Incubation of slices of immature *Malva parviflora* seeds with C^{14}-labeled one-carbon donors, such as methionine, formate, acetate, and malonate, leads mainly to the labeling of dihydrosterculic acid and only minor amounts of labeled dihydromalvalic, malvalic, or sterculic acid, despite the fact that in mature seeds malvalic acid greatly predominates. The methyl group of methionine was the most efficient source of the ring-methylene carbon. Whether dihydrosterculate is an intermediate in the synthesis of cyclopropenoid acids is not yet known.

In view of the disappointing yields of biologically labeled cyclopropenoid acids, an attempt is being made to obtain labeled acids by chemical synthesis via stearolic acid and labeled methylene iodide.

There are a number of interesting matters on which further knowledge is urgently needed. These include: the mechanism of synthesis of cyclopropenoids in plant cells, whether the compounds are deposited unchanged in the lipids of eggs and body organs, the molecular species into which they are incorporated, the mechanism of their action on cell membranes, the way in which they react with enzymes or coenzymes or substrates to change fatty acid metabolism, and why malvalic acid appears to have only about one half the biological activity of sterculic acid.

Acknowledgment

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WORLD-WIDE RESEARCH

Proteolytic Activity of Crystalline Rennin and Caseins Associations

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THE CLOTTING of milk by rennin is one of the key steps in cheese making. The cow does not always give the same milk, and the variations which are observed in milk composition will, in turn, affect the properties of the coagulum formed when rennet is added to milk.

These properties depend to a large extent upon the mineral substances present in the milk, particularly the calcium in its various forms (ionized, etc.). The proteins themselves do play a role which, very likely, is far from negligible. This is especially true when one considers the varieties of cooked cheese—such as Swiss cheese—in which the water content is relatively low. In these varieties, the physical properties of the curd during the cheese-making process and those of the cheese during the curing process are important because of their influence on the quality of the finished cheese.

The present cheese-making techniques and the use of automation show, better than past methods, the interest in and the need for a better knowledge of the asso-

A sensitive pH-stat showed that, during milk clotting, rennin hydrolyzes in κ-casein one ester or imide bond per mole (mol. wt. = 55,000) at a high rate, thus explaining the rapidity of the milk clotting process. Synthetic substrates have not yet been found for rennin, suggesting that a whole sequence of several amino acids might be involved in its specificity. Rennin is not inhibited by diisopropylfluorophosphate. Kinetic considerations have led to the conclusion that other caseins such as α- or β-caseins, when associated with κ-casein, prevent the dissociation of the carboxyl group of the ester or imide bond hydrolyzed by rennin. This is another proof of the reversible associations existing between caseins. Quantitative data for these associations are presented and discussed. The associations are relatively independent of temperature which is a characteristic of nonpolar interactions.

ciative properties of casein and of the mechanism by which rennin attacks casein during milk clotting.

Proteolytic Activity of Crystalline Rennin

By and large, we are far less advanced in the understanding of milk clotting than of blood clotting. Evidence indicates that the milk clotting activity of rennin might be related to its proteolytic activity. Alais and coworkers (*7*), in 1953, showed that a small amount of substances containing nitrogen (N.P.N.) appears in 2 and 12% trichloroacetic acid filtrates during rennin action in milk or sodium caseinate solutions.

More recently, Garnier (*3*, *5*) has developed a technique to follow rennin activity by measuring the number of bonds hydrolyzed by the enzyme. These experiments indicate that rennin might hydrolyze an ester or imide bond in κ-casein. This hypothesis has been reinforced by the recent work of Jollès, Alais, and Jollès (*8*).

After the splitting of the ester or imide bond, at neutral pH, the new COOH-terminal which appears dissociates, and a proton is set free. If the pH is kept constant by adding sodium hydroxide, the number of hydrolyzed bonds can be determined and then the amount of κ-casein which is hydrolyzed by rennin. When a molecular weight for κ-casein of 55,000 is assumed, during milk clotting, rennin hydrolyzes only one bond per molecule of κ-casein. Consequently, the hydrolysis appears to be very limited, and this explains why a special, manually operated pH-stat apparatus in which the pH is kept constant within one thousandth pH unit is necessary. With this special apparatus, kinetic measurements such as the initial rate of hydrolysis determinations, can be made (Figure 1). These experiments have been performed by using rennin concentrations of the same order of magnitude as the ones used to clot milk—i.e., 0.05 μg. per ml. for the lower curve and up to 0.9 μg. per ml. for the upper curve at 25° C. This is important to remember.

