927). It may be noted that, for a constant value of $\phi$, the variation of the right hand side of equation (3) with temperature is dominated by the variation in $\log_{10}\eta_0$ and as with this term, therefore, there is a linear dependence of $\log_{10}\eta$ for cream upon 1/temperature.

Clearly, the viscosity coefficient (in cP) of a cream at a chosen temperature may be determined by interpolating values for $A$ and $\log_{10}\eta_0$ with equations (4) and (5) and substituting these and the appropriate $\phi$ value into equation (3). The experimental results can be reproduced with the equations to within $\pm 3·6$ %.

There are only a few pertinent data in the literature with which to compare the above results. For milk, the data obtained by Caffyn (1951) on homogenized milk (no fat or SNF percentages given) have been considered. The effects upon the viscosity of milk of any fat clustering and of a reduction in fat globule sizes brought about by homogenization are likely to be very small at temperatures above 40 °C (cf. Leviton & Leighton, 1936, on cream) and may be neglected. Good agreement (within 3 %) was found between the viscosity values for milk computed with equation (3) at 40, 50,... 80 °C, assuming a fat content of 3·5 % ($\phi = 0·035$), and values determined with a polynomial expression (Cox, 1952) representing Caffyn's data. On the other hand, agreement is not so good in the case of the figure at 40 °C on whole milk given by Spöttel & Gneist (1941–3), which is 10 % lower than predicted by equation (3), while Tapernoux & Vuillaume's (1934) value at 40 °C is 4 % higher.

Comparisons with data on cream are less rewarding. The values for the viscosities of cream at different temperatures determined by Betscher (1960) must be highly suspect in view of the very large differences in the viscous behaviour of cream which he found with different instruments. All the viscosity values above 40 °C obtained with the one instrument that he favoured (Gardner Mobilometer) were very much lower for milk and cream of 10, 25 and 40 % fat content than would be predicted by equation (3). Examination of Leviton & Leighton's (1936) single set of results pertaining to normal cream shows that the continuous medium of skim-milk they used (no SNF % given) had a viscosity (0·644 cP) at 64 °C which is about 8 % lower than would be given by equation (5). By putting $\eta_0 = 0·644$ in equation (3) the anomaly is removed but, nevertheless, experimental and predicted viscosities show progressively worse agreement with increasing fat percentage. For example, relative to estimated values, their measured viscosities of cream containing 9·1, 18·3 and 36·5 % (w/w) fat are about 4, 7 and 16 % lower, respectively.

It would be difficult to find explanations for the numerical discrepancies revealed by these various comparisons but undoubtedly different experimental techniques would seem to play a large part.

*Density values*

Assuming an average and constant composition for the serum component of the cream samples, the density of a cream of fat content $\phi$ at a constant temperature will be given by

$$\rho = \rho_0 - (\rho_0 - \rho_f)\phi, \tag{6}$$

where $\rho_0$ and $\rho_f$ are the densities of the serum and the milk fat, respectively, appropriate to the temperature; at all temperatures considered $\rho_0 > \rho_f$. As in equation (3)

there are 2 temperature-dependent terms $(\rho_0 - \rho_f)$ and $\rho_0$ which can be shown to vary in a fairly simple way with temperature.

Linear plots of measured values of $\rho$ (g/ml) versus $\phi$ (w/w) at each temperature were obtained as predicted by equation (6). Scatter of the results about the best fitting lines was attributed mainly to small percentage changes in sample composition upon de-gassing and to the fact that the accuracy of the chemical analyses of the samples was inferior to that of the method employed for density measurements. Consequently, the slopes $(\rho_0 - \rho_f)$ of the lines over the limited range of $\phi$ values covered did not vary monotonically with temperature as might have been expected.
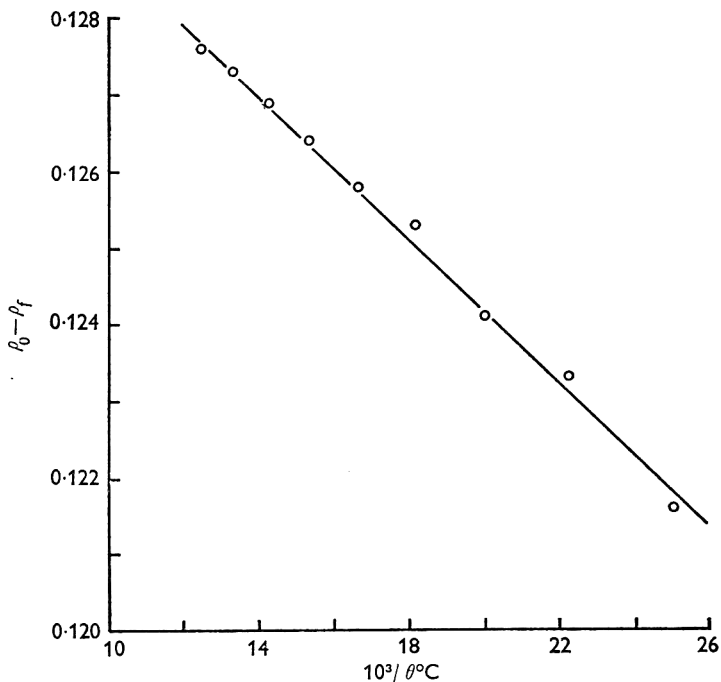


Fig. 3. Dependence of $\rho_0 - \rho_f$ in equation (6) on temperature.

However, by a very slight re-location of the lines so that they passed through the points representing the density values given by Whitaker *et al.* (1927) for $\rho_f$ at $\phi = 1\cdot0$ (by extrapolation) and $\rho_0$ at $\phi = 0$, convenient relations for $(\rho_0 - \rho_f)$ and $\rho_0$ were obtained.

A linear dependence of $(\rho_0 - \rho_f)$ on 1/temperature was found (Fig. 3) and being based on the results of Whitaker *et al.* (1927), their tabulated figures at intervals of 5 deg C have been used in calculating the following equation to the regression line

$$(\rho_0 - \rho_f) = 0\cdot1337 - 0\cdot4755/\theta, \tag{7}$$

where $\theta$ is in deg C.

Whereas $\rho_0$ is approximately proportional to $\theta$, a quadratic equation was found to fit the data more closely. The following relation was deduced using the method of least squares

$$\rho_0 = 1\cdot0382 - 0\cdot0017(\theta \cdot 10^{-1}) - 0\cdot0003(\theta \cdot 10^{-1})^2. \tag{8}$$

With this equation the figures of Whitaker *et al.* (1927) for separated milk can be reproduced to within $\pm 0.01\%$.

Using equation (6) in conjunction with equations (7) and (8), the experimental data for the densities (g/ml) of cream determined in the present investigation may be reproduced to within $\pm 0.45\%$.

*Nomograms*

Both equations (3) and (6) may be represented by nomograms of Class III, Genus 1 (Allcock & Jones, 1950), each of 3 loci comprising 2 parallel straight lines and a curve. These loci have been computed and are plotted in Figs 4 and 5. Thus, at the expense of a slight loss in accuracy the somewhat tedious calculations required to compute $\eta$ or $\rho$ when applying the equations may be avoided by a single alignment of a straight edge across the nomogram in the usual way to connect interrelated quantities.

A composite nomogram could be constructed to enable the kinematic viscosity $(\eta/\rho)$ to be read off, but this would require a multiple straight edge alignment and would be hardly more advantageous than separate determinations of $\eta$ and $\rho$ from Figs 4 and 5 and a calculation of their ratio.

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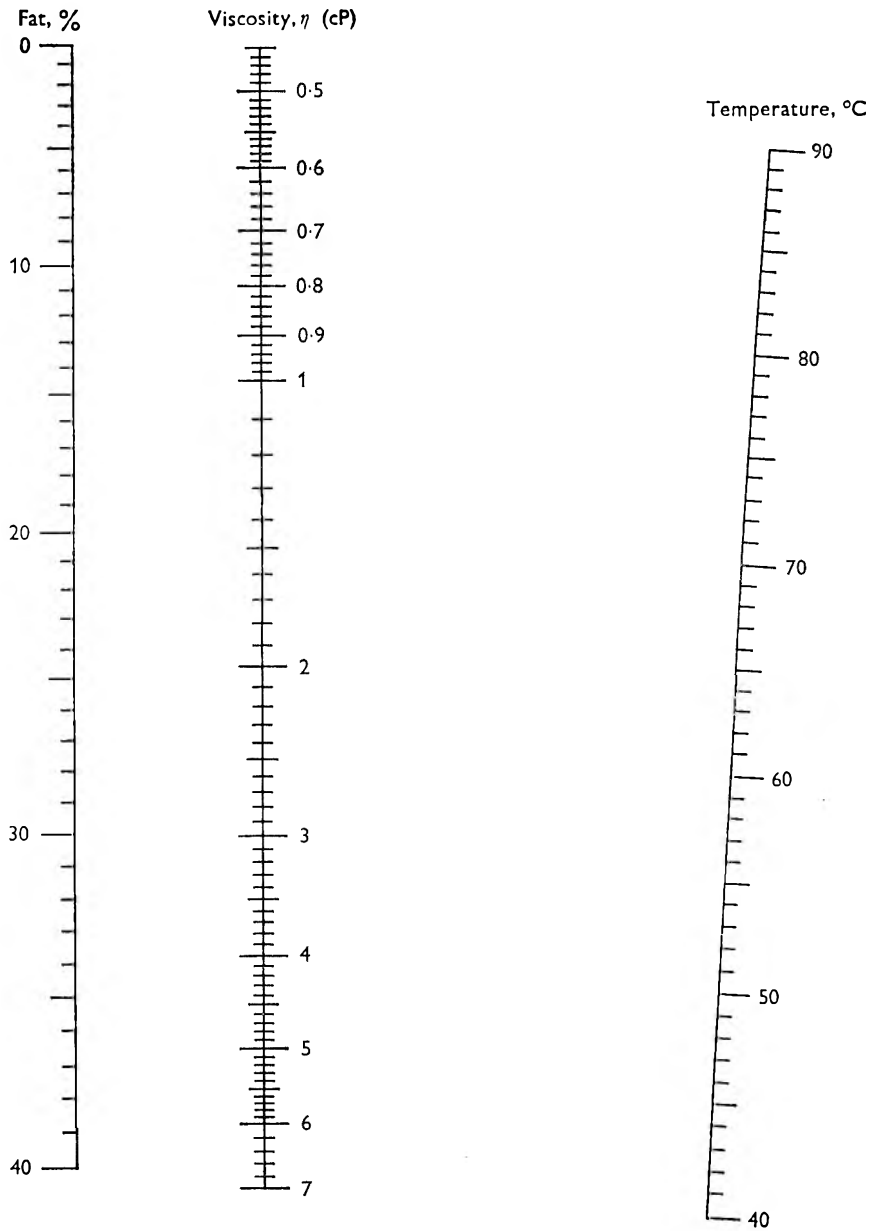
Fig. 4. Nomogram for computing viscosities of cream. A straight edge placed across the diagram passes through the 3 interrelated quantities, viscosity, fat percentage and temperature.
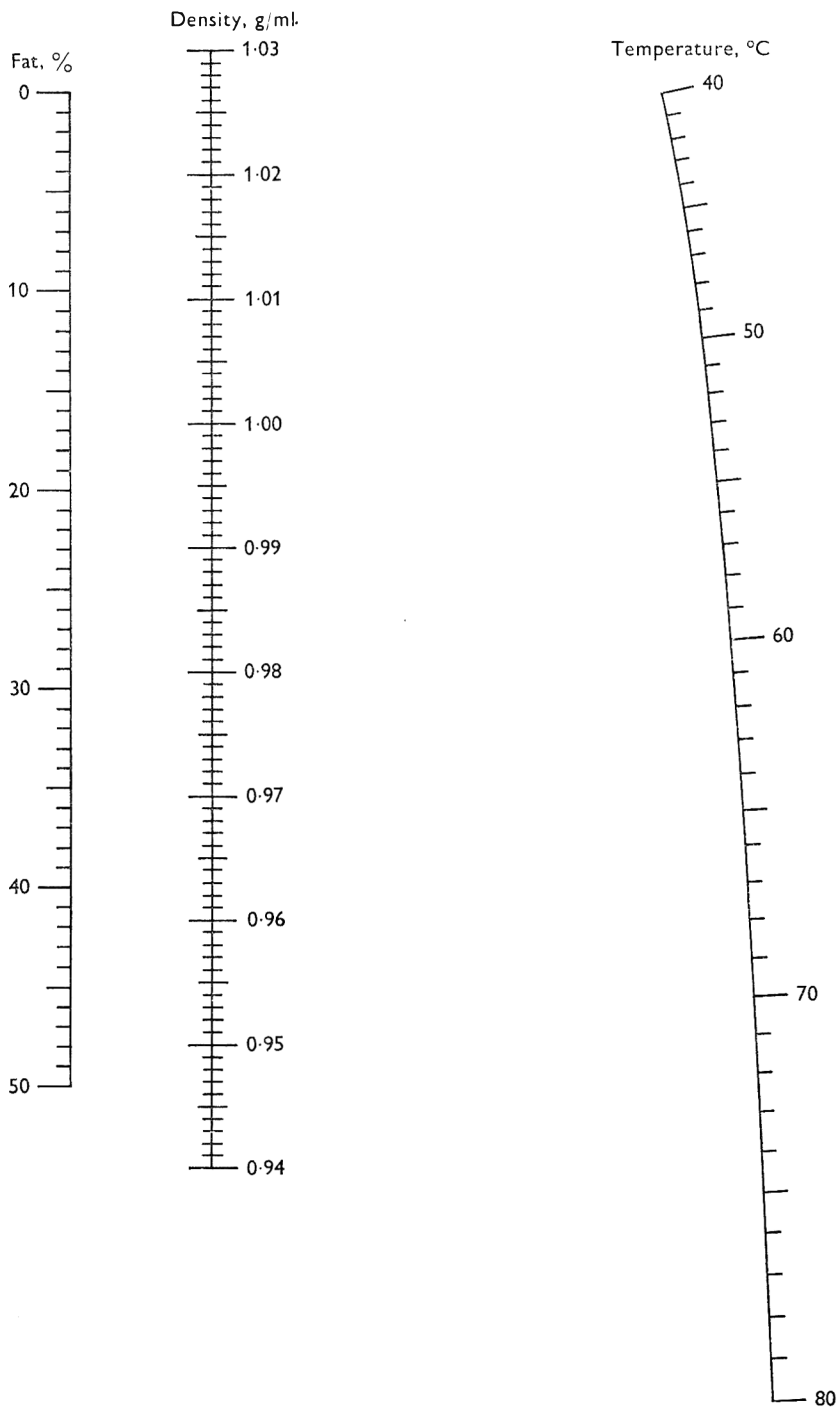
Fig. 5. Nomogram for computing the densities of cream. A straight edge placed across the diagram passes through the 3 interrelated quantities, density, fat percentage and temperature.

# Milk-clotting and proteolytic activities of rennet, and of bovine pepsin and porcine pepsin

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SUMMARY. The milk-clotting and proteolytic activities of rennet, bovine pepsin and porcine pepsin were compared. The milk-clotting activity of porcine pepsin was extremely pH-dependent around pH 6·6 and coagulation did not occur above pH 6·68. The clotting activity of bovine pepsin was slightly more dependent on pH than that of rennet but no rapid drop-off in activity occurred as with porcine pepsin. Temperature influenced the clotting activity of rennet and bovine pepsin similarly but the behaviour of porcine pepsin was markedly different.

For equal milk-clotting activity, the proteolytic activities of rennet and bovine pepsin were approximately equal and substantially lower than that of porcine pepsin. Electrophoretic examination showed that the proteolysis products of rennet and bovine pepsin were similar and quite different from those produced by porcine pepsin.

The suitability of bovine pepsin as a rennet substitute is discussed.

Rennet, extracted from the abomasa of young suckled calves, has traditionally been used for the coagulation of milk for cheese-making. The characteristic of rennet (rennin) which makes it so suitable for cheese manufacture is the high ratio of its milk-clotting to proteolytic activity. Rennin exhibits a high degree of specificity which results in its very limited ability to hydrolyse protein substrates (Fish, 1957; Bang-Jensen, Foltmann & Rombauts, 1964).

In recent years the supply of rennet has decreased while the demand for it has increased. Accordingly, scientists have been actively seeking an enzyme with high milk-clotting and low proteolytic activities which could be used satisfactorily as a rennet substitute. Most, if not all, proteases are capable of coagulating milk under the correct conditions but many break down the curd too quickly and too extensively. This does not apply to pepsin which, either alone but more frequently in admixture with rennet, has proved satisfactory and is now widely used in the manufacture of Cheddar and other varieties of cheese (Melachouris & Tuckey, 1964; Babel, 1967; Chapman & Burnett, 1968).

Pepsin replaces rennin as the principal gastric enzyme of the bovine within a relatively short time after birth. Although bovine pepsin in the form of 'rennet' from older calves or adult cattle has occasionally been used, the pepsin generally used in

cheese-making is that prepared from the stomach mucosa of the pig. It is reasonable to assume that bovine pepsin is at least as closely related to bovine rennin as is porcine pepsin. This paper reports the results of a preliminary study in which the milk-clotting and proteolytic activities of rennet, bovine pepsin and porcine pepsin were compared.

## EXPERIMENTAL

### Enzymes

*Rennet.* Hansen's commercial rennet was used, suitably diluted and standardized to give a coagulation time of about 6 min in milk at pH 6·6 and 32 °C.

*Porcine pepsin.* Powdered porcine pepsin (1:2500) obtained from British Drug Houses Ltd was used. It was dissolved in water at a concentration such that 50 $\mu$l of the resultant solution coagulated 5 ml of milk at pH 6·6 in about 6 min at 32 °C.

*Bovine pepsin.* A crude preparation of bovine pepsin was prepared by the following procedure: the mucosal lining of a washed bovine stomach was removed and extracted in a 10 % NaCl solution for 3 days at 3 °C. Insoluble material was removed by filtration. The pH of the filtrate was adjusted to 4·0 at which a heavy gelatinous precipitate formed and was removed by centrifugation. The supernatant was held overnight in the cold to activate the pepsinogen. The pH was then adjusted to 6·0 and the solution made to 25 % saturation with $(NH_4)_2SO_4$. The small amount of inactive precipitate which formed was removed by centrifugation and discarded. The concentration of $(NH_4)_2SO_4$ in the supernatant was increased to 50 % saturation. The precipitate which formed contained most of the milk-clotting activity. It was recovered by centrifugation, dissolved in 0·02 M phosphate buffer of pH 6·0, dialysed against the same buffer until free from $(NH_4)_2SO_4$ and stored at $-20$ °C. As yet, no attempt has been made to quantify the recovery obtained by this procedure.

### Measurement of milk-clotting activity

Fresh, skimmed herd milk was used. Unless otherwise specified, the pH of the milk was adjusted to 6·6 if necessary. Coagulation times were determined in a thermostatically controlled waterbath using a rocking device similar to that used by Sommer & Hart (1919).

### Measurement of proteolytic activity

Sodium caseinate, buffered in 0·1 M sodium phosphate, was used as substrate throughout. The caseinate was pasteurized at 60 °C for 30 min and saturated with toluene as a preservative. The enzymes were added at the levels described above, i.e. sufficient to give coagulation times of about 6 min in milk at pH 6·6 and 32 °C. Proteolytic activity at pH 5·5 and 32 °C was measured by changes in the nitrogen soluble in 2 % trichloroacetic acid (TCA), determined by the micro-Kjeldahl method (NPN). Changes in the electrophoretic patterns of casein in polyacrylamide gels were also used as an index of proteolysis.

### Gel electrophoresis

A vertical-gel electrophoresis apparatus (E. C. Apparatus Corp., 220, S. 40th Street, University City, Philadelphia, Pennsylvania) was used. Electrophoresis was carried out as described by Thompson, Kiddy, Johnston & Weinberg (1964). Samples

for electrophoresis were prepared by mixing equal volumes of incubated reaction mixture and 7 M urea sample buffer. Twenty microlitre samples were applied to the gel.

<div align="center">RESULTS</div>

<div align="center">*Effect of substrate pH*</div>

The pH-dependence of the milk-clotting activity of rennet, bovine pepsin and porcine pepsin is shown in Fig. 1. In agreement with the results of earlier workers (Tsugo & Yamauchi, 1959; Ernstrom, 1961), porcine pepsin failed to coagulate milk at pH values above 6·68. The milk-clotting activity of bovine pepsin was somewhat
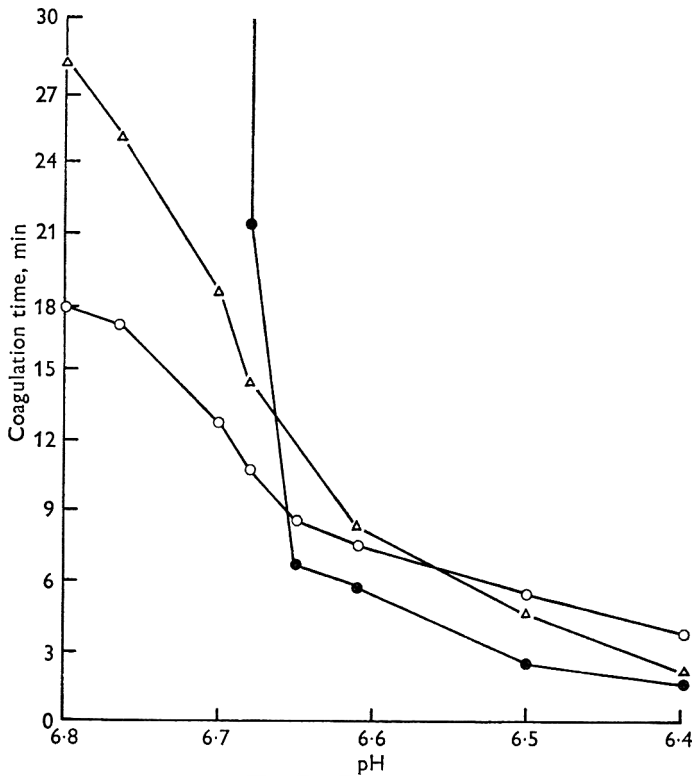


Fig. 1. The influence of pH on the milk-clotting activity at 32 °C of: rennet, ○; bovine pepsin, △; and porcine pepsin, ●.

more pH-dependent than was that of rennet but no sharp cut-off point like that for porcine pepsin was observed. Bovine pepsin was capable of coagulating milk at a fairly rapid rate up to pH 6·9.

<div align="center">*Effect of temperature*</div>

The incubation temperature influenced the milk-clotting activity of rennet, bovine pepsin and porcine pepsin at pH 6·55 as shown in Fig. 2. Activities at temperatures below 32 °C are not shown as the 3 enzymes behaved similarly below this temperature. It is apparent that rennet and bovine pepsin responded in a very similar manner to

changes in the incubation temperature but the behaviour of porcine pepsin was very different. At pH 6·55, porcine pepsin was inactive at 48 °C and above, whereas rennet and bovine pepsin were active at temperatures up to 55·6 °C.

The temperature at which the 3 enzymes became inactive was dependent on the pH of the substrate, an inverse relationship applying (Table 1). The inactivation temperature of porcine pepsin was more pH-dependent than that of the other 2 enzymes, which were quite similar.
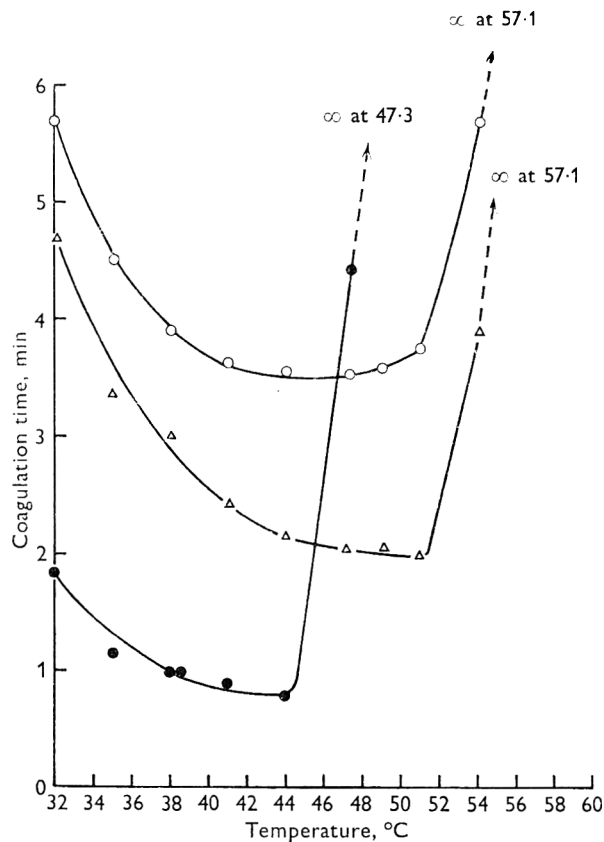


Fig. 2. The influence of temperature on the milk-clotting activity at pH 6·6 of: rennet, ○; bovine pepsin, △; and porcine pepsin, ●.

