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STUDIES ON THE ACTION MECHANISM OF RENNIN

BY

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The clotting mechanism of casein, catalyzed by rennin, is still unclear ([1] [2])). Neither is it established whether the peptidase activity of rennin plays a role in the process, nor whether phosphoric, amino, carboxyl or thiol groups of the substrate must be free for the reaction. In an attempt to solve these aspects of the problem, casein was modified by blocking some of its groups and used as substrate for rennin. Clotting and peptidase activities were measured and compared on natural and modified casein.

Experimental

Clotting of the substrate was checked by measuring at 540 mμ the gradually increasing turbidity. The reaction mixture contained 2.5 ml 0.04% substrate solution, 0.2 ml 0.1 M $CaCl_2$, 0.02 ml 0.04% rennin solution in 0.15 M KCl. Readings of the increase in optical density were made every 30 sec for 3 min in a 1 cm light path glass cuvette.

Peptidase activity of rennin was estimated measuring hydrolysis of casein: after incubation, the unhydrolyzed protein was precipitated with trichloroacetic acid and the phenolic groups of the hydrolyzed polypeptides were measured colorimetrically. The experiments were carried out at 37° C and stopped after 1 hour by addition of 1 ml of 1.2 M trichloroacetic acid. After centrifuging, the clear supernatant was assayed for phenolic groups ([3])). The colour was recorded in terms of the amount of tyrosine required to produce the same colour under the same experimental condition.

The following modifications of casein were effected.

Casein was methylated with methanol and hydrochloric acid according to FRAENKEL-CONRAT and OLCOTT[4]).

N-acetyl-casein was prepared by adding acetic anhydride to a 2% solution of the protein in Na-acetate by the procedure of FRAENKEL-CONRAT et al.[5]).

The hydroxymethylderivative was obtained by treating 1% casein

with 1% formaldehyde for 2 hours at room temperature and removing by dialysis the excess of the aldehyde.

The reaction between 1-fluor-2,4-dinitrobenzene and free amino groups of casein was carried out as described by HAMILTON[6]). The compound obtained was separated by centrifuging, washed, and dried with ethanol and ethyl ether.

Treatment with potassium cyanate formed the carbamyl derivative of casein ([7])). The unreacted cyanate was eliminated by dialysis against 0.085 M NaCl.

The mercaptide was obtained by treating 1% casein with 1.10^{-3} M *p*-chloromercuribenzoate at room temperature and removing the excess of the reactant by dialysis against distilled water.

Reduction of casein was performed by passing H_2S through the protein solution.

Dephosphorylated casein was prepared enzymatically from casein as suggested by HOFMAN[8]) with purified spleen phosphoprotein phosphatase prepared according to SUNDARARAJAN and SARMA[9]).

Phosphorylation of dephosphorylated casein was carried out with mitochondria of rat liver as described by BURNETT and KENNEDY[10]). The reaction mixture contained, in 3 ml, 5.10^{-3} M $MgCl_2$, 3.10^{-2} M sodium glutamate, 5.10^{-3} M adenosine triphosphate, $3.3.10^{-1}$ M tri (hydroxymethyl) aminomethane, pH=7.4, 0.83% dephosphorylated casein, and mitochondria prepared from 1 g of fresh tissue. After 1 hour incubation at 37° C, the mitochondria were removed by centrifuging 15 min at 30.000xg. The supernatant was promptly used for the assay of the clotting activity of rennin.

Results

A. Clotting activity of rennin.

Table I shows clotting activity of rennin on normal and modified casein. Treatment with H_2S and *p*-chloromercuribenzoate has no

TABLE I.

Clotting activity of rennin on casein and casein derivatives

Substrate	Optical density increase
casein	0.330
N-acetyl-casein	0
hydroxymethyl-casein	0
dinitrophenyl-casein	0
carbamyl-casein	0.050
acetyl-casein	0.024
p-mercuribenzoate-S-casein	0.318
reduced casein	0.335
dephosphorylated casein	0
phosphorylated dephospho-casein	0.260

effect on the rate of clotting of the substrate. When, however, amino groups are substituted, casein is not clotted any more by rennin. The enzyme can still act on the derivative obtained with cyanate, but at an extremely low rate. Even the esterification of carboxyl groups and the removal of phosphate modify the substrate in such a way that the enzyme can not coagulate it any more. When the dephosphorylated casein is, at least partially, phosphorylated by the mitochondrial phosphokinase, it serves again as substrate for the clotting activity of rennin. Actually, the shape of the casein molecule does not seem to be drastically changed by enzymatically removing phosphate ([9]), even if the two substrates are not hydrolyzed by trypsin at the same rate (Table II), contrary to what is claimed by SUNDARARAJAN and SARMA[11]).

TABLE II.

Hydrolysis of casein and dephosphorylated casein by trypsin

Substrate	µg tyrosine
casein	47
dephosphorylated casein	52

B. Peptidase activity of rennin.

In table III are shown the results obtained by measuring the peptidase activity of rennin on normal casein and casein derivatives. The figures indicate clearly that the reaction rate is not modified by the reduction of -S-S- bonds and by the oxydation of the -SH groups of the substrate, while it is reduced when the amino groups of the protein are substituted. The compound formed by treatment with fluorodinitrobenzene is the least susceptible to the rennin action: an 86% inhibition of the enzyme activity is observed. The hydrolysis of the hydroxymethylderivative is reduced by 45%, while the treatment with acetic anhydride and with cyanate reduces the rate of hydrolysis of substrate by only 15%. The esterification of carboxyl groups with methanol inhibits the activity by 80%. Rennin hydrolyzes dephosphorylated casein almost at the same rate as normal casein.

TABLE III.

Peptidase activity of rennin on casein and casein derivatives

Substrate	µg tyrosine
casein	34
N-acetyl-casein	29
hydroxymethyl-casein	19
dinitrophenyl-casein	5
carbamyl-casein	29
acetyl-casein	6
p-mercuribenzoate-S-casein	34
dephosphorylated casein	33

Discussion

The results demonstrate a correlation between rennin activity and presence of some groups of the casein molecule. In fact, while oxydation of -SH groups and reduction of -S-S-bonds do not affect the rate of hydrolysis, free amino and carboxyl groups seem to be necessary for clotting and hydrolysing casein by the enzyme. Treatment with acetic anhydride, formaldehyde and fluorodinitrobenzene destroyes completely or almost completely the clotting activity and markedly reduces the hydrolysis of the substrate. In the same way, esterified casein is not attacked by rennin. We have observed that the two activities of the enzyme behave differently only after removal of the phosphate groups: while hydrolysis of dephosphorylated casein is only slightly slower than that of normal protein, dephosphorylated casein is not coagulated any more by the enzyme, in agreement with the results of HSU et al.[12]). Furthermore, it is interesting to note that the process is reversible: if the dephosphorylated casein is phosphorylated, rennin is again able to clot it. Such a finding could point to a participation of the phosphate groups in the clotting by combining casein with Ca^{++}. These groups are, however, not necessary for the hydrolysis. Moreover, phosphate groups have a different function than carboxyl and amino groups, as casein derivatives having these groups substituted are hydrolyzed and clotted at a slower rate.

Insofar as the possibility of a relation between peptidase and clotting activity of rennin is concerned, their different behaviour on dephosphorylated casein could induce one to believe that they are independent or that hydrolysis takes place before, not after, clotting.

Summary

Oxydation of -SH groups and reduction of -S-S- bonds of casein do not affect clotting and peptidase activity of rennin. Clotting activity is completely, or almost completely, inhibited when casein derivatives obtained by treatment with methanol, fluorodinitrobenzene, formaldehyde or cyanate are used as substrates. Even hydrolysis of these compounds is reduced. By removal of phosphate groups from casein, derivatives are obtained which are hydrolyzed by rennin at the same rate as the normal protein, but which are not clotted any more. Rennin is again able to coagulate dephosphorylated casein which has been enzymatically phosphorylated.

Riassunto

L'ossidazione di gruppi -SH e la riduzione di legami -S-S- della caseina non hanno effetto sull'attività coagulante e peptidasica della rennina. L'attività coagulante è inibita completamente o quasi quando si usano come substrati derivati della caseina ottenuti da questa per trattamento con metanolo, fluorodinitrobenzolo, formaldeide o cianato. L'idrolisi di questi composti è pure ridotta. Per allontanamento dalla caseina di gruppi fostorici, si ottengono dei derivati che sono idrolizzati dalla rennina alla stessa velocità della caseina nativa, ma che non sono più coagulati. La rennina è capace di coagulare la caseina defosforilata previamente rifosforilata per via enzimatica.

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THE ROLE OF THYROID HORMONES IN TRANSSULPHURATION
I. INHIBITION OF CYSTATHIONASE BY THYROXINE*

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(with 6 figs.)

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The role of cystathionase in transsulphuration is well established([1])). It is also known that cystathionine is cleaved by this enzyme yielding cysteine and ketobutyrate ([2])). The enzyme has been crystallized from rat liver ([3])) and shown to require pyridoxal phosphate as coenzyme ([4])). Cystathionase preparations deaminate homoserine also and since the ratio of both activities, the cleavage of cystathionine and the deamination of homoserine, remain constant throughout the purification procedures, it was concluded that both are due to the same enzyme ([5])).

Thyroxine has been found to inhibit several enzymatic activities requiring pyridoxal phosphate, both in vivo and in vitro ([6])–[8])). HOPE[9]) made the interesting observation that vitamin B_6 deficient rats excrete cystathionine in their urine, and MASCIOLI et al.[10]) found that thyroxine treated rats have reduced pyridoxal phosphate levels in several tissues, including the liver.

It was of interest, therefore, to see whether thyroxine affects the activity of this enzyme and if so, whether the inhibition is an early effect or not, having in mind particularly the latent period, when oxygen consumption is still unaltered.

The swelling of mitochondria with diffuse cristae described in hyperthyroid tissues ([14])), induced us to combine biochemical determinations with a study of the mitochondria in the electrone microscope during the first 6 days of thyroxine administration.

Experiments in vitro were conducted with purified preparations using liver of dogs, a rather good source for cystathionase, as observed by FROMAGEOT[12]).

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RENNET: PAST, PRESENT AND FUTURE

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I-9428

Dr. Davis's cogent observations on rennet extend in his last paragraph to include insights on cheesemaking of great value to all dairy technologists.

I THE NATURE AND FUNCTION OF RENNET

The function of rennet in cheesemaking

Of the four materials used in all cheesemaking—milk, souring bacteria, rennet and salt—rennet is of particular interest because of the small quantity involved, only about 1 cc. rennet to 5,000 cc. of milk or 1 part of the pure enzyme to 5 million parts of milk, and the peculiar character of its effect. A very slight chemical change converts a liquid containing only about 2·6% casein into a gel, which on cutting, warming and souring can become, as in Parmesan (Grana) cheese, a food of almost indefinite keeping quality. This is a beautiful example of the economic way in which a valuable and highly perishable food can be converted by very simple means into a long-keeping product. Another unique property of rennin is the high ratio of clotting to proteolytic power, another essential feature in cheesemaking.

In addition to the purely physico-chemical effect, rennet may also be important in cheesemaking by virtue of its power to produce growth factors for starter streptococci, lactobacilli and other bacteria, and also because of its proteolytic power, although this is slight. Such proteolysis leads not only to ripening, by its effect on the curd, but also to the production of flavour.

It is universally accepted that rennet, the extract of the abomasum of the young suckling calf, is the best clotting agent for cheesemaking, but other agents have been recognised for centuries. The classical writers refer to calf, kid (goat) and lamb (sheep) rennets, and also to plant rennets, such as that from the fig tree[34,38].

In England the milk-clotting properties of plants were discovered centuries ago. Thus *Galium verum* (Lady's Bedstraw) was used in this way in 1577 and probably earlier. *Galium aparine* (Cleavers) was known colloquially as rennet-wort.

In addition to the juices of many plants, the extracts of stomachs of the pig and other mammals have been tried for cheesemaking. Numerous investigations have been published on the use of rennet substitutes and nearly all report that the cheese is inferior to that made with rennet, the most common criticism being of flavour, and the most common defect bitterness.

The nature of the rennin-milk reaction

It has been known for about 100 years that the action of rennet in clotting milk consists of two stages:

1. A purely enzymic action which can be effected and the enzyme then destroyed by heat.
2. A precipitation or gelling which requires calcium or similar ions and has a high temperature co-efficient.

A good historical review of the classical work is given by Effront[45].

It is only recently that the details of these changes have been revealed. The most interesting aspect is that a comparatively minor chemical reaction (hydrolysis of a bond and separation of a peptide) can change a liquid into a solid. Rennin produces this specific change extremely rapidly[44], the relevant bond being that between phenylalanine and methionine in κ-casein, the sensitive fraction in the casein micelle[42]. This attack appears to be catalysed by nearby serine and histidine sidechains[51]. Lysine sidechains also play a role[52].

Berridge[10] drew attention to the fact that the only similar reaction having a high temperature co-efficient was the denaturing of proteins and suggested that the clotting of milk was an essentially similar process. He developed this theory[13], succeeded in crystallising rennin[11,12] and made cheese with the pure enzyme[18].

Our knowledge of what happens when rennin acts on casein is still far from complete (*cf.* Sinkinson & Wheelock[78]). For general reviews of rennin and the rennin-casein reaction see Berridge[14,17,19].

The measurement of rennet strength

During the whole history of cheesemaking and the technological development of the subject, it has been the custom for cheesemakers to be interested only in the strength (milk-clotting power) of the rennet they use. Only research workers have studied other aspects, such as the nature and proportion of other enzymes, which may exert an effect on the ripening of the cheese even if the effect on the milk clotting is negligible. The retention of rennin and other enzymes in the curd has also received little attention.

The basic difficulty in all attempts to measure rennet strength lies in the fact that two uncontrollable materials —rennet and milk—are involved. In science an unknown always has to be measured in terms of the known. In practice fresh bulk milk is often used, but this varies. The British Standards Institution[20] has issued a standard method for measuring rennet strength based on a calcified reconstituted skim milk powder substrate[15,16] and a standard freeze-dried rennet preparation which is available to testing laboratories. A micro-method based on an agar diffusion slide technique has been described by Lawrence & Sanderson[58].

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II HISTORICAL

Commercial rennet

The first 'cheese' was probably made accidentally in an animal's stomach, used as a milk container. Later, pieces of calves' stomachs (vells) were used. The general history of commercial rennet is well known, and the great contribution made by the firm of Hansen to the problems of producing a stable, standardised and generally acceptable rennet is well recognised. Chr. Hansen started making rennet in Copenhagen in 1874, and in Reading, England, in 1918.

When we make cheese we largely imitate what happens when the young calf suckles its mother; here the milk is curdled, broken up and digested in the calf's stomach, and in cheesemaking we do the same under artificial conditions, the digestion of the curd being much slower, largely because the proportion of rennin is much lower than in the calf's stomach, and the temperature of maturing lower.

Alcohol-glycerol rennet

Following earlier work at the National Institute for Research in Dairying and at the West of Scotland College of Agriculture in the 1930s, the author studied the types of bacteria found in commercial rennets at that time. It had been alleged that anaerobes and possibly other undesirable types of bacteria found in rennet had been responsible for taints in Cheddar cheese. However, it must be stated that the evidence was extremely controversial. One of the factors possibly involved was the habit of small cheesemakers keeping surplus rennet supplies at room temperature from one cheesemaking season to the next. The preservatives normally added to commercial rennets at that time (boric and benzoic acids) would not keep the material at a low count indefinitely.

In his search for a solution to this problem the author investigated an alcohol-glycerol rennet or 'junket' preparation made by Bengers Ltd. for domestic use. Used at a suitable concentration, this produced a slightly soft but super-quality Cheddar curd, which matured to give an excellent cheese[35]. The rennet was naturally sterile. Unfortunately the rigid customs laws in the UK prevented this excellent rennet from becoming a commercial proposition. In countries where alcohol is not so rigidly controlled this type of rennet might have possibilities. It is not only permanently sterile, but as an enzyme preparation is remarkably stable. Whether the alcohol and glycerol exert any specific useful effect is unknown.

Propylene glycol is now used as a preservative for rennet.

'Sterren'

The advantages of an alcohol-glycerol rennet prompted us to explore the possibilities of a rennet which would have the advantages of the alcohol-glycerol preparation without actually using alcohol and glycerol. Research linked with development work by Bengers Ltd. led ultimately to a purified rennet which, by virtue of the method of manufacture, was not only sterile but a purer rennet (*i.e.* more free from pepsin and other enzymes than ordinary rennet). This was called *Sterren* and, like the parent alcohol-glycerol rennet, gave a rather soft delicate curd which at first led cheesemakers to pronounce it weaker than the ordinary rennet. However, the curd firmed up in the later stages, so that finally the curds from both rennets were of equal firmness.

Preparation of rennet from living animals

Milk and rennin are both secretions from certain organs of mammals, and it would be very convenient for cheesemakers if both could be obtained from the living animal. It is possible to do this by the calf fistula method[11] but the cost and small yield do not make the method a commercial proposition, quite apart from ethical considerations.

What are the essential requirements for any 'rennet'

The complexity of the whole cheesemaking operation and ripening makes it extremely difficult to establish what function each component exerts. Would a rennet containing only pure rennin make as good a cheese as ordinary commercial rennet?

Clearly the first stage in solving this problem was to isolate and study the pure rennin enzyme present in commercial rennet.

Pure (crystalline) rennet

The purified crystalline rennin prepared by Berridge[11] and Hankinson[50] has been used experimentally for cheesemaking[18]. Crystalline rennin clotted the milk quickly but the curd remained soft for a relatively long time. It developed less acidity and gave very short threads on the hot iron test. The cheese made with pure rennin plus the adventitious material discarded during purification gave a fuller flavour than that made with the pure rennin only, which was not so pleasant.

Rennet and lactic acid bacteria

These experiments draw attention to the possible useful function of the impurities (*i.e.* substances other than rennin) in commercial rennet. These may include growth factors for lactic acid and other bacteria responsible for flavour production in cheese[56]. It has long been known that rennin (or rennet) and lactic acid bacteria display an associated or synergistic effect. The former provides growth factors for the latter, and the acid produced by the latter accelerates the clotting and possibly the proteolytic action of the former[40]. The breakdown of protein is much greater by the combined action of rennet enzymes and lactic acid bacteria than by either alone[41].