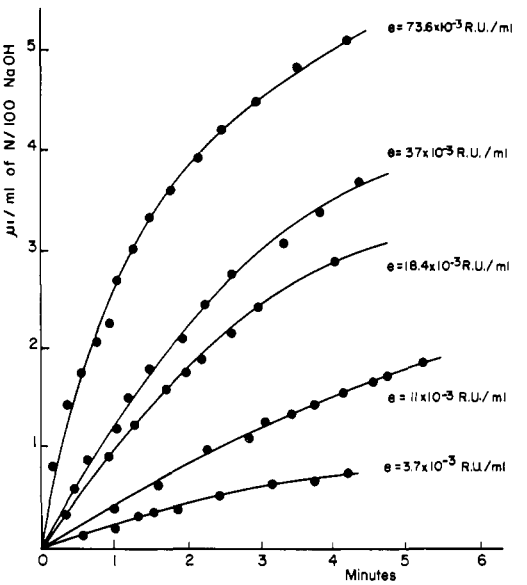

Figure 1. Proteolytic activity of rennin vs. time

Amounts of 0.01N sodium hydroxide added to keep the pH constant at 25° C. (Rennin concentration from e = 3.7 × 10^{-3} R.U./ml. to e = 73.6 × 10^{-3} R.U./ml.) (*4*). κ-Casein concentration: 3.9 mg./ml., 0.1M NaCl, pH 6.95

Proteolytic and Clotting Activities of Rennin

On the other hand, the results shown in Figure 2 indicate a linear relationship between the clotting activity of the enzyme (amount of *para*-casein) and the amount of N.P.N. released. A similar relationship between the number of hydrolyzed bonds and the amount of N.P.N. released is shown in Figure 3.

A combination of these two results shows that the number of hydrolyzed bonds is related to the clotting activity of the enzyme. These experiments show the desirability of following the proteolytic activity of crystalline rennin in the milk clotting process.

Kinetic Data and Rennin Specificity

Kinetic data obtained have been presented in Table I according to Garnier

Table I. Values of K_m and k_s at Different Temperatures (*4*)

	Temperature, °C.		
	25	35	40
V_M/e, H^+/min./ml./R.U./ml. × 10^7	4.6 ± 0.6	6.6 ± 0.7	8.4 ± 0.3
k_s,[a] s^{-1}	25 ± 3	36 ± 4	46 ± 2
K_m, mg./ml.	1.98 ± 0.3	1.55 ± 0.4	1.84 ± 0.03
K_m, M × 10^5	3.6 ± 0.5	2.8 ± 0.7	3.3 ± 0.05

[a] Value calculated on a basis of one H^+ liberated per kinetic unit of κ-casein (55,000 grams) and of 3 × 10^{-10} mole of rennin per rennin unit.

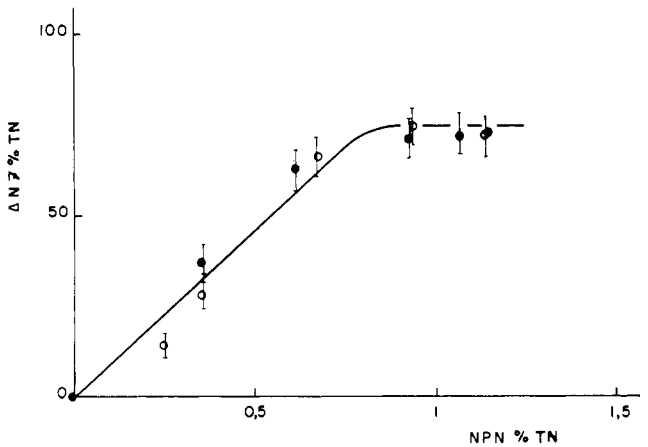

Figure 2. Clotting activity of rennin (amount of para-casein precipitated by Ca^{+2}, $\Delta N\nearrow$, in per cent of total nitrogen, NT) *vs.* the amount of N.P.N. soluble in 12% of T.C.A. expressed in per cent of total nitrogen, NT (3)