Table 1. *Effect of substrate pH on the temperature-inactivation of the milk-clotting activity of rennet, bovine pepsin and porcine pepsin*

| | Temperature at which the enzymes became inactive, °C | | |
|---|---|---|---|
| pH | Rennet | Bovine pepsin | Porcine pepsin |
| 6·65 | 52·5 | 48·8 | 39·9 |
| 6·55 | 55·6 | 55·6 | 48·2 |
| 6·45 | 59 | 57·6 | 52·6 |

### Proteolytic activity

A low proteolytic activity to milk-clotting ratio is an essential requirement in an acceptable rennet substitute. The proteolytic activity of rennet, bovine pepsin and porcine pepsin—standardized to equal milk-clotting activity at pH 6·6 and 32 °C—on casein at pH 5·5 and 32 °C are summarized in Fig. 3. Rennet showed the least proteolytic activity. Bovine pepsin was more active than rennet initially, but after about 6 h both enzymes produced NPN at approximately equal rates. Porcine pepsin was very active initially and continued to liberate NPN at a faster rate than either of the other 2 enzymes during the 48-h incubation period.
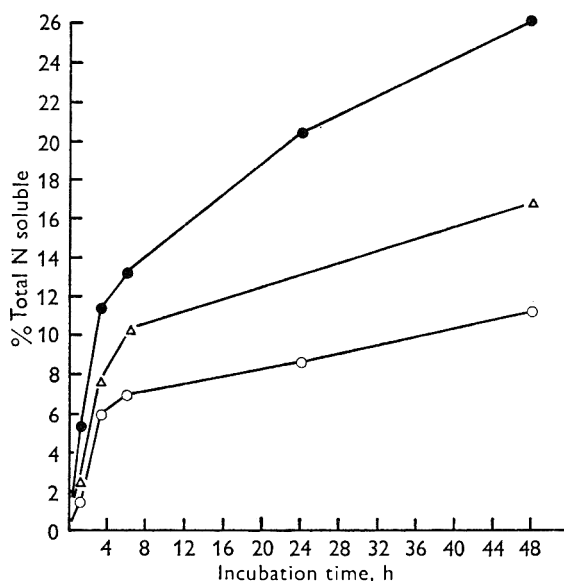


Fig. 3. The proteolytic activity on casein at pH 5·5 and 32 °C of: rennet, ○; bovine pepsin, △; and porcine pepsin, ●.

Electrophoretic examination of enzyme-treated casein confirmed that for equal milk-clotting activity at pH 6·6 rennet and bovine pepsin degraded casein to approximately the same extent in 24 h at pH 5·5, but porcine pepsin caused much more extensive degradation, especially of the $\beta$-casein (Plate 1). Bovine pepsin and rennet did not produce identical polypeptides in all cases but the differences were fairly minor at pH 5·5. At pH 6·5 bovine pepsin was considerably more active than rennet but this is of little importance in cheese ripening, where the pH value is normally 5·5 or below.

### DISCUSSION AND CONCLUSION

Porcine pepsin, usually in admixture with rennet, is now widely used in the manufacture of Cheddar cheese. It is accepted that a half and half mixture of rennet and pepsin produces a satisfactory cheese (Melachouris & Tuckey, 1964; Babel, 1967; Chapman & Burnett, 1968). Bovine pepsin has received little attention in recent years. It is generally accepted that it produces a cheese of inferior quality to rennet

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(Davis, 1965) but the relative merits of bovine and porcine pepsin for cheese-making have apparently not been investigated. Neither has the possibility of using mixtures of rennet and bovine pepsin been studied in detail.

The results of this investigation show that bovine pepsin resembles rennet much more closely than does porcine pepsin in many characteristics of importance for cheese-making. The extreme pH-dependence of the milk-clotting activity of porcine pepsin makes it unsuitable for the coagulation of milk of even moderately high pH and this is one of the reasons why mixtures of rennet and porcine pepsin, rather than pepsin alone, are used in cheese-making. From casual observation, the pH of bulk herd milk from Irish farms is frequently 6·8 and occasionally values of 6·85 are observed. At these pH values porcine pepsin contributes nothing to the clotting activity of rennet-pepsin mixtures and consequently larger quantities of the half and half mixture are required if an unaltered cheese-making schedule is to be maintained. As the pH decreases during cheese-making the pepsin becomes active and excessive proteolysis is to be expected. The clotting activity of bovine pepsin was influenced more by the pH of the substrate than was the clotting activity of rennet. However, while coagulation by bovine pepsin may be slowed somewhat at higher pH values, coagulation will occur at any pH which might normally be encountered in bulk milk. Mixtures of rennet and bovine pepsin, say 50:50 or 75:25, would approach the pH-dependent characteristics of rennet even more closely.

The temperature-dependence of the clotting activity of the 3 enzymes (Fig. 2) has little practical significance but it provides further evidence that bovine pepsin is more closely related to rennet than is porcine pepsin. This is particularly well demonstrated by the manner in which the inactivation temperature of the enzymes varies with the pH of the substrate (Table 1).

The most important finding to emerge from this investigation is that bovine pepsin had a lower proteolytic to milk-clotting ratio than porcine pepsin. In fact, the ratio for bovine pepsin was not very different from that for rennet. The present data are in disagreement with the results of Melachouris & Tuckey (1964) and of Babel (1967), who found that cheese made with pepsin had lower NPN values than cheese made with rennet. These differences may be explained by the fact that Melachouris & Tuckey standardized the enzymes at pH 6·3 and Babel at pH 6·5, whereas in the present study the enzymes were standardized at pH 6·6. It might be argued that the conditions employed were too severe on porcine pepsin but these conditions are reasonable if the conventional Cheddar cheese-making process is to be unaltered (Ernstrom, 1961). Moreover as the pH is increased from 6·4 to 6·6 the milk-clotting activity of bovine pepsin decreases at a slightly faster rate than that of porcine pepsin and the latter should therefore gain more by standardization at a lower pH but possibly the differences are hardly significant.

Electrophoretic examination of casein following incubation with each of the 3 enzymes sheds even more favourable light on bovine pepsin (Plate 1). Although not identical, the proteolysis products produced by bovine pepsin resembled those produced by rennet more closely than did those produced by porcine pepsin. The differences between the products produced by rennet and bovine pepsin may or may not be significant. The ability of porcine pepsin to degrade $\beta$-casein more quickly than either of the other 2 enzymes may be significant, as many enzymes which are very proteo-

lytic and unsuitable for cheese-making, e.g. ficin, papain, *Bacillus subtilis* protease, Olocast, etc. tend to degrade β-casein very quickly (Fox, 1968, 1969 unpublished) while rennet degrades β-casein slowly and only to a limited extent (Lindqvist & Storgårds, 1962; Ledford, O'Sullivan & Nath, 1966; Fox, 1969). It might be inferred that inability to degrade β-casein extensively is an essential characteristic of rennet and suitable rennet substitutes.
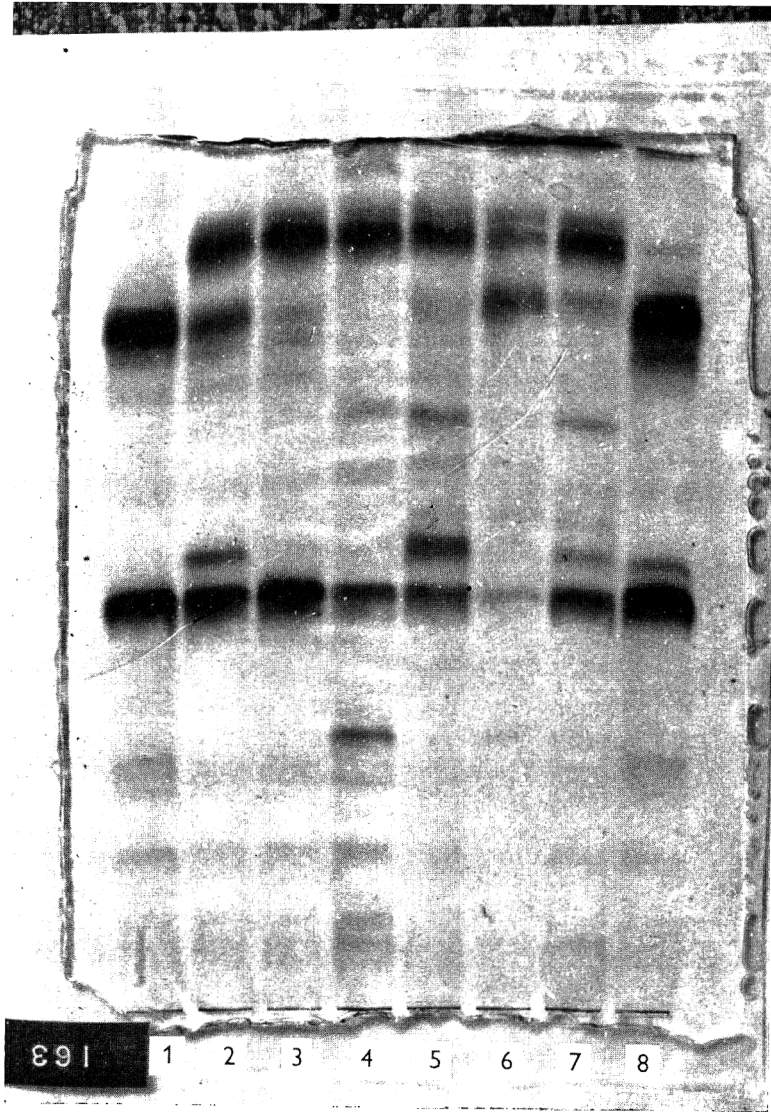
In the final analysis only a detailed comparison of cheese made with porcine pepsin and bovine pepsin will enable one to decide which is the better rennet substitute. However, the data presented in this paper indicate that bovine pepsin resembles rennet more closely than does porcine pepsin in many important characteristics. Other aspects, such as the ease and cost of production or stability on storage, may be the deciding factors but it is concluded that bovine pepsin deserves careful investigation as a rennet substitute.

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## EXPLANATION OF PLATE

Gel electrophoretograms of whole casein following proteolysis by rennet, bovine pepsin or porcine pepsin at pH 6·5 or 5·5 at 32 °C for 24 h. Slots 1 and 8, whole casein controls; 2 and 5, rennet, pH 6·5 and 5·5, respectively; 3 and 6, porcine pepsin, pH 6·5 and 5·5, respectively; 4 and 7, bovine pepsin, pH 6·5 and 5·5, respectively.

# The folate-binding protein in milk

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Summary. The folate in cow's milk was strongly and specifically bound to a minor whey protein (FP), forming a complex of primary M of about 38000, but exhibiting concentration-dependent reversible aggregation. The binding protein was present in excess, and the milk had the capacity to bind about 50 $\mu$g added folic acid/l. An enriched concentrate of FP was prepared by ammonium sulphate fractionation—FP was precipitated at between 50 and 60 % saturation—and further purified by chromatography in DEAE-cellulose and filtration in Sephadex gel G150. Its identity as a distinct minor whey protein was confirmed by comparative starch gel electrophoresis at various pH values.

Some properties of the protein are described, and its physiological significance discussed.

The folate in cow's milk occurs predominantly as $(-)N^5$-methyl-tetrahydrofolate, as judged by chromatography and differential microbiological assay. Ford & Salter (1967) fractionated the proteins of cow's milk by filtration in Sephadex gel G75 and found that the folic acid activity was associated with the fractions containing the $\beta$-lactoglobulin peak. The folate in human milk was associated with 2 distinct protein fractions, one of which was apparently similar to that in cow's milk. Salter & Ford (1968) later reported that the folate in cow's milk is specifically bound to a minor whey protein. On lowering the pH of the whey to 5·0 some free folate was present, and at pH 3·6 the folate-protein complex was wholly dissociated; on restoring the pH to 7·0 the folate and protein recombined. Heating for 10 min at 100 °C released the folate and destroyed the binding activity. Ghitis (1966, 1967) found that the folate in cow's milk did not adsorb to charcoal unless the milk had been previously boiled or autoclaved, and concluded that the vitamin was bound to a large molecule. He distinguished 2 different kinds of folate binding, and demonstrated that cow's milk has the capacity to bind added folic acid. Metz, Zalusky & Herbert (1968) and Ghitis, Mandelbaum-Shavit & Grossowicz (1969) described a technique for the separation of free and protein bound folate by the use of charcoal 'coated' with dextran or polyvinyl pyrrolidone, and employed it to investigate further the specificity of the folate binding.

Ford, Salter & Scott (1969) outlined a method for the partial purification of folate-protein from rennet whey. The present paper gives a fuller account of the procedures employed and describes some properties of the complex.

## MATERIALS AND METHODS

*Radioactive folates.* Folic acid-2-$^{14}$C, potassium salt, was purchased from the Radiochemical Centre, Amersham. Assay with *Lactobacillus casei* showed the biological activity to be 70 % of the stated folic acid potency. $^{14}$C($\pm$)$N^5$-methyl-tetrahydrofolic acid was kindly given to us by Dr M. A. Foster of the Department of Biochemistry, Oxford. It was formed by catalytic reduction of methyl-folate and was a mixture of ($+$) and ($-$)-stereo isomers. Only one of the isomers is biologically active, and the preparation had for *L. casei* 37·5 % of the activity of an equivalent weight of folic acid.

*Milk.* The cow's milk was bulked evening and morning milk from the Institute herd of Friesian cows. It was stored at 4 °C and processed as soon as possible after milking.

Samples of human milk were obtained from 4 mothers of babies aged about 6 weeks. The samples (about 25 ml) were obtained by manual expression at the time of the baby's feeding, usually in the morning. They were collected into 1-oz McCartney bottles in each of which had been freeze-dried 1 ml tris-ascorbate buffer of pH 7·3, containing 8 mg ascorbic acid. The bottles were filled brimful with milk and tightly stoppered, and stored overnight at 4 °C.

*Preparation of the albumin fraction.* The method used was that of Aschaffenburg & Drewry (1957), and it will be helpful to describe it briefly. Cow's milk was warmed to 40 °C and anhydrous sodium sulphate added slowly, with stirring, to a concentration of 20 g/100 ml. A precipitate formed which contained the cream, the casein and the immunoglobulins (Aschaffenburg & Drewry, 1955). The mixture was allowed to cool to room temperature and filtered through cheesecloth, and the filtrate further clarified by centrifugation. Ammonium sulphate (AR) was then added, with stirring, to a concentration of 20 g/100 ml filtrate. The precipitate which formed was recovered by filtration and dissolved in the minimum volume of 0·05 M-sodium phosphate buffer of pH 7·0, containing 50 mg ascorbic acid/l. The solution was freed from sulphates by dialysis for 24 h at 5 °C against 0·02 M-glycine that had been adjusted to pH 7·5 by addition of ammonia, and finally freeze-dried. The overall recovery of milk folate was only 19 %; 73 % was lost in the 20 % sodium sulphate precipitate.

*Preparation of the rennet whey fraction.* To 10 l. cow's milk at 37 °C were added 2 ml 2-mercaptoethanol (ME) and enough orthophosphoric acid solution (10 %, v/v) to lower the pH to 6·2. Commercial cheese-making rennet (1 ml) was then added and the mixture incubated for 30 min at 37 °C, with occasional mashing to break up the curd. The temperature was then raised to 45 °C and the whey filtered off through cheesecloth and cooled to 5 °C. Virtually all the milk folate was recovered in this filtrate. The whey was brought to pH 7·0 by addition of 4 N-sodium hydroxide and to it was added cold, saturated ammonium sulphate solution of pH 7·0, to 45 % saturation. A precipitate appeared and on standing it formed a surface layer which was removed and discarded. A further 2 ml ME were then added, and ammonium sulphate to 60 % saturation. The mixture was allowed to stand overnight at 5 °C and centrifuged. The residue was dissolved in 150 ml 0·005 M-sodium phosphate buffer of pH 7·2 containing 0·2 ml ME/l, and dialysed for 7 and 16 h against successive 10-l volumes of the same buffer.

The overall recovery of folate at this stage was about 70 %. Figure 1 shows that

nearly 80 % of the folate activity of rennet whey was precipitated at ammonium sulphate saturation of between 50 and 60 %.

In some preparations the ME was replaced by ascorbic acid (1 g AA/ml ME) added as tris-ascorbate solution of pH 7·2.

*Fractionation of whey proteins in anion exchange cellulose.* The general procedures employed were those described by Yaguchi, Tarassuk & Hunziker (1961). The whey preparations were dissolved and dialysed against 0·01 M-sodium phosphate buffer of pH 7·0 containing 50 mg ascorbic acid/l. The dialysed solution was applied to a column of DEAE-cellulose (Whatman, grade DE 52) and fractionated by gradient elution, or sometimes by stepwise elution, with NaCl in the 0·01 M-phosphate buffer.
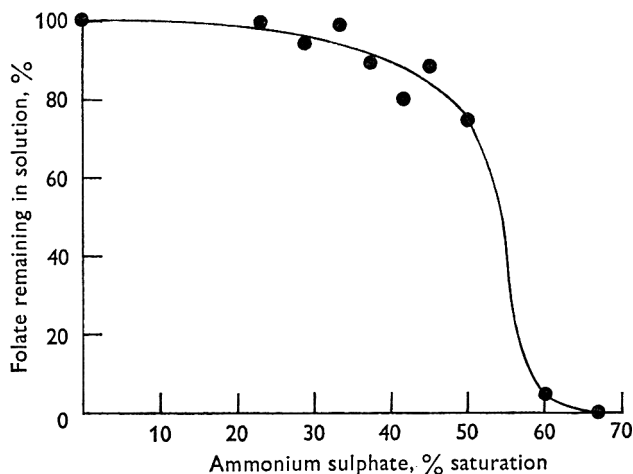


Fig. 1. Precipitation of folate-protein with ammonium sulphate.

Two columns were used. In one the cellulose bed measured 13 cm in length and 5 cm in diam., and in the other 45 × 2·5 cm. The shorter column was used for most of the preparations. The longer column gave better resolution and was used in the analysis of the albumin fraction (see Fig. 4).

*Filtration in Sephadex gel.* Filtration in Sephadex gel was employed for the separation and identification of whey proteins and also for the separation of free and protein-bound folate. Four grades of Sephadex—G 25, G 75, G 100 and G 150—were used in the conventional column fractionation procedure. The columns were eluted with buffer of pH 7·2 containing 0·15 M-NaCl and 0·02 M-sodium phosphate (buffer A), or 0·2 M-sodium phosphate, and 200 mg ME or ascorbic acid/l. Fuller details are given in the legends to the Figures.

*Determination of protein in the effluent fractions.* The fractions were monitored for protein by measurement of absorbance at 280 nm. In addition, a 1 ml portion of each fraction was taken and heated in a steam autoclave with 2 ml of 6 N-HCl for 3 h at 120 °C. To each hydrolysate were added 2·4 ml 4 M-NaOH and 1 ml of 4 M-sodium acetate buffer solution of pH 5·5, and finally water to 10 ml. A 1-ml portion was then taken for the estimation of amino N, by reaction with the modified ninhydrin reagent of Moore & Stein (1954). A standard curve was prepared with graded

concentrations of leucine and the protein content of the fractions was expressed in terms of 'leucine equivalent'.

*Measurement of folic acid activity.* Folic acid activity was assayed microbiologically with *L. casei*, by an adaptation of the procedure recommended by Herbert (1961) for the assay of folate in blood serum. Extracts of milk were made as follows. To each 2-ml sample of milk, in a $15 \times 150$ mm test tube, was added 1 ml of buffer solution, made by dissolving 1 g ascorbic acid in 100 ml of $1 \cdot 42 \% Na_2HPO_4$ solution and adding 4 N-NaOH to pH 7·8. The tubes with their contents were heated in a bath of boiling water for 2 min and cooled in cold water. To each was then added 1 ml of an extract of chicken pancreas, made by gently triturating 200 mg desiccated chicken pancreas (Difco Laboratories Inc., Detroit, U.S.A.) in 40 ml of ice-cold phosphate-ascorbate buffer and filtering through a plug of glass wool in the stem of a funnel. The tubes, containing milk samples and added enzyme, were incubated for 2 h in a water bath at 45 °C. The contents were then acidified to pH 4·8 by addition of 0·1 N-HCl, diluted with water to 50 ml and filtered through Whatman No. 42 paper. The filtrates were readjusted to neutral pH and further diluted as needed for test.

With the column effluent fractions the same extraction procedure was used, except that with fractions of low folate content a smaller amount of chicken pancreas extract was added, in order to reduce the 'enzyme blank' in the assay. With these more highly refined test preparations the filtration at pH 4·8, designed to clarify the milk extracts, was unnecessary and was omitted.

*Starch gel electrophoresis.* Starch gels were prepared from hydrolysed starch obtained from Connaught Medical Research Laboratories, University of Toronto, Canada. Horizontal electrophoresis was carried out using thin layers of the gel, 1·5 or 3·0 mm thick, 17·5 cm long and 9 cm wide, prepared as described by Aschaffen-burg & Thymann (1965). The following buffers were employed: (1), borate buffer of pH 9·0, containing 0·0230 M-boric acid, 0·0092 M-sodium hydroxide, and ascorbic acid 50 mg/l; (2), formic-acetic acid buffer of pH 2·0, containing 120 ml acetic acid and 30 ml formic acid/l; (3), acetate buffer of pH 5·0, ionic strength 0·1, containing 150 ml M-acetic acid and 100 ml M-sodium hydroxide/l. For electrophoresis in the presence of urea the gel contained 10 g starch, 12 g urea, 40 ml buffer and 2 drops ME. For some experiments the borate buffer was used in the absence of urea and ME, and the gel contained 12·5 g starch/100 ml buffer. Freeze-dried protein preparations were dissolved in the appropriate buffer. Pieces of Whatman No. 3 filter paper ($9 \times 1$ mm or $9 \times 2$ mm) were soaked in the protein solution and then inserted into a slit cut across the width of the gel 6 cm from one end. The best separations were obtained with a potential difference of 100 volts for 16–18 h at 4 °C.

Protein was detected by staining with amido black-nigrosine (amido black 0·25 g, nigrosine 0·5 g, water 800 ml, methanol 1000 ml, acetic acid 200 ml), followed by washing with methanol-acetic acid-water ($100:20:100$ by volume). The folate-binding activity was located by cutting the gels transversely into sections 0·5 cm in length and eluting with 3 ml portions of electrophoresis buffer. A 2·0 ml portion of each eluate was dialysed against buffer (containing, per litre, 3 g $K_2HPO_4$, 9 g NaCl, 0·5 ml ME and enough $H_3PO_4$ to lower the pH to 7·0) to remove urea and bring the pH value to 7·0. A quantity of $^{14}$C-folic acid estimated to be equal to the binding capacity of the protein was added to the solution in each dialysis sac, and dialysis was continued

to remove unbound folic acid. Residual $^{14}$C-folic acid in the sac contents was measured by liquid $\beta$-scintillation counting using the internal standard technique, and also by microbiological assay.