Plant rennets

There has recently been a revival of interest in plant rennets, used both to coagulate milk and as an additional enzyme to aid ripening[7]. The *Withania coagulans* protease, bromelin (pineapple) and papain (tropical melon tree) were used, and all induced bitterness. These findings confirm results obtained by nearly all other workers. It is clear that, if plant and other rennets are to become a commercial success, some method must be found to prevent this development of bitterness.

Other coagulating enzymes

It has long been known that all proteolytic enzymes can clot milk, with varying degrees of activity. Of these pepsin has been used most in experimental cheesemaking, but trials have also been made with papain, trypsin and many plant enzymes. In general, all these enzymes tend to give bitterness, and the usual explanation given is that this bitterness is due to 'peptones'[37,59,62]. However, the evidence as a whole is conflicting, which is not surprising in view of the

enormous number of uncontrollable factors involved in cheesemaking[43,67].

Pepsin has, quite naturally, received most attention because in mammals the gastric secretion of the neonate gradually changes from a rennin to a pepsin type of digestive enzyme. This change is parallel to the steadily increasing acidity of the stomach as the young mammal grows, and the gradual decreasing dependence on milk as the main nutrient.

Fox[47] has made the interesting observation that, for a given milk-clotting power, calf rennet and bovine pepsin were about equal in proteolytic power and much weaker than porcine pepsin. Moreover the products of proteolysis by rennet and bovine pepsin were similar, and different from those produced by porcine pepsin. This observation is clearly of interest from the point of view of the bitterness found in cheese made with pepsin (p. 136).

Non-coagulating-non-proteolytic enzymes

In addition to the purely proteolytic enzymes present in these commercial preparations, there are often other types of enzymes. Of these, probably the lipases are the most important from the point of view of cheesemaking. They may be present in stomach extracts by regurgitation from the duodenum. Experiments have shown that lipases can produce undesirable effects in cheesemaking[37,79].

III NEW RENNET PREPARATIONS

1. The modern 50:50 rennet-pepsin preparation

The increasing use of milk for cheesemaking, the steadily increasing yield per cow, and the development of dual purpose breeds and thus the decrease in the numbers of young calves (1-3 weeks old) available for making rennet, have led to a world shortage of the enzyme. In 1960 Marschall Dairy Laboratory developed a 50:50 mixture of rennet and pepsin, and in 1964 Chr. Hansen's Laboratory Ltd. also put a similar product on the market. It is claimed that this makes as good cheese as the ordinary rennet, although one may well ask, if this is so, why has it not been used before? This new preparation has now been used extensively for the last 3 years. In the USA and Canada 80%, and in the UK 40%, of the cheese has been made with this. In general the assessment has been favourable[7,31,46]. However, some firms have found that the flavour of cheese made in this way is not quite so good as that of cheese made with ordinary rennet[46].

Reports on Emmental, Edam, Gouda, Samsoe[43], Tilsit and Butterkäse[80] and soft cheese[2] indicate that only negligible differences have been observed. With Cheddar cheese some workers have commented on quicker clotting at the bottom of the vat, greater sensitivity to pH, and also to calcium ions. It is well known that 'new grass' milk may be more difficult to clot than usual, and that cold stored or bulk collected milk may give a soft clot as a result of proteolysis by the psychrotrophic bacteria, but Babel[7] reports that no difference in these respects has been found between ordinary rennet and the 50:50 mixture.

Chapman & Burnett[31] found that the mixture gave a softer curd initially but that this firmed up later. They found no need to change the cheesemaking method.

One important advantage is price. In the UK, ordinary rennet is £1·33 and the 50:50 mixture £1·10/l. An even more important advantage is that this new preparation allows us to maintain the production of normal cheese by standard methods of manufacture.

If the early experiments showed that pepsin tended to give bitter cheese, it is not surprising that the rennet-pepsin mixture gives a slightly inferior flavour. However we must also realise the fact that, in ordinary rennet, about half the clotting power is due to the pepsin content.

It is always possible that the criticisms which have been made of the new preparations really arise from other causes, and the inability to control all factors in cheesemaking (*cf.* p. 136). Bearing in mind the extensive use of the new preparation, its general acceptability and the fact that some prize-winning cheese have been made with it, we can reasonably conclude that it is a satisfactory product.

The findings in the UK generally on the *rennet-pepsin 50:50 mixture* can be summarised as follows:

1. The curd is softer but firms up later.
2. There may be a slightly greater loss of fat in the whey at certain seasons, especially in spring.
3. As a consequence of (2) the yield may be slightly less at times.
4. There is no deterioration on keeping, *e.g.* in Cheddar cheese after 12 months.
5. In general the flavour is not affected, although some workers have doubts on this aspect.
6. Summing up, the economic advantages outweigh the above very slight disadvantages.

2. Fungal rennets

The advantages of using micro-organisms for any purpose—enzymes, foods, chemicals, drugs *etc.*—are so tremendous that economically they appear in a very favourable light. No animals are involved, the fungi grow very quickly, and the process can be controlled as in a synthetic chemical plant.

The most important commercial preparations now on the market come from the USA and Japan. A number of moulds have been used and the main Japanese product comes from *Mucor pusillus*[54,55,70,81]. It is marketed in the UK as *Noury* rennet. In general, quite favourable results have been reported from the use of this fungal rennet[26,43,72,75,81]. However, some minor criticisms have been made, usually concerning bitterness[4,55,61,66,70,83].

Endothia parasitica is used in the USA as the source of a fungal rennet. This is marketed in the UK as *Suparen*. Favourable results have been reported by Alais & Novak[1], Anon[3], Bolliger & Schilt[28], Ramet *et al.*[68], Sardinas[73] and Shovers & Bavisotto[76], but certain criticisms have been made by Australia[5], Belgium[9], Martens[60], New Zealand[65], and Prapotnik[66], again largely centering on bitterness.

Babel[7] has worked on *Mucor pusillus* and found that Cheddar cheese sometimes had a coarse grainy texture. The American workers also found that if the preparation is not freed from lipase a rancid flavour develops in a short time (*cf.* Davis[36]). A further point of possible importance was the observation that a substance inhibitory to lactic cultures may be present[8].

3. Bacterial rennets

Early experiments in England to obtain an extra source of rennet for

use in war-time were based on extracts of *Bacillus* species, chiefly *subtilis*. However, at that time our yields were insufficient to justify this method commercially. For example, it was calculated that to produce enough rennet to convert 1,000 gal. of milk into Cheddar cheese would have required 1,000 gal. of culture[24]. The prospect of a cheese factory equipped with alternate vats of milk for cheesemaking and broth for bacterial rennet-making might be interesting but hardly feasible. Bengers Ltd. calculated that the cost of such a bacterial rennet would be about 10 times that of ordinary rennet.

Bacterial rennets have nearly all been made from *Bacillus* species, chiefly *subtilis*. In general the results of cheesemaking experiments have been favourable, with relatively few workers observing bitterness and other defects[52]. However, there appears to be no bacterial rennet yet on the market.

Babel[7] has reported that the protease of *Str. liquefaciens* produced a permanent bitterness in Cheddar cheese.

Microbial rennets act on casein differently from calf rennet. Hydrolysis of the casein components tends to be more rapid for a given clotting power, and particularly for β-casein[62].

The historical evolution of these advances in technique are summarised in Table 1.

TABLE I. Technological development of cheesemaking devices

Period	Souring of milk	Clotting of milk
1. Before 1874	Natural	By adding pieces of vell or home-made extract
2. 1874—1900	Natural	Commercial rennet (Hansens, etc.)
3. 1900—1945	Cultures or starters coming into use	"
4. 1945—1968	Increasing sophistication in selection of and use of starters	"
5. 1968—	"	Rennet-pepsin mixture introduced. Serious interest in fungal clotting enzymes.

4. Marketing of rennet substitutes

Ordinary consumers are not concerned with the nature of the rennet used for cheesemaking, but there is an important market for all manufacturers of 'rennets' of non-animal origin, for example the 'health food' stores and for kosher cheese. There is a small but very rigidly minded section of the community which refuses to eat any food involving the killing of animals. Thus the ovo-lacto vegetarians will accept milk and eggs, and foods made with them, but not meat and therefore not rennet. There is a still smaller and more extremist group who will not use milk but gladly eat a 'cheese' if made from non-animal foods. These dietetic principles give a complete monopoly to manufacturers of 'rennet' from plants and micro-organisms.

For reviews of rennet substitutes see Babbar *et al.*[6], Davis[38], Sardinas[74], Veringa[82], Ritter *et al.*[71].

5. Identification of rennets

Methods for identifying 'rennets' of various origins have been described by Cheeseman[32], Koning *et al.*[57], Garnier *et al.*[49], Melachouris and Tuckey[63], and Richardson[69].

The rennin in a rennin-pepsin mixture can be conveniently assayed by inactivation of pepsin at pH 7·3[77].

IV SOME PROBLEMS OF PRACTICAL IMPORTANCE IN THE USE OF THE NEW RENNETS

In addition to such obvious aspects as speed of clotting, effect of pH and concentration of calcium ions, firmness of curd, rate of drainage, rate of ripening and ultimate flavour, body and texture, there are other, less obvious aspects to be considered before any of the new rennets can be wholeheartedly accepted. This caution applies irrespective of their nature or origin.

1. Are they equally suitable for different varieties made in one factory?

The fluctuation in the cheese market and the control of milk utilisation by any authority may necessitate quite sudden changes in the type or variety of cheese to be made on any one day. For example, in the UK where we have a limited range of cheese varieties, an English factory may make any of the following: Cheddar, Cheshire, Double Gloucester, Derby, Leicester, Lancashire and/or Wensleydale, and make any proportion of these on any one day. In Scotland Dunlop may also be made, and in Wales and England Caerphilly. It is therefore most important that any rennet can be used for any of these varieties successfully, and without the need for any special modifications in manufacture.

2. Are they equally suitable for short and long maturing cheese?

The varieties of chief concern here are the long keeping cheese, such as Cheddar. For example, New Zealand and English Cheddar may be consumed quite early, *e.g.* at 2-3 months, but Canadian Cheddar may be held for 1-2 years to give the maturity commonly associated with this type. Would it be possible for the new rennets to give a cheese free from bitterness or other off-flavour at 3 months, and yet be at fault in this respect at 12 months? There are many interesting examples of this late fault development, such as slit-openness in New Zealand and the famous 'stinker' cheese in England, caused by *Cl. putrefaciens*, which passed the grader satisfactorily at 1 month but had to be buried after 6 months, being unfit for use even as animal feed. It is odd that, although more Cheddar is made than any other cheese in the world, flavour appears to be more unpredictable than with any other variety. The acknowledged higher ratio of proteolytic to clotting power in the new types of 'rennet', and the association between proteolysis and bitterness will make all cheesemakers particularly wary in respect of non-calf rennets for long keeping cheese.

3. What is the effect on yield?

Yield of cheese is obviously of the greatest importance, and there has been some suggestion of lower yields with the new rennets. This is quite possible when we consider that yield is controlled by amongst other factors:

(i) complete coagulation of the calcium-casein complex;

(ii) proteolysis between the times of addition of enzyme and final separation of curd;

(iii) loss of fat in whey, controlled by entanglement of fat globules in the gelled casein.

Yield is controlled mainly by residual moisture of whey in the curd, and this can vary appreciably. It follows that no criticism should be made on the score of yield without the most carefully controlled experiments, with due attention to the moisture content of the cheese.

The danger of generalising where different varieties are concerned is well brought out by the findings of Maubois and Mocquot[61] working with Camembert cheese. They found that the enzyme from *Endothia parasitica* gave a yield on average 1% less than with calf rennet, owing to the greater proteolysis during drainage of the curd. They also observed that the mould enzyme attacked the whey proteins, whereas calf rennet did not.

4. Compatibility of 'rennets' from different sources: inter-action between enzymes

One of the questions asked in classical physiology was why the gastric secretions did not digest the stomach itself. The same question may be applied to all digestive enzymes. We know now that the explanation may lie in the presence of anti-enzymes or special mechanisms which protect the host tissue.

Such problems may arise when digestive enzymes from different sources are mixed. All enzymes are proteins and so may be attacked by other proteases. It seems that rennin and pepsin from one animal are mutually compatible but when the enzymes from different animals, or from animals and plants or fungi are mixed, some mutual destruction may take place. It is therefore not possible to put out mixtures of certain enzymes for sale as a stable product. However, it would presumably be quite feasible to pack the enzymes separately and add them to the milk in the cheese vat.

One problem worthy of exploration would be to investigate the value of different properties of these enzymes for particular varieties of cheese. For example, calf rennet might be best for hard and unripened soft cheese, and other more complex 'rennets' better for blue-veined and surface-bacterial ripened varieties, such as Limburg.

5. What is the fate of rennin and other coagulating enzymes in the cheese?

An enzyme is defined as an organic catalyst, *i.e.* it brings about a change without being changed itself. Presumably rennin breaks down the κ-casein but is not itself destroyed, nor are other enzymes used for the same purpose. Do they remain attached to the protein, or are they leached out with the whey, which constitutes about 90% of the original milk by volume? Apparently this point has never been investigated, but it is of interest and may even be of importance. Different types of clotting enzymes may behave differently.

V MEDICAL ASPECTS

Dermatitis and allergies caused by enzymes

The rapid development of enzyme-based washing powders has led to complaints of dermatitis or allergies (the two are sometimes difficult to separate) by users, usually housewives and women employed in laundries and canteens. Whether these are real or not, it is well established that contact through the skin or in the lungs with foreign proteins can lead to unpleasant reactions. Thus it has been reported that those engaged in the preparation or handling of powdered fungal enzymes have suffered from an eczema. It is advisable, therefore, for all engaged in any way with powder enzymes to wear masks and suitable protective clothing.

Liquid preparations are unlikely to be responsible for this type of complaint, unless they frequently come into contact with the skin.

Amines in cheese and tranquillising drugs

Cheese is a favourite subject for jokes on indigestibility, nightmares, and other physiological and psychological disturbances. Large portions of any concentrated food can lead to indigestion, but there has recently come to light an important medical aspect linked with certain tranquillising drugs, inhibiting mono-amine oxidase, and the relatively high concentration of mono-amines, such as tyramine, in cheese[27]. If the new rennets are more proteolytic and produce more amines in cheese, this may be a point to watch.

We know of no case where ordinary rennet or any new type has *of itself* in the cheese produced an allergic reaction in man.

VI LEGAL AND SCIENTIFIC DEFINITIONS OF RENNET

Legal

The word *rennet* is derived from the Old English word *run*, meaning to move or flow. Its use as a word to describe the clotting of milk is presumably derived from the sense of 'running together' or 'uniting' a liquid to form a solid mass (1715).

In the UK the *Cheese Regulations 1970 Stat. Instr. No. 94* define 'cheese' and 'starter' but not rennet. Out of 24 countries, eight require normal or traditional rennet (basically rennin) to be used for cheesemaking and in nine (including the UK) there is no prohibition on the use of substitutes (IDF 1969).

The contemporary use of milk coagulating enzymes from a wide variety of sources raises the question as to whether a definition of rennet is desirable, and, if desirable, possible. From the stomach of the suckling calf to bacteria and fungi represents a considerable range in biological origins, and the following definitions are tentatively suggested:

Rennets

1. Type A—true rennet derived from stomachs of suckling calves.
2. Type B—rennet derived from the stomachs of older bovines.
3. Type C—rennet derived from other herbivorous mammals, especially if used for cheesemaking with their specific milk.

Pepsin

4. Enzyme preparations derived from stomachs of other named mammals.

Milk coagulating enzymes

Enzyme preparations derived from other living organisms.

5. Plants
6. Fungi
7. Bacteria

} identified by name

The following justifications are offered for these proposed definitions:

1. Cheesemakers should know the origin and nature of 'rennets' which they are offered.
2. Successful cheesemaking depends not only on using a rennet of standard strength, but also one of standard properties.
3. Manufacturers of genuine rennet should be protected in a commercial sense.

Scientific description of 'rennets'

The above requirements may cover the legal aspects, but in addition there is the purely scientific side to be considered. The advent of different types of rennet will probably produce new problems for the cheesemaker and research worker, and it would be most helpful if the manufacturers of the new types of rennet were to give information on the nature and strength of the enzymes in their products. For example, the following data might be given:

Enzyme	How measured
Rennin or milk clotting enzyme	Clotting power on standard milk at pH 6.5
Proteases	Breakdown of casein at pH 5.5
Lipases	Breakdown of milk fat at pH 5.5

VII THE FUNDAMENTAL PROBLEMS IN CHEESE RESEARCH

The fundamental difficulty in tyrology, or the scientific study of cheese, is the virtual impossibility of standardising all factors and varying only the one being studied. No two milk supplies, rennets, starters and series of reactions in the cheesemaking process are ever identical, and this is the reason why there is so much conflicting evidence in the literature when such factors as rennin and other enzymes are being studied and information sought. Steps which are being taken in this direction include attempts to use:

(i) a sterile milk, undamaged physico-chemically;
(ii) pure enzymes;
(iii) pure cultures of starter and other organisms;
(iv) aseptic conditions.

It will not be possible to evaluate completely beyond doubt any of the factors involved until a way has been found to standardise all of them.

The rennet of the future

If the ever increasing demand for food in the world requires more cheese to be made, it is obvious that the supply of rennet will become more restricted. One possibility would be the development of blended rennets, such as the 50:50 rennet-pepsin, but utilising the fungal rennets now coming on to the market. If these rennets are to be successful, it would be desirable to have the fullest possible knowledge of the nature of all the enzymes in the rennets and their precise functions in the cheese. This is a matter of considerable difficulty because of the inter-relationship between the numerous factors involved. Once the exact value of the different enzymes has been determined, it would be possible to separate the enzymes and recombine them as required.