Whole casein: 29 mg./ml.
Rennin concentration: ○ 0.008 R.U./ml.
● 0.16 R.U./ml.
Temperature: 30° C., pH 6.9

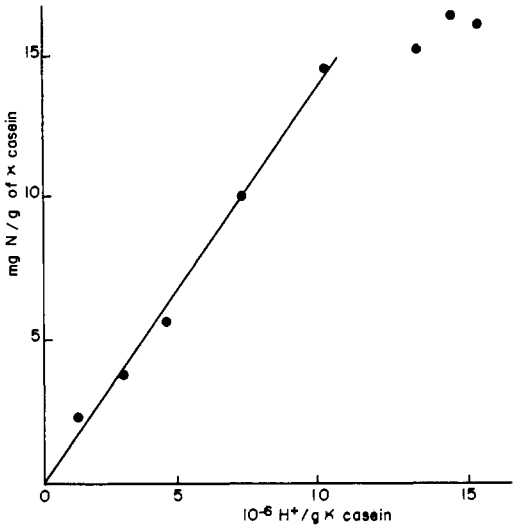

Figure 3. Relationship between the amount of N.P.N. released and the liberated protons at pH 6.95 (3)

Temperature 40° C.
Rennin concentration, 0.008 R.U./ml.
κ-Casein concentration, 5 mg./ml.

(*4*). It has been verified that the Michaelis constant, K_m, represents the dissociation of the κ-casein–rennin complex as is usually the case in enzymatic proteolysis. Within the limits of experimental error, K_m does not vary with the temperature between 25° and 40° C.

Study of the variation with temperature of the constant of decomposition of the intermediary complex enzyme-substrate has given the thermodynamic data presented in Table II. Such low values of ΔH^* are often observed for the hydrolysis of ester bonds. The value of k_s, which measures the rate of rennin action, is high which means that, at 35° C., one molecule of rennin hydrolyzes one molecule of κ-casein in about three hundredths of a second—200 times faster than the proteolysis of β-lactoglobulin by trypsin. This corresponds to the well known experimental fact that the clotting reaction can be very rapid or that minute amounts of enzyme are enough to obtain a clot.

Rennin is not inhibited by diisopropyl-fluorophosphate as it is found with some esterases such as carboxypeptidase and A esterases.

Table II. Thermodynamic Constants of the Reaction of Activation of the Rennin–κ-Casein Complex at 35° C. (4)

Constant	*Value*
k_s (sec.$^{-1}$)	36 ± 4
ΔF^* (cal./mole)	+15,800 ± 100
μ (cal./mole)	+ 6,900 ± 400
ΔH^* (cal./mole)	+ 6,300 ± 400
ΔS^* (cal./deg./mole	−31 ± 1.5

Until now, there has not been a substrate which could be hydrolyzed by rennin with the same conditions as κ-casein—i.e., with a low concentration of enzyme. For instance, according to Fish (*2*), high amounts of enzyme acting during several hours are needed to hydrolyze to a certain extent the B chain of insulin. The same observations have been made in the authors' laboratory with glucagon and *N*-benzoyl-DL-phenylalanine-α-naphthyl ester. All of these results prevent the drawing of any conclusion about the exact specificity of rennin.

Rennin must be very specific, as easily hydrolyzed synthetic substrates have not yet been found, κ-casein being the only easily hydrolyzed substrate known until now. The specificity of rennin should be opposed to the broader specificities of other proteolytic enzymes such as trypsin, chymotrypsin, or pepsin. It is not impossible, as seems to be the case for thrombin, that a certain sequence of several amino acids is required around the ester or imide bond to make it hydrolyzable by rennin. Among the caseins, κ-casein is the only one to be hydrolyzed in such a way by rennin, in the milk clotting process (Figure 4). These results do not mean, of course that the others are not partially hydrolyzed during cheese ripening, but this hydrolysis has nothing to do with the coagulation of milk.