*Reference proteins.* $\beta$-Lactoglobulins 'A' and 'B', $\alpha$-lactalbumin, bovine $\alpha_1$-globulins (Cohn fraction IV$_1$), bovine $\alpha_4$-globulins (Cohn fraction IV$_4$), transferrin from pooled bovine plasma, and ovalbumin, were obtained from Koch–Light Laboratories Ltd, Colnbrook, Bucks, England; bovine $\beta$-globulins (fraction III, B grade), from Cal-biochem, Los Angeles, U.S.A.; bovine $\gamma$-globulin (fraction II), from Armour Pharma-ceutical Co. Ltd, Eastbourne, England; crystalline bovine serum albumin, from Sigma Chemical Co., 3500 De Kalb Street, St Louis 18, Missouri, U.S.A.; and whole skeletal muscle myoglobin from Seravac Laboratories (Pty) Ltd, Moneyrow Green, Holyport, Maidenhead, Berks, England.
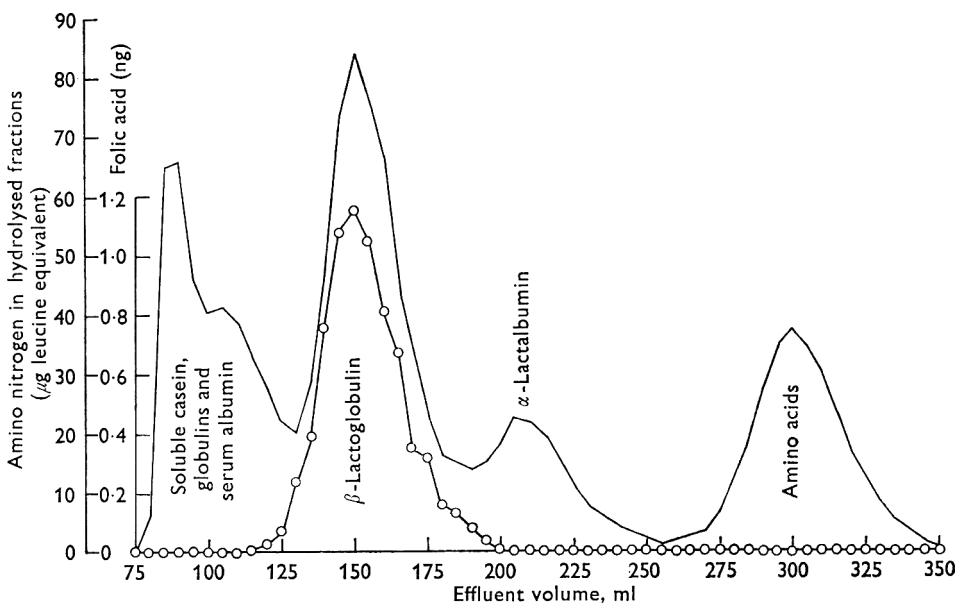


Fig. 2. Filtration of cow's milk freed from micellar casein and fat, in Sephadex G75.
———, Amino nitrogen; ○—○, folate activity (*L. casei*).

## RESULTS AND DISCUSSION

*Filtration of milk in Sephadex gel G75.* Cow's milk (30 ml) was centrifuged at $40\,000 \times g$ for 2 h at 2 °C. A deposit of micellar casein was formed, and a surface layer of solidified milk fat. Both were separated from the aqueous phase by decantation followed by filtration through Whatman No. 42 filter paper. A 5-ml portion of the filtrate was applied to a $58.5 \times 2.6$ cm column of Sephadex gel G75 and eluted with sodium chloride-phosphate buffer (p. 437). Ten ml fractions were collected and analysed for folate activity and protein content. Figure 2 shows the results obtained in a typical run. All the folate activity was associated with the $\beta$-lactoglobulin peak, indicating an association between the milk folate and a protein of M about 38000.

Figure 3 shows the results obtained for the human milk. The large $\beta$-lactoglobulin

peak was not present here, but a peak of folate activity was again measured in the same effluent fractions as in the cow's milk. A second peak of folate activity was eluted in the void volume, showing the presence of folate in association with protein of high M, probably > 75000. As in the cow's milk, no free folate was detected.

*Chromatography of the whey protein preparations in anion exchange cellulose.* A preparation of albumin fraction (p. 436) was applied to a 45 × 2·5 cm column of DE 52 and eluted with graded concentrations of NaCl in 0·02 M-sodium phosphate buffer of pH 7·0. The results are shown in Fig. 4. All the folate activity moved with a minor protein peak near the solvent front, at near-zero NaCl concentration.
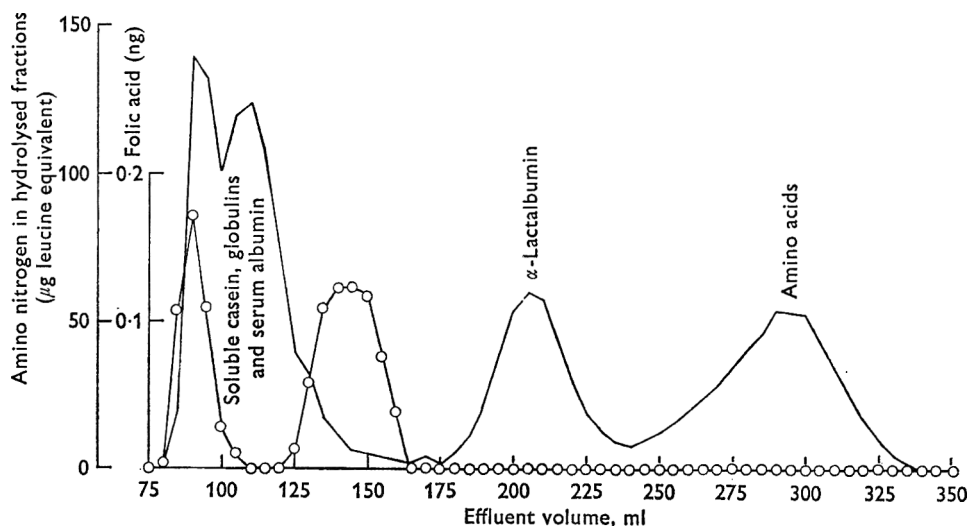


Fig. 3. Filtration of human milk freed from micellar casein and fat, in Sephadex G 75.
———, Amino nitrogen; ○———○, folate activity (*L. casei*).

Closer examination by chromatography with 0·005 M-phosphate showed this peak to be made up of 2 components—the front absorbing strongly at 420 nm and the later fractions absorbing only weakly at this wavelength. Szuchet-Derechin & Johnson (1965) observed this same peak in a chromatograph of the bovine milk albumin fraction. They identified it as a red, iron-containing protein and later resolved it into several component fractions (Szuchet-Derechin & Johnson, 1966).

A broadly similar elution pattern was obtained for the rennet whey preparations chromatographed in the 13 × 5 cm column, and here again the folate activity was associated with the first protein peak.

Further investigation was confined to this folate-protein peak obtained from the rennet whey preparation (RW-DE 52).

*Filtration of folate-protein in Sephadex-gel G 150.* Fractions containing the folate protein peak from a DE 52 column run were combined and freeze-dried, and then redissolved in 2 ml of buffer A. The solution, which contained 5 μg folate, was fractionated in a 51·0 × 2·6 cm column of Sephadex gel G 150 that had been calibrated with marker proteins of known molecular weight. The fractions were assayed for folate and for protein content, and those containing the folate peak were combined. A sample containing 0·05 μg folate was taken and the remainder was freeze-dried.

The sample was diluted to 2 ml and was then again fractionated in the G150 column. The results are shown in Fig. 5. The folate activity peak from the first column emerged in an effluent volume of about 140 ml, from which a M of about 100000 for the folate-protein was calculated. This contrasts with the value of about 38000 obtained for the folate-protein in fresh milk (Fig. 2). The peak was markedly unsymmetrical, and the slow decline in folate activity from the peak value suggested reversible concentration-dependent aggregation of the folate-protein complex. On
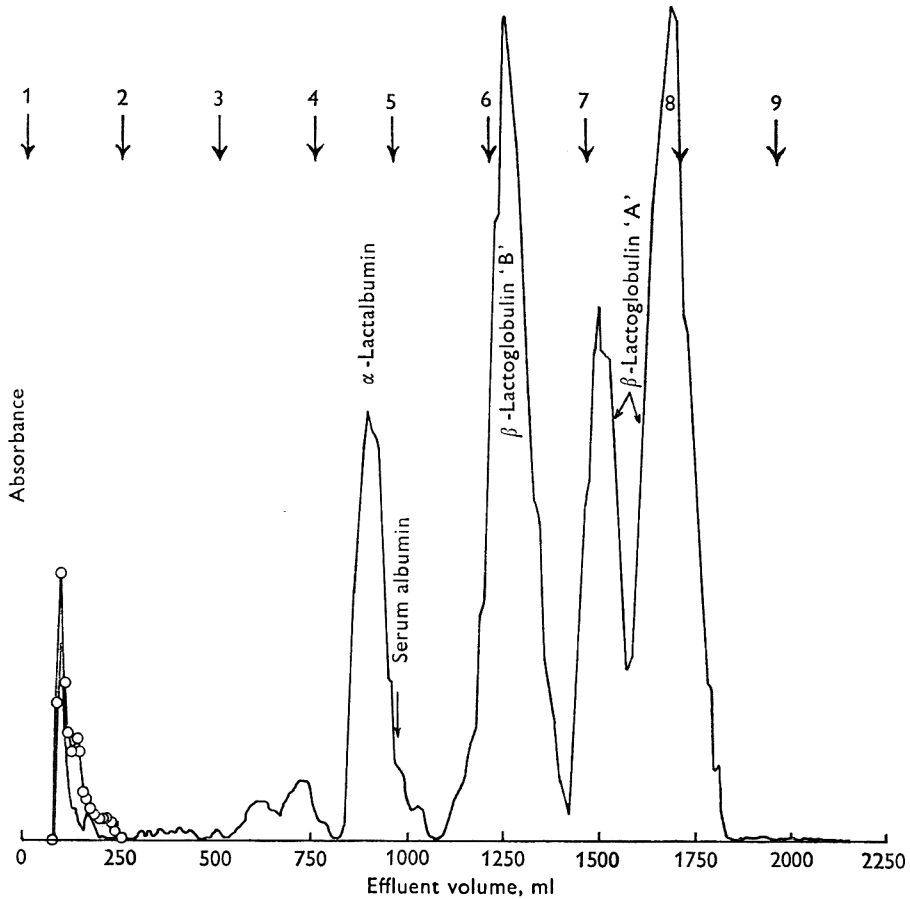


Fig. 4. Chromatography of milk albumin fraction on DEAE-cellulose. ——, Protein; ○——○, folate activity (*L. casei*). Arrows indicate points at which gradient buffer was changed. Mixing vessel contained 0·02 M-phosphate buffer of pH 7·0. Concentration of NaCl in 0·02 M-phosphate, pH 7·0, added to reservoir: (1) 0·05 M, (2) 0·075 M, (3) 0·100 M, (4) 0·125 M, (5) 0·150 M, (6) 0·175 M, (7) 0·200 M, (8) 0·225 M, (9) 0·250 M. α-Lactalbumin, serum albumin and β-lactoglobulins 'A' and 'B' were identified by starch gel electrophoresis of samples alongside authentic markers at pH 9. The heterogeneity of β-lactoglobulin 'A' as evidenced by the 2 peaks was probably an artifact arising from the stepwise change in the NaCl gradient (cf. Szuchet-Derechin & Johnson, 1965).

filtration of the folate-protein preparation after 100-fold dilution the folate activity peak in the column effluent was found to be at 180 ml, corresponding to a molecular weight of about 35000.

Ford *et al.* (1969) described a preparation containing folate-protein of molecular

weight about 76 000 as judged by sedimentation analysis and filtration in Sephadex gel G 150. This preparation was presumably composed largely of the dimer.

*Starch gel electrophoresis.* On starch gel electrophoresis at pH 9, in absence of urea, the folate activity was associated with a protein band that moved slightly towards the anode. A second, diffuse band moved towards the cathode. No better resolution was obtained at this pH in the presence of urea. Electrophoresis at acid pH showed more clearly the heterogeneity of the preparation. Plates 1(a) and 1(b) show the patterns obtained for folate-protein and RW-DE 52 for a variety of serum and whey
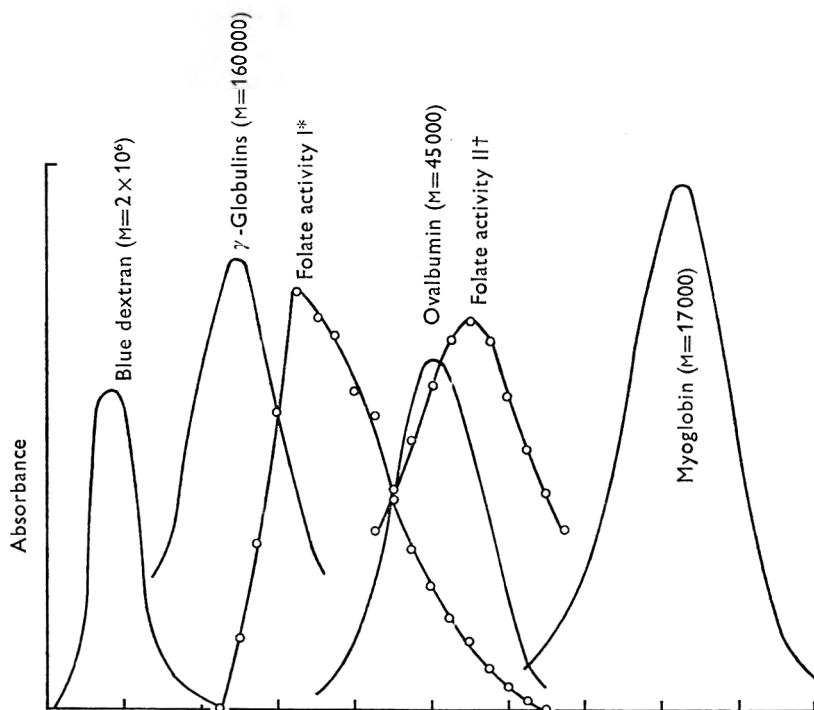


Fig. 5. Gel filtration of folate-protein in Sephadex G150. *, 5·0 μg Folate activity applied to column; †, 0·05 μg folate activity applied to column.

proteins in presence of urea and ME, at pH 2 and pH 5. At pH 2, the folate protein was resolved into 5 bands. Two main bands (C and D) ran in the same position as bovine serum transferrin and α-globulin. Two slower moving bands (A and B) corresponded with the positions of 2 bands obtained for γ-globulin. A minor, faster moving band (E) moved at pH 2 to about the same extent as β-lactoglobulin, but at pH 5 was clearly differentiated from this protein. It was also clearly different from all the other reference proteins employed as markers.

The problem now remained to identify one of these bands as the folate binding protein. To do this the preparation of folate protein RW-DE 52 was further purified by repeated chromatography in DE 52 and filtration in Sephadex-gel G 150. This further purification largely removed the γ-globulin components A and B. The preparation was then subjected to starch gel electrophoresis at pH 2 and the folate binding protein located as described on page 438. Plate 2 shows that folate-binding

activity was almost entirely associated with the minor protein band E, which we think represents the folate-binding protein, or an active fragment of it split off under these strongly dissociating conditions.

*Influence of pH on folate binding.* Portions of a solution of folate protein RW-DE 52 were dispensed into buffer solutions of pH 8·8, 7·1, 6·0, 5·0 and 3·6 and the pH values adjusted to correspond closely with those of the buffers. The solutions were allowed to stand at room temperature for 10 min and then applied to calibrated columns of Sephadex gel G 25 and eluted, each with its appropriate buffer solution.



| [¹⁴C]folic acid, $\mu$g | | Bound, |
|---|---|---|
| Added | Bound | % |
| 0·0114 | 0·0114 | 100 |
| 0·0228 | 0·0228 | 100 |
| 0·0456 | 0·0456 | 100 |
| 0·0913 | 0·0768 | 84·3 |
| 0·183 | 0·108 | 59·0 |
| 0·365 | 0·167 | 45·9 |
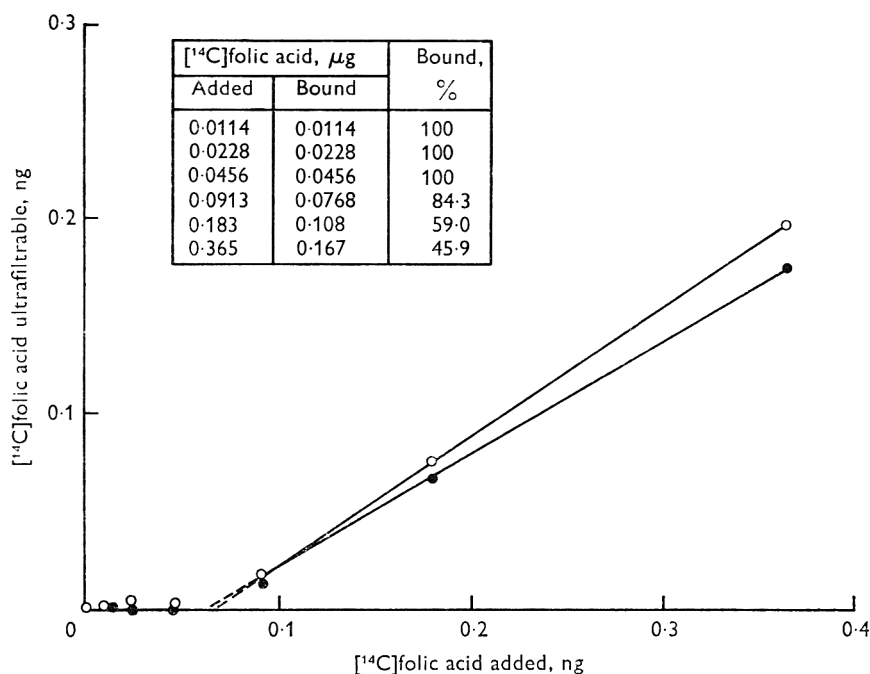
Fig. 6. Binding of folic acid added to a preparation of folate protein. ○——○, Determined by radioactivity; ●——●, determined microbiologically.

The effluent fractions were analysed for protein content by measurement of absorbance at 280 nm, and for folate activity. At pH values of 8·8, 7·1 and 6·0 the folate activity was wholly associated with protein, which was eluted from the columns in the void volume. At pH 5·0, only 61 % of the folate emerged with the protein and 39 % was recovered as free folate. At pH 3·6, only free folate was present in the eluate. The dissociation at pH 3·6 was reversed on readjustment of the pH value to 7·1.

In the presence of 8 M-urea free folate was present at pH 7·1, and at pH 6·0 the complex was completely dissociated.

*Binding of folic acid added to folate protein RW-DE 52.* To 0·25-ml portions of the folate protein preparation were added graded amounts of ¹⁴C-folic acid, and NaCl-phosphate buffer of pH 7·0 (p. 437) to 20 ml. A sample (1 ml) of each solution was taken for folate assay and the remainder ultra-filtered at 2 °C through Visking cellulose tubing, until about 2 ml ultrafiltrate had been collected from each. The ultrafiltrates were assayed for folate activity. The results are illustrated in Fig. 6.

Ultrafiltration of a 'blank' series of dilutions of folic acid in the buffer solution showed that little or no folate was lost by adsorption in the filter membrane, whereas folate in aqueous solution at these great dilutions was largely retained in the membrane.

Figure 6 shows that of folic acid added in amounts up to 70 ng, none was ultra-filtrable and it was presumed to be firmly bound to the folate protein. However, recovery of folic acid added in excess of this amount was far from complete and it seems probable that, in addition to the firm binding of folic acid to the folate protein, other weaker-binding sites are present on this or other proteins in the preparation and retain folic acid against ultrafiltration.

Table 1. *The influence of* $(\pm)N^5$*-methyl tetrahydrofolate on the binding of* $^{14}C$*-folic acid*

Folates ($\mu$g) added to binding protein

| $^{14}C$ folic acid | Methyl folate | $^{14}C$ folic acid bound, $\mu$g* |
|---|---|---|
| 0·0400 | 0 | 0·0400 |
| 0·0400 | 0·050 | 0·0400 |
| 0·0400 | 0·100 | 0·0353 |
| 0·0400 | 0·200 | 0·0345 |
| 0·0400 | 0·500 | 0·0261 |
| 0·0400 | 1·00 | 0·023 |

\* Measured by ultrafiltration (see p. 443).

*Influence of* $(\pm)$*-$N^5$-methyl-tetrahydrofolate on the binding of folic acid.* To 0·25-ml portions of RW-DE 52 were added graded amounts of $(\pm)N^5$-methyl-tetrahydrofolate together with 0·04 $\mu$g $^{14}C$-folic acid, and NaCl-phosphate buffer to 20 ml. The solutions were then ultrafiltered as described above. The levels of $^{14}C$-folate in the ultra-filtrates were measured, and the amounts of $^{14}C$-folate bound were calculated by difference. The results are shown in Table 1. It is apparent that folic acid and $N^5$-methyl-tetrahydrofolate compete for the binding protein, and that folic acid was preferentially bound. This confirms the findings of Ghitis *et al.* (1969).

*Separation of free and bound folate by dialysis.* In a further experiment, dialysis was employed instead of ultrafiltration to separate free and protein-bound folate. To portions (0·5 ml) of RW-DE 52 were added graded increments of folic acid or $(\pm)N^5$-methyl-tetrahydrofolate, and NaCl-phosphate buffer (p. 437) to 5 ml. A 3-ml sample of each was transferred to a dialysis sac and dialysed for 24 h against 5 successive 100-ml portions of buffer. The residual folate activity in the sacs was then assayed microbiologically with *L. casei*. It was found that the preparation of folate protein bound 0·18 $\mu$g added folic acid/ml, and about 0·14 $\mu$g folate activity as $(-)N^5$-methyl-tetrahydrofolate, on the assumption that only the natural stereoisomer is bound. Our experience has been that the potency of the $(\pm)N^5$-methyl-tetrahydro-folate relative to that of folic acid, measured microbiologically with *L. casei*, varied rather widely between assays and ranged between 30·5 and 50%. Assuming 40% (80% for the L-isomer) we can correct the above value of 0·14 to 0·175, which is close to the value of 0·18 obtained for folic acid. In view of the imprecision of the micro-biological assays, this close agreement may be fortuitous, but it does seem to con-tradict the report by Ghitis *et al.* (1969) that, compared with folic acid, only half the

amount of $(-)N^5$-methyl-tetrahydrofolate becomes bound when added to milk. The reason for this wide difference is not apparent but it is no doubt related to the different methods employed for assessing binding. Ghitis *et al.* (1969) used coated charcoal to remove excess unbound folate and determined folate by measurement of radioactivity. The discrepancy adds point to these authors' conclusion that further studies are required on the kinetics of folate-binding to the protein of milk.

<div align="center">GENERAL</div>

Metz & Herbert (1967) and others have reported that human blood serum does not contain a strong folic acid-binder, and patients undergoing regular dialysis treatment with the artificial kidney suffer considerable loss of plasma folate during dialysis (Mackenzie & Ford, 1969). Normal plasma folate levels are relatively very low and the physiological effect of the binding protein in milk is presumably to accumulate folate into the milk against a considerable concentration gradient. Metz *et al.* (1968) observed that in lactating women with folate deficiency severe enough to provoke megaloblastic anaemia, orally administered folic acid appeared to be taken up by breast milk in preference even to the haemopoietic system.

Besides acting to concentrate plasma folate into the milk, it seems possible that the folate binder might influence the nutritional availability of the milk folate to the suckling infant by making the vitamin inaccessible to gut microorganisms, or perhaps by directly facilitating uptake. Little or nothing is known on the absorption of folate during the neonatal period.

It may be that folate-binding proteins identical with that in milk occur in all the body tissues, where they may constitute a mechanism underlying folate homoeostasis. Johns, Sperti & Burgen (1961) demonstrated the remarkable avidity with which folic acid is removed from the plasma by the body tissues, and suggested that a high affinity for folic acid must be a property of most tissues. They commented that the very high intracellular concentrations of folic acid points to an accumulative process with a high affinity and capacity. Similarly in the gut, the uptake of folate may be mediated through the agency of folate binding protein. The content of folate binder in the mucosal cells might well determine the rate of absorption of food folate, and the relative avidities of different natural folates for the binder might determine the differences (cf. Butterworth, 1968) in their biological availability. In this connexion, it is of interest that Perry & Chanarin (1968) reported that heptaglutamate forms of folate constitute about three-quarters of the folate in a normal mixed diet and yet were absorbed and utilized only to about one-third of the extent of monoglutamate forms. This conclusion was based on a comparison of the uptake of pteroylglutamic acid and of yeast folate—mainly heptaglutamate—given orally to healthy subjects in doses equivalent to 20 $\mu$g folic acid/kg body weight—a dosage of folic acid that is considerably in excess of the supposed nutritional requirement. It is arguable that the observed difference in nutritional availability was more apparent than real. If the level of folate binder in the gut mucosa were sufficient to facilitate the uptake of only a small proportion of this large dose, then the smaller uptake of the heptaglutamate might reflect simply the relatively slower uptake of the larger molecule by passive diffusion. A clear implication of these findings is that the yeast heptaglutamate was

not extensively hydrolysed by folic acid conjugase present in human intestinal juice (Klipstein, 1967).