Equally important will be our knowledge of these enzymes in various rennets, the natural enzymes in milk and the role of the bacteria present in the milk. Pasteurisation of milk may kill 99% of the organisms present, but cheesemaking is, from the microbiological point of view, a highly specialised enrichment culture of those organisms able to grow *in a particular type of cheese*. Each type of cheese therefore presents a problem of its own. Cheesemaking has always been based on the souring of milk by fortuitous or added bacteria, followed by addition of rennet or some other coagulating enzyme. In other words, we use living organisms to sour the milk and an enzyme extract to clot it. It would be theoretically possible to grow a rennin-producing organism in the milk and then clot it by acidification. Obviously control would be difficult, but acid production by starter is controlled and there would seem to be no reason why rennin production should not similarly be controlled. For example, a psychrotrophic rennin-producing organism could be allowed to grow slowly overnight at neutral pH, and then the milk acidified to a suitable pH the next day and warmed in order to kill the rennin-producing bacteria (if this should be necessary) and initiate the coagulation of the milk. The starter could be added immediately after the warming and before coagulation set in. Both souring and rennin-clotting are biochemical processes and there is no reason why they should not both be produced by bacteria. The extensive research carried out in the last 20 years on genetics in bacteriology and the manipulation and control of genes points the way to the production of rennin and the most suitable bacteria for cheesemaking in the future. The time may even come when we have trained an organism to produce rennin, lactic acid and a desirable flavour for a particular type of cheese. Already yeasts have been cultured and adapted for their suitability for a particular type of wine, beer or cider, and certain strains of lactic acid bacteria, used as starter cultures for Cheddar cheese, are known to produce certain flavours in the mature cheese (Fryer 1969).

This idea may appear to be rather visionary at the present time, but I may point out that there is already a similar phenomenon operating in the dairy world, although in an adverse way. The faults known as 'bitty cream' and 'sweet curdling' of milk in Britain are produced respectively by the lecithinase and rennin-type enzymes of *B. cereus*. We have here therefore an exact analogy of the mechanism I have suggested.

The role of starter and other bacteria in the production of flavour is still largely an unsolved problem, largely due to the instability of bacterial metabolisms in cheese. Future research will have to be concentrated not only on the nature of cheese flavour substances and the organisms producing them, but also on the stabilisation of these biochemical processes.

Rennet and new techniques in cheesemaking

Our fast developing knowledge of the nature of rennin and its mode of action has enabled workers to apply this knowledge to devise new methods for making cheese. Probably the most interesting and important research for the future of the cheese industry is the continuous curd method for Cheddar cheese developed at Shinfield by Berridge and Chapman and their colleagues (Berridge[9,21,22,23], Cheeseman and Chapman[24], Budd and Chapman[30]).

The particular value of these

methods is economic; any new procedure which saves time and labour has a definite market value. What still has to be established is whether these new methods can give the characteristic true-to-type varieties in the UK (p. 138) and other countries. In the author's opinion, the chief danger in the food technology of the future will be that of lack of variety in our foods and so monotony in our diet (Davis[39]).

The many varieties of cheese throughout the world constitute one of the most interesting parts of our diet, and the subtle differences between them are important for those who are seriously concerned with diet, nutrition and other aspects of food.

The author believes that there is a great danger that advances in cheese technology, as for other food technologies, may steadily lead to the mass automated and mechanised production of ever fewer and fewer genuine varieties, so that by the year 2000 A.D. we shall finish up with only one grana type, one Cheddar type, one Port Salut type, one blue-veined type, one washed or spun curd type, one surface-bacteria ripened or Limburg type, one Camembert type and one soft cheese, throughout the world. The dairy technologists in all countries should fight to maintain the integrity of their national varieties.

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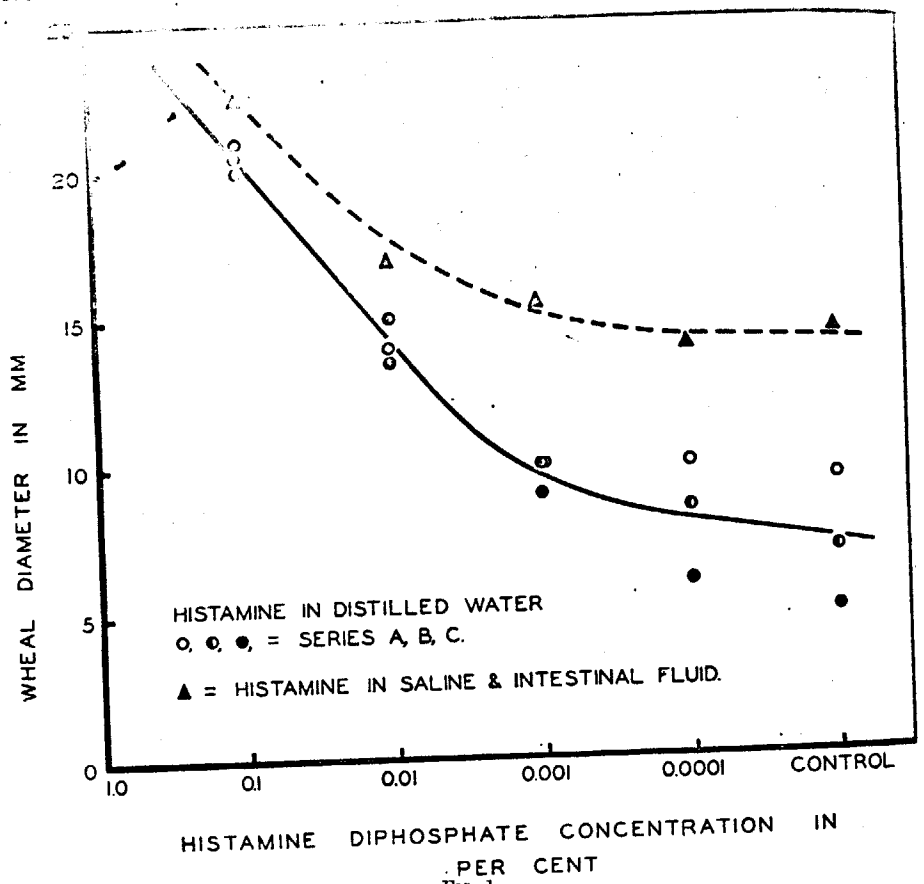

FIG. 1.

The relation of wheal diameter to concentration of histamine diphosphate injected intradermally in the goat. Solid line represents histamine in distilled water; broken line, in a mixture of physiological saline and intestinal fluid from a fasted cat.

the basis of uncontrolled factors causing variation of response to trauma incident to the injection, where histamine is no longer a limiting factor.

Measurement of histamine in the presence of a substance which also produces whealing is feasible providing the concentration of histamine is the limiting factor. Whereas the [illegible] histamine alone is 0.001%, [illegible] amine-like substance, [illegible] by the broken [illegible] phosphate [illegible] relative [illegible] of the wheal becomes increasingly dependent on the former, the 2 curves tending to converge.

These experiments suggest the importance of proper selection of laboratory animals for all dermatological investigations involving a local skin reaction. The response to histamine diphosphate injected intradermally provides a means of selecting animals which have a peripheral vascular mechanism similar to that existing in man. The use of dogs or goats would seem to be more logical than the time worn reliance on guinea pigs and rabbits for the study of response to local irritants and allergenic substances. The quantitative relationship existing between histamine concentration and skin wheal size suggests the possibility of using histamine as a standard reference material in comparing various skin irritants.

Summary. There is a species variation in the local reaction to intradermal injections of histamine. The dog, goat, and man show a marked skin whealing, but no such response is obtainable in the guinea pig, rabbit, or cat.

The diameter of the skin wheal produced by [illegible] is a [illegible] concentration [illegible] hold being 0.001%. In the presence of an extraneous histamine-like substance, this relationship still [illegible], but the threshold of histamine sensitivity is elevated to 0.01%.

A practical application to the field of experimental dermatology is suggested involving the proper selection of test animals and the use of histamine as a standardizing agent.

15057

Ulcer Production in Intestines of Dogs by Various Enzymes Under Hydrostatic Pressure.

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In a previous paper from this laboratory the effect of hydrostatic pressure on the production of ulcers was described.[1] In another paper it was reported that ulcers could be produced in the intestines of dogs by an alkaline solution of pancreatin under a hydrostatic pressure of 90 cm H_2O.[2]

The purpose of the experiments reported here was to study the effect produced on the intestines by the following enzymes: rennin, trypsin, erepsin, steapsin, and amylopsin. The enzyme solutions were at pH ranges corresponding to those under which the enzymes act in the normal animal.

The procedure was the same as that described in [1] except that in these experiments only one pressure (90 cm H_2O) was used. The severity of damage to the loops was graded according to a scheme adopted in this laboratory which is described here: (1) represents a condition in which there is a slight surface necrosis of the mucosa; (2) designates a deeper necrosis involving at least ⅓ of the thickness of the mucosa of the entire area with smaller areas (from ½ cm^2 to 2 cm^2) showing necrosis as deep as the submucosa; (3) indicates necrosis of practically all the mucosa with some patches extending into the circular muscle; (4) was applied to a loop having areas of necrosis extending to the serous coat or to a frank perforation. Each loop was scored in this manner and the average taken for each group of experiments. The results are given in Table I.

Discussion. Rennin (0.1%) in N/10 HCl produced perforating ulcers in 114 minutes while N/10 HCl alone caused perforation in 174 minutes and pepsin (0.1%) in N/10 HCl in 82 minutes.[2] These data show that rennin has a marked digestive effect on the mucosa of the intestine but not so great an effect as pepsin.

Of the other enzymes studied only the proteolytic ones were active. The necrosis produced by trypsin was about twice as severe as that caused by erepsin, but not even trypsin digested the mucosa to an extent comparable to that of rennin and pepsin, and was only about one-half as effective as pancreatin.[2]

Neither steapsin nor amylopsin caused any necrosis of the mucosa. Alkaline solutions of these enzymes [illegible] the loops of intestine were exposed to the

[1] Driver, R. L., Chappell, R. H., and Carmichael, E. B., *Am. J. Dig. Dis.*, 1945, **12**, 168.

[2] Driver, R. L., *Arch. Path.*, in press.

TABLE I.
Ulcer Formation in Intestines by Enzyme Solutions under 90 cm H_2O Pressure.

Dog No.	0.1% solution of	pH	Time exposed (min.)	Remarks	Severity
1-12	rennin* in N/10 HCl	1.2	60-180	perforated ulcer in all dogs	4.0 in all dogs
13	trypsin† in N/10 $NaHCO_3$	8.1	580	necrosis through ½ thickness of mucosa entire area	2.0
14	,, ,, ,, ,,	,,	698	necrosis through ⅓ thickness of mucosa over entire area, 10 small spots into submucosa	2.0
15-17	,, ,, ,, ,,	,,	326-384	normal mucosa	0
18	,, ,, ,, ,,	,,	696	necrosis through ⅓ thickness of mucosa entire area, 5 small spots into submucosa	2.0
19	,, ,, ,, ,,	,,	694	surface necrosis over ¾ area of loop	1.0
20-21	,, ,, ,, ,,	,,	690-692	normal mucosa	0
22	erepsin† in N/10 $NaHCO_3$	,,	640	surface necrosis	1.0
23-24	,, ,, ,, ,,	,,	636-638	normal mucosa	0
25	,, ,, ,, ,,	,,	634	necrosis entire mucosa, some patches into muscle	3.0
26-30	,, ,, ,, ,,	,,	284-632	normal mucosa	0
31-41	steapsin† in N/10 $NaHCO_3$	,,	458-810	,, ,,	0
42-53	Amylopsin† in N/10 $NaHCO_3$	,,	290-745	,, ,,	0
54-65	N/10 HCl	1.0	91-320	perforated ulcer in all dogs	4.0 in all dogs

* Armour, Rennet, N.F.
† Pfanstiehl.

alkaline solution alone.

Summary. 1. The proteolytic enzymes, rennin, trypsin, and erepsin, under hydrostatic pressure of 90 cm H_2O produced necrosis of the intestines of dogs, rennin being the most active of the three. 2. The average time required to produce a perforation with N/10 HCl alone was about 50% more than with rennin in N/10 HCl. 3. Neither steapsin nor amylopsin caused necrosis under the above condition.

15058

Effect of Infused Penicillin in the Bovine Mammary Gland.*

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Reports[1-3] have been published dealing with the therapy of bovine mastitis by infusion of penicillin but none has been found that deals with the physiologic behavior of this antibiotic when introduced into the mammary gland. This report deals primarily with studies on the rate of decline and irritating effects of infused penicillin. A knowledge of both of these aspects is essential to a rational approach to the use of the drug in therapy.

For the majority of experiments the sodium salt of penicillin was made up with sterile distilled water so that 1 ml of solution contained 5,000 Oxford Units. Immediately after milking infusions were made via the teat canal, in 5, 10, 30, 40, 50, 100, and 200 thousand unit doses in normal glands and in 10, 30, 50, and 100 thousand unit doses in pathologic glands. All glands were carefully palpated before and after treatment. Milk samples were taken before and for varying periods after treatment and examined grossly and for bacterial, chloride and cell content. Yields of milk before and after treatment were also ascertained. Blood and urine samples were taken periodically for penicillin determination.

Sampling of milk for penicillin determination varied. In some trials small samples were drawn at hourly intervals, in others the intervals were longer. Complete milking varied from 10 to 72 hours after the infusions were made.

In one series the penicillin was infused in quantities of water equal to the volume of milk withdrawn.

The method used for assaying the amount of penicillin in milk was a slight modification of the cylinder-plate procedures described by Schmidt and Moyer[4] and Beadle, Mitchell, and Bonner.[5] It was noted early in the work that the zones of inhibition due to penicillin in milk stimulated the growth of the test organism (*Staphylococcus aureus* No. 209) at the periphery of the zone. In order to standardize the appearance of the zones of inhibition of the unknown and the standard solutions the following procedure was adopted. Sterile skim milk was used instead of 1% phosphate buffer for diluting the 50 Oxford Units per ml penicillin standard to 0.25, 0.5, 1.0, 2.0 and 3.0 Oxford Units per ml. These skim milk standards were run, in duplicate, on each day of assay for the standard curve[4] and all dilutions of milk samples for assay were made in sterile skim milk.

Culture dishes[5] were covered with a piece of celotex instead of a glass plate during the incubation period in an incubator humidified so that the relative humidity was between 40 and 50%.

Penicillin assay of urine samples was carried out in the same manner as for milk except 1% phosphate buffer was the diluent instead of skim milk.

Several methods were utilized at various times to assay the penicillin content in blood. The method of choice was the serial dilution assay employing *Bacillus subtilis* described by Hickey.[6] This method, in our experience, usually detects 0.031 Oxford Units per ml in blood plasma.

To determine the length of time a therapeutic level of penicillin would remain in the milk after its infusion into normal glands, small samples of milk were taken at varying intervals for penicillin determination. All of the milk was removed at intervals from 10 to 72 hours following infusion and penicillin therein determined. Twelve normal quarters were infused with solutions containing 5,000 Oxford Units per ml water and 7 normal quarters with solutions containing 100 and 200 Oxford Units per ml.

The rate of decline in concentration of penicillin per ml of milk for infusions of 5,000 and 40,000 Oxford Units in 1 and 8 ml of water respectively, is presented in Fig. 1. The rate of decrease is the greatest during the first 10 hours after which the disappearance of penicillin is at a slower rate. The rate of decrease is greater for each increment of the dose administered over the range studied which was up to 200,000 Oxford Units per quarter. In all cases where 30,000 Oxford Units or more were infused the milk contained 0.5 or more units of penicillin per ml 24 hours after the infusion was made. At no time was there sufficient penicillin in the blood to be detected by the assay methods used, but that some of the drug found its way into the blood is evidenced by its presence in the urine. The urine levels are presented in Fig. 1 for a period of 24 hours when a total of 100,000 Oxford Units were infused in all 4 quarters. The highest level of 11 Oxford

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[illegible] A., [illegible] D. W., and Moor, J. W., [illegible]
[illegible] and Huffman,

4 Schmidt, W. H., and Moyer, A. J., *J. Bact.*, 1944, **47**, 199.

5 Beadle, G. W., Mitchell, H. K., and Bonner, D., *J. Bact.*, 1945, **49**, 101.

6 Hickey, R. J., Commercial Solvents Corporation, according to information furnished in communication March 14, 1945.

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In the second case, why has it been apparently cured for four years without the administration of alkalis? These cases were both duodenal ulcer.

In some obstinate and resistant cases, my son, Dr. Leland Russell, has within the past few months been doing a sub-total gastrectomy and resection of the first portion of the duodenum, thus removing the ulcer bearing area. He has not done this operation in a sufficient number of cases as yet to determine the results.

STUDIES ON THE DIGESTION OF MILK IN VIVO*

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In 1935, the author published a series of studies on the effect of adding rennin as found in junket to untreated cow's milk and some of its modifications in regard to the speed of digestion of these foods. Studies in vitro[1] showed that cow's milk digested most rapidly at a pH between 4 and 5 when rennin (junket) was added to the mixture of a substrate and enzyme. Under these conditions most of the other milk modifications digested most rapidly. In vivo studies[2] showed that the time after ingestion of food at which gastric analysis revealed the greatest quantity of soluble nitrogen per 100 c.c. gastric contents was shorter when cow's milk and junket were fed than when cow's milk alone was fed. This time was assumed to show the period after ingestion at which digestion was at its peak, and as less soluble nitrogen was found in succeeding gastric analysis, it was assumed that digestion was decreasing. Accordingly it was concluded that gastric digestion was faster when milk plus junket was fed than when milk alone was fed. The addition of rennin in this form (junket) had no effect on the speed of digestion of other milk modifications in vivo.

The author believes that these results from the in vivo studies may be misleading since no attempt was made to control the dilution factor of the gastric contents by outpouring of gastric juice from the stomach wall or of loss of substrate by its passage into the intestine. A small amount of soluble nitrogen per 100 c.c. might be due to a great dilution of the food by gastric juice while actually more of the total amount of nitrogen may be a soluble form than was observed in another reading where merely a higher concentration of the ingested material might result in a greater figure for the soluble nitrogen per 100 c.c. of gastric contents. A truer measure of digestion would be the proportion of soluble nitrogen to the total nitrogen of 100 c.c. of gastric contents.

The following studies were made with the determination of the changing concentration of substrate. The effect of pH on the digestion of milk was also taken into account, and the gastric digestion of subjects with little HCl in the gastric juice was made. This was to see whether the effect of pH as noted in the in vitro studies was applicable to the human stomach.