Some Experimental Facts on Associations of Caseins

A study of the associative properties of caseins has been undertaken after an early observation that the normal clotting of κ-casein by rennin, in the absence of calcium ions, can be prevented if a certain amount of α_s- or β-caseins is added to the reaction medium.

An example of associations that occur between κ-casein and α_s-casein is given with the ultracentrifugal patterns presented in Figure 5. Although it was impossible previously to envisage a study of the association through the use of the ultracentrifuge, owing to the fact that the state of polymerization of the caseins is unknown, the authors have nevertheless been able to obtain quantitative data on these associations. This has been achieved by taking advantage of the fact that the carboxyl group of the ester or imide bond split off by rennin is no longer dissociated at neutral pH when κ-casein is associated to α_s- or β-caseins. But the enzymatic activity of rennin on κ-casein or on the complexes of κ-α_s-caseins or κ-β-caseins is unchanged, the hydrolysis taking place at the same rate. The dissociation constant K_{α_s} or K_β has been determined assuming that one molecule of κ-casein associates stoichiometrically and reversibly with one molecule of α_s- or β-caseins. By plotting the initial rate of the reaction v_0 *vs.*, for instance, $-\log(\alpha_s)$, (α_s) being the concentration of α_s-casein which has not formed a complex with κ-casein, the expected S-shaped curve was obtained (Figure 6). At the inflection point of the curve, the concentration in free α_s-casein or free β-casein is equal to the dissociation constant of the complex formed with κ-casein.

The values for the dissociation con-

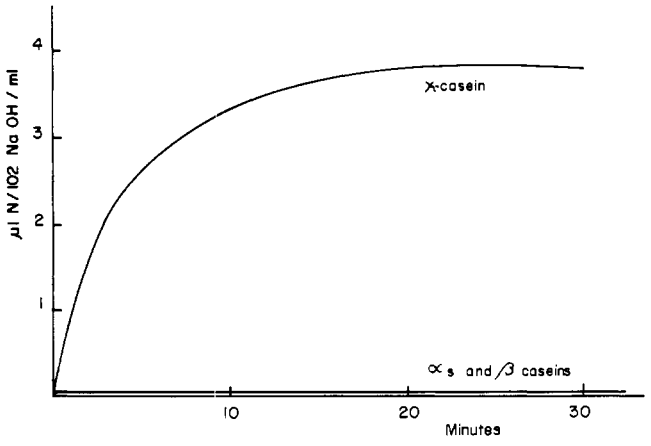

Figure 4. Activity of rennin on α_s-, β-, and κ-caseins followed with the pH-stat

The amount of liberated protons is expressed in microliters of 0.0102N sodium hydroxide added per ml. of solution to keep the pH constant. Temperature: 35° C., 0.1M NaCl, pH 6.95. κ-Casein concentration: 2 mg./ml. Rennin concentration: 1.6×10^{-2} R.U./ml.

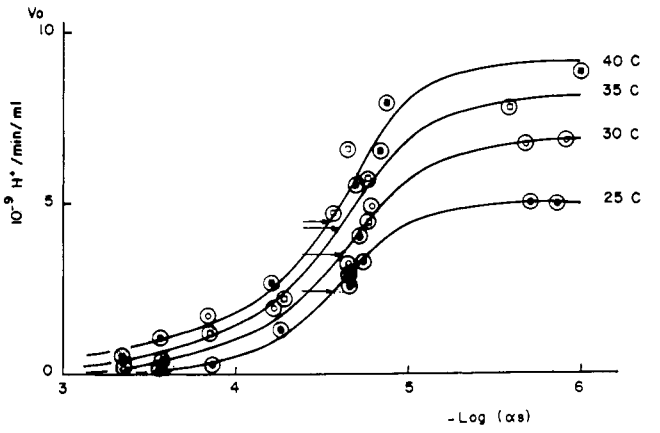

Figure 6. Formation-dissociation of the α_s-κ-caseins complex *vs.* free α_s-casein concentration (in moles/liter) at various temperatures followed with the pH-stat (7)

κ-Casein concentration: 4 mg./ml. Rennin concentration: 14×10^{-3} R.U./ml. or 0.17 μg./ml.