Our best preparations of the folate protein were still grossly impure, and the scale of the preparation needs now to be enlarged to make further purification possible. The content of folate protein in cow's milk is about 10 mg/l—assuming that each molecule of the protein binds one molecule of folate—and the overall recovery was low. Mell, Whiteley & Huennekens (1968) have described an ingenious method for the isolation of dihydrofolate reductase from chicken liver, by the use of amethopterin that had been linked covalently to an amino-ethyl derivative of soluble starch. This material was mixed with the crude enzyme at pH 6, and the resulting enzyme-amethopterin-starch complex was isolated by Sephadex gel filtration. The complex was then dissociated by raising the pH and the enzyme recovered by filtration through Sephadex. It seems likely that this procedure could be adapted for the efficient isolation of folate protein, by the use of dextran- or starch-bound folic acid, and this possibility is now being investigated.

We thank our colleagues Dr G. C. Cheeseman and Mrs J. Jeffcoat for examining several of our preparations by sedimentation analysis, and Dr J. W. G. Porter for helpful discussions.

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EXPLANATION OF PLATES

PLATE 1

Comparative starch gel electrophoresis of folate protein and other whey proteins (a) at pH 2, (b) at pH 5.

PLATE 2

Location of the folate-binding component in folate-protein preparation RW-DE 52. After electrophoresis at pH 2, the gel was cut transversely into 0·5 cm sections which were tested for folate-binding activity as described on pp. 438–439.

Plate 1

(a)

E    DC    B    A

Folate-protein

$\alpha_1$-Globulin

$\alpha_4$-Globulin

$\beta$-Globulin

Transferrin

$\gamma$-Globulin

Serum albumin

$\alpha$-Lactalbumin

$\beta$-Lactoglobulin 'A'

$\beta$-Lactoglobulin 'B'

(b)

Folate-protein

$\gamma$-Globulin

$\alpha$-Lactalbumin

Serum albumin

$\beta$-Lactoglobulins
('A'+'B')

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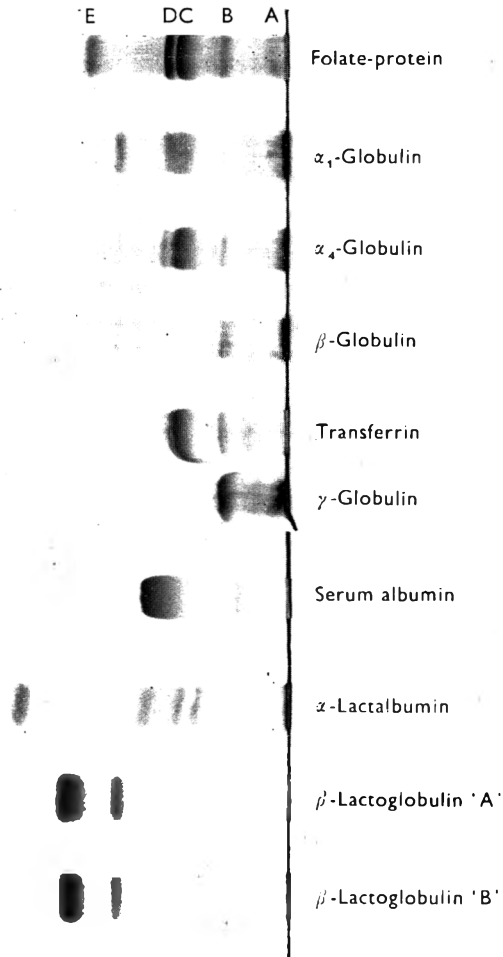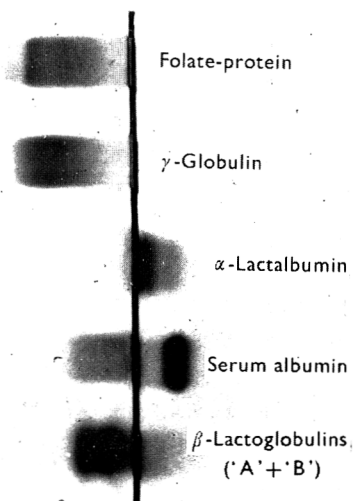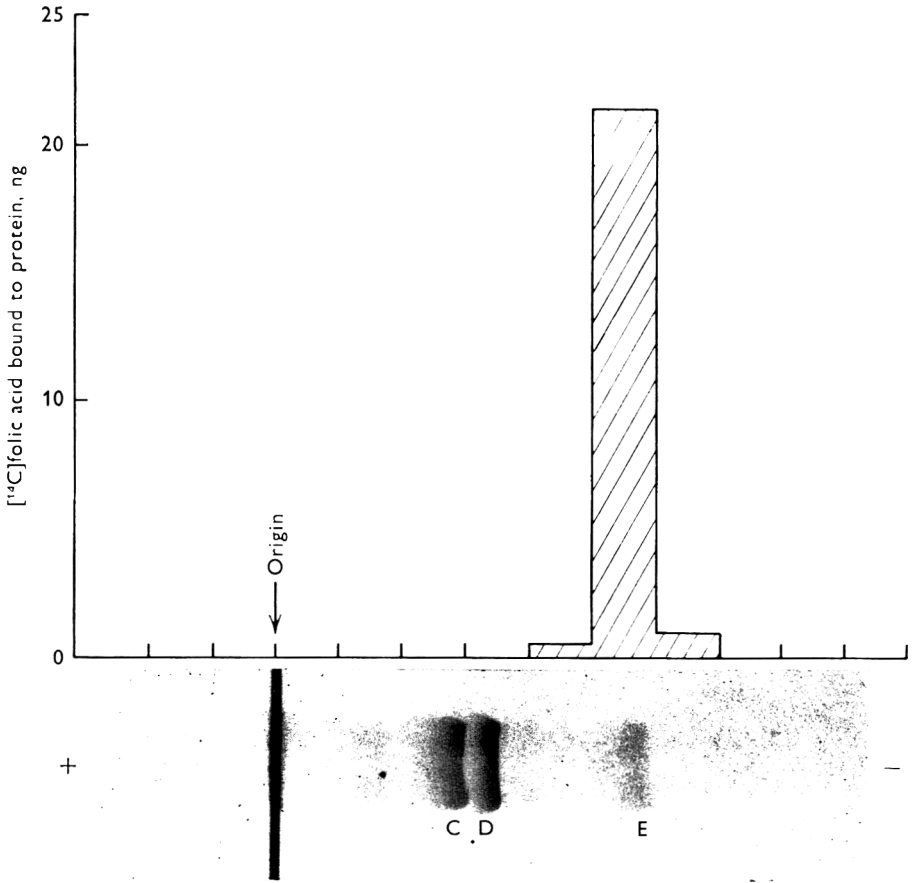
# Effects of ultra-high-temperature (UHT) processing and of subsequent storage on the vitamin content of milk

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SUMMARY. The vitamin content of ultra-high-temperature (UHT) processed milk was compared with that of the original raw milk. Three processes were used. In the first, which caused no change in oxygen content, the milk was heated and cooled in a plate-type heat exchanger. In the second, the milk was again heated indirectly and then evaporatively cooled, leaving in the milk about one-third of the initial oxygen content. In the third process the milk was heated by direct steam injection and cooled by evaporation and contained little or no residual oxygen.

On processing and during subsequent storage for 90 days there was no loss of vitamin A, carotene, vitamin E, thiamine, riboflavine, pantothenic acid, biotin or nicotinic acid. There was little or no loss of vitamin $B_6$ or vitamin $B_{12}$ on processing, but up to 50 % of each of these vitamins was lost during 90 days' storage. All the dehydroascorbic acid (DHA) and about 20 % of the ascorbic acid (AA) was lost on processing. There was no further loss of AA during 90 days' storage when no residual oxygen was present, but in milks containing more than about 1 ppm oxygen all the AA was lost within 14 days. About 20 % of the folic acid was lost on processing; thereafter, as with ascorbic acid, the extent of the loss on storage depended on the residual oxygen content of the milk: in the absence of oxygen the folic acid was stable.

Milk sterilized by an ultra-high-temperature (UHT) process and aseptically filled into aluminium foil-lined Tetra Pak cartons is commercially available in several European countries. Such milk remains in good condition for several months, even when stored without refrigeration. It is of interest to users, manufacturers and health authorities to know whether losses of vitamins occur during such storage.

Earlier studies of the vitamin content of UHT milk (Chapman *et al.* 1957; Nagasawa, Tanahashi, Kuzuya & Shigeta, 1960; Lhuissier, Hugo & Biette, 1962; Gregory & Burton, 1965) have shown that small losses of several vitamins may occur during processing but that the UHT process was considerably less destructive than in-bottle sterilization. These studies have now been extended and a detailed investigation has been made of the losses of vitamins during the processing of milk in directly and indirectly heated UHT plants and during subsequent storage for up to 90 days. Preliminary accounts of some of the findings were given by Ford, Porter, Thompson, Toothill & Edwards-Webb (1968) and Porter & Thompson (1969).

### Milk

Indirectly heated UHT milk in 1-pint Tetra Pak cartons and samples of the corresponding raw milks were supplied by 2 commercial dairies in the South of England; milk from the direct heating process in 0·5 l-Tetra Pak cartons and a sample of the corresponding raw milk were obtained from a commercial dairy in Sweden. The raw milk samples were taken from the bulk tank feeding each plant during the production run so that they represented the milk being treated as closely as possible. The raw milk samples from the British plants were protected from light and transported to the laboratory within 3–4 h of collection; those taken in Sweden were frozen, packed in solid $CO_2$ and transported by air.

For the first experiment, sets of cartons of UHT milk were taken from each plant on a single day in October 1966. For the second experiment sets of cartons were taken from the British plants on each of 3 successive days in October 1967 and from the Swedish plant on a single day in December, 1968. Analyses for ascorbic acid—and, in the second experiment, for oxygen content—were carried out immediately on receipt of the milks. The test samples for microbiological assay were put into cold store at $-30$ °C until all the samples representing the first 14 days had been taken, and were then assayed together. A second set of samples representing milk cartons stored up to the end of the storage period was collected and kept at $-30$ °C and also assayed all together. At each sampling time the contents of 2 cartons were bulked before samples were taken for analysis. The remaining cartons were kept at room temperature (15–19 °C) until required for testing. A sample was taken aseptically from each carton for bacteriological examination. The results confirmed that all the cartons were sterile.

### Types of plant

*Indirect heating.* One dairy used an Alfa Laval plant (cf. Clark, 1966) in which indirect heating at about 138 °C for 2 sec was followed by evaporative cooling (process I/E). The other dairy used an APV ultramatic plant (cf. Burton, 1965) in which indirect heating at about 138 °C for 2 sec was followed by indirect cooling (process I/I).

*Direct heating.* The Swedish dairy used an Alfa Laval VTIS plant in which direct heating at about 145 °C for 3–4 sec was followed by evaporative cooling (process D/E).

### Chemical methods

*Vitamin C* was determined by indophenol titration using a bromine/$H_2S$ procedure for the differential assays of AA and DHA (Toothill, Thompson & Edwards-Webb, unpublished).

*Riboflavine* was determined essentially by the fluorescence method of Emmerie (1938), but omitting the potassium permanganate-hydrogen peroxide oxidation procedure which is unnecessary with cow's milk and its products (Thompson unpublished). Fluorescence was measured in a fluorimeter constructed in this

laboratory, using light of wavelength 436 nm from a mercury vapour lamp for excitation, isolating the emitted fluorescence through a Wratten no. 12 filter and measuring its intensity with an EMI photomultiplier connected to a DC amplifier driving a meter having a circular scale about 0·5 m long.

*Vitamin A, biologically active carotenoids and vitamin E.* The lipids were extracted from 200 ml of milk by the method of Olson, Hegsted & Peterson (1939) and saponified by heating on a steam bath with 4·4 ml of 60 % (w/w) KOH, 20 ml of ethanol and 100 mg of sodium ascorbate. After saponification the mixture was diluted with 80 ml of water and the whole extracted with 3 successive portions of 80 ml of peroxide-free diethyl ether. The combined ether extracts were washed with 3 successive 80-ml portions of water. One-fifth of the washed ether extract was taken for the estimation of vitamin A and carotene and the remainder for the measurement of tocopherols.

Vitamin A and inactive carotenoids were separated from the biologically active carotenes by column chromatography using aluminium oxide. Vitamin A was measured colorimetrically after the addition of antimony trichloride and carotene by direct colorimetry, both essentially as described by Kon & Thompson (1957). α-Tocopherol was determined by the method of the Analytical Methods Committee (1959), except that the purification step using Floridin Earth × S was replaced by a simpler procedure using Florisil; further details of the application of the method to milk are described by Thompson, Henry & Kon (1964).

### Microbiological methods

Folic acid activity was assayed with *Lactobacillus casei*, by an adaptation of the procedure recommended by Herbert (1961) for the assay of folate in blood serum (Ford, 1967). For the assay of folate in milk, 2 ml samples were taken and diluted with 1 ml of buffer solution, made by dissolving 1 g ascorbic acid in 100 ml 1·4 % (w/v) $Na_2HPO_4$ solution and adding 4 N-NaOH to pH 7·8. Samples so treated showed no loss of folate during prolonged cold storage.

Vitamin $B_{12}$ was assayed with *L. leichmannii* as described by Gregory (1954), and thiamine with *L. viridescens* as described by Deibel, Evans & Niven (1957). Vitamin $B_6$ was measured with *Kloeckera brevis* as described by Barton-Wright (1963), except that the test samples were heated with 0·055 N-HCl for 30 min at 100 °C as recommended by Gregory (1959) and were not predigested with takadiastase. Nicotinic acid and biotin were assayed by standard microbiological procedures that have been fully described in earlier publications (Ford, Gregory, Porter & Thompson, 1953; Chapman *et al.* 1957). All the milk samples were assayed at least twice, and some as many as 4 times. The between-assay standard deviation was < 10 %.

### RESULTS AND DISCUSSION

### First experiment

The results for the content of all the vitamins measured in the milks collected and stored in the first experiment are shown in Table 1. There was little or no change during processing and during storage for up to 90 days in the content of vitamin A, carotene, vitamin E, thiamine, riboflavine, pantothenic acid, biotin and nicotinic acid. These findings are in agreement with those of most of the other reports on the effect

Table 1. *The content of vitamins in freshly processed and in stored UHT milks, and in the corresponding raw milks*

| Process* | Period of storage, days | Vit. A, μg/g fat | Carotene, μg/g fat | Vit. E, μg/g fat | Vit. C, mg/100 ml | Riboflavine, μg/ml | Folic acid, μg/ml | Vit. B$_{12}$, μg/ml | Vit. B$_6$, μg/ml | Pantothenic acid, μg/ml | Thiamine, μg/ml | Biotin, μg/ml | Nicotinic acid, μg/ml |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Raw  I/E | 0 | 10 | 8·4 | 30 | 1·7 | 2·0 | 0·073 | 0·0048 | 0·40 | 2·5 | 0·44 | 0·020 | 0·90 |
| I/I |  | 9·9 | 8·1 | 28 | 1·5 | 1·9 | 0·062 | 0·0042 | 0·43 | 3·0 | 0·49 | 0·021 | 0·88 |
| D/E |  | 7·0 | 4·9 | 17 | 1·5 | 1·8 | 0·045 | 0·0040 | 0·38 | 3·0 | 0·59 | 0·018 | 0·76 |
| UHT  I/E |  | 10 | 8·1 | 27 | 1·1 | 2·0 | 0·050 | 0·0043 | 0·38 | 2·4 | 0·48 | 0·022 | 0·83 |
| I/I |  | 10 | 7·9 | 29 | 1·2 | 1·9 | 0·055 | 0·0045 | 0·40 | 3·1 | 0·48 | 0·022 | 0·90 |
| D/E |  | — | — | — | — | — | — | — | — | — | — | — | — |
| UHT  I/E | 2 | 10 | 8·6 | 31 | 0·40 | 2·0 | 0·043 | 0·0036 | 0·36 | 2·4 | 0·48 | 0·019 | 0·81 |
| I/I |  | 9·9 | 7·8 | 24 | 1·2 | 1·9 | — | — | --- | — | — | — | — |
| D/E |  | — | — | — | — | — | — | --- | --- | — | — | — | — |
| UHT  I/E | 14 | 10 | 7·6 | 33 | 0·17 | 2·0 | 0·041 | 0·0035 | 0·32 | 2·5 | 0·16 | 0 022 | 0 87 |
| I/I |  | 9·7 | 8·0 | 27 | 0·04 | 1·9 | 0·019 | 0·0039 | 0·31 | 2·9 | 0·46 | 0·021 | 0·84 |
| D/E |  | 7·0 | 4·9 | 19 | 1·0 | 1·8 | 0·048 | 0·0035 | 0·35 | 3·3 | 0·53 | 0·021 | 0·80 |
| UHT  I/E | 30 | 10 | 7·8 | 30 | 0·15 | 2·0 | — | — | — | — | — | — | — |
| I/I |  | 9·8 | 7·9 | 28 | 0·04 | 2·0 | 0·0023 | 0·0042 | 0·27 | 3·4 | 0·43 | 0·021 | 0·85 |
| D/E |  | 7·3 | 4·7 | 18 | 1·2 | 1·8 | 0·048 | 0·0027 | 0·29 | 2·8 | 0·49 | 0·020 | 0·79 |
| UHT  I/E | 60 | 9·8 | 7·5 | 30 | 0·03 | 2·1 | 0·047 | 0·0028 | 0·25 | 2·4 | 0·48 | 0·019 | 0·85 |
| I/I |  | 9·8 | 7·6 | 24 | 0·05 | 2·0 | 0·0006 | 0·0039 | 0·24 | 2·8 | 0·47 | 0·024 | 0·79 |
| D/E |  | 7·1 | 4·8 | 21 | 0·80 | 1·9 | 0·051 | 0·0028 | 0·24 | 2·9 | 0·55 | 0·021 | 0·85 |
| UHT  I/E | 90 | 10 | 7·8 | 30 | 0·16 | 2·1 | — | 0·0038 | 0·25 | — | 0·48 | — | 0·83 |
| I/I |  | 10 | 7·7 | 24 | 0·05 | 2·0 | — | 0·0028 | 0·25 | 2·7 | 0·44 | — | 0·88 |
| D/E |  | 7·0 | 4·9 | 16 | 0·90 | 1·8 | 0·045 | 0·0025 | 0·21 | 3·3 | 0·43 | 0·020 | 0·86 |

* I/E, indirect heating, evaporative cooling; I/I, indirect heating and cooling; D/E, direct heating and evaporative cooling.

of UHT processing on these vitamins (cf. van Eekelen & Heijne, 1965; Burton, 1969), and lend no support to the report by Lembke, Frahm & Wegener (1968) that significant losses of vitamin A and riboflavine occurred during UHT processing of milk by direct and indirect heating methods and that the content of these vitamins fell further during 28 days' storage of the processed milk. Our finding that there was no change in the content of vitamin A on processing and during subsequent storage was confirmed in further experiments.

Processing caused no loss of vitamin $B_6$, but there was a considerable and progressive loss during storage, with the result that about half the vitamin had been destroyed after 90 days. It seems unlikely that the UHT processing was a contributory factor in these storage losses since similar losses during storage were found in raw milk held at $-30$ °C. It is probable that the contradictory claims of earlier workers about the effect of the UHT process on the vitamin $B_6$ content of milk were confounded by this effect of storage (cf. Burton, 1969).

Processing and subsequent storage caused losses of vitamin C, folic acid and vitamin $B_{12}$ though, as is apparent from the results, the losses were smaller with milk processed either by direct heating or by indirect heating followed by evaporative cooling. It is known that the stability of folic acid and vitamin $B_{12}$ in milk is closely associated with the presence of AA (Ford, 1957, 1967) and that the loss of AA is determined ultimately by the concentration of oxygen dissolved in the milk. In the present study, the observed changes in the content of these 3 vitamins were clearly related to the oxygen content of the milk. Evaporative cooling in the direct heating plant removed the oxygen from the milk almost completely. Partial-evaporative cooling, as used in one indirect heating plant, reduced the oxygen in the milk to 1–2 ppm, as measured at 4 h after processing (personal communication from the processor); no evolution of oxygen could occur during processing in the indirectly cooled milk from the other plant.

### Second experiment

To investigate further the interrelationship of these vitamins, a second series of samples was collected and assays for vitamin C, folic acid, vitamin $B_{12}$ and vitamin $B_6$ were complemented by measurement of the oxygen content of the milk at each time of sampling. The results in Table 2 amplify and extend those in Table 1 and the findings of both experiments can conveniently be discussed together.

*Oxygen content.* Measurement of oxygen content showed that, as expected, oxygen was virtually completely removed during the process using direct heating. The oxygen content of milk from the process using indirect heating and partial-evaporative cooling showed some day-to-day variation but was markedly higher than that in milk from the same plant examined in the first series of experiments, and this was reflected in a more rapid loss of folic acid and AA than was found previously. The oxygen content of milk from the process using indirect heating and cooling was essentially unchanged.

During storage the dissolved oxygen content of the samples from the indirect heating plants fell but it is noteworthy that about half the initial oxygen was still present when all the vitamin C had been destroyed. Thus, it is evident that to conserve vitamin C the processed milk should have a very low oxygen content. Certainly

Table 2. *The content of vitamin C, folic acid, vitamin $B_{12}$ and vitamin $B_6$ in freshly processed and in stored UHT milks, and in the corresponding raw milks*

| Age of sample, days | Process* | Production day → Oxygen ppm 1 | 2 | 3 | Vitamin C Ascorbic acid, mg/100 ml 1 | 2 | 3 | Dehydroascorbic acid, mg/100 ml 1 | 2 | 3 | Folic acid, µg/ml 1 | 2 | 3 | Vitamin $B_{12}$, µg/ml 1 | 2 | 3 | Vitamin $B_6$, µg/ml 1 | 2 | 3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Raw 0 | I/E | 10.4 | 10.8 | 11.0 | 1·15 | 0·97 | 1·15 | 0·11 | 0·16 | 0·53 | 0·075 | 0·069 | 0·059 | 0·0059 | 0·0052 | 0·0045 | 0·63 | 0·67 | 0·57 |
|  | I/I | 8·4 | 7·4 | 7·7 | 0·81 | 0·92 | 0·59 | 0·64 | 0·61 | 0·69 | 0·073 | 0·059 | 0·062 | 0·0051 | 0·0049 | 0·0051 | 0·62 | 0·62 | 0·57 |
|  | D/E | 9·5 | — | — | 1·71 | — | — | 0·22 | — | — | 0·030 | — | — | 0·00325 | — | — | 0·43 | — | — |
| UHT 0 | I/E | 6·9 | 6·3 | 3·9 | 0·62 | 0·69 | 0·75 | 0·17 | 0·06 | 0·24 | 0·050 | 0·061 | 0·056 | 0·0052 | 0·0044 | 0·0036 | 0·60 | 0·60 | 0·51 |
|  | I/I | 8·2 | 8·4 | 8·3 | 1·39 | 1·13 | 0·82 | 0·07 | 0·06 | 0·14 | 0·068 | 0·057 | 0·059 | 0·0051 | 0·0040 | 0·0046 | 0·54 | 0·60 | 0·55 |
|  | D/E | 0·1 | — | — | 1·78 | — | — | 0·07 | — | — | 0·030 | — | — | 0·0034 | — | — | 0·43 | — | — |
| UHT 2 | I/E | 5·6 | 5·2 | 2·3 | 0·13 | 0·35 | 0·21 | 0·05 | 0·12 | 0·04 | 0·052 | 0·050 | 0·054 | 0·0050 | 0·0043 | 0·0032 | 0·49 | 0·61 | 0·47 |
|  | I/I | 6·2 | 5·6 | 6·7 | 0·36 | 0·26 | 0·34 | 0·09 | 0·18 | 0·15 | 0·064 | 0·059 | 0·057 | 0·0048 | 0·0039 | 0·0042 | 0·54 | 0·57 | 0·49 |
|  | D/E | <0·1 | — | — | 1·68 | — | — | 0·07 | — | — | 0·0345 | — | — | 0·0031 | — | — | 0·38 | — | — |
| UHT 7 | I/E | 4·7 | 3·8 | 1·8 | 0·11 | 0·00 | 0·01 | 0·01 | 0·03 | 0·11 | 0·0085 | 0·037 | 0·031 | 0·0052 | 0·0042 | 0·0033 | 0·42 | 0·54 | 0·47 |
|  | I/I | 4·9 | 4·4 | 5·3 | 0·10 | 0·12 | 0·00 | 0·00 | 0·00 | 0·05 | 0·022 | 0·035 | 0·030 | 0·0050 | 0·0038 | 0·0043 | 0·41 | 0·47 | 0·46 |
|  | D/E | <0·1 | — | — | 1·56 | — | — | 0·08 | — | — | 0·031 | — | — | 0·0027 | — | — | 0·34 | — | — |
| UHT 14 | I/E | 4·1 | 3·5 | 1·0 | 0·02 | 0·04 | 0·07 | 0·04 | 0·05 | 0·01 | 0·001 | 0·0076 | 0·01 | 0·0048 | 0·0040 | 0·0032 | 0·37 | 0·50 | 0·46 |
|  | I/I | 4·2 | 4·2 | 4·3 | 0·09 | 0·04 | 0·09 | 0·03 | 0·04 | 0·01 | 0·0 | 0·0058 | 0·0058 | 0·0049 | 0·0038 | 0·0040 | 0·46 | 0·52 | 0·43 |
|  | D/E | <0·1 | — | — | 1·64 | — | — | 0·09 | — | — | 0·0315 | — | — | 0·0023 | — | — | 0·30 | — | — |
| UHT 30 | I/E | 3·6 | 2·4 | 0·8 | 0·08 | 0·06 | −0·02 | 0·04 | 0·04 | 0·09 | 0·0 | 0·0 | 0·0 | 0·0036 | 0·0039 | 0·0032 | — | — | — |
|  | I/I | 3·4 | 3·8 | 3·7 | 0·07 | 0·03 | 0·04 | 0·03 | 0·06 | 0·05 | 0·0 | 0·0 | 0·0 | 0·0037 | 0·0033 | 0·0039 | — | — | — |
|  | D/E | <0·1 | — | — | 1·56 | — | — | 0·07 | — | — | 0·0305 | — | — | 0·0020 | — | — | 0·29 | — | — |
| UHT 60 | I/E | 3·4 | 1·7 | 0·7 | 0·04 | 0·04 | 0·02 | 0·08 | 0·02 | 0·06 | — | — | — | 0·0035 | 0·0042 | 0·0027 | — | — | — |
|  | I/I | 2·5 | 2·3 | 2·8 | 0·02 | 0·08 | 0·06 | 0·06 | 0·03 | 0·03 | — | — | — | 0·0035 | 0·0034 | 0·0035 | — | — | — |
|  | D/E | <0·1 | — | — | 1·48 | — | — | 0·02 | — | — | 0·0305 | — | — | 0·0013 | — | — | 0·28 | — | — |

\* I/E, indirect heating, evaporative cooling; I/I, indirect heating and cooling; D/E, direct heating and evaporative cooling.

at 1 ppm (see above) there was sufficient oxygen present to destroy all the vitamin C, which was clearly oxidized in preference to folic acid (Table 1).