The method used for these studies was as follows: The subject swallowed a Rehfuss tube and through it we injected into the stomach, 200 c.c. of milk into which we had stirred 10 grams of dextrose. On alternate days, one gram of commercial rennin was added to the mixture. Fifteen minutes after ingestion, a sample of the gastric contents was withdrawn. Samples were also taken every

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*From the University of Minnesota.

half hour after the first one, for a period of two hours. These samples, as well as one from the milk mixture originally fed, were subsequently tested for pH, for soluble nitrogen according to the Sorenson method of titration for amino acids[3], and for sugar according to Benedict's method[1]. The total nitrogen of the milk mixtures as originally fed was calculated from that of a sample determined by the micro kjeldahl method. Since the dextrose (and lactose of milk) is subjected to no chemical change in the stomach, the concentration of sugar in a sample of gastric contents compared to that of the sugar in the milk mixture as originally prepared was assumed to represent the concentration or dilution of all the materials present in the milk, as all these substances were subjected to the same amount of mixing with gastric juice, and loss through the duodenum.

The actual amount of soluble nitrogen in 100 c.c. of gastric contents then was multiplied by the dilution factor so determined, in order to find the concentration of soluble nitrogen in a volume which had not been made greater by dilution. The undiluted soluble nitrogen then was calculated as the percentage of total nitrogen which the sample would contain if undiluted, and the results compared.

The results on five subjects are given. Since each subject had different peculiarities, each subject is treated separately. Full tables of results will be given in two cases; in all cases the results will be discussed.

Case 1:—A man aged 50. Diagnosis: peptic ulcer. Seven experiments were run on this subject in three cases of which milk alone was given and in four of which rennin was added to the milk. During one experiment while milk without the enzyme was given, the patient complained of pain, so one half gram of sodium bicarbonate was given at intervals raising the pH of the gastric contents. In this experiment, the digestion of milk was slight. In the other two cases the digestion of milk was considerable, all the nitrogen being in the soluble form in one hour. In some of the experiments with milk and rennin, the pH was altered to a higher value by the administration of sodium bicarbonate. This was in order to see if rennin increased the speed of digestion at a higher pH than that of the usual gastric contents as found in the experiment cited[1]. In one of these latter experiments, the highest per cent of soluble nitrogen to total nitrogen was 24, reached one half hour after the ingestion of the food, and in the second experiment the highest per cent of soluble nitrogen was 14, reached one half hour after the ingestion of milk. In a third experiment wherein rennin was added but the pH of the gastric contents was not altered, the highest percentage of soluble nitrogen to total nitrogen was 7.5, reached one hour after feeding and in another similar experiment the percentage of soluble nitrogen to total nitrogen rose to 50 in one half hour and 66 in one hour. Thereafter, both the soluble nitrogen and its proportion to the total decreased.

A table of results follows. The percentage of soluble nitrogen to total nitrogen was calculated as explained above.

Experiment 1. — Milk without rennin ingested. ½ gm. $NaHCO_3$ given every half hour. Total N in 200 c.c. milk—2,120 milligrams.

	pH	Mg. sol N/100 c.c.	% sol N to total N
milk	6.40	13.3	1.2
15 min. sample	3.51	3.15	0.2
½ hour sample	5.94	1.68	0.17
1 hour sample	4.45	70.0	6.7
1½ hour sample	6.73	—	—

Experiment 2.—Milk without rennin ingested. Total N in 200 c.c. milk—1,160 milligrams.

	pH	Mg. sol N/100 c.c.	% sol N to total N
milk	6.55	30.8	5.3
15 min. sample	2.28	199.5	34.0
½ hour sample	5.21	210.0	58.0
1 hour sample	1.67	1062.0	100.0
1½ hour sample	6.00	—	—

Experiment 3.—200 c.c. milk without rennin ingested. Total N in 200 c.c. milk—1,160 milligrams.

	pH	Mg. sol N/100 c.c.	% sol N to total N
milk	6.69	21.7	3.7
15 min. sample	4.06	147.0	25.0
½ hour sample	6.24	—	—
1 hour sample	1.70	1011.5	100.0
1½ hour sample	2.76	7.4	1.2
2 hour sample	6.90	—	—

Experiment 4.—200 c.c. milk with 1 gram rennin ingested. Total N in 200 c.c. milk—1,160 milligrams. ½ gram $NaHCO_3$ given every half hour.

	pH	Mg. sol N/100 c.c.	% sol N to total N
milk	6.64	2.8	0.4
15 minute sample	4.10	31.92	5.2
½ hour sample	5.90	140.0	24.0
1 hour sample	5.62	—	—

Experiment 5.—200 c.c. milk with 1 gram rennin ingested. Total N in 200 c.c. milk—1,040 milligrams. ½ gram $NaHCO_3$ given every half hour.

	pH	Mg. sol N/100 c.c.	% sol N to total N
milk	6.39	14.7	2.8
15 min. sample	5.45	40.5	7.8
½ hour sample	6.63	70.0	14.0
1 hour sample	1.82	4.6	0.8

Experiment 6.—200 c.c. milk with 1 gram rennin ingested. Total N in 200 c.c. milk—2,000 milligrams.

	pH	Mg. sol N/100 c.c.	% sol N to total N
milk	6.55	7.0	1.0
15 min. sample	1.99	50.96	7.2
½ hour sample	1.60	9.7	1.4
1 hour sample	1.55	52.5	7.5
1½ hour sample	1.44	3.5	0.4

Experiment 7.—200 c.c. milk with 1 gram rennin ingested. Total N in 200 c.c. milk—1,080 milligrams.

	pH	Mg. sol N/100 c.c.	% sol N to total N
milk	6.66	20.3	3.6
15 min. sample	2.55	57.4	10.0
½ hour sample	2.22	274.4	50.0
1 hour sample	1.77	308.0	66.0
1½ hour sample	1.65	4.9	0.6

The results on this patient show most speedy and complete change of the nitrogen to the soluble form when rennin is not added to the milk and the gastric contents follow the normal acidity curve.

Case 2:—A man aged 19. Diagnosis: Hodgkin's disease. A series of seven experiments was run on this patient. In four of the experiments, milk without the enzyme was given, and in two of these four, no attempt was made to change the pH of the gastric contents. In two experiments, the pH of the gastric contents was raised by the administration of one fourth gram of $NaHCO_3$ every half hour.

In three other experiments, rennin was added to the milk. In two of these latter experiments no attempt was made to control the pH of the gastric contents; while in the third, the pH was raised as before.

In the first of the experiments with milk alone, the percentage of nitrogen changed to its soluble form reached 100 in one hour, and thereafter very little soluble nitrogen was found in the gastric contents indicating complete digestion in an hour. In the second experiment performed under the same conditions, the percentage of soluble nitrogen rose steadily but gradually for two hours, to a high point of 30 per cent of the whole.

In the two experiments wherein rennin was added to the milk, and the pH of the gastric contents in no way interfered with, the percentage of soluble nitrogen to total nitrogen reached a high point of 100, in the first experiment at one and one-half hours after ingestion, and in the second at two hours after ingestion.

In all those experiments wherein the pH was raised to a point between 5 and 6 by the administration of sodium bicarbonate, very little or no soluble nitrogen was found in the gastric contents. At such a high pH of the gastric contents then, this patient was unable to digest the protein of milk, whether or not rennin was ingested.

The experiments on this patient then are not sufficient in number to be conclusive, but they indicate that the addition of rennin to milk does not change its digestibility.

Case 3:—A woman aged 36. No diagnosis. She entered the hospital because of nutritional disturbances. Gastric analysis showed an achlorhydria.

In four of the experiments in which this woman served as a subject, the pH of the gastric contents was lowered by the administration of 5 c.c. 0.5 per cent HCl by mouth, at one half hour intervals during the test. In five of the experiments the pH of the gastric contents was in no way controlled.

Gastric digestion appeared to be poor in this patient, since all samples of gastric contents showed that only a small proportion of nitrogen was changed to the soluble form. In two experiments in which milk without the addition of the enzyme was fed, and the patient treated with 0.5 per cent HCl, the highest percentage of soluble nitrogen to the total was 11 in one case and 16 in the other. These peaks of maximum soluble nitrogen occurred one and one-half hours after ingestion. In two experiments in which the conditions differed from those described above by the addition of rennin, the maximum percentage of nitrogen in the soluble form was 3.3 and 17 reached respectively at two and one and one-half hours after ingestion.

In one experiment where milk was given and no HCl administered, the highest percentage of soluble nitrogen was 33 and was reached one half hour after ingestion of the food. In another such experiment, the maximum soluble nitrogen was 41 per cent at one and one-half hours after ingestion.

With the addition of rennin to the milk and no interference with the pH of the gastric contents the point of greatest concentration of soluble nitrogen was reached fifteen minutes after ingestion and was 1.6 per cent. In another experiment, it was reached in one hour and amounted to 17 per cent.

The addition of rennin to milk did nothing to increase the already poor gastric digestion of this subject in regard to either the amount of soluble nitrogen formed from this milk protein, or the time at which maximum digestion occurred.

A table of the results for case 3 is given.

Experiment 1.—Milk without rennin ingested. 5 c.c. of 0.5% HCl given every half hour. Total N in 200 c.c. milk—1,000 milligrams.

	pH	Mg. sol N/100 c.c.	% sol N to total N
milk	6.66	19.6	3.7
15 min. sample	6.56	21.0	4.2
½ hour sample	5.80	36.4	7.2
1 hour sample	3.15	36.4	7.2
1½ hour sample	3.37	84.2	11.0

Experiment 2.—Milk without rennin ingested. 5 c.c. of 5% HCl given every half hour. Total N in 200 c.c. milk—1,080 milligrams.

	pH	Mg. sol N/100 c.c.	% sol N to total N
milk	6.69	37.2	3.5
15 minute sample	6.72	27.37	4.9
½ hour sample	4.82	62.37	11.0
1 hour sample	2.47	69.30	12.0
1½ hour sample	1.64	91.00	16.1
2 hour sample	1.54	4.7	0.7

Experiment 3.—Milk without rennin ingested. Total N in 200 c.c. milk—1,240 millgrams.

	pH	Mg. sol N 100 c.c.	% sol N to total N
milk	6.35	16.10	2.6
15 min. sample	6.88	21.00	3.3
½ hour sample	5.80	196.84	33.0
1 hour sample	5.00	36.00	5.8
1½ hour sample	6.45	84.00	13.0

Experiment 4.—Milk with rennin ingested. 5 c.c. of 0.5% HCl given every half hour. Total N in 200 c.c. milk—1,240 milligrams.

	pH	Mg. sol N/100 c.c.	% sol N to total N
milk	6.62	20.3	3.2
15 min. sample	6.78	27.3	7.0
½ hour sample	5.18	5.6	0.8
1 hour sample	6.27	12.92	1.9
1½ hour sample	2.14	25.20	1.3
2 hour sample	2.18	93.8	3.3

Experiment 5.—Milk with rennin ingested. 5 c.c. of 0.5% HCl given every half hour. Total N in 200 c.c. milk—1,400 milligrams.

	pH	Mg. sol N/100 c.c.	% sol N to total N
milk	6.44	21.0	5.2
15 min. sample	6.74	15.9	3.8
½ hour sample	2.72	24.5	6.1
1 hour sample	4.30	53.2	13.0
1½ hour sample	2.13	71.4	17.0
2 hour sample	1.34	26.18	2.4

Experiment 6.—Milk with rennin ingested. Total N in 200 c.c. milk—2,000 milligrams.

	pH	Mg. sol N/100 c.c.	% sol N to total N
milk	6.52	18.2	0.9
15 min. sample	6.26	32.34	1.6
½ hour sample	—	—	—
1 hour sample	—	13.72	0.7

Experiment 7.—Milk with rennin ingested. Total N in 200 c.c. milk—1,320 milligrams.

	pH	Mg. sol N/100 c.c.	% sol N to total N
milk	6.49	9.1	1.4
15 min. sample	6.20	0	0
½ hour sample	6.70	31.36	5.0
1 hour sample	6.98	107.80	17.0
1½ hour sample	7.00	32.2	5.3
2 hour sample	7.29	6.5	0.9

Case 4:—A woman 25 years of age. Diagnosis: tuberculosis of the lung. Accompanying this condition was an unexplained low gastric acidity. Four experiments were run on this subject, in two of which no attempt was made to control the pH of the gastric contents and in two of which the pH was lowered by the injection into the stomach every half hour of 5 c.c. of 0.5 per cent HCl.

In the one case in which milk without rennin was fed to the patient and the pH uncontrolled, the digestion, as found by comparing the soluble nitrogen to the total nitrogen was found to be 100 per cent at two hours after ingestion. When milk with rennin was fed under the same conditions, this percentage of soluble nitrogen to total nitrogen was reached at the one and one-half hour and two hour readings.

When the pH was lowered by the administration of HCl, the maximum percentage of soluble nitrogen was 54 and was reached one and one-half hours after ingestion of milk alone. In a similar experiment in which rennin was added to the milk, the maximum percentage of solubility of nitrogen was 12 and occurred one and one-half hours after ingestion.

The experiments show no definite change in digestibility of milk after addition of rennin.

Case 5:—A man aged 53 entering the hospital for circulatory disorders. Accompanying these was an unexplained achlorhydria. A series of 10 experiments was performed using this subject, in five of which no attempt was made to control the pH but in five of which the pH was lowered by the administration of 5 c.c. of 0.3 per cent HCl every half hour. In one of the experiments in which no HCl or rennin was given, the greatest proportion of soluble nitrogen to total nitrogen was 39 per cent and was reached one-half hour after ingestion of food. In a second such experiment the high point of digestion occurred two hours after ingestion and reached 60 per cent. In three experiments made under similar conditions with the exception of the addition of rennin, the highest percentage of soluble nitrogen to total nitrogen was 6, 23 and 41 occurring at one-half, one, and one and one-half hours after ingestion respectively.

In the series of experiments in which the pH of the gastric contents had been lowered to a normal value, the high percentage of soluble nitrogen was 25, 2.6, and 6.2 in the three cases in which no enzyme was given. These high percentages were reached one half hour after ingestion of food in all three experiments. In two similar experiments with the exception of the addition of rennin to the food ingested, the maximum digestion showed a 40 per cent and 45 per cent conversion of the nitrogen to the soluble form, occurring at one and one-half and one hours after ingestion of food.

In Case 5, then, a slightly favorable effect on digestion by the addition of rennin to milk is indicated by the higher degree of conversion of nitrogen to a soluble form when the pH was lowered to the normal value. Otherwise, the figures showed no significant difference.

Study of these results shows that each individual digests milk at about the same rate and to approximately the same degree whether or not rennin is added to the milk. In the two subjects in whom the normal pH of the gastric contents was raised by the ingestion of $NaHCO_3$ the digestion appeared to be less complete than under normal conditions as shown by the lowered percentage of conversion of total nitrogen to soluble nitrogen. In one subject (Case 2) gastric digestion was almost completely inhibited by such treatment.

The subject in which hypochlorhydria occurred (Case 4), showed complete digestion of milk after it had remained in the stomach for a relatively long time

(1½ hours). Administration of HCl apparently inhibited this complete conversion of the nitrogen to its soluble form.

The two subjects whose gastric juice showed no free acid (Cases 3 and 5) also showed poor gastric digestion according to the percentage of total nitrogen converted to its soluble form. Lowering the pH by the administration of acid in no way increased the percentage of soluble nitrogen. Presumably this gastric juice may also be deficient in pepsin.

We may summarize the results in regard to rennin by saying that rennin added to milk has no effect on its digestion either in regard to the proportion of nitrogen liberated from its peptid linkage or in regard to the time at which the maximum liberation is accomplished after ingestion, i.e., in amount or speed of digestion.

This work was done at the University of Minnesota. The author wishes to thank Dr. Cecil Watson for permission to use the clinical material, and Dr. J. F. McClendon for laboratory facilities.

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THE GASTROENTEROLOGIST AND THE CHOLESTEROL PROBLEM*

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Joslin[1], in his article on "Fat and the Diabetic", published in 1933 was the first clinician to voice interest in this subject. Timothy Leary[2], the pathologist, deserves credit for his masterful work "Experimental Atherosclerosis in the Rabbit Compared with Human (Coronary) Atherosclerosis". Leary recently summarized his work in the subject as follows. "All the lesions of aortic atherosclerosis, save the early mucoid change, are due to the presence of cholesterol. In old age cholesterol metabolism ceases, globular lipophages accumulate in masses, with inadequate nutrition and support and a primary atheromatous "abscess" is the typical lesion. Atherosclerosis is a disease due to disturbances of the cholesterol metabolism. Stresses appear to be responsible for the localization of the lesions. Advanced atherosclerosis is the result of years of progressive accretion. The fact that the cholesterol content of the blood is low is not evidence that it was not high at times during its development. Thyroid and iodine are cholesterol solvents".

Rabinowitch[3] concludes that excess blood cholesterol is an important etiologic factor in the production of arteriosclerosis in the young diabetic. Also that treatment with the high carbohydrate-low fat diet has delayed development of cardiovascular disease in the cases investigated.

A recent important contribution was made by Messinger and Hawkins[4]. They found that arsphenamine liver injury was profoundly modified by dietetic control. A protein diet was most effective in repairing damage as well as protecting dogs from arsphenamine liver injury. Fat diet proved to be extremely deleterious.

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efficacious, whereas vagotomy alone was without effect in modifying the average minimal fatal dose.

These facts are interpreted to mean that in the intact rabbit, respiratory embarrassment must exist, either through spasm of the glottis or other obstruction, in such a way that the asphyxial element thereby induced very markedly contributed to the central injury produced by cocaine.

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Sulphur metabolism in yeast.

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The studies of Swoboda[1] in this laboratory indicate that cystin added to certain media stimulates yeast growth. This suggested a more intensive study on the actual utilization of various forms of sulphur in yeast growth.

The forms of sulphur studied were sulphate, sulphide, cystine, cystein, cysteinic acid, taurine and taurocholic acid. These were added to the usual synthetic medium as employed by Williams,[2] Miller,[3] and Swoboda,[1] with and without the "biose vitamine" and without the usual sulphate content. The yield of yeast was determined by weighing the yeast obtained after a growth period of eighteen hours. The saccharose and asparagin used in the media were purified to remove sulphur containing substances as completely as possible.

The results obtained were as follows:

1. Inorganic sulphate is the best form of sulphur for yeast growth, especially if magnesium is present with an ample amount of the biose vitamine. Neither magnesium nor sulphate could be substituted for the biose vitamine.