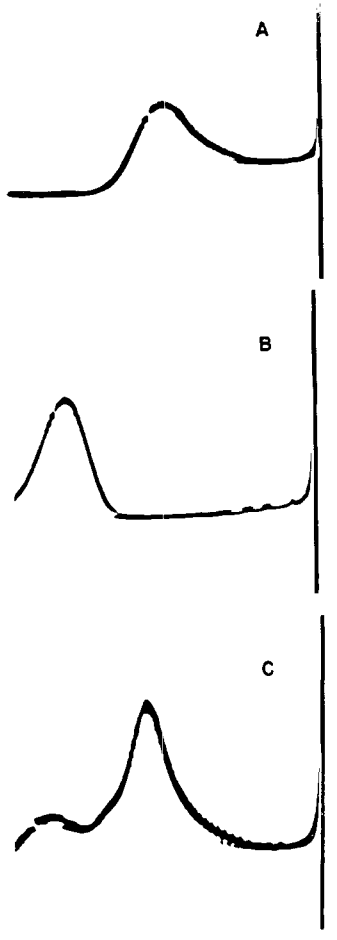

Figure 5. Ultracentrifugation diagrams of κ-, α_s-caseins, and the mixture of both caseins (7)

A. κ-Casein: 3.8 mg./ml.
B. α_s-Casein: 3.2 mg./ml.
C. The mixture of κ- and α_s-caseins at the same concentration as A and B
Temperature, 25.5° C.; 0.1M NaCl; pH 6.95

stants of both complexes are found in Table III, together with the corresponding thermodynamic data. All the variation of the standard free energy, ΔF, is linked to a positive variation of entropy (ΔS).

Table III. Thermodynamic Constants of the Formation of the Caseins Complexes

κ-α_s-Caseins Complex (7)	κ-β-Caseins Complex (6)
$K_{\alpha_s} = 2.5 \pm 0.5 \times 10^{-5} M$	$K_\beta = 12 \pm 5 \times 10^{-5} M$
$\Delta H \sim 0$	$\Delta H \sim 0$
$\Delta F_{35° C.} = -6350 \pm 150$ cal./mole	$\Delta F_{35° C.} = -5500 \pm 300$ cal./mole
$\Delta S_{35° C.} = +21 \pm 0.5$ cal./deg./mole	$\Delta S_{35° C.} = +18 \pm 1$ cal./deg./mole

Discussion of Associative Properties of Caseins

Among the assumptions made, the one corresponding to the association between one molecule of κ-casein and only one molecule of α_s- or β-caseins can be proved also by the fact that a great variation of initial rate is observed with a relatively low ratio of α_s/κ or β/κ. Although one assumes a 1-to-1 ratio for α_s-κ-caseins complex, Waugh (*10*) in his experiments found a value of 3 and some authors found even more. These results are compatible with our own results if we assume that κ-casein can link several molecules of α_s-casein, but that only one molecule is necessary to prevent the dissociation of the carboxyl group. The current method (pH-stat) will detect only the latter type of association. To avoid a competitive effect of the other binding sites, an association constant has to be assumed much smaller than the association constant corresponding to the inhibitory effect. The binding site may be close to the ester or imide bond split off in κ-casein but different from the binding site of rennin, as no enzymatic inhibition is observed either with α_s- or with β-caseins. The association constant is high, of the same order of magnitude as the enzyme-substrate association. Experimental results suggest that the rate of association is very rapid, more rapid than between rennin and κ-casein. This association prevents *para*-κ-casein from precipitating, and it might be inferred that the carboxylic group of the ester or imide bond split off plays a role in the formation of fibrils by *para*-κ-casein.

κ-Casein bonds α_s-casein more firmly than β-casein in these experimental conditions (neutral pH, 0.1M NaCl). This result could have been expected if one remembered that, using the electrophoresis apparatus of Tiselius, a rather large amount of substance, mostly composed of β-casein, can be separated from the α_s-κ-caseins complex which migrates as a single peak.

In both complexes, the heat of formation is close to zero. The preponderance of the interactions between nonpolar groups can be expected to stabilize the complex. As was pointed out by Waugh (*11*) several years ago, α_s- or β-caseins are remarkable for their very high content of nonpolar side chains compared with other known proteins.