*Vitamin C.* Milk as secreted contains about 2 mg/100 ml of the heat-stable AA, but by the time the milk reaches the processing plant a part of the vitamin is present as DHA, which is completely destroyed by UHT processing. Our results show also a loss of about 20 % of the AA. No further DHA was found in the stored milks. The vitamin C content of the milks prepared by the indirect heating processes fell rapidly, except in the samples in the first experiment from the plant with partial-evaporative cooling in which the initial oxygen content was much lower than in later samples from the same plant.

There was little or no loss of vitamin C from milk prepared by the direct heating process, even after storage for 90 days.

*Folic acid.* Processing caused small, variable losses of up to 20 % of the original content of folic acid. During storage, stability was clearly and closely related to the content of AA. Thus, in the milk processed by direct heating and evaporative cooling, in which AA persisted, there was no loss of folic acid during 90 days' storage, whereas in milk from the indirect heating and cooling plant all the folic acid was lost within 14 days. The rate of loss in milk processed by indirect heating and evaporative cooling was markedly influenced by the extent of evaporation allowed and hence by the residual oxygen content. In the first series of experiments there was little loss of folic acid at 14 days, whereas in the second series the vitamin was completely destroyed within 14 days.

*Vitamin $B_{12}$.* Processing generally caused only small losses ($< 10 \%$) of vitamin $B_{12}$ but further progressive loss occurred in all samples during storage, and after 90 days nearly one-half of the vitamin had been lost. These results are broadly in line with those of Lembke *et al.* (1968), but other workers (cf. Burton, 1969) have reported results for the loss of vitamin $B_{12}$ that tend to vary with the type of UHT plant and the details of milk handling and may, as with vitamin $B_6$, confound the effects of processing and of storage.

### CONCLUSIONS

Our results confirm previous reports that relatively small losses of vitamins occur during the UHT treatment of milk, whether by the direct or the indirect process, but they show also that there may be considerable losses of vitamin C, folic acid, vitamin $B_6$ and vitamin $B_{12}$ on subsequent storage of the milk. For 2 vitamins, vitamin C and folic acid, these losses can be greatly reduced by rigorously removing oxygen from the milk during processing and, in so far as the milk might be used in infant feeding as the sole source of nutriment, the preservation of these vitamins is clearly desirable. However, an important consideration is the relationship of the content of dissolved oxygen to milk flavour. It is recognized that the persistence of the cooked flavour arising during UHT processing is related to the residual oxygen content of the milk and, in as much as this cooked flavour reduces palatability, there may be commercial advantage in allowing the presence of a higher level of residual oxygen. To this extent the preservation of vitamins and the rapid dispersal of the cooked flavour—both highly desirable—may prove to be incompatible.

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# The effects of acetate and of pyruvate on the pathways of glucose catabolism in lactating mammary tissue

## 1. Rat tissue

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SUMMARY. A study was made of the changes in the pathways of glucose catabolism in slices of lactating rat mammary gland which occurred when acetate or pyruvate was added to the medium.

When acetate was added there was an increase in the rates of oxidation of the $C_{(2)}$ and $C_{(6)}$ atoms of glucose but little change in that of the $C_{(1)}$ atom. The amounts of these carbon atoms which were incorporated into fatty acids, lactic acid and glyceride glycerol were reduced, but the results showed that the relative proportion of the glucose converted to these substances which passed through the pentose phosphate pathway was increased.

When pyruvate was added, the rates of oxidation of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ atoms were all reduced to about the same extent. The incorporation of the glucose carbon into fatty acids was almost abolished and its incorporation into lactic acid and glyceride glycerol was very much reduced. The results showed that most of the glucose incorporated into these compounds under these conditions passed through the pentose phosphate pathway.

Evidence is presented which suggests that the triose phosphates are not in complete isotopic equilibrium in lactating rat mammary tissue.

These results are discussed in relation to the existence of 2 pools of acetyl coenzyme A, one intra- and the other extra-mitochondrial, and to the possible inhibition of certain steps of the glycolytic pathway.

Information about the pathways of glucose metabolism in lactating rat mammary tissue has been obtained largely from experiments with tissue slices *in vitro*. It has been shown for instance that the pentose phosphate pathway is very active during lactation (McLean, 1958; Abraham & Chaikoff, 1959) and that there is a close relationship between this pathway and the synthesis of fatty acids (McLean, 1960). However, there is only a limited amount of information about the extent to which this pattern of glucose catabolism *in vitro* may be modified by the presence of additional substrates as, of course, occurs *in vivo*. Hirsch, Baruch & Chaikoff (1954) reported that the addition of acetate had very little effect on the rate of oxidation of uniformly labelled glucose but reduced its incorporation into fatty acids by about 50 %. This latter result agreed with the results of Balmain, Folley & Glascock (1954). Duncombe

& Glascock (1956) also found that acetate was without effect on the rates of oxidation of both uniformly labelled and 1-$^{14}$C-glucose. However, in a more recent study Green_ baum & Darby (1964) found that the addition of acetate gave rise to a small increase in the production of $^{14}CO_2$ from uniformly labelled glucose and also increased its incorporation into fatty acids. Hirsch *et al.* (1954) and Duncombe & Glascock (1956) also investigated the effects of the addition of pyruvate and found that it caused a decrease in the rate of oxidation of uniformly labelled glucose by slices of lactating rat mammary tissue. Hirsch *et al.* also showed that the incorporation of glucose into fatty acids was very greatly reduced in the presence of pyruvate. The present paper describes experiments in which the effects of acetate and of pyruvate on glucose catabolism by lactating rat mammary tissue were examined in more detail and with particular regard to the relationship between the glycolytic and pentose phosphate pathways.

## MATERIALS AND METHODS

*Chemicals.* Diphenyl oxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene for liquid scintillation counting were obtained from Thorn Electronics Ltd., Tolworth, Surrey. Silicic acid for column chromatography was Mallinckrodt 100-mesh grade and sup- plied by Kodak Ltd., Kirby, Liverpool. [1-$^{14}$C]glucose, [2-$^{14}$C]glucose and [6-$^{14}$C]- glucose were bought from the Radiochemical Centre, Amersham, Bucks. Sodium pyruvate was prepared by the method of Price & Levintow (1952).

*Animals.* Primiparous hooded Norway rats from the Institute colony were used at the fourteenth–sixteenth day of lactation.

*Preparation of slices.* The animals were killed by breaking their necks and the abdominal mammary glands were quickly dissected out. Tissue slices approximately 0·3 mm thick were cut on a mechanical tissue chopper (H. Mickle, Gomshall, Surrey), and washed in ice-cold Ringer's saline (Krebs & Henseleit, 1932) to remove the retained milk.

*Incubation technique.* Washed slices (500 mg) were incubated in a unit similar to that described by Duncombe & Glascock (1956) with 5 ml of Krebs–Ringer bicar- bonate medium and 100 $\mu$moles of glucose containing 1 $\mu$c of either [1-$^{14}$C]glucose, [2-$^{14}$C]glucose or [6-$^{14}$C]glucose. When required, 100 $\mu$moles of sodium acetate or sodium pyruvate were also added. The gas phase was $O_2 + CO_2$ (95:5) and the flasks were incubated at 37 °C for 3 h with shaking.

### Isolation of metabolites

*Respiratory* $^{14}CO_2$. At the end of the incubation period the $CO_2$ in the flasks was extracted as described by Duncombe & Glascock (1956). The tissue slices were separated from the medium by filtration, well washed with water, freeze-dried and their dry weight determined.

*Fatty acids.* Total lipids were extracted from the dry tissue with chloroform- methanol (2:1, v/v). The lipid extract was concentrated to dryness, redissolved in chloroform and applied to a chromatography column prepared from 2 g of silicic acid. Triglycerides and other neutral lipids were eluted with 20 ml of chloroform. This eluate was concentrated to dryness and the residue was hydrolysed by heating it with 0·5 N-ethanolic KOH. The saponification mixture was shaken with hexane to

remove non-saponifiable material which was discarded. The aqueous phase was then acidified with 10 $N$-$H_2SO_4$ and the fatty acids were extracted by shaking again with hexane. This hexane extract was washed with water to remove mineral acid, and titrated with saturated $Ca(OH)_2$ after the addition of sufficient 95 % ethanol to maintain a one-phase system. It was then concentrated to dryness and the calcium salts were submitted to combustion as described by Glascock (1954). The $^{14}CO_2$ was collected and counted.

*Glyceride glycerol.* The solution which remained after the extraction of the fatty acids was made up to a known volume and duplicate samples were counted after the addition of 10 ml of the scintillator solution described below. The glycerol content of this solution was determined by the method of Moore (1962).

*Lactic acid.* Carrier lactic acid (100 $\mu$moles in the form of zinc lactate trihydrate) was added to the incubation medium and washings which were then concentrated to about 2 ml. This solution was adjusted to pH 2, and mixed with anhydrous $Na_2SO_4$ (20 g) to give a dry powder which was extracted with diethyl ether. The ether was removed by evaporation and the lactic acid was purified by partition chromatography on silicic acid. The method of Ladd & Nossal (1954) was used except that the column was prepared from 3 g of silicic acid and the aqueous phase was 1·8 ml of 0·5 $N$-$H_2SO_4$. All the solvents were equilibrated with 0·5 $N$-$H_2SO_4$ before use. Fractions (2 ml) were collected at the rate of 36/h and titrated with 0·01 $N$-NaOH (carbonate-free) with phenol red as indicator. The fractions which contained lactic acid were pooled and concentrated to dryness. The residue was redissolved in a known volume of water and duplicate samples were counted.

### Measurement of radioactivity

All measurements of radioactivity were done by liquid scintillation counting in a Packard-Tri-Carb model 3314 Liquid Scintillation Spectrometer. The efficiency of counting was determined by the internal standard technique for which [$^{14}C$]toluene was used. The scintillator solution used for counting the solutions of lactic acid and glycerol consisted of 2,5 diphenyl oxazole (7 g/l), 1,4-bis-(5-phenyloxazol-2-yl) benzene (0·3 g/l) and naphthalene (100 g/l) in a 3:2 (v/v) mixture of 2-methoxyethanol and anisole. The absorption in ethanolamine and subsequent counting of the $CO_2$ extracted from the incubation flasks or obtained by combustion of fatty acids was carried out as described by Smith & Phillips (1969).

### RESULTS

#### Oxidation of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ atoms of glucose to $CO_2$

*Expression of results.* Hourly rates of oxidation are expressed as $\mu$g C/mg dry weight of tissue and are designated by the symbol q with the appropriate subscript. Thus, $q_{G1}$, $q_{G2}$ and $q_{G6}$ refer to the rates of oxidation of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ atoms.

*The effect of acetate.* The rates of oxidation of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ atoms of glucose in the presence and absence of acetate are shown in Table 1. When glucose was the only substrate the mean values of $q_{G1}$ and $q_{G2}$ were, respectively, 23 times and 11 times that of $q_{G6}$. On the addition of acetate to the medium, $q_{G2}$ increased by 41 % and $q_{G6}$ by 107 %. The increase in the value of $q_{G1}$ was only 14 % although still

**Table 1.** *The effect of unlabelled acetate on the hourly rate of oxidation† of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ atoms of glucose by slices of lactating rat mammary gland*

| | $q_{G1}$ | | $q_{G2}$ | | $q_{G6}$ | | Ratio $q_{G1}/q_{G6}$ | |
|---|---|---|---|---|---|---|---|---|
| | Acetate absent | Acetate present | Acetate absent | Acetate present | Acetate absent | Acetate present | Acetate absent | Acetate present |
| No. of rats... | 8 | | 8 | | 8 | | 8 | |
| Range | 0·94–1·66 | 1·17–2·25 | 0·43–0·90 | 0·62–1·17 | 0·030–0·093 | 0·10–0·14 | 13·0–31·8 | 8·7–18·8 |
| Mean | 1·35 | 1·54 | 0·63 | 0·89 | 0·058 | 0·12 | 24·6 | 12·7 |
| Difference ± standard error of difference | 0·19 ± 0·079 | | 0·26 ± 0·046 | | 0·062 ± 0·0066 | | 11·9 ± 1·98 | |
| Significance of difference | * | | *** | | *** | | * | |

† Rate of oxidation expressed as $\mu$g C/mg dry wt of tissue.    Levels of significance: * $P < 0.05$.    *** $P < 0.001$.

**Table 2.** *The effect of unlabelled pyruvate on the hourly rate of oxidation† of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ atoms of glucose by slices of lactating rat mammary gland*

| | $q_{G1}$ | | $q_{G2}$ | | $q_{G6}$ | | Ratio $q_{G1}/q_{G6}$ | |
|---|---|---|---|---|---|---|---|---|
| | Pyruvate absent | Pyruvate present | Pyruvate absent | Pyruvate present | Pyruvate absent | Pyruvate present | Pyruvate absent | Pyruvate present |
| No. of rats... | 5 | | 5 | | 5 | | 5 | |
| Range | 0·91–1·19 | 0·20–0·43 | 0·38–0·57 | 0·17–0·25 | 0·024–0·075 | 0·010–0·036 | 14·8–37·9 | 11·8–27·9 |
| Mean | 1·01 | 0·32 | 0·51 | 0·22 | 0·048 | 0·019 | 24·1 | 19·1 |
| Difference ± standard error of difference | 0·69–0·068 | | 0·29 ± 0·053 | | 0·029 ± 0·0048 | | 5·0 ± 4·04 | |
| Significance of difference | *** | | ** | | *** | | NS | |

† Rate of oxidation expressed as $\mu$g C/mg dry wt of tissue.    Levels of significance: NS $P > 0.05$.    ** $P < 0.01$.    *** $P < 0.001$.

significant at the 5 % level. Since the relative increase in the value of $q_{G6}$ was greater than that of $q_{G1}$ the ratio $q_{G1}/q_{G6}$ was halved.

*The effect of pyruvate.* When the additional substrate in the medium was pyruvate, the values of $q_{G1}$, $q_{G2}$ and $q_{G6}$ were all reduced to between one third and one half of the values observed when glucose was the only substrate (Table 2). There was no significant change in the value of the ratio $q_{G1}/q_{G6}$.

### *Pathways of glucose catabolism*

The changes in the rates of oxidation of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ carbon atoms of glucose resulting from the addition of a second substrate may have been associated with a change in the relative extents to which the pentose phosphate and glycolytic pathways participated in the catabolism of glucose to triose phosphate. The incorporation of these carbon atoms of glucose into 3 derivatives of triose phosphate (fatty acids, lactic and glyceride glycerol) were therefore measured. The results were used to calculate the fraction of the glucose converted to these substances which was catabolized by the pentose phosphate pathway. The method of calculation used was that proposed by Abraham, Hirsch & Chaikoff (1954) in which the ratio ($^{14}$C incorporated from 6-$^{14}$C-glucose–$^{14}$C incorporated from 1-$^{14}$C-glucose)/($^{14}$C incorporated from 6-$^{14}$C glucose) represents the fraction of glucose catabolized to triose phosphate by way of the pentose phosphate pathway.

### *Effect of acetate and of pyruvate on the contribution of the pentose phosphate pathway as measured by the incorporation of glucose carbon into:*

#### (a) FATTY ACIDS

*Effect of acetate.* Table 3 shows that when acetate was added to the medium the incorporation into fatty acids of the $C_1$ atom of glucose was reduced by 43 % and of the $C_{(6)}$ atom of glucose by 22 %. The incorporation of the $C_{(2)}$ atom of glucose was not significantly changed.

When glucose was the only substrate the proportion converted into fatty acids by the pentose phosphate pathway was about 50 % (Tables 3 and 4). This result is in good agreement with those of other workers (Abraham & Chaikoff, 1959; Greenbaum & Darby, 1964; McLean, 1964). Although the incorporation of glucose into fatty acids was decreased when acetate was present in the medium the relative contribution of the pentose phosphate pathway was increased from 47 to 62 % (Table 3).

*Effect of pyruvate.* The results presented in Table 4 show that when the pyruvate was added to the medium the incorporation of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ atoms of glucose into the fatty acids was almost completely abolished. The table also shows that the small amounts of glucose which were converted to fatty acids in the presence of pyruvate were catabolized largely by the pentose phosphate pathway.

#### (b) LACTIC ACID

*Effect of acetate.* Table 5 shows that on the addition of acetate the incorporation of the $C_{(1)}$ and $C_{(6)}$ atoms of glucose into lactic acid was reduced by about 80 % and of the $C_{(2)}$ atom of glucose by 47 %. A smaller effect had also been observed on the incorporation of the $C_{(2)}$ atom into fatty acids (Table 3).

Table 3. *The effect of unlabelled acetate on the incorporation† of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ atoms of glucose into fatty acids of lactating rat mammary tissue, and on the proportion of glucose catabolized by the pentose phosphate pathway*

| | $C_{(1)}$ | | $C_{(2)}$ | | $C_{(6)}$ | | Pentose pathway, % | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | Acetate absent | Acetate present | Acetate absent | Acetate present | Acetate absent | Acetate present | Acetate absent | Acetate present |
| No. of rats... | 6 | | 6 | | 6 | | 6 | |
| Range | 0·36–1·36 | 0·20–0·94 | 0·46–1·68 | 0·32–1·35 | 0·62–2·58 | 0·62–2·21 | 36–60 | 56–70 |
| Mean | 0·90 | 0·51 | 0·98 | 0·72 | 1·65 | 1·28 | 47 | 62 |
| Difference ± standard error of difference | 0·39±0·092 | | 0·26±0·107 | | 0·37±0·139 | | 15±3·7 | |
| Significance of difference | ** | | NS | | * | | ** | |

† Incorporation expressed as mg labelled C/100 mg fatty acid C.
Levels of significance: NS $P > 0.05$.  * $P < 0.05$.  ** $P < 0.01$.

Table 4. *The effect of unlabelled pyruvate on the incorporation† of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ atoms of glucose into fatty acids of lactating rat mammary tissue, and on the proportion of glucose catabolized by the pentose phosphate pathway*

| | $C_{(1)}$ | | $C_{(2)}$ | | $C_{(6)}$ | | Pentose pathway, % | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | Pyruvate absent | Pyruvate present | Pyruvate absent | Pyruvate present | Pyruvate absent | Pyruvate present | Pyruvate absent | Pyruvate present |
| No. of rats... | 4 | | 4 | | 4 | | 4 | |
| Range | 0·57–0·86 | 0·0028–0·0035 | 0·95–1·23 | 0·0026–0·0069 | 1·44–1·93 | 0·016–0·061 | 30–65 | 82–93 |
| Mean | 0·70 | 0·003 | 1·06 | 0·005 | 1·66 | 0·038 | 52 | 88 |
| Difference ± standard error of difference | 0·70±0·064 | | 1·05±0·059 | | 1·62±0·098 | | 36±5·0 | |
| Significance of difference | ** | | *** | | *** | | ** | |

Table 6. *The effect of unlabelled pyruvate on the incorporation† of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ atoms of glucose into lactic acid by lactating rat mammary tissue, and on the proportion of glucose catabolized by the pentose phosphate pathway*

| | $C_{(1)}$ | | $C_{(2)}$ | | $C_{(6)}$ | | Pentose pathway, % | |
|---|---|---|---|---|---|---|---|---|
| | Acetate absent | Acetate present | Acetate absent | Acetate present | Acetate absent | Acetate present | Acetate absent | Acetate present |
| No. of rats… | 5 | | 3 | | 5 | | 5 | |
| Range | 1·31–2·38 | 0·23–0·74 | 1·13–3·06 | 0·38–1·87 | 2·49–5·13 | 0·67–1·43 | 31–60 | 46–67 |
| Mean | 1·86 | 0·52 | 1·78 | 0·94 | 3·83 | 1·03 | 50 | 52 |
| Difference ± standard error of difference | 1·34 ± 0·153 | | 0·84 ± 0·184 | | 2·80 ± 0·339 | | 2 ± 9·0 | |
| Significance of difference | *** | | * | | ** | | NS | |

† Incorporation expressed as $\dfrac{\text{specific activity of lactic acid by wt} \times 100}{\text{specific activity of substrate glucose by wt}}$.

Levels of significance: NS $P > 0·05$.  * $P < 0·05$.  ** $P < 0·01$.  *** $P < 0·001$.

| | $C_{(1)}$ | | $C_{(2)}$ | | $C_{(6)}$ | | Pentose pathway, % | |
|---|---|---|---|---|---|---|---|---|
| | Pyruvate absent | Pyruvate present | Pyruvate absent | Pyruvate present | Pyruvate absent | Pyruvate present | Pyruvate absent | Pyruvate present |
| No. of rats… | 4 | | 4 | | 4 | | 4 | |
| Range | 1·63–2·80 | 0·16–0·44 | 3·20–6·31 | 0·27–0·34 | 4·35–8·20 | 0·48–1·42 | 40–66 | 50–89 |
| Mean | 2·39 | 0·26 | 4·45 | 0·30 | 5·52 | 0·87 | 55 | 64 |
| Difference ± standard error of difference | 2·13 ± 0·235 | | 41·5 ± 0·679 | | 4·65 ± 1·05 | | 9 ± 8·9 | |
| Significance of difference | ** | | ** | | * | | NS | |

specific activity of lactic acid by wt × 100

*...by lactating rat mammary tissue, and on the proportion of glucose catabolized by the pentose phosphate pathway*

| | C(1) | | C(2) | | C(16) | | Pentose pathway, % | |
|---|---|---|---|---|---|---|---|---|
| | Acetate absent | Acetate present | Acetate absent | Acetate present | Acetate absent | Acetate present | Acetate absent | Acetate present |
| No. of rats... | 5 | | 3 | | 5 | | 5 | |
| Range | 6.96–12.30 | 5.38–10.40 | 11.04–13.20 | 9.75–15.50 | 8.39–13.55 | 8.10–20.00 | 5–26 | 26–48 |
| Mean | 9.69 | 8.05 | 12.38 | 12.65 | 11.25 | 13.08 | 14 | 37 |
| Difference ± standard error of difference | 1.64 ± 0.446 | | 0.27 ± 1.187 | | 1.83 ± 1.294 | | 23 ± 3.6 | |
| Significance of difference | * | | NS | | NS | | ** | |

† Incorporation expressed as $\dfrac{\text{specific activity of glycerol by wt} \times 100}{\text{specific activity of substrate glucose by wt}}$.