[1] Swoboda, F. K., *J. Biol. Chem.*, 1922, lii, 91.
[2] Williams, R. J., *J. Biol. Chem.*, 1920, xlii, 259.
[3] [illegible], *J. Biol. Chem.*, 1921, xlviii, 329.

2. Sulphide sulphur added as hydrogen sulphide in concentrations of 0.0022 mg. per 125 cc. may be used as a sulphur source by growing yeast. In higher concentrations it retards yeast growth, especially when a good sulphate containing medium is employed.

3. Cystine stimulates yeast growth slightly in concentrations of 1 to 4 mg. per 125 cc. medium. In higher concentrations and especially in a good sulphate containing medium it retards yeast growth. Cysteine behaves similarly. Possibly the harmful effect of these is in part due to their partial conversion into hydrogen sulphide.

4. Cystine sulphur is partly utilized in synthesizing new yeast protein, but a part of it also remains in the medium as sulphate.

5. In otherwise sulphur free media cysteinic acid stimulates yeast growth very slightly, but in a sulphate containing medium it retards the same.

6. Taurine in concentrations of 2 to 20 milligrams per 125 cc. has no appreciable effect on yeast growth.

7. Taurocholic acid in concentrations of 1 mg. or more per 125 cc. was toxic even in sulphate media.

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Specific absorption studies upon calf rennin.

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The question of the identity of pepsin and rennin has been under consideration for many years, Pekelharing,[1] Pawlow[2] and others, holding to the view that pepsin and rennin activities were not distinct, with Hammarsten,[3] and more recently Fenger,[4] claim that the two activities are different in character.

[1] Pekelharing, C. A., *Z. Physiol. Chem.*, 1896, xxii, 233-44.
[2] Pawlow, J. P., *Z. Physiol. Chem.*, 1904, xlii, 415; Schmidt-Nielsen, S., *Z. Physiol. Chem.*, 1906, xlviii, 92.
[3] Hammarsten, O., *Z. Physiol. Chem.*, 1911, lxxiv, 142-68; *Ibid.*, 1918, cii, 33-77.
[4] Fenger, F., *J. Am. Chem. Soc.*, 1923, 249-55.

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The study made in this laboratory was carried on in order to help bring further proof as to the identity of these two activities.

A very active preparation of rennin was made by the method of Fenger.[4] The dried product from this method of preparation showed on the average a milk-curdling power of 1:1,000,000 in ten minutes on fresh pasteurized milk at 40° C. Reprecipitation of this material gave a final product 41.66 times as active as Armour's commercial rennin. It was more active than could be consistently prepared by the sodium chloride salting out process and it had the advantage of being free from absorbed sodium chloride. The product can be kept for a long time without losing activity.

Studies were then carried out in order to determine if a more active product could be prepared. This was attempted by absorbing the rennin activity from the calf preparation, on solid purified casein suspended in solutions at various pH values. In these studies it was observed that the rennin activity could be absorbed to the extent of 90 to 95 per cent when the pH value of the solution was from 4.4 to 4.6. This was shown to be true by testing the filtrates, after absorption, for milk curdling power. It was also observed that the absorbed rennin activity could be recovered from the casein to the extent of 75 per cent to 80 per cent by bringing the solution to a pH value of from 6.4 to 6.6 by means of $NaHCO_3$. The recovered rennin, however, contained considerable casein, which prevented the exact determination of the purity thereof, hence it was thought that another protein, of different solubilities than casein, might be used to advantage.

Dry, powdered, coagulated, acid treated egg white was then used as the absorbing medium and it was found that only from 17 per cent to 20 per cent of the rennin activity was absorbed by the egg white. It was known from work being carried on in the laboratory by Dr. T. L. McMeekin that hog's pepsin could be absorbed practically quantitatively on coagulated egg white. Studies were then carried out by the method of McMeekin[5] to determine if the absorbed material on the egg white possessed peptic activity. It was found that solutions of the calf rennin

[4] Fenger, F., *J. Am. Chem. Soc.*, 1923, xlv, 249-55.

[5] McMeekin, T. L., Doctorate Dissertation, U. of Chicago, 1925, "Studies on the Purification of Pepsin."

preparation when incubated with egg white in 0.3 per cent HCl for two hours at 40° C. showed digestive powers from 36 per cent to 37 per cent of that of 1:3,000 hog pepsin solution of the same per cent strength. These experiments also showed that this pepsin or pepsin-like material contained in the calf rennin preparation can be removed from its solution to the extent of 84 per cent to 92 per cent by absorption on egg white without appreciable removal or destruction of the rennin activity. The filtrates resulting from the egg white absorption process possessed only slight pepsin proteolytic power, but had from 87.5 per cent to 91 per cent of the milk curdling power of the original rennin solutions.

It was also observed that a mixture of hog preparation and calf preparation when incubated with egg white in 0.3 per cent HCl showed digestive powers equal to the sum of the two. The study shows, therefore, that practically complete separation of hog pepsin and calf rennin can be made by absorption on acid treated egg white and that the calf preparation contains two activities which can be separated from each other without destruction of either one, by absorbing the respective activities upon the proper proteins at properly controlled pH values.

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Effect of visual impulses on the posture of the head.

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It is a well known fact that the normal position of the head is maintained through impulses coming mainly from the otoliths (labyrinthine righting reflexes on the head). An animal with both labyrinths destroyed will not be able to preserve the normal posture of the head when held in the air in various abnormal body positions. According to Magnus,[1] the cat, the dog, and the monkey are exceptions to this rule, in that through visual im-

[1] Magnus, R., *Koerperstellung*, Berlin, 1924, p. 19, 224, 261, 267.

their specific activities, in their pH optima and ranges, and in heat sensitivity. All highly purified proteases which have been studied show specificity for certain peptide bonds and have little or no action on other peptide bonds. For example, trypsin will rapidly hydrolyze only those bonds linking the carboxyl group of the two basic amino acids, lysine and arginine, to the amino group of any other amino acid. Depending upon the individual enzymes present in commercial proteases they may range from quite specific to fairly general in their actions on proteinaceous substrates. Hence, in practical applications of proteases in food processing it is necessary to select the appropriate protease complex or combination of enzymes. Usually this can be determined only by empirical methods. With proper selection of enzymes, and with appropriate conditions of time, temperature and pH, either limited proteolysis or nearly complete hydrolysis of most proteins to amino acids can be brought about. For some uses the proteases of different origin are directly competitive, in other cases the special properties of one may give it advantage over others.

The important uses for proteases in the food industries are shown in Table 3.

TABLE 3
Uses of Proteases in Food Industries

Use	*Enzymes Employed*
Oriental condiments	Fungal
Dairy—cheese	Animal, bacterial, fungal
Baking—bread, crackers	Fungal, bacterial
Brewing—chillproofing	Plant, fungal, bacterial
Meat—tenderizing	Plant, fungal
Fish—solubles	Bacterial, plant

Fungal protease has been used for centuries in the Orient for production of soy sauce, tamari sauce, and miso, a breakfast food. The application is a rather complex art, although simple in operation. Proteolytic strains of *Aspergillus oryzae* are grown on rice or soybeans to produce a heavily sporulated koji which is added to vats containing roasted soybeans, cereal grains and salt brine. Over a period of many months proteolysis, solubilization and extraction occurs. The soy sauce is then recovered by straining and pressing out the liquid with hydraulic presses.

One of the oldest uses of an enzyme is that of rennet for coagulating milk in cheesemaking. Most proteolytic enzymes will clot milk, but on further contact will dissolve the curd by hydrolysis of the insoluble proteins to peptides and amino acids. Rennin is a unique enzyme which after clotting milk has no further rapid or extensive action on the curd. It seems quite clear, however, that rennin acts as a protease, splitting peptide bonds in one particular casein fraction. The mechanism of curd formation has been postulated to involve limited proteolysis of κ-casein by rennin forming soluble glycopeptide and para-κ-casein. The para-casein precipitates and leads to the precipitation of other casein fractions which have been exposed to calcium ions released as a result of the κ-casein hydrolysis. This is probably an oversimplification of the process and alternate theories have been proposed.

The production of cheese is still very much of an art. The procedures

vary depending upon the types of cheese, and somewhat from plant to plant for the same type of cheese. In general the milk is heat treated or pasteurized and than lactic starter culture is added. After a period of about an hour to allow the culture to develop, rennet is added and the curd allowed to form. The curd is then cut into small pieces, and the mass "cooked" at a temperature a little over 100°F. After the whey is drained off, the slabs of curd are piled and turned, and finally salted and pressed. The cheese is then aged or ripened by holding under controlled moderate temperature. During the ripening processes enzymatic reactions occur producing desirable and characteristic flavors in the cheese. Proteolytic activity during the ripening of cheeses is extensive. This proteolysis is in part due to enzymes produced by the lactic starter cultures and in part by continuing slow proteolytic action of the rennet-coagulating enzyme system.[200] The proteolytic breakdown in cheeses results in production of peptides and free amino acids.[110]

A search for substitutes for rennet for milk coagulation in cheesemaking has been underway for many years. In certain countries such as India ritual requirements make a "vegetable" rennet desirable. Also because the source of commercial rennet is the stomach mucosa of young calves, rennet has become increasingly expensive and in short supply. While there is a continuing increase in demand for cheese, and hence rennet, there is at the same time a decrease in the number of young calves being slaughtered and hence a shortage of rennet.

Milk can be clotted by most of the known proteases but with the exception of rennin and pepsin, proteolysis proceeds beyond the slight rise in non-protein nitrogen (NPN) observed with rennet. In an investigation with eight different proteases Tsugo and Yamauchi reported that rennin and pepsin produced the largest amount of curd after 15 minutes and no loss of yield after 60 minutes in contact with the whey, whereas all the other proteases produced less curd and continuing proteolysis led to further loss on holding in the whey.[173] Continued proteolysis also had a profound effect on the strength of the curd. Rennin gave the firmest curd while pepsin and protease from *Pseudomonas myxogenes* gave firmer curds than the other enzymes. Pepsin has in fact come into commercial use in combination with rennet for cheesemaking. There are differences in the behavior of the two enzymes, but pepsin when properly used has proved to be a satisfactory extender for rennet. The effect of rennet extract and commercial pepsin preparation has been followed in great detail throughout the entire production period of cheddar cheeses by Melachouris and Tuckey.[110] The amount of NPN formed during ripening of the cheeses was slightly greater with rennet.

Veringa reported earlier work in India where enzymes from *Ficus carica* (ficin) and from *Streblus asper*, a wild shrub, yielded curds only slightly less in weight than rennet curd. The curd made with ficin was slightly softer than the rennet curd and that with the *S. asper* enzyme was much softer.[186] Of all plant enzymes, ficin has been used more often than any of the other proteases for cheesemaking.

At present, research effort to obtain rennet substitutes has shifted to microbial sources. Milk-clotting enzymes for cheesemaking from the fungi *Mucor pusillus* and *Endothia parasitica* have been patented.[11,149] In the author's laboratory, a useful milk-coagulating enzyme has been developed from a strain

of *Bacillus cereus*. These new microbial enzyme preparations compare favorably with rennet in cheesemaking with respect to curd yield and firmness of curd. There have been reports, however, that some of them lead to more bitterness in young cheese. The bitterness may remain, increase or decrease during prolonged aging of the cheese, depending upon the enzyme preparation, conditions of use and of aging. It seems quite certain that suitable "microbial rennets" will soon become accepted in the cheese industry.

One of the largest uses for microbial proteases is in baking bread and crackers. The unique and variable properties of bakery doughs are due largely to the insoluble or gluten proteins of the flour. When wheat flour is mixed with water a complex dough system is formed. Because of the hydrated gluten the dough has extremely viscous flow properties and is therefore said to be extensible. However, it is at the same time elastic and resists deformation. With protease enzyme supplementation, gluten hydrolysis can be controlled to achieve better properties in the dough.

Cereals normally contain a very low level of protease. The protease content increases somewhat when wheat or barley is malted. However, the malts are still poor sources of protease, and cereal proteases provided by malt supplementation of flour have relatively little effect on dough properties.

The special viscoelastic properties of doughs are provided by the wheat gluten. The so-called strength of flours is determined by the quantity and quality of the gluten. Strong flours give doughs which have good tolerance to extensive mixing. The use of oxidizing agents—bromates, iodates, peroxides—as dough improvers has a strengthening effect while reducing agents have a weakening effect.[131] The effect of proteolytic enzymes resembles in some respects that of reducing agents. Mixing time and viscosity of doughs are reduced, and the doughs are more pliable and extensible. In practice, the combined use of oxidizing agents and of proteolytic enzymes has shown considerable benefits. In present day baking, flours and doughs which have not been treated with oxidizing dough improvers are very rare. Probably fungal protease derived from *Aspergillus oryzae* is added by the baker for more than half of the bread baked in the United States. Recently, the use of bromelain in bread baking has been permitted but data on its effectiveness have not been published.

A major benefit of adding fungal protease to sponge doughs is the marked reduction in mixing time which results.[32,140] The proper use of fungal protease reduces the mixing time by about one third, and improves the handling properties of the doughs. The protease increases extensibility of doughs, and is thus valuable in controlling the pliability of doughs, eliminating buckiness, and ensuring proper machinability. Improved loaf characteristics, including better volume, greater symmetry, and improved grain, texture and compressibility are also observed. These probably come as secondary benefits resulting from the greater extensibility and better machinability; the primary benefits are unquestionably the reduction in mixing time and better handling properties of the dough.[140]

The action of protease seems to be limited to the mixing and fermentation periods since the very low inactivation temperatures (50–60°C) preclude any appreciable action during baking.[114] The initial process in dough formation must be hydration of the flour particles to bring out the cohesive properties

THE POSSIBILITY FOR USE OF COW'S MILK CURDLED WITH PEGNIN IN INFANT NUTRITION

Ulrich Grüninger and Carl Hünermann

Introduction

In 1901, Siegert reported on his experiences with curdled cow's milk, and some authors, who will be mentioned in more detail later, retested the method, sometimes with good results and sometimes with poor results. However, nothing has appeared in the pediatric literature of the past 20 years concerning the use of cow's milk curdled with pegnin. Even in the textbooks, this is only mentioned infrequently (e.g. see Finkelstein: *Lehrbuch der Säuglingskrankheiten* [Textbook of Pediatric Diseases]).

If the large number of infant diets suggested up to the present time is reviewed, and the often complicated methods of preparing them are considered, and if on the other hand the possibility for successful use of pegnin-curdled cow's milk and especially the great ease of preparing this are considered, it seems unfortunate to us that pegnin milk has been relegated to oblivion in pediatrics.

Recently, one of us (Grüninger) described the use of curdled cow's milk in the medical treatment of pylorospasm. In addition, Rösch recently recommended pegnin milk in the diet of the adult. He reported on certain differences in the gastric digestion of milk on the one hand and of pegnin milk on the other hand, and pointed out the better tolerance of pegnin milk compared to ordinary milk in many gastrointestinal disorders.

In the following, the possibilities for use of sweetened, curdled cow's milk (whole milk and cow's milk mixtures) in infant dietetics will be discussed, based on all of the clinical material gathered within the

past one and one-half years. First, the experience of previous authors will be discussed briefly.

Siegert was the first, in 1901, to recommend cow's milk curdled with pegnin, sometimes as whole milk and sometimes diluted with 10% sugar solution, based on his experiences, particularly with patients from the polyclinic; he recommended this in cases of atrophy, vomiting, regurgitation, and even in some forms of dyspepsia. He administered it for only a few weeks; generally, non-curdled whole milk became well tolerated within four weeks.

In 1902, Langstein from Siegert's Clinic in Strassburg reported on 25 polyclinic cases which he treated with pegnin milk. These included 20 infants with chronic gastrointestinal disturbances. In almost all cases he observed a rapid regression of gastrointestinal symptoms, and he emphasized in particular that the vomiting disappeared rapidly, whereas a normal consistency of the stools was only gradually achieved. He also considered this diet advantageous in that it made it possible to administer a more concentrated nutritional form than is conventionally given to young infants.

Czerny in 1903 had Oppler check the results obtained by Siegert. He also primarily studied polyclinic patients. Out of 222 cases, 214 were observed in the polyclinic and only 8 in the clinic. All cases received exclusively undiluted, curdled cow's milk without sugar. The results were poor in all of the eight clinic cases, with one questionable exception. On the basis of the entire body of material, Oppler comes to the following conclusions: contraindications against curdled cow's milk:

1. First month of life;

2. Diseases resulting from excessive feeding of milk.

Indications:

1. Damage to health caused by feeding exclusively with meal or gruel;

2. Certain, not further specified acute gastrointestinal diseases;

3. Habitual vomiting.

In is advised that this type of nutrition be maintained for only a few weeks.

In 1904, Reinach reported on his experiences with 51 patients, some of whom were treated in the polyclinic. He obtained the following results: in chronic nutritional disturbances, he saw no advantage in pegnin milk compared with non-curdled cow's milk with respect to healing of the intestinal disturbance and improvement of the nutritional state. More favorable results were obtained in the healing of acute digestive disturbances. Reinach administered the curdled milk in diluted form, and only for a short time. Pegnin milk was always readily accepted. "In a large series of chronic and acute disturbances, dominated by vomiting and restlessness after drinking, the latter symptoms were always affected most favorably."

Since at that time Siegert believed chronic nutritional imbalance in the young infant was caused by an enzyme deficiency, in addition to curdling the milk with pegnin, he also recommended the administration of pancreas preparations (e.g., pancreon and pancreatin). Trichetta found elevated obstruction and retention of nitrogen in dogs given a mixture of pepsin, trypsin, and emulsin or a single one of these enzymes. As was previously mentioned, Czerny had already rejected enzyme therapy, based

on his clinical experiences and on the prevailing knowledge of infant digestive physiology at the time. On the basis of Trichetta's work, he had Philipps retest the circumstances in the infant again in metabolic experiments. Although the experimental period was very short, Philipps found no effect on nitrogen retention and no effect on the general health of four children given a mixture of pepsin, emulsin, and pancreon. Based on these experimental results, Czerny decisively rejected enzyme therapy and the use of pegnin milk in infant nutritional disturbances.