The association between κ- and α_s-caseins gives a new property: formation with calcium of high polymers or micelles of a molecular weight between 10^7 to 3×10^9 according to Nitschmann (*9*). Their great stability is unique among micelle suspensions and makes very remarkable the stability of milk *vs.* different factors—such as the length of time and heating—well known to dairy scientists and technologists.

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WORLD-WIDE RESEARCH

Recent Studies on the Microbial Degradation of Cotton

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Recent work at the Shirley Institute funded by a grant from the United States Department of Agriculture is summarized. A detailed study of the mechanism of action of the cellulase from *Myrothecium verrucaria* has shown that only relatively few sites in the cotton hair are accessible to the enzymes and that attack proceeds by solubilization of the cotton in the immediate vicinity of these sites. This suggested successful trials in which cotton was protected from degradation at very low levels of substitution with a bulky substituent when care was taken to minimize swelling. Some of the properties of the individual enzymes that contribute to the cellulolytic action of this fungus have been investigated.

The Shirley Institute has long been interested in the microbial degradation of cotton textiles, and this communication summarizes the recent advances made during the tenure of a grant from the United States Department of Agriculture. Microbial degradation causes considerable losses both to the industry and to the consumer, especially under tropical or subtropical conditions. During World War II, the average life of tentage in certain parts of the Far East was reduced by fungal attack to less than six weeks. A great many rot-proofing agents have been made available over the years. Shirlan (salicylanilide) was produced in 1929 and others in common use include metallic naphthenates, organic mercurials, and chlorinated phenolic compounds. However, even today, there is probably no completely satisfactory rot-proofing agent available.

A very large volume of largely *ad hoc* work has already gone into the production of presently available rot-proofing agents, but it seemed that a study of the mechanism of deterioration, by providing a better understanding of it, would form a basis for a more rational approach to the development of improved methods of prevention.

Extracellular Attack by *Myrothecium verrucaria*

It seemed reasonable to suppose that the degradation of cotton was caused by extracellular enzyme systems produced by the organisms. It was, therefore, surprising that cell-free filtrates obtained from microorganisms, which were themselves extremely active, had been found, by all the workers in this field, to have little action on native cotton (*13*), although they were well able to attack cotton that had been swollen (*5*). The differences in rate and extent of attack produced by the organism and by its extracellular extract were well illustrated in the case of *Myrothecium verrucaria*. After a lag phase of about two days, this organism would reduce the strength of a scoured cotton yarn to nothing in an additional two to three days. On the other hand, a filtrate from the same organism, prepared after periods of up to 14 days, or even longer, would at best reduce the strength by about one third even after incubation for many days (Figure 1). If, however, the cotton was swollen by alkali before exposure to the filtrate, both the rate and extent of attack were comparable with those obtained when the organism grew on unswollen cotton.

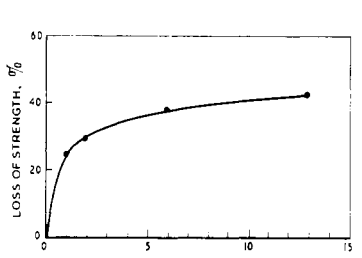

Figure 1. The degradation of cotton yarn by treatment with a culture filtrate from *M. verrucaria*

Most investigators, although aware of the problem posed by these differences, have concentrated their attention on the use of substrates made more accessible than unswollen cotton. On the assumption that the cell-free filtrates contain the extracellular enzymes involved in the cellulose breakdown, this apparent lack of activity is of considerable interest. For present purposes, however, it was pointless to investigate the degradation of easily accessible substrates, and it was desirable to use enzyme preparations with degradative powers similar to the living organisms. The purpose of this work was, first, to try to explain the low activity of the enzyme preparations and, second, to determine how the organism causes cellulolytic degradation with the ultimate objective of seeking fresh methods for its prevention.

Reese suggested (*8*) that hyphal penetration of the cotton hairs was an essential preliminary to the enzyme attack. Past experience with cotton degraded by microorganisms did not support this view. Cotton hairs had often been seen displaying damage typical of attack by microorganisms with no trace of the organism in their immediate vicinity