Levels of significance: NS $P > 0.05$. * $P < 0.05$. ** $P < 0.01$.

Table 8. *The effect of unlabelled pyruvate on the incorporation† of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ atoms of glucose into glyceride glycerol by lactating rat mammary gland, and on the proportion of glucose catabolized by the pentose phosphate pathway*

| | C(1) | | C(2) | | C(6) | | Pentose pathway, % | |
|---|---|---|---|---|---|---|---|---|
| | Pyruvate absent | Pyruvate present | Pyruvate absent | Pyruvate present | Pyruvate absent | Pyruvate present | Pyruvate absent | Pyruvate present |
| No. of rats... | 5 | | 4 | | 5 | | 5 | |
| Range | 8.50–14.80 | 0.38–0.50 | 17.30–21.30 | 0.54–1.72 | 11.05–15.60 | 4.20–8.10 | 0–36 | 90–94 |
| Mean | 10.72 | 0.45 | 18.90 | 0.93 | 13.09 | 5.63 | 18 | 92 |
| Difference ± standard error of difference | 10.27–1.100 | | 17.97–0.845 | | 7.46–0.871 | | 74 ± 7.3 | |
| Significance of difference | *** | | *** | | ** | | *** | |

specific activity of glycerol by wt ×100

When glucose was the only substrate the mean value for the proportion of glucose catabolized to lactic acid by the pentose phosphate pathway was 52 %, a result which agrees well with that based on the incorporation of these carbon atoms into fatty acids. The addition of acetate resulted in an increase in the relative participation of this pathway which, however, was not significant (Table 5).

*Effect of pyruvate.* Table 6 shows that on the addition of pyruvate the incorporation of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ atoms of glucose into lactic acid, as into fatty acids (Table 4), was markedly reduced. In contrast with the results obtained with fatty acids, the incorporation of glucose carbon into lactic acid in the presence and absence of pyruvate indicates that this substrate had no significant effect on the proportion of glucose catabolized by the pentose phosphate pathway.

### (c) GLYCERIDE GLYCEROL

*Effect of acetate.* The incorporation of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ atoms of glucose into glyceride glycerol is shown in Table 7. When glucose was the only substrate the mean incorporation of the $C_{(2)}$ atom of glucose was higher than that of the $C_{(6)}$ atom, whereas Tables 3, 4, 5 and 6 show that the level of incorporation of the $C_{(2)}$ atom into both fatty acids and lactic acid was less than that of the $C_{(6)}$ atom.

The addition of acetate had no significant effect on the incorporation of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ atoms of glucose into glycerol. Although the contribution of the pentose pathway as calculated from the data for this derivative was tripled on the addition of acetate, all the values obtained (Table 7) were very much lower than those calculated from the data for fatty acids and lactic acid.

*Effect of pyruvate.* As observed with the other 2 derivatives, the incorporation into glycerol of the $C_{(1)}$ and $C_{(2)}$ atoms of glucose was markedly reduced on the addition of pyruvate (Table 8). The incorporation of the $C_{(6)}$ atom, however, was only halved. Calculations of the participation of the pentose phosphate pathway indicate that, as for fatty acids, the small amounts of glucose converted to glycerol in the presence of pyruvate are metabolized largely by that pathway.

### DISCUSSION

#### Evaluation of pathways

No attempt has been made to make a quantitative assessment of the activity of the pentose phosphate pathway on the basis of the relative conversion of the $C_{(1)}$ and $C_{(6)}$ atoms of glucose to $CO_2$. This method is particularly inadequate when there is an extensive re-cycling of some of the carbon atoms of glucose (Katz & Wood, 1960). The high rate of oxidation of the $C_{(2)}$ atom relative to that of the $C_{(6)}$ atom found both in this and other work (McLean, 1964) indicates that there is a considerable re-cycling of glucose carbon in lactating rat mammary tissue. Even when used as a qualitative guide to changes in the activity of the pentose phosphate pathway this ratio must be interpreted with caution. Thus, the decrease in the ratio observed when acetate was added to the medium (Table 1) was the result of a 2-fold increase in $q_{G6}$ (which is converted to the methyl carbon atom of 'acetyl' by the glycolytic pathway), and seems to reflect an increase in the amount of labelled glucose which is completely

oxidized in the tricarboxylic acid cycle rather than any change in the activity of the pentose phosphate pathway.

Katz & Wood (1960) examined the assumptions made in the development of the method used in this study to evaluate the participation of the pentose phosphate pathway. They showed that it is inadvisable to place too much weight on the absolute values of the results obtained. Nevertheless, it seems justifiable to apply the method to studies in which changes in the general pattern of metabolism are being investigated, as here. Thus, Greenbaum & Darby (1964) used this type of calculation in their study of the effect of adrenalectomy on mammary gland metabolism, and Walters & McLean (1967) used it in a similar study of the effects of thyroidectomy.

One of the assumptions on which the method is based is that the 2 'triose phosphates' (glyceraldehyde 3-phosphate and dihydroxyacetone phosphate) are in isotopic equilibrium. However, there is evidence obtained from studies of liver and adipose tissue that this is not always so (Marks & Horecker, 1956; Katz, Landau & Bartsch, 1966).

The results obtained when the relative participation of the pentose phosphate pathway is calculated from the incorporation of the $C_{(1)}$ and $C_{(6)}$ atoms of glucose into the 3 derivatives used (fatty acids, lactic acid and glycerol) are not in agreement. Glycerol consistently indicates a lower participation of the pentose phosphate pathway than do the other 2 derivatives. Statistical analysis of the results ('t' test) shows that when glucose is the only substrate, there is no significant difference ($P > 0.05$) between those obtained from fatty acids and lactic acid, respectively. The difference between the results obtained from fatty acids and lactic acid on the one hand and glycerol on the other are, however, highly significant ($P < 0.001$). In the presence of acetate the results obtained from glycerol are still significantly different from those obtained from either fatty acids or lactic acid ($P < 0.001$, $P < 0.05$). Under these conditions, however, there is also a significant difference at the 5 % level between the results obtained from fatty acids and lactic acid.

Both fatty acids and lactic acid derive carbon from glucose via glyceraldehyde-3-phosphate whereas glycerol is derived from dihydroxyacetone phosphate. In spite of the difference in the results obtained from fatty acids and lactic acid in the presence of acetate, the best explanation of the results as a whole would appear to be that in the rat mammary gland as in liver and adipose tissue the 2 'triose phosphates' are not in isotopic equilibrium. The observation that the $C_{(2)}$ carbon of glucose appears in glyceride glycerol to a greater extent than the $C_{(6)}$ carbon provides further evidence to support this view because it indicates that dihydroxyacetone phosphate is a better precursor of glycerol than glyceraldehyde 3-phosphate.

### The effect of acetate

The increase in $q_{G6}$ observed when acetate was added to the medium (Table 1) may be due to a disturbance of the balance between the oxidation of 'C-2 units' derived from glucose and their utilization for fatty acid synthesis. It is now accepted that fatty acid synthesis in mammary gland tissue is largely an extra-mitochondrial process (Dils & Popják, 1962) and since the pyruvate oxidase system is known to be intramitochondrial, it follows that there must be 2 pools of acetyl coenzyme A, one extra-mitochondrial and the other intra-mitochondrial. Glucose carbon can there-

fore contribute to the extra-mitochondrial pool of acetyl coenzyme A only by way of the intra-mitochondrial pool. Smith, Easter & Dils (1966) showed that acetate:CoA ligase in rabbit mammary gland tissue was largely extra-mitochondrial, and it there- fore follows that exogenous acetate can contribute to the extra-mitochondrial pool directly.

Although in rat mammary tissue acetate does not contribute to the synthesis of fatty acids in the absence of glucose, it is readily utilized when glucose is present (Balmain, Folley & Glascock, 1952). Under these conditions almost equal amounts of the 2 sub- strates are incorporated into fatty acids (Balmain *et al.* 1954) and they must therefore contribute almost equally to the extra-mitochondrial pool of acetyl co-enzyme A.

The mechanism by which acetate produces its effect on glucose oxidation must, however, be to some extent a matter for conjecture. The addition of acetate to a pre- paration metabolizing only glucose will make available an additional source of C-2 units to the extra-mitochondrial pool. This will tend to reduce the flow into that pool of C-2 units from the intra-mitochondrial pool, thus leaving more glucose carbon in that pool available for oxidation in the tricarboxylic acid cycle. Acetate will also, however, contribute to the intra-mitochondrial pool and dilute the labelled glucose carbon in it. Whether a net increase or decrease in the rate of oxidation of glucose carbon then results will depend on the balance of these 2 opposing tendencies. The fact that an increase in the rate of oxidation of the $C_{(6)}$ atom of glucose is observed when acetate is added suggests that the 'sparing effect' on the intra-mitochondrial pool is greater than the effect of dilution by the $C_{(2)}$ units derived from acetate.

As already noted, the high rate of oxidation of the $C_{(2)}$ atom of glucose relative to that of the $C_{(6)}$ atom (Table 1) must have been largely due to re-cycling in the pentose phosphate pathway and the 40 % increase observed on the addition of acetate must for the same reasons have been due to increased re-cycling. This idea is supported by the results of 2 of the 3 methods of evaluation of the pentose phosphate pathway in the presence and absence of acetate.

In addition to a stimulation of the pentose phosphate pathway as suggested above, acetate may also have an inhibitory effect upon the glycolytic pathway. Williamson (1965), in a study of the metabolism of glucose by the perfused rat heart, found that the levels of glucose 6-phosphate were raised and hexokinase activity was decreased by the addition of acetate. He attributed these effects to an inhibition of phosphofructokinase and showed that an increased synthesis of glycogen occurred, presumably by diversion of glucose into this pathway. In rat mammary tissue a similar inhibition of some step or steps in the glycolytic pathway may occur. How- ever, both Williamson (1965) and Bethencourt, Matos & Shipp (1966) have shown that in the perfused rat heart the oxidation of uniformly labelled glucose was greatly decreased by the addition of acetate. Thus, the arguments put forward by Williamson are not an adequate explanation of the effect of acetate on glucose metabolism in rat mammary tissue, nor do the present experiments resolve this problem.

### *The effect of pyruvate*

The decreased rate of oxidation of the $C_{(6)}$ atom of glucose which is observed in the presence of unlabelled pyruvate may be due to the dilution of labelled carbon derived from the $C_{(6)}$ atom of glucose at the pyruvate stage of the glycolytic pathway. However,

this explanation cannot account for the large reductions in $q_{G1}$ and $q_{G2}$ because the conversion of these carbon atoms to $CO_2$ is largely the result of the reactions of the pentose phosphate pathway and thus occurs by a route which does not involve pyruvate. Although isotope dilution by carbon derived from pyruvate cannot be excluded as the cause of the marked reduction in the incorporation of glucose carbon into lactic acid and glycerol, it seems improbable. First, a similar effect is observed on the incorporation into fatty acids into which pyruvate can pass only by way of acetate, and the effect of acetate was very much smaller. Secondly, when it has been observed isotope dilution produced by an exogenous substrate produces a very much smaller effect than that observed here (Duncombe & Glascock, 1956). The very large reduction caused by pyruvate in the incorporation of glucose carbon into the 3 derivatives studied makes the apparent increase in the relative participation of the pentose phosphate pathway of doubtful biochemical significance.

Work in other laboratories has shown that in the perfused rat heart pyruvate inhibits glucose uptake and oxidation by the inhibition of one or more steps of the glycolytic pathway (Newsholme, Randle & Manchester, 1962; Williamson, 1965). The effects of pyruvate on the oxidation of glucose by lactating rat mammary gland tissue may be the result of a similar inhibition, and the large depression of $q_{G1}$ suggests that it must occur before the hexose phosphate stage, i.e. either glucose entry into the cell or its phosphorylation by hexokinase are inhibited. The apparent change in the relative contributions of the 2 pathways may mean that a further step which occurs before the formation of triose phosphate is affected. It is obvious, however, that the mechanism by which pyruvate exerts its effect is different from that involved when acetate is the additional substrate and perhaps different also from those investigated in the studies with the perfused rat heart.

The very much reduced synthesis of fatty acids from glucose in the presence of pyruvate remains to be explained. Although the addition of pyruvate reduces the oxidation of glucose, it is by no means completely abolished. Thus, although one step at which pyruvate exerts its effect may be located at a point where the glucose that is oxidized and the glucose that is incorporated into fatty acids follow a common pathway, it is possible that pyruvate also inhibits the chain elongation process.

The existence of an active pentose phosphate pathway in lactating rat mammary tissue and its high capacity to synthesize fatty acids make the effects of additional substrates more far-reaching than in heart muscle where neither of these pathways has any significant role. The effects are consequently more difficult to elucidate.

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# The effects of acetate and of pyruvate on the pathways of glucose catabolism in lactating mammary tissue

## II. Sheep tissue

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SUMMARY. A study was made of the changes in the rates of oxidation of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ atoms of glucose and in the pathways of glucose catabolism in sheep udder tissue *in vitro* which occurred when acetate and pyruvate were added.

Whereas in rat mammary tissue the rate of oxidation of the $C_{(1)}$ atom of glucose was very much greater than that of the $C_{(6)}$ atom, the ratio of the rates of oxidation of these 2 atoms in sheep tissue was less than 2 when glucose was the only substrate.

The addition of acetate resulted in an unequal stimulation of the oxidation of these 2 atoms, with the result that the ratio of their rates of oxidation was about doubled. The rate of oxidation of the $C_{(2)}$ atom was also increased.

Acetate also increased the participation of the pentose phosphate pathway in glucose catabolism as measured by the incorporation of the $C_{(1)}$ and $C_{(6)}$ atoms of glucose into fatty acids, lactic acid and glycerol.

Pyruvate produced little effect on the rate of oxidation of the $C_{(1)}$ atom but somewhat depressed that of the $C_{(6)}$ atom of glucose. At the same time, it caused a large increase in the participation of the pentose phosphate pathway.

These results are discussed with reference to re-cycling of glucose carbon in the pentose phosphate pathway and to the relationship between that pathway and fatty acid synthesis. It is noted that the incorporation of glucose carbon into the 3 intermediates used gave values for the participation of that pathway which were in better agreement than was obtained in rat tissue. It is concluded that triose phosphates are more nearly in equilibrium in sheep than in rat mammary tissue.

The role of glucose in the metabolism of the ruminant udder, other than its importance as the precursor of lactose, is not well defined. Although it makes only a small contribution to the milk fatty acids, Hardwick, Linzell & Price (1961) found that it was essential for the maintenance of milk secretion and they concluded that contrary to earlier ideas it is an important metabolite.

Although the participation of the pentose phosphate pathway in the metabolism of glucose by lactating rat mammary gland tissue has been examined in detail, the function of this pathway in the udder of the lactating ruminant has received much less attention. The only studies *in vitro* which are relevant to this problem are those reported by Duncombe & Glascock (1956), who demonstrated that the oxidation of

glucose by sheep udder slices was increased 3-fold on the addition of acetate to the medium. However, their results were inconclusive as to the operation of a non-glycolytic pathway.

Wood and his colleagues have obtained evidence for the existence of this pathway in udder tissue both from experiments *in vivo* (Wood, Gillespie, Joffe, Hansen & Hardenbrook, 1958) and, more recently, from studies with perfused udders from lactating cows (Wood, Peeters, Verbeke, Lauryssens & Jacobson, 1965). In the present paper we report the results of a further approach to the problem using the *in vitro* technique. The pathways of glucose catabolism by sheep udder slices and the changes which occur when acetate or pyruvate are added to the incubation medium have been studied.

## MATERIALS AND METHODS

*Chemicals.* The purity of all chemicals and the sources of supply were as described in the preceding paper (Smith & Glascock, 1969).

*Animals.* The animals used in this study were purchased from local farmers and were in the third or fourth week of lactation at the time of the experiment. They were taken to the abbatoir for slaughter, where thin portions of the udder were quickly removed and stored in ice-cold Ringer's solution for transport to the laboratory.

*Experimental procedures.* The preparation of tissue slices, conditions of incubation and substrates were as described by Smith & Glascock (1969). The methods for the collection of respiratory $CO_2$, the isolation of fatty acids, glyceride glycerol and lactic acid and the measurement of radioactivity have also been described by Smith & Glascock (1969).

## RESULTS

### Oxidation of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ atoms of glucose to $CO_2$

*Expression of results.* Hourly rates of oxidation are expressed as $\mu g$ C/mg dry weight of tissue and are designated by the q notation defined in the preceding paper (Smith & Glascock, 1969). Thus, for example, $q_{G1}$ refers to the rate of oxidation of the $C_{(1)}$ atom.

*The effect of acetate.* When glucose was the only substrate $q_{G1}$ was in most experiments between 1·5 times and twice as great as $q_{G6}$, and $q_{G2}$ was also slightly greater than $q_{G6}$ (Table 1). This pattern of glucose oxidation is quite different from what had been observed repeatedly in experiments with lactating rat mammary tissue. These experiments had shown that in rat tissue the rate of oxidation of the $C_{(1)}$ atom may be 20 times as great as that of the $C_{(6)}$ atom (see, for example, Smith & Glascock, 1969).

When acetate was added to the medium the values of $q_{G1}$ and $q_{G2}$ were increased by as much as a factor of 5 but $q_{G6}$ was only doubled. The unequal stimulation of the 2 carbon atoms resulted in an increase in the value of the ratio $q_{G1}/q_{G6}$. In similar experiments with lactating rat mammary tissue, which have been reported in the preceding paper, it was shown that the addition of acetate resulted in an increase in the rate of oxidation of the $C_{(6)}$ atom but had little effect on that of the $C_{(1)}$ atom, thus reducing the value of the ratio $q_{G1}/q_{G6}$.

*The effect of pyruvate.* Duncombe & Glascock (1956) found that the addition of pyruvate to the medium did not affect the rate of oxidation of the $C_{(1)}$ atom of

| | $q_{G1}$ | | $q_{G2}$ | | $q_{G6}$ | | Ratio $q_{G1}/q_{G6}$ | |
|---|---|---|---|---|---|---|---|---|
| | Acetate absent | Acetate present | Acetate absent | Acetate present | Acetate absent | Acetate present | Acetate absent | Acetate present |
| No. of sheep... | 11 | | 11 | | 11 | | 11 | |
| Range | 0·046–0·19 | 0·13–0·77 | 0·038–0·10 | 0·088–0·57 | 0·023–0·076 | 0·056–0·22 | 1·09–3·90 | 2·29–7·00 |
| Mean | 0·091 | 0·40 | 0·070 | 0·26 | 0·053 | 0·11 | 1·82 | 3·54 |
| Difference ± standard error of difference | 0·31 ± 0·055 | | 0·19 ± 0·045 | | 0·057 ± 0·012 | | 1·72 ± 0·280 | |
| Significance of difference | *** | | ** | | *** | | *** | |

† Rate of oxidation expressed as $\mu$g C/mg dry wt of tissue.
Levels of significance: ** $P < 0.01$. *** $P < 0.001$.

Table 2. *The effect of unlabelled pyruvate on the hourly rate of oxidation† of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ atoms of glucose by slices of lactating sheep udder*

| | $q_{G1}$ | | $q_{G2}$ | | $q_{G6}$ | | Ratio $q_{G1}/q_{G6}$ | |
|---|---|---|---|---|---|---|---|---|
| | Pyruvate absent | Pyruvate present | Pyruvate absent | Pyruvate present | Pyruvate absent | Pyruvate present | Pyruvate absent | Pyruvate present |
| No. of sheep... | 7 | | 7 | | 7 | | 7 | |
| Range | 0·046–0·12 | 0·067–0·12 | 0·038–0·089 | 0·015–0·037 | 0·023–0·074 | 0·007–0·034 | 1·00–2·10 | 3·53–12·30 |
| Mean | 0·072 | 0·094 | 0·067 | 0·031 | 0·048 | 0·017 | 1·61 | 6·60 |
| Difference ± standard error of difference | 0·022 ± 0·009 | | 0·036 ± 0·0072 | | 0·031 ± 0·053 | | 4·99 ± 1·018 | |
| Significance of difference | * | | ** | | *** | | ** | |

† Rate of oxidation expressed as $\mu$g C/mg dry wt of tissue.

glucose. In the present studies a variable effect was observed when pyruvate was added to the medium, but there was little change in the mean value of $q_{G1}$ (Table 2). However, the rates of oxidation of both the $C_{(2)}$ and $C_{(6)}$ atoms were reduced and the ratio $q_{G1}/q_{G6}$ was increased. This result is again different from that observed with rat mammary tissue in which the rates of oxidation of the $C_{(1)}$ and $C_{(6)}$ atoms were reduced to an approximately equal extent and the ratio $q_{G1}/q_{G6}$ thus remained unchanged.

### Pathways of glucose catabolism

In the experiments with rat mammary tissue (Smith & Glascock, 1969) it was shown that the changes in the rates of oxidation of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ atoms of glucose which were observed on the addition of acetate or pyruvate were associated with a change in the relative extents to which the pentose phosphate and glycolytic pathways participated in the catabolism of glucose to triose phosphate. The incorporation of these carbon atoms into 3 derivatives of triose phosphate (fatty acids, lactic acid and glyceride glycerol) by sheep udder slices was therefore measured. The results were used to calculate the proportion of the glucose converted to these substances which was metabolized by the pentose phosphate pathway. The method of calculation and its reliability are discussed in the preceding paper.

### Effect of acetate and of pyruvate on the contribution of the pentose phosphate pathway as measured by the incorporation of glucose carbon into:

#### (a) FATTY ACIDS

*Effect of acetate and of pyruvate.* Balmain, Folley & Glascock (1954) found a very low incorporation of glucose total carbon into the fatty acids of sheep udder slices and that this level was further depressed by the addition of acetate. In agreement with those findings the present work shows that the incorporation of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ atoms of glucose were approximately equal, less by a factor of 100 than that observed in rat mammary tissue, and further reduced by the addition of acetate (Table 3). The inhibitory effect of acetate, however, was greater on the $C_{(1)}$ atom than on the $C_{(6)}$ atom, with the result that the calculated participation of the pentose phosphate pathway increased from a mean value of 28 % to one of 61 %. The addition of pyruvate (Table 3) reduced even further the incorporation of the $C_{(1)}$ and $C_{(6)}$ atoms of glucose but in such a way that the calculated participation of the pentose phosphate pathway was about the same as in the presence of acetate. The addition of pyruvate reduced the incorporation of the $C_{(2)}$ atom to a smaller extent than that of the other 2 carbon atoms studied.

#### (b) LACTIC ACID

*Effect of acetate.* The addition of acetate produced a 25 % depression in the incorporation of the $C_{(1)}$ atom and a somewhat smaller increase in the incorporation of the $C_{(6)}$ atom of glucose into lactic acid. This resulted in an increase in the calculated participation of the pentose phosphate pathway from 17 to 44 %. Acetate had no significant effect on the incorporation of the $C_{(2)}$ atom (Table 4).