According to the current theories in infant nutritional physiology, no functional loss of enzymes exists, although the quantities of digested enzymes may be reduced. Thus we do not, for example, wish to revise Siegert's theory of enzyme therapy for nutritional disturbances as a simple replacement therapy for enzyme deficiency, but instead we wish to ask how the physiology of digestion in the infant explains the curdling process in the stomach, and whether it provides an explanation for the favorable effect of preliminary curdling of cow's milk, as that which takes place in pegnin milk. In the following, we will limit ourselves to a discussion of infant gastric digestion, as provided by Freudenberg in his book on "The Physiology and Pathology of Digestion in the Infant".

Curdling is an enzymatic process, which changes the caseinogen of milk by degradation in such a manner that substances readily precipitating with lime are formed, i.e., casein in the case of milk. In addition, milk protein is formed. Many proteolytic enzymes can curdle milk, e.g., pepsin. It is not necessary at this point for us to discuss whether the curdling enzyme and pepsin are identical, since we know that the optimum hydrogen ion concentration for the curdling process in the stomach is

different from that for pepsin digestion in the stomach. These two processes in the stomach can be strictly distinguished from one another. The optimum for curdling of cow's milk is pH 6.0-6.4; pepsin acts only below pH 4.0. The true acidity of the gastric juice at the peak of digestion is about pH 4.5-5.0 [Davidsohn, Hess, Salge, Scheer, cited from Meyer-Nassau]. Since pH 3.0 in the stomach is only attained after two to three hours, and during this time the milk has mostly left the stomach already, very little pepsin digestion takes place in the stomach. Only after the end of infancy does pepsin digestion in the stomach play a role (cited from Meyer-Nassau). Thus although peptic digestion in the infant stomach does not play a role, we know today that the most favorable hydrogen ion concentrations for the activity of gastric lipase exist in the stomach, namely at pH 4.0-5.0. Since during the preliminary curdling and collection of the curd, the milk fat is retained by refined casein curds and exposed to the action of gastric lipase in the form of a fine colloidal solution with large surface area, we view this as the exertion of a favorable influence on gastric lipolysis. Based on these facts about digestive physiology in the infant stomach, we can expect the following for cow's milk curdled with pegnin:

1. Preliminary curdling with pegnin save the stomach a significant work of curdling.

2. The fine colloidal solution produced with pegnin will promote gastric lipolysis.

3. The separation of solid and liquid parts accomplished in the case of pegnin curdling will offer the possibility for more rapid passage of the liquid portion of the milk through the pylorus. In this manner,

excessive distension of the stomach by a meal will be avoided (see the results in the case of vomiting and regurgitation of the infant after meals).

We will not consider for the present time whether curdling with pegnin may promote the further digestion beyond the pylorus.

Experimental Part

Using the experimental setup of Rosch--determinations of the nitrogen content in the aspirated gastric contents after identical times in the case of curdled and uncurdled cow's milk mixtures--we attempted experimentally to find an explanation for the favorable effect of curdling the milk with pegnin. Experiments performed in 10 children did not yield any clear results, so that the details will not be reported here. In order to resolve this question, another experimental arrangement will have to be developed first.

In addition, in order to demonstrate the frequently observed better growth of the infant on curdled than on uncurdled milk, we performed exchange experiments, by administering in 8 cases curdled and non-curdled milk alternately for several cycles. Nothing else was changed in the diet. Care was taken that these experimental periods were not disturbed by infections.

First experiment (see Fig. 1): case 20, E. L., 2 1/2 months old. dystrophia, duration of cycle: 4 days. Weight changes in grams: 1. curdled = 110 g, uncurdled = -40 g; 2. curdled = +120 g, uncurdled = -50 g.

Second experiment (see Fig. 2): Case 26, R. M., 2 months old, healthy intestine. Duration of cycles: 9 and 7 days. 1. curdled = + 240 g, noncurdled = + 80 g; 2. curdled = + 190 g, noncurdled = + 50 g.

Third experiment: Case 5. H. Kr., 3 months old: chronic dyspepsia

and dystrophia. Duration of cycles: 10 days. Curdled = + 340 g, uncurdled = - 60 g.

Fourth experiment: Case 23. H. Tr., 4 1/2 months old; atrophy and regurgitation. Duration of cycles: 4 days. 1. Curdled = + 160 g, uncurdled = + 30 g; 2. curdled = + 170 g, uncurdled = + 30 g.

Fifth experiment: case 31. K. Z., 2 months old; acute alimentary dyspepsia and pertussis. Duration of cycle: 5 days. Curdled =+120 g, noncurdled = - 100 g.

Sixth experiment: Case 62. D. B., 10 weeks old; pertussis and cystopyelitis. Duration of cycle: 6 days. Curdled = + 140 g, uncurdled = - 100 g.

Seventh experiment: Case 76. F. I., 4 1/2 months old; dystrophia. Duration of cycles: 4 days. 1. curdled = + 190 g, uncurdled = - 20 g; 2. curdled = + 180 g, uncurdled = + 40 g.

Eighth experiment (see Fig. 3). R. M., 4 months old: pylorospasm. Duration of cycle: 3 days. Curdled: + 90 g, uncurdled = - 110 g.

Note: As this article was going to press, an additional exchange experiment was carried out, which impressibly confirmed the results of the above experiments:

Ninth experiment: F. B., 3 months old: treated acute parenteral dyspepsia. Duration of cycle: 5 days. 1. curdled = + 160 g, uncurdled = + 30 g. Duration of cycle: 3 days. 1. curdled = + 190 g, uncurdled = + 60 g. 2. curdled = + 130 g.

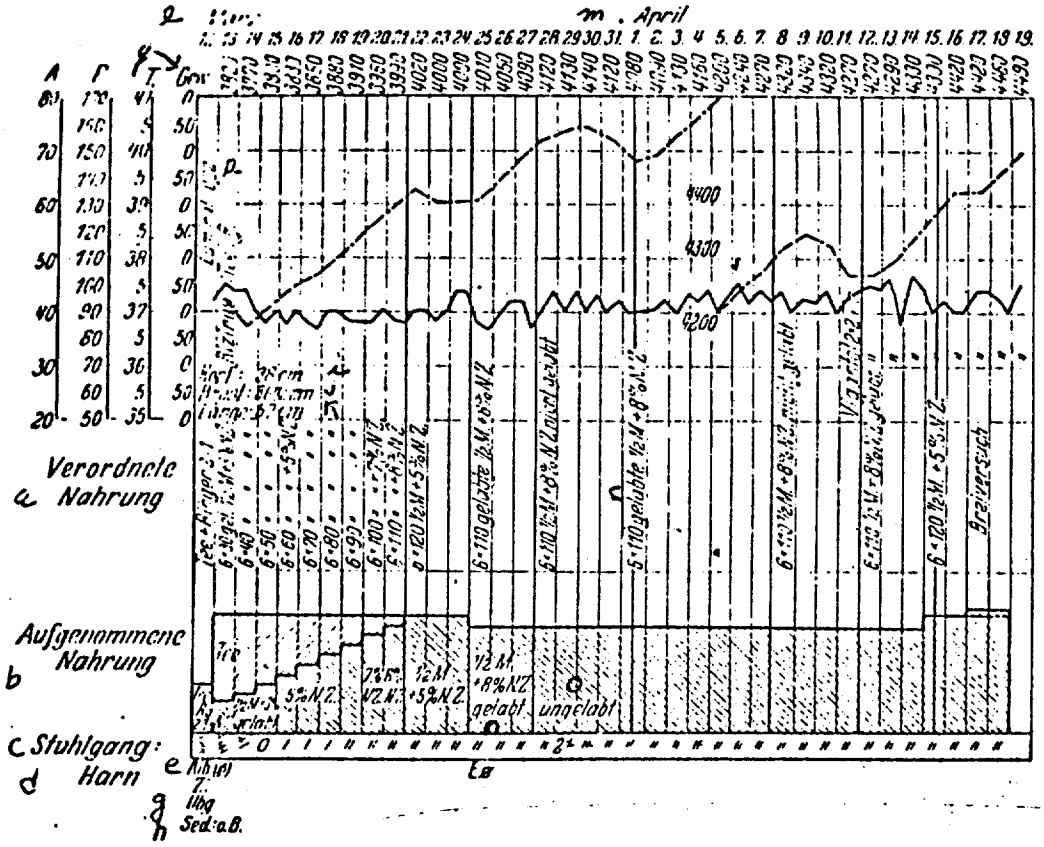

Fig. 1.

a. diet prescribed, b. diet consumed, c. bowel movements, d. urine, e. sugar, g. urobilinogen, h. sediment: no abnormalities, i. head, j. chest, k. length, l. March, m. April, n. curdled, o. not curdled, p. serial test, q. weight

[Note: Remainder of translation illegible--Tr.]

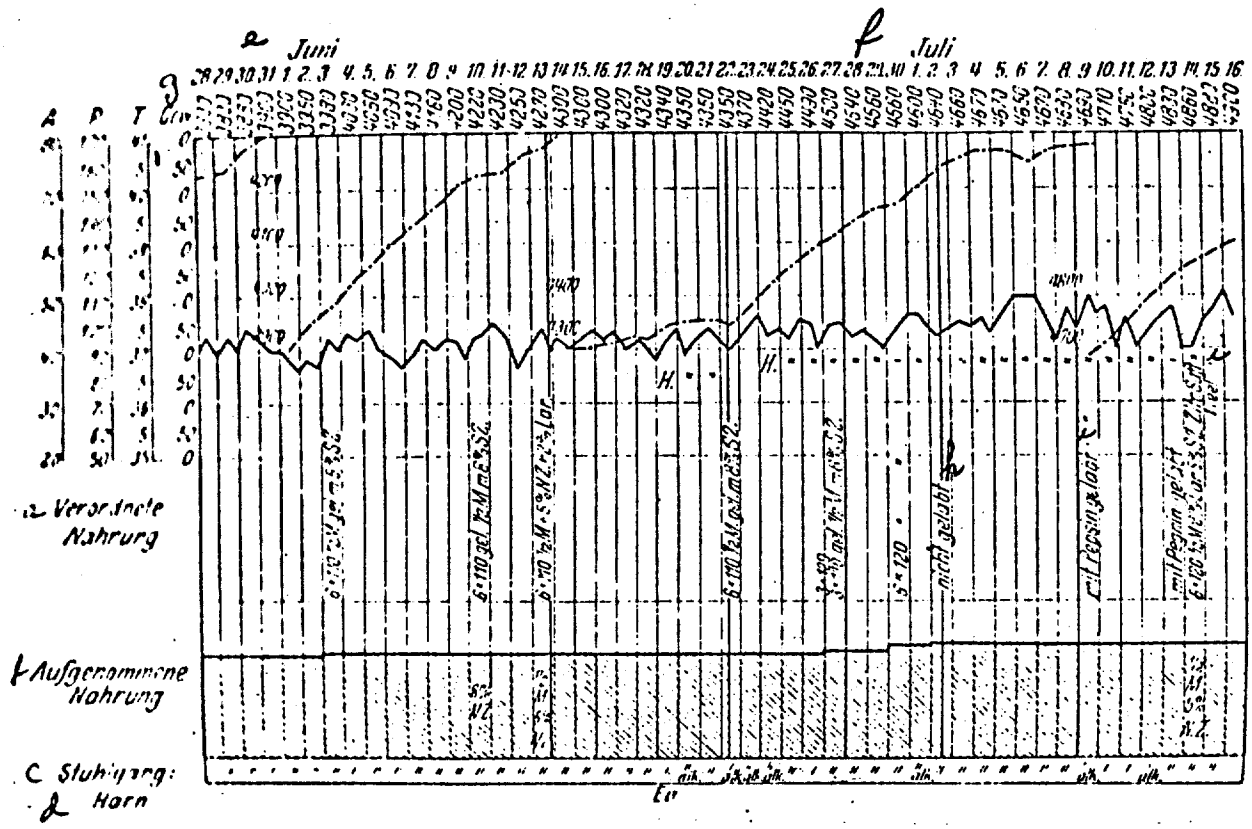

Fig. 2.

a. diet prescribed, b. diet consumed, c. bowel movements, d. urine, e. June, f. July, g. weight, h. not curdled, i. curdled with pepsin.

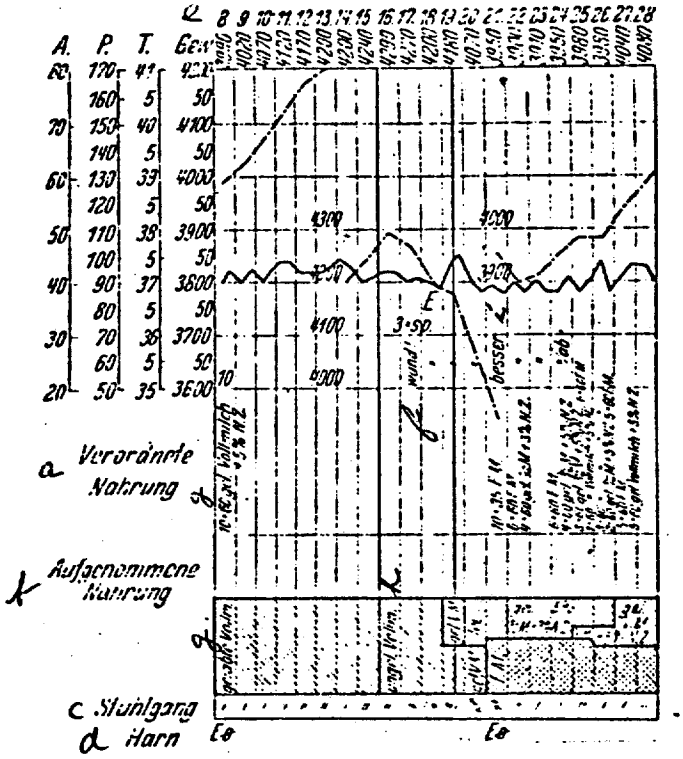

Fig. 3.

a. diet prescribed, b. diet consumed, c. bowel movements, d. urine, e. weight, f. chafed, g. curdled whole milk, h. noncurdled whole milk, i. better

[*Note: Remaining terms are illegible.--Tr.*]

These replacement experiments, some of which were performed on children whose intestines were still sensitive, uniformly resulted in good weight gains during the period of feeding the curdled milk, compared to a standstill or loss of weight in the period of feeding noncurdled milk. An additional impressive finding in these experiments, which we have often observed before, was that the children were reluctant to drink the noncurdled milk. Some of them developed diaper rash and bowel difficulties on this diet. When we again changed over to curdled milk, appetite and stool conditions improved again immediately. Fig. 3, which shows a section from a pylorospasm curve, clearly demonstrates the

extensive disturbances which may occur if the milk is not curdled, once the infant has become accustomed to curdled milk.

Since pegnin powder (according to von Dungern) contains salt as well as lactose, it is necessary to inquire, for example, whether the infant's weight increase might not be due to the salt content in pegnin. This presumption was not confirmed in an experiment in which a quantity of salt corresponding to that contained in a suspension with 1 g of pegnin was added.

Clinical Section

Originally, Siegert used the curdled cow's milk as unsweetened whole milk in some cases, and as 1/2 or 2/3 milk diluted with 10% sugar solution in others. Basically, we gave pegnin milk only in the form of whole milk with added sugar (Soxhlet's nutrient sugar was used). As a curdled whole milk, pegnin milk was only prescribed when a concentration diet was desired in an infant with a healthy intestine (e.g., in pertussis and its complications and in pylorospasm). In all other cases, we gave pegnin milk diluted as 1/2 or 2/3 milk. If the stool conditions permitted it, the children were also given cereal and vegetables appropriate for their ages, and adequate vitamin levels were provided.

Pegnin milk is very easy to prepare:

The boiled cow's milk, enriched with nutrient sugar (5-8-10-12%), is heated in a water bath to 40°, and pegnin is added (2 g pegnin = 1 measuring spoon enclosed in the package per 200 g). The mixture is shaken vigorously. Fine coagulation begins after 2 to 3 minutes. The curdled milk is permitted to cool before using. It must be shaken briefly before using. If pegnin milk is to be given in diluted form, the appropriate quantity of boiled water is added to the curdled, sugared whole milk, and the mixture is shaken well.

We also convinced ourselves that the customary 1/2 to 2/3 milk semolina gruel mixtures can be curdled, even with 2% larosan added, and the diet curdled in advance in this manner often produced the same success as was described previously for pegnin milk.

We now come to the question of deciding on the infants for whom the use of curdled cow's milk is indicated, how it is used, and the clinical results obtained.

The 130 infants to whom we gave the pegnin milk were divided into the following groups:

1. Dystrophy and atrophy
2. Pylorospasm
3. Febrile diseases without nutritional disorder
4. Various other diseases
5. Acute dyspepsia (parenteral and alimentary).

1. Dystrophy and Atrophy

These tests involved 41 dystrophic and 18 atrophic infants, four weeks to 12 months old. The average age was four to five months. Uniform, undisturbed well-being was attained in 36 of the dystrophic patients and 12 atrophic patients. Limited success was achieved in two of the dystrophic children, one of whom was removed from the clinic prematurely, and the other of whom had to be changed over to human milk because of severe cystitis and parenteral nutritional disorders. In two other cases in this group with chronic dyspepsia, the curdled cow's milk had to be stopped temporarily because of recurrent, severe infections. There were also two cases among the atrophic children in whom healing was unsatisfactory. The one child had severe purulent cystopyelitis and furunculosis, and the other died from bronchial pneumonia after a brief improvement.

We encountered one absolute failure among the cases of dystrophy. This was a three-month-old child (case 46) with congenital syphilis. In this instance, recovery was obtained only after specific treatment was carried out, as is generally true in the case of syphilis. There were four failures in the atrophy cases: in case 2, the curdled whole milk was administered improperly. This was one of the first cases, in which unsweetened, curdled whole milk was given exclusively to the 10-month-old infant. In the other three cases, the stool conditions became worse after addition of cereal simultaneously with the occurrence of infection, so that it was necessary to change over to protein milk.

In the great majority of cases, we saw only favorable results from the use of pegnin milk. The appetites of the infants were strikingly affected. It is fair to say that all of the children drank the curdled cow's milk more readily than any other diet. This was true to such an extent that we often had difficulties when attempting to change to milk which was not curdled. Renewed introduction of curdled milk restored the infant's eagerness to drink the milk in every case (according to the replacement experiments). When curdled cow's milk was given in addition to human milk, the children often preferred the curdled milk to such an extent that they refused to drink the human milk, and would only drink it after it was mixed with curdled cow's milk. The skin condition of the infants remained good; turgor and tone were good. Children with a tendency to intertrigo often underwent improvements in skin conditions, especially diaper rash, on the buttocks. The latter finding is attributable to the fact that when curdled milk was given, the stools were generally dry and formed, whereas they were previously loose and full of mucus.

Acid stools became alkaline.

As is mentioned above, pegnin milk was given with sweetening in undiluted as well as diluted form. As undiluted, sweetened whole milk, it was found useful as a simple, concentrated form of diet in the case of children with vomiting and whooping cough, thus in conditions where concentrated diet is used. There were 18 cases of this type in the group, and one ruminant. A fatty diet is not recommended for such children in particular here in Cologne.

Pegnin milk is generally given for several weeks, and in the case of children with whooping cough, even for several months.

We emphasize that we would like to see pegnin milk used primarily in cases of dystrophy and atrophy.

The mode of application of curdled cow's milk in atrophy and dystrophy will be illustrated on the basis of three examples.

First case. No. 14. E. Fl., 7 weeks old. Weight on admission 3030 g. Infant admitted to the clinic because of dystrophia on a mixed milk diet. Pharyngitis and bronchitis present on admission. Diet: beginning with human milk alone, on the third day 4 × 60 g human milk and 4 × 60 g curdled half milk with 5% nutrient sugar. On the sixth day, 4 × 75 g human milk and 4 × 75 g curdled half milk with 7% nutrient sugar. The stools remained firm. Very good, uniform weight increase. Released after 26 days with a weight gain of 1030 g.

Second case. No. 68, Kl. Gr., 3 months old. Weight on admission 3600 g. Child admitted to the clinic because of very severe whooping cough. 12 to 15 attacks of whooping per day. Subconjunctival hemorrhages. Treatment with lobeline-cardiazole, oxygen, and stronchin. Initially fed with human milk and curdled whole milk with 8% nutrient sugar. A

parenteral disturbance developed after 10 days. The whole milk was stopped immediately. Tea with Ringer solution was given, and 10 × 30 g human milk plus 2% plasmon started. After another 14 days, the stools were improved, but still numerous. The child had not yet gained weight. The whooping cough remained severe. Now the child was converted from 8 × 80 g of mother's milk, one bottle at a time, to 8 × 80 g curdled half milk with 5% nutrient sugar. After conversion was complete, the child gained regularly and well. The stools rapidly became more infrequent, and remained firm.

Third case. No. 107. I. B., 5 months old. Weight on admission 2450 g. Typical ruminant. Admitted to the clinic in very poor general condition. Subnormal temperature and melena on admission. First given human milk in small quantities. Beginning on the sixth day, 8 × 50 g of mother's milk and 1 × 40 g of curdled whole milk with 8% nutrient sugar. At the same time, Siegert's balloon catheter was used for 12 days. On the 11th day, 3 × 90 g human milk and 3 × 85 g curdled whole milk with 8% nutrient sugar. Despite nasal pharyngitis and bronchitis, undisturbed, sharp weight increase with good bowel condition. The human milk was further replaced gradually by curdled whole milk up to 6 × 100 g curdled whole milk and 8% nutrient sugar. After 6 weeks, released from the clinic with a weight increase of 1400 g. At first, failure to thrive at home on gruel and vegetables. Only after the milk had been curdled with pegnin again did the child begin to gain weight again.

II. Pylorospasm

Based on the reports "Curdled cow's Milk and Pylorospasm" we will briefly report that of 14 instances pylorospasm, only 2 were treated with

curdled whole milk (with and without sugar). The other 12 received as follows:

4 in the first week
3 in the second week
2 in the third week
2 in the sixth week
1 in the seventh week

the treatment with sugar-enriched, curdled whole milk added to human milk as a concentrated secondary food. In the meantime, an additional, severe case of pylorospasm, who received curdled whole milk with 5 and 8% nutrient sugar beginning in the third week, was discharged after healing. Another infant, in whom whole milk with 5% nutrient sugar was added to human milk as early as the second week of treatment, is still in the hospital and is thriving well. The details of treatment of pylorospasm with curdled whole milk will not be discussed again here. We refer to the report cited. Here, the simple form of concentrated diet will only be mentioned once again; this also has a favorable effect on vomiting.

III. Febrile Diseases without Nutritional Disturbances

This group involves 8 infants, one to nine months old. All of these were seriously ill, and we found it necessary to administer the most concentrated, well tolerated diet possible, which would be readily taken. Good results were attained in seven cases. One baby died of bronchial pneumonia. We obtained very good results with the use of curdled milk in pertussis-bronchial pneumonia, although intubation was more often necessary. The duration of use was several weeks.

IV. Various other Diseases without Nutritional Disturbances

In order to gain an idea concerning the acceptability of pegnin milk in children without nutritional disturbances, we gave pegnin milk to 25 babies, one to eight months old. These included infants who weighed 2000 g (premature birth). Good tolerance and thriving were apparent in all of these cases.

V. Acute Dyspepsia (alimentary and parenteral)

A special chapter remains for describing the use of pegnin milk in acute nutritional disturbances in infancy.

After we saw in the dystrophic infants that loose, mucus stools often became dry and formed after the use of curdled milk was initiated, it seemed obvious to try pegnin milk in the cases of acute nutritional disturbance where a lack of success was apparent in treatment with protein or better milk, even with large amounts of carbohydrates added, with an absence of improvements in the stool by the third or fourth week. Thus 20 infants with acute parenteral dyspepsia, from 6 weeks to 18 months old, and 2 with alimentary dyspepsia, 2 and 8 1/2 months old, were treated in this manner. The latter two cases were milder: dyspepsia after a fatty diet, and one drug-induced dyspepsia. The disturbances were eliminated by immediately changing to curdled half-milk. In the 20 infants with acute parenteral dyspepsia, we saw success in 18 cases: the stools became firm, and the children began to thrive. It was once again striking to note how eagerly the children drank the curdled half milk. In 5 cases, the curdled half milk was given as a therapeutic diet immediately upon development of the disturbance, four times successfully and once unsuccessfully. In two cases, curdled half milk was successfully used following

the conventional rice gruel diet. We saw a second failure in a 3 1/2 month old infant, in whom we gave the pegnin half milk as a tolerance test after healing was completed. Otherwise, the cases were usually transferred to pegnin milk only when no improvement occurred after 3 to 4 weeks of the conventional treatment with buttermilk.

Satisfactory results are seen in mild parenteral dyspepsia. Success can be anticipated if a 12-to-24 hour interval with only tea is allowed after the disturbance begins, then curdled half milk is given, at reduced levels at first.

However, we are far from trying to recommend curdled half milk in general as a therapeutic diet in acute dyspepsia. Our cases were well selected and clinically very well known. Otherwise, we have the same good clinical impression of the use of pegnin milk in this group of infants as in dystrophic and atrophic individuals.

In this group also, the use of pegnin milk will be illustrated on the basis of three examples.

First case. No. 24. A. R., one month old. Weight on admission, 3150 g. Admitted to the clinic in moderate nutritional state because of acute parenteral dyspepsia. Pharyngitis and bronchitis present on admission. Treated with human milk and buttermilk. Only buttermilk given from the tenth day on. At first, good weight gain. During the fourth week, the child developed diarrhea again during febrile bronchitis. Weight gain arrested, diaper rash developing. After a brief interval on tea, changed to 6 × 100 g curdled half milk with 5% nutrient sugar. The stools immediately became less frequent, and the diaper rash improved. After another two days, the sugar added was increased to 8%. The stools were normal after 8 days. Good weight gain.

Second case. No. 80, A. Gl., 2 months old. Weight on admission 4200 g. After correction of a mild weaning dyspepsia with buttermilk and after changing to larosan-1/2 milk, the infant developed an influenza infection during the fourth week, with numerous thin-mucus stools and severe diaper rash on the buttocks. After a 12 hour interval of tea administration, changed to 6 × 100 g curdled half milk with 5% nutrient sugar. The stools were immediately improved, the intertrigo healed. Undisturbed course of healing with further increase in pegnin milk given.

Third case. No. 89. C. R., 3 months old. Weight on admission 5300 g. Admitted to the clinic because of pertussis and dyspepsia. Heavy child with severe bronchitis. Had three to four thin mucus stools per day and severe diaper rash. Typical protein milk diet. The very severe whooping cough was treated with lobeline-cardiazole, stronchin and transpulmin. After 3 weeks, no weight increase, and a further increase in number of thin, pulpy stools. The protein milk was drunk reluctantly. Therefore, the child was changed to a diet of curdled half milk with 5% nutrient sugar. A little gruel had been mixed in before this. The stools became perfect immediately, and the child gained weight, despite severe whooping cough.

Summary

1. The dietary experiments of previous authors using pegnin milk, mostly carried out on polyclinic patients, are discussed.

2. An attempt is made to explain the favorable effect of preliminary curdling of cow's milk, based on current knowledge on the physiology of digestion in the infant.

3. Based on replacement experiments with curdled and noncurdled cow's milk, the favorable effect of pegnin milk on the well being of the infant

demonstrated.

4. The technique for use of pegnin milk in infants is described.

5. Based on experiences with a group of 130 clinically observed infants, the following indications were found for the use of pegnin milk in infants:

a) dystrophy and atrophy,

b) loss of appetite in febrile infants without nutritional disturbances,

c) as concentration diet in pylorospasm and other disease pictures accompanied by vomiting (pertussis, regurgitation, and rumination).

d) certain forms of milk parenteral dyspepsia.

6. The ease and low cost of pegnin milk production makes it preferred by experienced persons for the indications mentioned above, over the other types of diets which might come into question.

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Rennet

(Aus der Universitäts-Kinderklinik Köln. — Direktor: Geh.-Rat Prof. Dr. *Siegert*.)

Über die Anwendungsmöglichkeiten mit Pegnin gelabter Kuhmilch in der Ernährungstherapie des Säuglings.

Von

Ulrich Grüninger und Carl Hünermann,
Assistenten der Klinik.

Mit 3 Textabbildungen.

(*Eingegangen am 2. Mai 1931.*)

Einleitung.

Nachdem *Siegert* im Jahre 1901 über seine Erfahrungen mit gelabter Kuhmilch berichtet hatte, und einige Autoren, auf die weiter unten noch näher eingegangen werden soll, teils mit günstigem, teils mit ungünstigem Erfolge das Verfahren nachgeprüft hatten, ist in der pädiatrischen Literatur der letzten 20 Jahre von der mit Pegnin gelabten Kuhmilch nicht mehr die Rede. Auch in den Lehrbüchern wird ihrer nur noch ganz vereinzelt Erwähnung getan (z. B. bei *Finkelstein*: Lehrbuch der Säuglingskrankheiten).

Wenn man die große Zahl der bisher angegebenen Säuglingsnahrungen übersieht und ihre zum Teil komplizierte Herstellungsart berücksichtigt, und wenn man andererseits sich von der erfolgreichen Anwendungsmöglichkeit mit Pegnin vorgelabter Kuhmilch überzeugt hat und besonders die einfache Art ihrer Herstellung bedenkt, so scheint uns die *Pegninmilch* zu Unrecht in der Pädiatrie in Vergessenheit geraten zu sein.

Vor kurzem hat nun der eine von uns (*Grüninger*) die Anwendung der gelabten Kuhmilch bei der internen Behandlung des Pylorospasmus beschrieben. Ferner hat neuerdings *Rösch* in der Diätetik des Erwachsenen die Pegninmilch empfohlen. Er berichtete dabei über gewisse Unterschiede in der Magenverdauung der Milch einerseits und der Pegninmilch andererseits und wies auf die bessere Verträglichkeit der Pegninmilch gegenüber der einfachen Milch bei vielen Krankheitszuständen des Magens und Darms hin.

Im folgenden sollen an Hand des gesamten *klinischen* Materials der letzten 1½ Jahre die Verwendungsmöglichkeiten gezuckerter gelabter Kuhmilch (Vollmilch und Kuhmilchmischungen) in der Säuglingsdiätetik erörtert werden. Zunächst seien die Versuche früherer Autoren kurz mitgeteilt.

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Siegert hat zuerst im Jahre 1901 auf Grund seiner Erfahrung an vorwiegend poliklinischem Material mit Pegnin gelabte Kuhmilch teils als Vollmilch teils mit 10%-Zuckerlösung verdünnt empfohlen: bei Atrophie, bei Erbrechen und Spucken und sogar bei bestimmten Formen von Dyspepsie. Er gab sie nur einige Wochen, meist wurde schon nach 4 Wochen ungelabte Vollmilch gut vertragen.

Langstein berichtete 1902 aus *Siegerts* Ambulatorium in Straßburg über 25 poliklinische Fälle, die er mit Pegninmilch behandelte. 20 Säuglinge hatten chronische Magen- und Darmstörungen. Er sah fast in allen Fällen einen schnellen Rückgang der gastro-intestinalen Symptome, insbesondere betonte er, daß das Erbrechen schnell wiche, während die Stühle erst allmählich die normale Beschaffenheit erhielten. Ferner sah er einen Vorteil dieser Ernährung auch darin, daß sie gestattete, die Nahrung konzentrierter als üblich auch dem jüngsten Säugling zu geben.

Czerny ließ 1903 durch *Oppler* die günstigen Erfahrungen *Siegerts* nachprüfen. Es handelte sich auch bei ihm hauptsächlich um *poliklinisches* Beobachtungsmaterial. Von 222 Fällen wurden 214 poliklinisch und nur 8 klinisch beobachtet. Alle Fälle bekamen ausschließlich unverdünnte gelabte Kuhmilch ohne Zucker. Bei den 8 klinischen Fällen waren die Resultate mit einer fraglichen Ausnahme durchweg schlecht. Auf Grund des gesamten Materials kommt *Oppler* zu folgendem Schluß:

Gegenindikationen gegen gelabte Kuhmilch:

1. Erster Lebensmonat;
2. Erkrankungen bei Überernährung mit Milch.

Indikationen:

1. Schädigungen des Organismus durch ausschließliche Mehl- oder Schleimernährung;
2. Gewisse noch nicht näher abgegrenzte akute Magen- und Darmerkrankungen;
3. Habituelles Erbrechen.

Es wird empfohlen, diese Ernährungsart nur wenige Wochen beizubehalten.

Reinach berichtete 1904 über seine Versuche an 51 zum Teil poliklinisch behandelten Kindern. Er hatte folgende Ergebnisse: bei chronischen Ernährungsstörungen sah er bezüglich Ausheilung der Darmstörung und Hebung des Ernährungszustandes keinen Vorzug der Pegninmilch gegenüber ungelabter Kuhmilch. Günstiger gestaltete sich die Ausheilung akuter Verdauungsstörungen. *Reinach* gab die gelabte Kuhmilch verdünnt und nur kürzere Zeit. Die Pegninmilch wurde durchweg gern genommen. „In einer größeren Reihe von chronischen und akuten Störungen mit vorwiegendem Erbrechen und Unruhe nach

dem Trinken wurden letztere Symptome in der günstigsten Weise durchweg beeinflußt."

Da *Siegert* damals die chronischen Ernährungsstörungen der Säuglinge durch einen Fermentmangel verursacht sich vorstellte, empfahl er außer der Labung der Milch mit Pegnin die Verabreichung von Pankreaspräparaten (z. B. Pankreon und Pankreatin). *Trichetta* hatte beim Hunde die Resorption und Retention von Stickstoff unter dem Einflusse eines Gemisches von Pepsin, Trypsin und Emulsin oder eines einzelnen von diesen Fermenten erhöht gefunden. Wie schon erwähnt, hatte *Czerny* bereits nach seinen klinischen Erfahrungen und nach den damaligen Kenntnissen der Verdauungsphysiologie des Säuglings die Fermenttherapie abgelehnt. Auf Grund der Arbeit von *Trichetta* ließ er die Verhältnisse beim Säugling nochmals durch *Philipps* im Stoffwechselversuch nachprüfen. *Philipps* fand bei 4 Säuglingen — bei denen zwar die Versuchsperioden sehr kurz gewählt waren — durch Zufuhr eines Pepsin-, Emulsin- und Pankreongemisches keine Wirkung auf Resorption von Stickstoff, Fetten und Kohlehydraten, keine Wirkung auf die Retention von Stickstoff und keine Wirkung auf das Gedeihen der Kinder. Nach diesen Versuchsergebnissen lehnte *Czerny* endgültig die Fermenttherapie und die Pegninmilch bei Ernährungsstörungen der Säuglinge ab.

Auch nach dem heutigen Stande der Ernährungsphysiologie des Säuglings liegt bei den Ernährungsstörungen kein Funktionsausfall der Fermente vor, wenn auch die Mengen der Verdauungsfermente verringert sein können. Wir wollen also nicht etwa erneut die Vorstellung *Siegerts* von der Fermenttherapie der Ernährungsstörungen als einer einfachen Substitutionstherapie eines Fermentmangels aufnehmen, sondern wir wollen uns fragen, wie erklärt uns heute die Physiologie der Verdauung im Säuglingsalter den Labungsvorgang im Magen und gibt sie uns eine Erklärung für die günstige Wirkung einer Vorlabung der Kuhmilch, wie sie bei der Pegninmilch stattfindet. Wir halten uns im folgenden an die Darstellung der Magenverdauung des Säuglings, wie sie *Freudenberg* in seinem Buch über „die Physiologie und Pathologie der Verdauung im Säuglingsalter" gegeben hat.