*Effect of pyruvate.* As was observed in rat mammary tissue (Smith & Glascock, 1969), the addition of pyruvate caused a reduction by a factor of about 10 in the incorporation of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ atoms of glucose into lactic acid. The effect on the

Table 3. *The effect of unlabelled acetate and pyruvate on the incorporation† of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ atoms of glucose into fatty acids of lactating sheep udder tissue, and on the proportion of glucose catabolized by the pentose phosphate pathway*

| $C_{(1)}$ | | | $C_{(2)}$ | | |
|---|---|---|---|---|---|
| Substrate No. of sheep... | Glucose | Glucose + Acetate 5 | Glucose + Pyruvate | Glucose | Glucose + Acetate 5 | Glucose + Pyruvate |

| | $C_{(1)}$ | | | $C_{(2)}$ | | |
|---|---|---|---|---|---|---|
| Substrate No. of sheep... | Glucose | Glucose + Acetate 5 | Glucose + Pyruvate | Glucose | Glucose + Acetate 5 | Glucose + Pyruvate |
| Range | 5·20–13·05 | 1·20–2·40 | 0·46–1·03 | 8·20–14·90 | 1·98–4·77 | 3·20–5·30 |
| Mean | 8·29 | 1·66 | 0·62 | 10·54 | 3·70 | 4·12 |
| ‡Difference ± standard error of difference | | 6·63 ± 1·181 | 7·67 ± 1·181 | | 6·84 ± 1·289 | 6·42 ± 1·289 |
| Significance of difference | | *** | *** | | *** | ** |

| | $C_{(6)}$ | | | Pentose pathway, % | | |
|---|---|---|---|---|---|---|
| Substrate No. of sheep... | Glucose | Glucose + Acetate 5 | Glucose + Pyruvate | Glucose | Glucose + Acetate 5 | Glucose + Pyruvate |
| Range | 8·74–14·67 | 2·60–8·75 | 0·91–2·21 | 11–41 | 51–73 | 50–77 |
| Mean | 11·36 | 4·74 | 1·71 | 28 | 61 | 63 |
| ‡Difference ± standard error of difference | | 6·62 ± 1·672 | 9·65 ± 1·672 | | −33 ± 8·6 | −35 ± 8·6 |
| Significance of difference | | ** | *** | | ** | ** |

† Incorporation expressed as $\mu$g labelled C/100 mg fatty acid C.

‡ The difference is the mean value with glucose as the only substrate minus the mean value with glucose + acetate or glucose + pyruvate as substrates

Levels of significance:   ** $P < 0.01$.   *** $P < 0.001$.

Table 4. *The effect of unlabelled acetate and pyruvate on the incorporation† of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ atoms of glucose into lactic acid by lactating sheep udder tissue, and on the proportion of glucose catabolized by the pentose phosphate pathway*

$C_{(1)}$

| Substrate | Glucose | Glucose + Acetate | Glucose + Pyruvate |
|---|---|---|---|
| No. of sheep... | | 5 | |
| Range | 2·10–4·70 | 1·50–3·60 | 0·11–0·57 |
| Mean | 3·70 | 2·78 | 0·25 |
| ‡Difference ± standard error of difference | | 0·92 ± 0·358 | 3·45 ± 0·358 |
| Significance of difference | | * | *** |

$C_{(2)}$

| Substrate | Glucose | Glucose + Acetate | Glucose + Pyruvate |
|---|---|---|---|
| No. of sheep... | | 2 | |
| Range | 2·60–4·50 | 3·10–4·90 | 0·72–0·77 |
| Mean | 3·55 | 4·00 | 0·74 |
| ‡Difference ± standard error of difference | | −0·45 ± 0·736 | 2·81 ± 0·736 |
| Significance of difference | | NS | NS |

$C_{(6)}$

| Substrate | Glucose | Glucose + Acetate | Glucose + Pyruvate |
|---|---|---|---|
| No. of sheep... | | 5 | |
| Range | 3·00–5·60 | 3·30–6·50 | 0·14–0·77 |
| Mean | 4·42 | 4·96 | 0·40 |
| ‡Difference ± standard error of difference | | −0·54 ± 0·458 | 4·02 ± 0·458 |
| Significance of difference | | NS | *** |

Pentose pathway, %

| Substrate | Glucose | Glucose + Acetate | Glucose + Pyruvate |
|---|---|---|---|
| No. of sheep... | | 5 | |
| Range | 6–30 | 37–55 | 21–59 |
| Mean | 17 | 44 | 40 |
| ‡Difference ± standard error of difference | | −27 ± 8·9 | −23 ± 8·9 |
| Significance of difference | | * | * |

† Incorporation expressed as $\dfrac{\text{specific activity of lactic acid by wt} \times 100}{\text{specific activity of substrate glucose by wt}}$.

‡ The difference is the mean value with glucose as the only substrate minus the mean value with glucose+acetate or glucose+pyruvate as substrates. Levels of significance: NS $P > 0.05$.  \* $P < 0.05$.  \*\*\* $P < 0.001$.

Table 5. *The effect of unlabelled acetate and pyruvate on the incorporation† of the $C_{(1)}$, $C_{(2)}$ and $C_{(6)}$ atoms of glucose into glyceride glycerol by lactating sheep udder tissue, and on the proportion of glucose catabolized by the pentose phosphate pathway*

|  | $C_{(1)}$ | | | $C_{(2)}$ | | |
|---|---|---|---|---|---|---|
| Substrate | Glucose | Glucose + Acetate | Glucose + Pyruvate | Glucose | Glucose + Acetate | Glucose + Pyruvate |
| No. of sheep… | | 5 | | | 5 | |
| Range | 1·6–4·1 | 1·7–4·9 | 1·0–3·1 | 2·4–4·6 | 2·8–6·7 | 1·4–3·8 |
| Mean | 3·02 | 3·30 | 1·94 | 3·28 | 4·72 | 2·40 |
| ‡Difference ± standard error of difference | | −0·28 ± 0·311 | 1·08 ± 0·311 | | −1·44 ± 0·417 | 0·88 ± 0·417 |
| Significance of difference | | NS | ** | | ** | NS |

|  | $C_{(6)}$ | | | Pentose pathway, % | | |
|---|---|---|---|---|---|---|
| Substrate | Glucose | Glucose + Acetate | Glucose + Pyruvate | Glucose | Glucose + Acetate | Glucose + Pyruvate |
| No. of sheep… | | 5 | | | 5 | |
| Range | 2·2–5·5 | 4·3–11·5 | 3·0–5·2 | 8–27 | 30–65 | 35–67 |
| Mean | 3·74 | 7·18 | 4·10 | 19 | 53 | 55 |
| ‡Difference ± standard error of difference | | −3·44 ± 0·768 | −0·36 ± 0·768 | | −34 ± 6·5 | −36 ± 6·5 |
| Significance of difference | | ** | NS | | *** | *** |

† Incorporation expressed as $\dfrac{\text{specific activity of glycerol by wt} \times 100}{\text{specific activity of substrate glucose by wt}}$.

‡ The difference is the mean value with glucose as the only substrate minus the mean value with glucose + acetate or glucose + pyruvate as substrate

Levels of significance: NS $P > 0.05$. ** $P < 0.01$. *** $P < 0.001$.

calculated participation of the pentose phosphate pathway was to increase it from a mean value of 17 % to one of 40 %, which is about the same result as was observed on the addition of acetate (Table 4).

### (c) GLYCERIDE GLYCEROL

*Effect of acetate.* The addition of acetate had no significant effect on the incorporation of the $C_{(1)}$ atom, but nearly doubled the incorporation of the $C_{(6)}$ atom into glycerol, with the result that the calculated participation of the pentose phosphate pathway increased from 19 to 53 %. Acetate also produced a 43 % increase in the incorporation of the $C_{(2)}$ atom (Table 5).

*Effect of pyruvate.* The effect of pyruvate was quantitatively very different from that observed on the incorporation of glucose carbon into fatty acids and lactic acid, where it produced a 90 % reduction in the incorporation of the $C_{(1)}$ and $C_{(6)}$ atoms. The incorporation of the $C_{(1)}$ atom into glycerol was reduced by less than 50 % and that of the $C_{(6)}$ was not significantly changed. This had the result that the calculated participation of the pentose phosphate pathway was increased from 19 to 53 % by the addition of pyruvate, which therefore had about the same effect as acetate. The small depression in the mean incorporation of the $C_{(2)}$ atom in glycerol was not significant, which again was different from the effect of pyruvate on the incorporation of this atom into fatty acids and lactic acid. There the incorporation was very much reduced.

### DISCUSSION

Duncombe & Glascock (1956) concluded that, contrary to earlier ideas, appreciable amounts of glucose are oxidized by sheep udder tissue, especially when, as in the living animal, acetate is present. Further evidence to support this conclusion was provided by Hardwick, Linzell & Mepham (1963), who found that in the perfused goat udder about 40 % of the expired $CO_2$ came from added glucose. The results presented here support this conclusion and show that the rate of oxidation of the $C_{(6)}$ atom of glucose, which must occur by the reactions of the tricarboxylic acid cycle, is equal to or greater than that found in lactating rat mammary slices (Smith & Glascock, 1969).

When glucose was the only substrate there was a marked species difference in the rates of oxidation of the $C_{(1)}$ and $C_{(2)}$ atoms relative to that of the $C_{(6)}$ atom. Thus, it was found that although the rate of oxidation of this atom was approximately the same in both species, that of the $C_{(1)}$ and $C_{(2)}$ atoms was very much lower in sheep than in rat mammary tissue. This was probably due to a lower participation of the pentose phosphate pathway in glucose catabolism in sheep tissue than in rat tissue with a consequent smaller degree of re-cycling of the $C_{(2)}$ atom. Furthermore, the results presented in Tables 3, 4 and 5 show that this pathway did not play a major role in the catabolism of glucose to triose phosphate when glucose was the only substrate. The difference between the rates of oxidation of the $C_{(2)}$ and $C_{(6)}$ atoms may also have been partly due to unequal rates of oxidation in the tricarboxylic acid cycle (Duncombe & Glascock, 1956).

### Evaluation of pathways

Tables 3, 4 and 5 show that the values for the relative participation of the pentose phosphate pathway as calculated from the incorporation of the $C_{(1)}$ and $C_{(6)}$ atoms

of glucose into the 3 chosen derivatives were in better agreement than was observed for rat tissue (Smith & Glascock, 1969). In that work, glycerol always gave a lower value than did fatty acids and lactic acid, and this was attributed to triose phosphates' not being in isotopic equilibrium. This possibility was supported by the fact that the $C_{(2)}$ atom of glucose was incorporated into glycerol to a greater extent than the $C_{(6)}$ atom. As this was not observed in the present work it appears that triose phosphates are more nearly in equilibrium in sheep than in rat mammary tissue.

## The effect of acetate

Incorporation of the $C_{(1)}$ and $C_{(6)}$ atoms of glucose into all the 3 intermediates studied indicated that the addition of acetate resulted in an increased participation of the pentose phosphate pathway in glucose catabolism (Tables 3, 4 and 5) and this conclusion is supported by the increased rate of oxidation of the $C_{(1)}$ atom (Table 1). There is little doubt that the interaction between these substrates in sheep udder tissue is quite different from that in rat mammary tissue. In addition to the stimulation of the pentose phosphate pathway observed on the addition of acetate, there must also have been an increase in the activity of the tricarboxylic acid cycle because the increased oxidation of the $C_{(6)}$ atom of glucose observed in this work (Table 1) is known to be accompanied by an increased oxidation of acetate carbon (Duncombe & Glascock, 1956).

It is possible that the increased participation of the pentose phosphate pathway produced by the addition of acetate was due to a partial inhibition of the glycolytic pathway as suggested for rat tissue (Smith & Glascock, 1969). It seems more likely, however, that the factor responsible for this effect is the close relationship which exists in mammary tissue between the pentose phosphate pathway and the systems for the synthesis of fatty acids, these being dependent on the supply of NADPH produced by the pentose phosphate pathway (see Folley & Greenbaum, 1960). Although sheep udder tissue incorporates acetate into fatty acids when it is the only substrate, it is particularly relevant that there is a considerable increase in this incorporation when glucose is added to the medium (Balmain, Folley & Glascock, 1952). Glucose, on the other hand, is not an important precursor of fatty acids in ruminant udder tissue (Balmain *et al.* 1954; Hardwick *et al.* 1963) presumably because of the absence of ATP-citrate lyase (Hardwick, 1966; Hanson & Ballard, 1967). Thus, when glucose is the only substrate there is no synthesis of fatty acids and no demand for NADPH, hence the low activity of the pentose phosphate pathway. When acetate and glucose are both available the demand for NADPH created by the synthesis of fatty acids from acetate is met by an increase in the activity of the pentose phosphate pathway. The 2 pathways probably stimulate one another, each requiring for its continued function the co-enzyme in the state of oxidation produced by the other, i.e. they are regulated by the equilibrium between NADP and NADPH.

The source of NADPH when acetate is the only substrate is a matter for speculation. Lowenstein (1961*a*, *b*) suggested that an NADP-dependent isocitrate dehydrogenase present outside the mitochondria may provide NADPH for fatty acid synthesis in rat liver and it is possible that a similar enzyme system is present in sheep udder tissue. Wise & Ball (1964) concluded that in some tissues the reaction catalysed by the malic enzyme may function to provide NADPH for synthetic purposes. High

levels of this enzyme are found in rat mammary tissue during peak lactation (Matthes, Abraham & Chaikoff, 1963) but its occurrence in ruminant udder tissue has not been reported.

Because the blood of ruminants contains appreciable levels of acetate, both glucose and acetate are available to the ruminant udder *in vivo*. It is likely therefore that, as found by Wood *et al.* (1965) in their studies on perfused udders from lactating cows, the pentose phosphate pathway has an important function in the metabolism of the ruminant udder *in vivo*. Hardwick (1965) has suggested that in the perfused goat udder the output of NADPH by the pentose phosphate pathway is adequate for fatty acid synthesis.

### The effect of pyruvate

In general, the changes in the catabolism of glucose which occurred when pyruvate was added to the medium were similar to those reported for rat mammary gland tissue (Smith & Glascock, 1969). The decrease in the rates of oxidation of the $C_{(2)}$ and $C_{(6)}$ atoms may be explained by a dilution of labelled carbon derived from glucose by unlabelled carbon from pyruvate at the point of entry into the tricarboxylic acid cycle. Again, as in rat mammary gland tissue, the results for the incorporation of the $C_{(1)}$ and $C_{(6)}$ atoms of glucose into the various derivatives of triose phosphate suggest that, although the absolute amounts of glucose incorporated into these compounds may be decreased, there is an increase in the fraction of this glucose which is metabolized via the pentose phosphate pathway.

The possibility that pyruvate inhibits some step or steps of the glycolytic pathway in rat mammary tissue was discussed in the preceding paper and it is equally possible that in sheep udder tissue there is a similar inhibition of this pathway. The point at which such an inhibition occurs must be different, however, because pyruvate causes no depression of the rate of oxidation of the $C_{(1)}$ atom of glucose similar to that observed in rat mammary gland tissue.

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# Non-nutritional factors affecting milk yield in dairy cattle

## Section G. General

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### INTRODUCTION

In research on the development and function of the mammary gland, many different mammals have been used. Most of our understanding has come from experiments with laboratory animals, but for technical reasons these experiments rarely give information on the yields and composition of the milk produced. Through selection the dairy cow has become very highly developed for milk production, and although it has been used as an experimental animal for research on the physiology of lactation, this has been limited for economic reasons. Nevertheless, the dairy cow has many advantages for the investigation of lactation and in recent years studies have been made on factors affecting the rate of secretion of milk and of various constituents in milk. In some of this work milking machines, designed for the separate collection of milk from the individual quarters, have been used to measure the effects of environmental factors such as the degree of milk removal, bacterial infections of the udder and the influence of the timing and duration of the non-lactating (dry) periods between lactations. All of these factors can influence the secretion of milk in an individual quarter relative to other quarters within the same gland, and so the effects are not directly due to factors which affect the whole animal such as the supply of hormones or of precursors for synthesis. Consequently, it seems likely that their influence must be on the total number of secretory cells, the activity of the individual cell or on the transfer of constituents across the mammary epithelium of the particular gland.

A consideration of the results of these experiments shows that some of the non-nutritional factors affecting milk yield fall conveniently into 2 groups:

1. Those which are accompanied by specific characteristic changes in the composition of the milk, of increases in the concentrations of sodium and chloride and decreases in those of lactose and potassium. These occur: (a) with abnormal accumulation of milk within the mammary gland, (b) in late lactation, (c) during and after bacterial infections of the mammary gland.

2. Those which are not accompanied by any change in the concentrations of sodium, chloride, lactose and potassium in the milk. These occur: (a) during a lactation when milking has been continued throughout the whole of the previous pregnancy, with the result that there is no dry period between lactations, (b) as a result of injections of oxytocin during the preceding dry period, (c) as a result of a temporary suspension of milking in the course of the previous lactation, (d) when milking precedes the first parturition.

With the exception of the accumulation of milk within the mammary gland for comparatively short periods, all of the factors that are associated with a change in milk composition cause or are associated with a permanent depression in the yield of milk. Also, all the factors are applied or occur within the lactation. On the other hand, the second group of factors are all applied before the start of the lactation in which the changes in milk yield are observed, though it is possible that husbandry factors applied in the period immediately after parturition, such as particular methods of stimulating milk ejection, may influence milk production in a similar way (Whittlestone, 1968). We believe that the pattern of the results described is interesting and must ultimately be explained in terms of the fundamental physiology of lactation. The present review attempts to summarize the results of experiments on dairy cows and where possible to relate the results to studies carried out with other species. We realize that all the non-nutritional factors influencing lactation have not been covered and that other factors may be economically more important. For example, there is ample evidence that methods of milking affect milk yield, either through their influence on milk ejection or by other mechanisms. The significance of this in dairy cattle has recently been reviewed (Brandsma, 1968).

## LACTATION

It is usually accepted that milk secretion is initiated by hormonal changes that occur at parturition. However, with dairy cattle, the potential for continuous secretion may be present for several weeks before parturition, since high yields of milk of normal composition may be obtained by pre-partum milking (Vanlandingham, Weakley, Ackerman & Hyatt, 1949; Rowland, Roy, Sears & Thompson, 1953). Nevertheless, lactation usually starts at parturition, as this normally coincides with the beginning of milk removal. The part played by pre-partum milking in the initiation of secretion is of some interest and may be due to a direct stimulus similar to that suggested by Benson & Folley (1957). Alternatively, the presence of milk in the unmilked gland may inhibit further secretion so that the full secretory activity is developed only when the milk is regularly removed.

Once lactation is established milk secretion is not only continuous (Gaines &

Sanmann, 1927), but at least over periods of days it is also approximately constant. The changes in the rate of secretion, due to advance in lactation, are gradual and more rapid only when there are either sudden changes in the environment, e.g. in level of feeding, or when the animal becomes diseased. Normally the composition of the milks (i.e. concentrations of constituents such as sodium, chloride, lactose and potassium) from each quarter of an individual cow is identical, and with the usual milking intervals the rate of secretion of milk is unaffected or only slightly modified by the accumulation of milk within the storage spaces of the mammary gland (Turner, 1955; Elliott, Dodd & Brumby, 1960; see also review by Elliott, 1959a).

After milk ejection has occurred most, but not all, of the milk can be removed by milking or suckling. The residual milk which cannot be removed by normal milking is retained within the secretory tissue by capillary forces and is normally 10–25 % of the total secretion in the gland at the end of the milking interval (Johansson, 1952). Most of this residual milk can be removed by milking again after injections of oxytocin, but it must be recognized that even then small residues of milk particularly rich in fat are retained within the gland (Elliott *et al.* 1960).

Over periods of weeks the quantity of milk removed from the mammary gland must be equal to the amount secreted, but for a single milking interval this assumption cannot be made (Johansson, 1940; Turner, 1953; Bailey, Clough & Dodd, 1955). The yield of milk obtained at a given milking is described by the following relationship:

$$y = r_1 + s - r_2,$$

where $y$ = yield of milk, $r_1$ = residual milk retained at the end of the previous milking, $s$ = milk secreted since the previous milking and $r_2$ = residual milk retained at the end of the current milking.

Clearly, the yield at a milking will equal the quantity secreted in the preceding interval only when $r_1 = r_2$, and because residual milk is proportional to milk yield (Turner, 1953) the yield of milk at a given milking may be more than the amount secreted since the previous milking if the milking interval is much shorter than the previous milking interval. The difference between the yield obtained and the amount secreted in an interval will be much greater for fat because of the very high concentration of fat in the residual milk. In estimating the rate of secretion of milk, bias due to residual carry-over can be eliminated by removing the residual milk following injection of oxytocin at the end of the normal milking procedure so as to obtain a second ejection of milk. A limitation of this method is that the administered oxytocin may affect the rate of secretion of milk (Morag, 1968) or its composition (Mackenzie & Lascelles, 1965; Wheelock, Rook & Dodd, 1965a). However, Linzell (1967) considers that these results may be due to the high doses of oxytocin used since he found that in goats the composition of the milk was not affected by lower doses. The possibility that removal of residual milk from the udder affects the secretory processes should not be ignored (Azimov, Orlov & Belugina, 1962). An alternative method of eliminating bias due to residual carry-over by an arrangement of the variable length milking intervals without the use of oxytocin injection is described by Bailey *et al.* (1955).

Over longer periods of lactation the trends in milk production must reflect changes in the development, structure and function of the mammary gland. Detailed study

of these can be made in a number of ways, particularly by histological and histo-chemical techniques and also by measurement and analysis of the products of secretion.

Chemical determinations of nucleic acids and enzymes are now frequently used to obtain an index of the state of development and of the secretory activity of the mammary gland. Details of results obtained with these methods have been the sub-ject of a comprehensive review by Munford (1964).

The content of DNA in the mammary gland is used as an indication of the number of secretory cells (Kirkham & Turner, 1953). This method is based on the belief that the amount of DNA/cell nucleus is constant under a variety of conditions and that therefore changes in the DNA content of the mammary gland represent changes in the number of secretory cells. This basis has been questioned by Sod-Moriah & Schmidt (1968). They consider that the determination of total DNA of the mammary gland is limited because of a lack of standardization due to differences in the number of glands/animal, size of the animal and its glands and the quantity of milk in the gland. They criticize the use of chemical methods on the grounds that they do not discriminate between other cellular components, such as connective tissue and vascular elements. Information obtained by Sod-Moriah & Schmidt (1968) on rabbits shows that the concentration of DNA/cell is not constant, which confirms their criticisms. It follows that results from experiments carried out for example by Tucker (1966) and Ôta, Yokoyama & Shinde (1962), who used chemical methods to determine the DNA content of the mammary gland, must be interpreted with caution. Ôta et al. (1962) allowed for any milk contained in the gland by determining the phosphoprotein P, which is largely in the milk casein. The above experimental pro-cedures are normally used with small animals and so it is difficult to obtain an accurate assessment of the milk yield. Usually milk yield is determined by weighing the young before and after suckling, and it is normally not possible to obtain a value for the milk produced by individual glands or to obtain a representative sample of milk for analysis.

FACTORS DURING LACTATION AFFECTING CURRENT MILK YIELD

The results of experiments on dairy cows have been reviewed by Rook & Wheelock (1967), to which the reader is referred for a detailed analysis.

*Abnormal accumulation of milk within the mammary gland*

Milk accumulates within the gland when the interval between milking or suckling is extended or when all the available milk is not removed when suckling or milking occurs at the usual intervals.

The effect of suspension of suckling in rats has been studied by Ôta (1964). He observed that the size of the mammary gland increased markedly if the litters were removed on the twelfth day of lactation. Between 1 and 3 days after removal of the litter the total content of DNA in the gland started to decrease. The RNA decreased at a much greater rate, so that the RNA:DNA ratio decreased.

In subsequent experiments, Ôta & Yokoyama (1965) restored suckling by intro-ducing a foster litter. As before, the litters were removed on the twelfth day of lactation and re-suckling commenced 3, 5 and 9 days later. They found that milk

secretion was resumed and the DNA in the gland and the RNA:DNA ratio increased again but did not recover completely.

Tucker & Reece (1963) observed that if milk removal in half of the mammary glands of rats was prevented by teat ligation and suckling continued, there was a depression in the DNA and RNA content and in the RNA:DNA ratio of the ligated glands. Their results also showed that in both mice and rats the DNA content of the 3 contra-lateral non-ligated glands was higher than that of the corresponding glands of normal animals at comparable stages of lactation. A similar finding has been obtained with mice (Mizuno, 1961).

Tucker (1966) ligated the thoracic teats of lactating rats on the third day of lacta-tion. From then on, 2, 4, or 6 pups were permitted to suckle the 6 intact abdominal-inguinal glands to achieve 3 intensities of suckling. Groups of rats on each suckling intensity were killed at intervals throughout the lactation. Mammary glands were analysed for nucleic acids and the litter weight gain over a period was used to esti-mate the cumulative milk production. The highest contents for DNA and RNA were obtained with the highest suckling intensity and the lowest values with the lowest suckling intensity. From this information it is not possible to determine whether the high DNA and RNA values obtained for the highest suckling intensity are due to more frequent suckling with consequent release of oxytocin, or whether the low values were obtained with low suckling intensity because of incomplete removal of milk. Tucker also showed that mammary RNA and RNA:DNA ratios were highly correlated with litter weight gain.

*Frequency of milking*

It has been known for many years that cows milked 3 or 4 times daily produce higher milk yields than when milked twice daily (see review by Elliott, 1959b). Traditionally, this effect was ascribed to a decline in the rate of secretion of milk with the rise in intramammary pressure that occurs with increasing milking intervals but, following the demonstration by Turner (1955), Elliott *et al.* (1960) and others that the relationship between the quantity of milk secreted and the duration of the milking interval is virtually linear for at least 16 h, other explanations have been sought. The possibility that the effect of a higher frequency of milking is due to an increased milking stimulus (Benson & Folley, 1957) can be discounted, at least in the sense that it is hormonal, since the effect of frequency of milking can be measured when the experimental comparisons are between quarters within udders. It is to be expected that the levels of residual milk and fat when milking is twice daily will be greater than when milking is thrice daily. But Elliott (1961) was unable to demonstrate that differences in residual milk levels of this order would influence milk yield sufficiently to account for the milking frequency effect. In the latter experiment, the treatments were applied to different halves of the same udder. It was found that the udder halves which were milked 3 times daily produced, on average, 12 % more milk than the halves milked twice daily. There were marked differences in response between animals. The effect was apparent on the first day of the experiment and continued throughout the whole of the 39-day experimental period. The fat concentration of the milk was not affected by the treatments.

Essentially similar results were obtained by Linnerud, Caruolo, Miller, Marx &

Donker (1966). In experiments carried out on a whole udder basis they compared twice daily with 4 times daily milking and found that there was a significant increase in milk yield with 4 times daily milking. These authors failed to obtain any significant increase when every second milking was replaced by an injection of oxytocin (10 i.u.) or hand stimulation.

These results suggest that the increase in milk yield is due to the removal of milk and not to the increased frequency of stimulation with the consequent release of oxytocin. However, the results of Linnerud et al. (1966) may be confused by the effect of the hand stimulation or oxytocin injections on the efficiency of the subsequent natural milk ejection. This would not be so with Elliott's results, as the frequency of stimulation was the same for both treatments.

### Stage of lactation

After the initiation of lactation in cows the milk yield gradually increases for up to 3–6 weeks and thereafter declines approximately linearly although the rate of decline increases after about the fifth month of pregnancy (Gaines & Davidson, 1926). A detailed investigation into the effect of stage of lactation on the yield of milk and on the concentration of a range of constituents has been carried out by Rook & Campling (1965), who also reviewed the previous literature. Their results showed typical changes in the yield of milk throughout the course of a lactation. At the beginning of lactation the concentrations of lactose and potassium were comparatively low and that of sodium comparatively high. Within a few days, however, values were attained which remained remarkably constant for the greater part of the lactation. At the point where the decrease in the milk yield is accelerated due to pregnancy there was a progressive decrease in the concentrations of lactose and potassium and an increase in that of sodium.

It has been shown with mice (Chikamune & Mizuno, 1958; Brookreson & Turner, 1959) and rats (Tucker, 1966) that the DNA content of the mammary gland increases during the first part of the lactation and then declines again as the lactation advances. There is a similar trend for the RNA:DNA ratio. However, the results of Tucker (1966) show that the increase is not observed if the number of pups suckling is reduced to 2 pups/6 intact glands. This suggests that the increase in DNA content of the gland may depend on the release of oxytocin at frequent intervals in response to the suckling stimulus. Alternatively, the incomplete removal of all the available milk may prevent maximum cellular development and secretory activity.

Using rats, Paape & Tucker (1969a) showed that after day 20 of lactation the mammary DNA and RNA was lower in the pregnant than in the non-pregnant animals, provided that a sufficiently strong suckling stimulus is maintained. However, with mice Mizuno (1961) showed that the DNA content was significantly higher in mammary glands from pregnant lactating mice. The RNA content was also greater in the pregnant group than in the non-pregnant group.

The changes in DNA concentration of the mammary gland during late pregnancy and early lactation have been investigated in first lactation cows and in several other species by Baldwin (1966). He found that in the guinea-pig there was a rapid rise in the DNA concentration and in the enzyme activities, but that in the cow the DNA concentration stayed relatively constant between 2·5 and 3·0 m/g mammary tissue

from 14 days before parturition to the fortieth day of lactation. The enzymes, which included glucose-6-*P*-dehydrogenase, malate dehydrogenase and fatty acid synthetase, showed marked increases in activity in the smaller animals, but no significant increase in the cow. Baldwin suggests that there may be significant interspecies differences in the processes associated with the initiation of lactation although he does recognize that the results could be due to differences in the methods of sampling used. From the cows relatively pure samples of secretory tissue were collected by surgical biopsy, but from the guinea-pigs the whole mammary gland was taken and would therefore be expected to contain some adipose tissue. The results, however, do not exclude the possibility that there is an increase in the DNA content of the mammary gland during the period of study because of the general increase in its size. Nevertheless, this work does demonstrate the need for caution in using results from small animals to obtain an understanding of lactation in cows.

The changes in the histology of the mammary gland throughout lactation have been reviewed by Munford (1964). Using a number of different methods it has been shown that the total number of alveolar cells increases during late pregnancy and continues to increase in the early part of lactation. As lactation in the cow advances the lobular volume is diminished (McFarlane, Rennie & Blackburn, 1949). It has been postulated that the decrease in milk yield with advancing lactation is partly due to a decrease in the number of actively secreting cells (Turner, 1952).

### Oxytocin injection

In view of its role in milk ejection, oxytocin is of particular interest as it has frequently been used to overcome difficulties in experimental procedure when the residual milk has to be removed and in cases of impaired efficiency of milk ejection. As it is now known that oxytocin may have an effect on milk secretion in addition to its effect in causing milk ejection, it is necessary to re-evaluate the results of experiments in which oxytocin has been used as an experimental tool to cause or to improve milk ejection.

Administration of oxytocin for a period after suckling was suspended retarded involution of the mammary gland in rats (Benson & Folley, 1957). However, this treatment did not maintain the integrity of the mammary glands indefinitely—a finding which was in agreement with the work of Selye & McKeown (1934) and Williams (1945). Benson & Folley (1957) suggested that the administration of oxytocin during lactation might cause an increase in the secretion of milk.

When investigations have been carried out to determine if the administration of oxytocin during the lactation affects the milk yield, the results have been rather confused. Morag (1968) has shown that oxytocin can increase milk yield and has suggested an explanation for the discrepancies between the results of previous workers. He found that when oxytocin (2·5 i.u./h, 2·5 i.u./2 h and 2·5 i.u./4 h) was administered between milkings and the usual milking routine adopted, the milk yield was depressed. However, if a similar experiment was performed in which the residual milk was removed at the end of each normal milking after 2 injections of oxytocin (5 i.u. followed by 2·5 i.u.) to give a further milk ejection, he found that the milk yield was increased. Consideration of the fat content of the milk obtained suggested that the administration of oxytocin between milkings had impaired the natural milk ejection

reflex so that the galactopoietic effect was masked. When the residual milk was removed, the effect was observed. Morag (1968) also showed that the response to the oxytocin was most marked with a 16-h milking interval as compared with 8- or 12-h intervals.

Injections of oxytocin (20 i.u.) to permit removal of residual milk after the end of the usual milking procedure have been found to affect the composition of the milk at subsequent milkings: the concentrations of sodium and chloride increased and those of lactose and potassium decreased (Wheelock et al. 1965a). These effects were more marked when the injections were given at several successive milkings. After the injections were discontinued, there was a progressive return to the original composition. Mackenzie & Lascelles (1965) showed that when, at a single milking, the residual milk was removed in portions after successive injections of oxytocin (5 i.u.) the later portions showed changes in composition similar to the above, except that the potassium content was virtually unaffected. In the goat, milked 1–4 times/h with the aid of oxytocin (0·05–0·4 i.u.), removal of the milk by cannula or by gentle hand milking caused a slight fall in potassium content only. With more vigorous milking, the changes in potassium content were even more marked and were accompanied by a decrease in the lactose content and increases in the contents of sodium and chloride (Linzell, 1967). The changes could not be explained by assuming that the act of milking causes exudation of tissue fluid into milk and it was considered possible that the doses of oxytocin used caused some changes in the composition of the milk.

It appears that there is a lack of information concerning the action of oxytocin in cows, with particular reference to the effects of different dose levels, on milk ejection and on milk secretion. It is possible that the normal amounts of residual milk can be obtained with much smaller doses of oxytocin than have been commonly used without affecting the secretion of milk. Linzell (1967) considers that with goats it is possible to obtain efficient milk ejection at hourly intervals using low doses of oxytocin (0·05–0·4 i.u.).

### Bacterial infections of the udder

It is now well established that an infection of pathogenic bacteria in an individual udder quarter normally depresses the milk yield of that quarter, the effect varying from a few per cent of production to complete cessation of secretion. This depression in yield persists for the whole of the lactation, even when the bacteria are eliminated by antibiotic therapy, but there is recovery, which may not be complete, in the following lactation (Crossman, Dodd, Lee & Neave, 1950; Rowland, Neave, Dodd & Oliver, 1959). In addition, the depression in milk yield is accompanied by a fall in the concentrations of lactose and potassium and by increases in those of sodium, chloride and whey proteins (see e.g. McDowall, 1945). Recently experiments have been carried out to obtain more complete and quantitative information on the effect of induced bacterial infections on the composition of the milk (Wheelock, Rook, Neave & Dodd, 1966). These workers showed that immediately after the development of an infection there was invariably a depression in milk yield. In some cases there was a partial recovery shortly after the infection developed but otherwise the depression in yield persisted for the remainder of the lactation, even when the infection was eliminated. A recovery in milk yield in the next lactation was also observed but this was not com-

plete. The extent of the changes in concentration of the individual constituents was roughly related to the depression in milk yield. It has been shown that a bacterial infection is associated with a much greater degree of involution than is normally observed at a given stage of lactation (Waite & Blackburn, 1963). The recovery of milk yield in the subsequent lactation suggests that the mammary gland has the ability to regenerate damaged tissue between lactations. Naturally this regeneration would occur automatically if all the secretory tissue is renewed for each lactation.

### FACTORS BEFORE PARTURITION AFFECTING SUBSEQUENT MILK YIELD

Except where stated, all the results described in this section were obtained with cows.

#### *Milking throughout the whole of pregnancy*

Using twins, Swanson (1965) demonstrated that milking throughout the whole of pregnancy causes a decrease in the milk yield during the next lactation. In a similar type of experiment Smith, Wheelock & Dodd (1967) applied the treatments to different quarters of the same animal. They obtained similar results to those of Swanson (1965) and also observed that the extent of the depression in milk yield was roughly related to the amount of milk produced by the quarters in the period immediately before parturition. The concentrations of fat, solids-not-fat (SNF), sodium, chloride, lactose potassium and protein in milks from experimental and control quarters were identical. The milk production of rats was also depressed in the second lactation when suckling was continued throughout the whole of the previous pregnancy (Paape & Tucker, 1969b).

#### *Administration of oxytocin during the dry period*

Injections of oxytocin (5 i.u.) have been given to cows twice daily throughout a 60-day period (Gorman & Swanson, 1968). In the next lactation these animals produced, on average, 20 % less than a group of untreated control animals. The depression in lactation in individual animals was roughly related to the maintenance of secretion by the oxytocin treatment. The concentrations of fat and SNF in the milk were not significantly affected by the treatment.

In another experiment (Swanson & Claycomb, 1968) the treatment commenced when it was judged that the cows had ceased to lactate, in fact from 8 to 19 days after the end of lactation. The depression in milk yield in the following lactation was again found.

#### *Temporary suspension of milking in the previous lactation*

In an experiment performed by Wheelock, Smith & Dodd (1967), when milking in 2 quarters of each cow was suspended for a period of 14 days in mid-lactation, these 2 quarters produced more milk in the next lactation than did the 2 control quarters in which milking was not suspended. The result was observed whether the animals were given a dry period immediately prior to parturition or not. In the next lactation the concentrations of fat, SNF, sodium, potassium, lactose and chloride were identical in the milk of all 4 quarters.

*Milking before parturition after a normal dry period*

In view of the fact that milking throughout the whole of pregnancy can have a marked effect on milk secretion in the subsequent lactation, milking prior to parturition might be expected to affect milk secretion during lactation. From time to time claims are made that inducing lactation by pre-partum milking increases subsequent lactation milk yields, but as yet the claim has not been substantiated. However, Walsh & Downey (1967) milked 2 quarters of each of 2 heifers for 20 and 40 days before parturition and observed that during the lactation these quarters produced about 24 % more milk than the corresponding quarters in which milking commenced on the day of parturition. From 5 days after parturition the concentrations of sodium and protein in the milks of all 4 quarters of each cow were similar.

RELATIONSHIP BETWEEN MILK SECRETION AND STRUCTURE OF
THE MAMMARY GLAND

The necessity for exercising caution in applying to dairy cows conclusions drawn from experiments on the structure and function of the mammary gland in small animals is emphasized by the experiments of Baldwin (1966). In addition, Sod-Moriah & Schmidt (1968) have found that the DNA content of mammary secretory cells in rabbits is not constant. This finding questions the basic assumption of many workers that the amount of DNA in the mammary gland can be used as an index of the number of secretory cells. Sod-Moriah & Schmidt (1968) also obtained results, based on the incorporation of labelled thymidine into DNA, which confirm that in the rabbit there is rapid cellular proliferation during pregnancy. Taking all the results, there is general agreement that considerable cellular proliferation occurs during the first pregnancy.

It is of interest that it is also possible for milk secretion to occur before parturition. This is demonstrated by the fact that there may be considerable milk present in the gland at the time of parturition. If cows are milked regularly before parturition, substantial quantities of milk, similar in composition to that obtained during the lactation, may be obtained. Although the effect of milking prior to parturition on the cellular proliferation has not been investigated, it is possible that pre-partum milking may affect the secretory potential during the lactation. The experiment of Walsh & Downey (1967) showed that milking 2 quarters of heifers prior to parturition resulted in these quarters producing more milk than the corresponding quarters which were not milked before parturition.

After parturition, and when suckling or milking is carried out at sufficiently frequent intervals to provide an adequate milking stimulus and prevent accumulation of milk, there is an increase in the daily yield of milk. This is apparently accompanied by an increase in the DNA content of the mammary gland. At present it is not possible to determine how much of this increase in DNA is due to an increase in the number of secretory cells or how much is due to an increase in the DNA content of secretory cells as suggested by Sod-Moriah & Schmidt (1968).

Later in lactation, when milk yield is decreasing, there is also a depression in the DNA content of the mammary gland. This is probably due to a decrease in the number of active cells, as shown by histological studies, although there may also be a

decrease in the DNA content of individual cells. In cows, the decline in the number of alveoli is reflected by a reduction in the storage space of the mammary gland (Matthews, Swett & Fohrman, 1949) and as a result there is a rise in the intra-mammary pressure/unit of milk secreted during a milking interval (Korkman, 1953).

At the end of lactation, when milking or suckling has ceased, the involution of secretory tissue is more rapid but the extent of involution in cows has not been measured. It is not known if the tissues revert to a state similar to that found in the virgin but there is evidence that the changes which do occur during the dry period can be influenced by environmental and physiological factors. The effect of preg-nancy on mammary involution is likely to be of considerable interest but has not been investigated in detail. The experiments of Swanson (1965) and Smith *et al.* (1967) show that a dry period between consecutive lactations is essential for maxi-mum secretory activity in the following lactation. They also show that if milking continues throughout pregnancy the alveoli probably can remain active for con-secutive lactations and that some renewal of secretory tissue occurs even when milking is continuous.

The evidence available on the changes that occur in the usual dry period of dairy cattle is fragmentary, but on balance it seems more likely that secretory tissue is regenerated for each lactation. The characteristic shape of the lactation curve applies to all lactations and not only the first, though the maximum daily production and the rate of decline are greater in the second and subsequent lactations. If the depres-sion in milk yield with advancing lactation is partly attributed to a decrease in the number of active secretory cells, then it must be accepted that some secretory cells have to be replaced for each lactation. Certainly regeneration of secretory tissue can occur since Crossman *et al.* (1950) showed that quarters damaged by intramammary infection and cured by antibiotic therapy show no recovery in milk yield as lactation progresses, but marked and often complete recovery is found, after a dry period, at the beginning of the next lactation. Further, it is established with dairy cattle that at least for the first lactation secretory tissue is mainly developed in mid preg-nancy. If this pattern occurs in older cows, then secretory cells must be formed in the later part of one lactation which do not function until after the next parturition. This would mean that development of secretory cells can occur while the cells associated with the current lactation are actively functioning. However, the development of the secretory tissue in mid-pregnancy but during lactation may be retarded by stimuli associated with the regular milking, so that the complete regenera-tion of secretory tissue does not occur until after the involution that follows the sus-pension of milking. This hypothesis is supported by the evidence that lactation milk yields of dairy cattle are depressed if they follow dry periods of less than about 40 days (Johansson & Hansson, 1940).

In rats, the depression in mammary DNA during lactation is more marked if the animals are pregnant (Paape & Tucker, 1969a). This suggests that one effect of pregnancy is to increase the rate of involution of secretory cells during lactation. If this is the case, there must subsequently be development of new cells in preparation for the next lactation. Eventually it would be expected that pregnant animals would have a higher DNA content in the mammary glands than the non-pregnant ones, as observed by Mizuno (1961).

Although there is a marked depression in the DNA content of mammary glands after the end of lactation in non-pregnant animals, the situation is less clear for animals which are pregnant. In view of the fact that the degree of development of the secretory tissue must be related to the secretory potential in the following lactation, information on the factors affecting this development could have a marked significance in milk production.

<div align="center">CONCLUSIONS</div>

Abnormal accumulation of milk within the udder, advance in stage of lactation and bacterial infections of the udder are all factors which are associated with a decrease in the yield of milk and with characteristic changes in the composition of the milk. With the exception of abnormal accumulation of milk for short periods and briefly at the start of infection periods, the effects continue throughout the lactation in which they occur. These appear to be the result of a decline in the number of actively secreting cells and possibly also a depression in the activity of the remaining secretory cells. These effects could therefore be associated with a depression in mammary DNA content and possibly a depression in RNA:DNA ratio. A decrease in mammary DNA has been observed in lactating rats as the lactation advances and when milk accumulates in the mammary gland (Tucker, 1966). Histological studies have shown that bacterial infections increase the degree of mammary involution.

The cause of the decline in active secretory cells and secretory activity that follows accumulation of milk for relatively short intervals is not known. In cows, as the milk accumulates during a normal interval (say 12 h) the intramammary pressure increases (Korkman, 1953). Further accumulation causes greater increases in the intramammary pressure (J. V. Wheelock, unpublished) which may be responsible for the movement of lactose and proteins into the blood (Wheelock & Rook, 1966; Lyster & Wheelock, 1967). Petersen & Rigor (1932) demonstrated that under certain conditions induced high intramammary pressures inhibit secretion, but it is evident from the work of Smith et al. (1967) that following an extended milking interval of 14 days the inhibition of secretion is not due to pressure since milk production can be restored by regular milking, even though the initial yields may be less than 100 g. The increase in the amount of milk in the mammary gland as such is unlikely to have an effect on the secretory activity, since the concentration of synthesized constituents, with the exception of fat, does not increase. But fat is a discontinuous phase and there may be a marked increase in the fat concentration in the ducts of the mammary gland. It has been shown that the concentrations of fat in the residual milk obtained after a long interval are much higher than those obtained after a 12-h interval (Wheelock, Rook & Dodd, 1965b). Levy (1964) has demonstrated that fat synthesis in mammary gland slices is inhibited by fatty acids present in the milk. Lack of milking stimulus is unlikely to be the important factor in the depression of secretion since the effect has been observed in one quarter when the others were milked at regular 12-h intervals (Wheelock et al. 1965b). However, it would be expected that for long periods of suspension of milking the presence of a milking stimulus would affect the extent of recovery.

For short periods of accumulation the inhibition of secretion is reversible, but for

longer periods the inhibition is usually permanent. The work of Ôta & Yokoyama shows that in rats the depression in the DNA content of the mammary gland which accompanies suspension of suckling is also partly reversible. These reversible changes in DNA may be due to variation in the DNA content of the secretory cells as suggested by Sod-Moriah & Schmidt (1968). The reduction in DNA/cell could, therefore, be the initial stage of involution, which would occur if milk were to accumulate for a sufficiently long period.

It has previously been postulated (Barry & Rowland, 1953; Rook & Wheelock, 1967) that the increases in the concentration of sodium and chloride and the decreases in those of lactose and potassium in the milk are the result of an increase in the proportion of an extra-cellular transudate in the milk. Since this situation is likely to arise if the integrity of the mammary epithelium is adversely affected, it is entirely consistent with an increase in the degree of involution. Further support for this view is shown by the observations that whenever abnormal accumulation of milk occurs within the gland there is a movement of synthesized constituents out of the gland (Wheelock & Rook, 1966; Lyster & Wheelock, 1967; Wheelock, Smith, Dodd & Lyster, 1967).

It has also been shown (Wheelock, Smith, Dodd & Lyster, 1967) that at the beginning of the dry period the composition of the fluid present in the gland alters so that the concentrations of the water-soluble constituents approach those of the extra-cellular fluid. Since involution is almost complete at this stage, there is virtually free movement of milk constituents between the extra-cellular fluid and the mammary gland.

On the other hand, those differences in yield which are the result of factors which occur or are applied prior to the lactation probably arise in a different way. We suggest that these changes in milk yield are the result of a change in number of secretory cells but not in the secretory activity of individual cells. These differences would be reflected in the total mammary DNA but the RNA:DNA ratio would be expected to be about the same. All these effects persist throughout the entire lactation. It is reasonable to suggest that changes in yield not associated with changes in the composition of the milk are due to specific environmental factors affecting the development and therefore the amount of secretory tissue.

Milking throughout pregnancy resulted in a decrease in the milk yield of cows in the following lactation and since the gland continued to produce milk until the beginning of that lactation, some of the secretory cells must have continued to function throughout the entire pregnancy (Smith *et al.* 1967). It is suggested that the failure of the gland to involute completely before parturition inhibited development of the secretory tissue in preparation for the next lactation. In the quarters which were allowed a dry period of adequate length, there would be complete involution and therefore the development of secretory tissue would not be impaired. The administration of oxytocin in the dry period (Gorman & Swanson, 1968; Swanson & Claycomb, 1968) would retard involution and a similar explanation for the results would apply.

Suspension of milking in mid-lactation was associated with an increase in milk yield in the following lactation when compared with the contralateral control quarters (Wheelock, Smith & Dodd, 1967). This effect was observed whether or not all 4

quarters had a dry period. It was noted that the control quarters, in which milking continued normally, had a slight compensatory increase in the yield which presumably was associated with an increase in the DNA of the quarter as observed for smaller animals (Mizuno, 1961; Tucker, 1966). This increase may have an adverse effect on the mammary development for the next lactation.

Milking heifers before first parturition resulted in an increase in milk yield during the lactation from the milked quarters as compared with that from the corresponding quarters which were first milked at parturition (Walsh & Downey, 1967). However, it was not possible to determine whether this effect was due to an increase in the yield of the quarters which were milked before parturition or a decrease in the yield of the quarters which were not milked at parturition. In an experiment of this type, the stimulus of milking 2 quarters may also affect the quarters which are not milked. This would result in accumulation of milk within the mammary gland to a greater extent than would occur in the absence of the experimental treatment. Further experiments using the same treatments would have to be performed using monozygous twins to determine the effects precisely.

Our conclusions draw attention to the possibility that factors other than those actually applied during a lactation can have a marked effect on milk secretion. Up to now it has been generally assumed that the mammary secretory tissue develops in response to hormones. While this is undoubtedly true, some of the experimental results described here indicate a strong possibility that other factors can interact with the hormones involved in mammary development. In particular, there is a need for careful investigation to determine much more precisely the effect of factors applied before the first parturition on the development of mammary secretory tissue. If mammary development does continue during the first few weeks of lactation, then the management of dairy cows at this time may be critical in determining the performance throughout the lactation.

Finally, there is a need for information on how the mammary gland develops in the second and subsequent lactations, with special emphasis on the interaction of one lactation with development for the next.

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# JOURNAL OF DAIRY

# RESEARCH

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ASSISTED BY

VOLUME 36, 1969

CAMBRIDGE UNIVERSITY PRESS

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