Die Labung ist ein fermentativer Vorgang, der das Caseinogen der Milchen durch Abbau derart verändert, daß mit Kalk leichter fällbare Substanzen entstehen, in der Milch das Casein. Nebenbei wird Molkeneiweiß gebildet. Labungsvermögen haben zahlreiche proteolytische Fermente, z. B. auch das Pepsin. Auf die Streitfrage, ob Labferment und Pepsin identisch sind oder nicht, einzugehen, erübrigt sich deshalb, weil wir wissen, daß sicher das Optimum der Wasserstoffionenkonzentration für den Labungsvorgang im Magen ein anderes ist als das für die Pepsinverdauung im Magen. Diese beiden Vorgänge im Magen

sind voneinander streng zu scheiden. Das Optimum der Labfällung für Kuhmilch liegt bei p_H 6,0—6,4, das Pepsin wirkt erst unter p_H 4,0 ein. Die wahre Acidität des Magensaftes beträgt auf der Höhe der Verdauung etwa p_H 4,5—5,0 (*Davidsohn*, *Hess*, *Salge*, *Scheer* zit. nach *Meyer-Nassau*). Da p_H 3,0 im Magen erst nach 2—3 Stunden erreicht wird, und in dieser Zeit die Milch zum größten Teil bereits den Magen verlassen hat, findet im Magen nur eine ganz geringe Pepsinverdauung statt. Erst am Ende des Säuglingsalters spielt die Pepsinverdauung im Magen eine Rolle (zit. nach *Meyer-Nassau*). Während also die peptische Verdauung im Magen des Säuglings keine Rolle spielt, wissen wir heute, daß für die Tätigkeit der Magenlipase die günstigen Verhältnisse der Wasserstoffionenkonzentration im Magen gegeben sind, nämlich bei p_H 4,0—5,0. Da bei der Labgerinnung und der Vorlabung das Milchfett von den feinen Caseingerinnseln zurückgehalten und als feinkolloidale Lösung mit großer Oberfläche der Magenlipase angeboten wird, sehen wir darin einen günstigen Einfluß auf die Magenlipolyse. Aus diesen Tatsachen der Verdauungsphysiologie im Magen des Säuglings können wir für die mit Pegnin vorgelabte Kuhmilch folgendes erwarten:

1. Die Vorlabung mit Pegnin erspart dem Magen die wichtige Arbeit des Labens.

2. Die mit Pegnin hergestellte feinkolloidale Lösung wird die Magenlipolyse begünstigen.

3. Die bei der Pegninlabung erfolgte Trennung von festen und flüssigen Teilen wird die Möglichkeit eines rascheren Verlassens des flüssigen Teiles der Milch durch den Pylorus geben. Einer Überfüllung des Magens durch eine Mahlzeit wird so vorgebeugt werden (vgl. die Erfolge bei Spucken und Erbrechen des Säuglings nach der Mahlzeit).

4. Ob jenseits des Pylorus die Labung mit Pegnin die weiteren Verdauungsvorgänge begünstigen kann, sei dahingestellt.

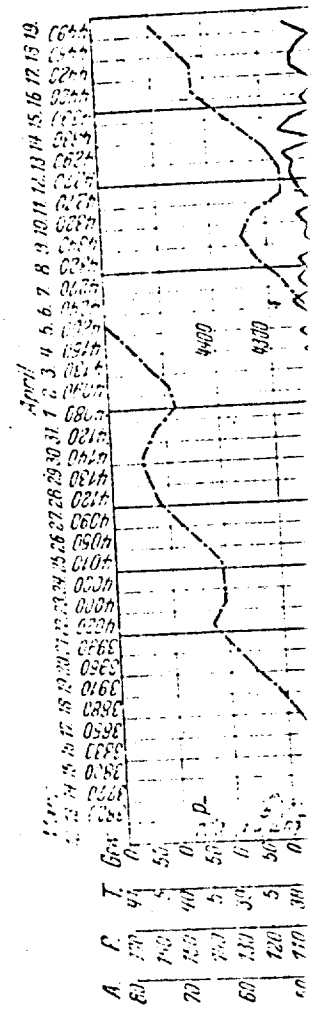
Experimenteller Teil.

Unter Anwendung der Versuchsanordnung von *Rösch* — Bestimmung des Stickstoffgehaltes im ausgeheberten Mageninhalt nach gleichen Zeiten bei gelabten und ungelabten Kuhmilchmischungen — haben wir versucht, experimentell eine Erklärung für die günstige Wirkung der Vorlabung der Milch mit Pegnin zu finden. Bei 10 Kindern angestellte Versuche ergaben kein eindeutiges Resultat, so daß wir auf die Wiedergabe der Versuchsergebnisse verzichten. Zur Klärung dieser Frage muß zunächst eine andere Versuchsanordnung ausgearbeitet werden.

Weiterhin haben wir, um das von uns oft beobachtete bessere Gedeihen der Säuglinge bei gelabter statt bei ungelabter Milch zu zeigen, *Austauschversuche* in der Art gemacht, daß wir bei 8 Fällen in mehreren

mit Pegnin gelabter Kuhmilch

Perioden abwechselnd gelab
nichts an der Nahrung geä
Versuchsperioden nicht du



1. *Versuch* (vgl. Abb
Periodendauer: 4 Tage.
ungelabt — — 40 g. 2. ge

2. *Versuch* (vgl. Abb.
dauer: 9 bzw. 7 Tage. 1. g
ungelabt — — 50 g.

3. *Versuch*: Fall 5. H.
Periodendauer: 10 Tage.

Perioden abwechselnd gelabte und ungelabte Milch gaben. Sonst wurde nichts an der Nahrung geändert. Es wurde darauf geachtet, daß die Versuchsperioden nicht durch Infekte gestört wurden.

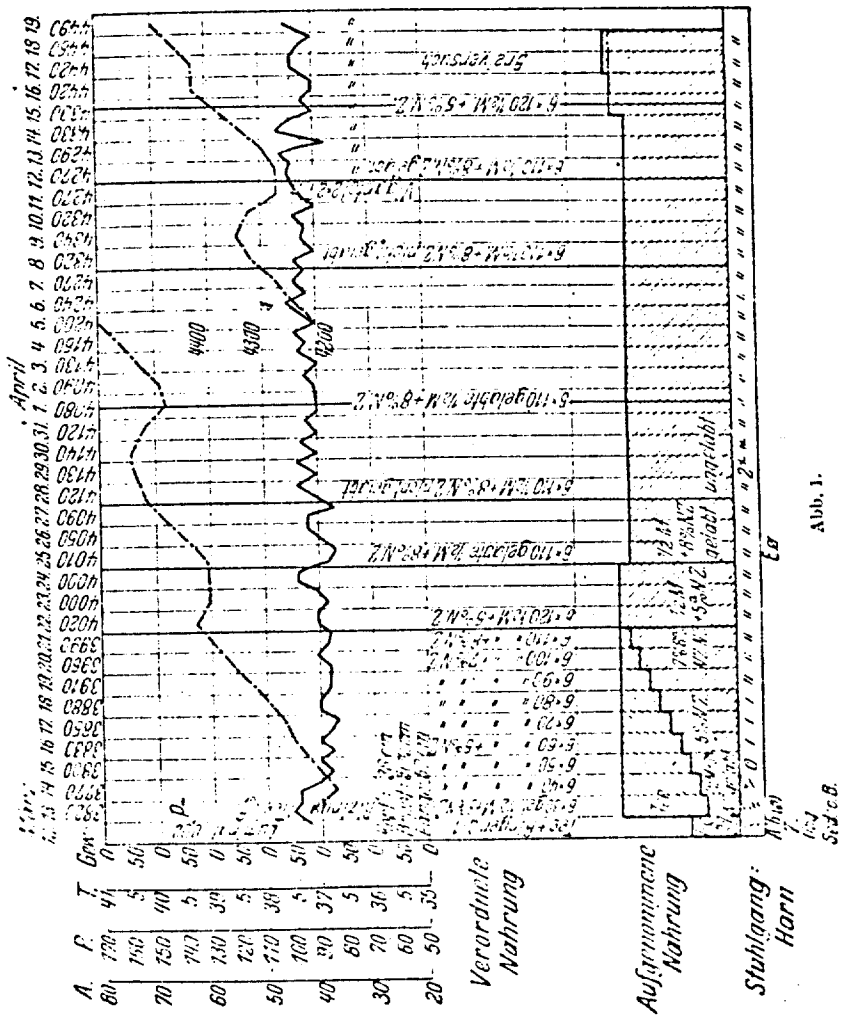


Abb. 1.

1. *Versuch* (vgl. Abb. 1): Fall 20, E. L., 2½ Monate alt. Dystrophie. Periodendauer: 4 Tage. Gewichtsverhältnisse in Grämm: 1. gelabt = + 110 g, ungelabt = — 40 g. 2. gelabt = + 120 g, ungelabt = — 50 g.

2. *Versuch* (vgl. Abb. 2): Fall 26. R. M., 2 Monate alt, darmgesund. Periodendauer: 9 bzw. 7 Tage. 1. gelabt = + 240 g, ungelabt = + 80 g. 2. gelabt = + 190 g, ungelabt = + 50 g.

3. *Versuch:* Fall 5. H. Kr., 3 Monate alt; chronische Dyspepsie und Dystrophie. Periodendauer: 10 Tage. Gelabt = + 340 g, ungelabt = — 60 g.

4. *Versuch:* Fall 23. H. Tr., 4½ Monate alt; Atrophie und Spucker. Periodendauer: 4 Tage. 1. gelabt = — 160 g, ungelabt = + 30 g. 2. gelabt = + 170 g, ungelabt = + 30 g.

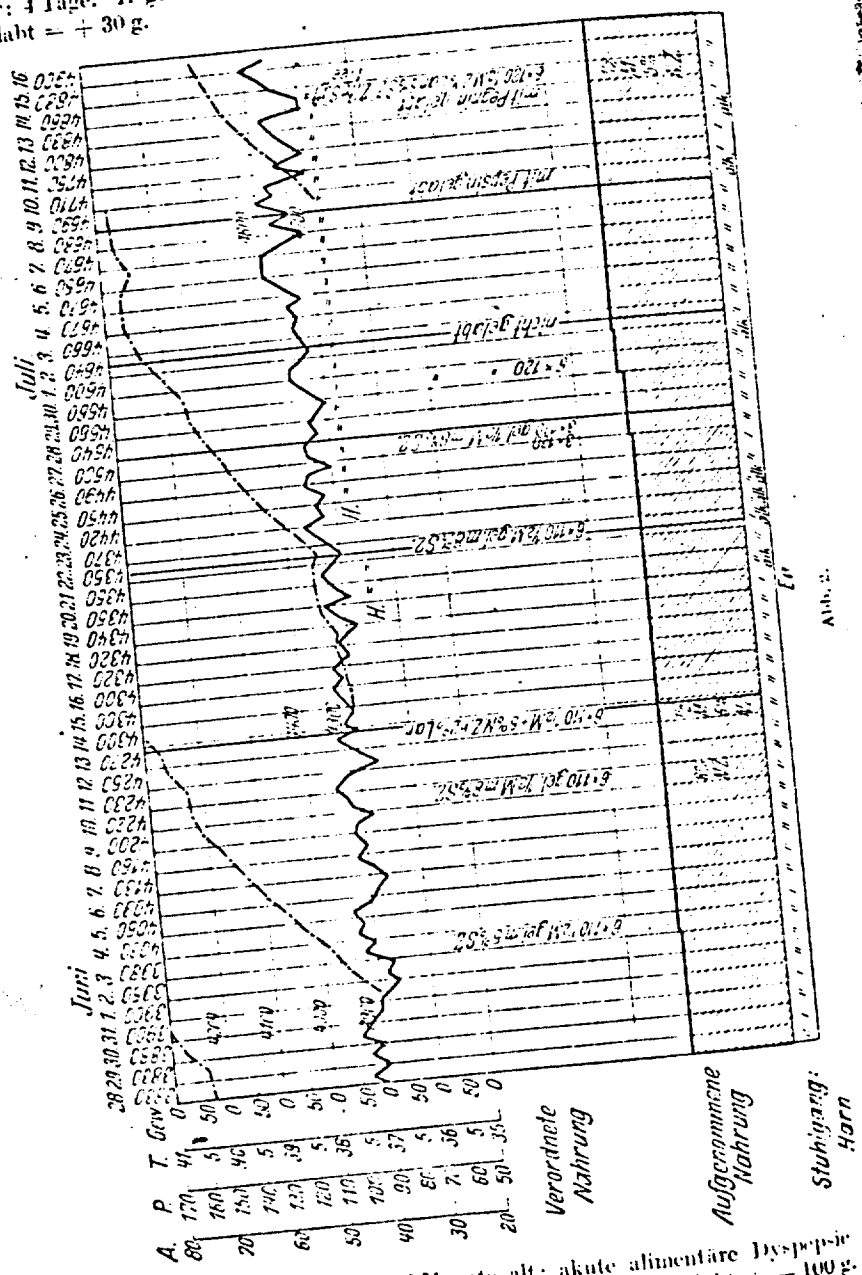


Abb. 2.

5. *Versuch:* Fall 31. K. Z., 2 Monate alt; akute alimentäre Dyspepsie und Pertussis. Periodendauer: 5 Tage. Gelabt = + 120 g, ungelabt = — 100 g.

mit Pegnin gelabter Kuhmil

6. *Versuch:* Fall 62. D.
Periodendauer: 6 Tage. Gela
7. *Versuch:* Fall 76. F. L.
1. gelabt = + 190 g, ungelabt
8. *Versuch* (vgl. Abb. 3)
dauer: 3 Tage. Gelabt = +

Anmerkung: Während d
ausgeführt, der eindrucksvo

9. *Versuch:* F. B., 3 M
Periodendauer: 5 Tage. 1.
dauer: 3 Tage. 1. Gelabt =

A.	P.	T.	Gew.
80	170	41	4200
	160	5	50
70	150	40	4100
	140	5	50
60	130	39	4000
	120	5	50
50	110	38	3900
	100	5	50
40	90	37	3800
	80	5	50
30	70	36	3700
	60	5	50
20	50	35	3600

Verordnete Nahrung

Aufgenommene Nahrung

Stuhlgang Harn

Diese Austauschv
Kindern angestellt v
nahme in der gelab
stand oder einer G
Ferner war bei die
häufig beobachtet h
tranken. Manche w
Gegen wir wieder
wieder Appetit un

6. Versuch: Fall 62. D. B., 10 Wochen alt: Pertussis und Cystopyelitis. Periodendauer: 6 Tage. Gelabt = + 140 g, ungelabt = — 100 g.

7. Versuch: Fall 76. F. L., 4½ Monate alt: Dystrophie. Periodendauer: 4 Tage. 1. gelabt = + 190 g, ungelabt = — 20 g. 2. gelabt = + 180 g, ungelabt = + 40 g.

8. Versuch (vgl. Abb. 3). R. M., 4 Monate alt: Pylorospasmus. Periodendauer: 3 Tage. Gelabt = + 90 g, ungelabt = — 110 g.

Anmerkung: Während der Drucklegung wurde ein weiterer Austauschversuch ausgeführt, der eindrucksvoll das Ergebnis der obigen Versuche bestätigt:

9. Versuch: F. B., 3 Monate alt; reparierte akute-parenterale Dyspepsie. Periodendauer: 5 Tage. 1. Gelabt = + 150 g, ungelabt = + 30 g. Periodendauer: 3 Tage. 1. Gelabt = + 190 g, ungelabt = + 60 g. 2. Gelabt = + 130 g.

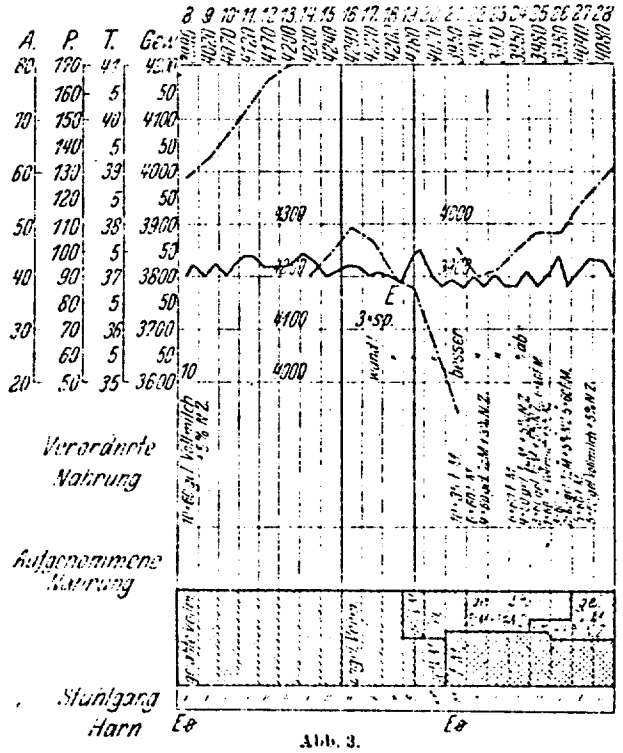


Abb. 3.

Diese Austauschversuche, die zum Teil bei noch darmempfindlichen Kindern angestellt wurden, ergaben einheitlich eine gute Gewichtszunahme in der gelabten Milchperiode gegenüber einem Gewichtsstillstand oder einer Gewichtsabnahme in der ungelabten Milchperiode. Ferner war bei diesen Versuchen eindrucksvoll, was wir auch sonst häufig beobachtet haben, daß die Kinder die ungelabte Milch ungern tranken. Manche wurden dabei wund und bekamen schlechtere Stühle. Gingen wir wieder auf gelabte Milch über, so besserten sich sogleich wieder Appetit und Stuhlverhältnisse. Abb. 3, die einen Abschnitt

aus einer Pylorospasmuskurve wiedergibt, demonstriert deutlich, wie stark die Störung nach Nichtlaben der Milch sein kann, wenn der Säugling an die gelabte Milch gewöhnt war.

Da das Pegninpulver (nach v. *Dungern*) neben Milchzucker Kochsalz enthält, mußte daran gedacht werden, ob die Gewichtszunahme der Säuglinge etwa auf den Kochsalzgehalt des Pegnins zurückzuführen war. In einem Kontrollversuch durch Zusatz einer entsprechenden Menge Kochsalz, wie sie die Aufschwemmung von 1 g Pegnin ergeben hatte, wurde diese Vermutung nicht bestätigt.

Klinischer Teil.

Siegert hat ursprünglich die gelabte Kuhmilch teils als ungezuckerte Vollmilch, teils als mit 10%-Zuckerlösung verdünnte $^1/_2$ und $^2/_3$ Milch verwendet. Wir haben grundsätzlich die Pegninmilch auch als Vollmilch nur mit Zuckerzusätzen (verwendet wurde Soxhlets Nährzucker) gegeben. Als gelabte *Vollmilch* wurde die Pegninmilch nur dann verordnet, wenn wir bei einem *darmgesunden* Säugling eine konzentrierte Ernährung anstrebten (z. B. bei Pertussis und seinen Komplikationen und bei Pylorospasmus). In allen übrigen Fällen gaben wir die Pegninmilch verdünnt als $^1/_2$ oder $^2/_3$ Milch. Wenn die Stuhlverhältnisse es gestatteten, wurde den Kindern dem Alter entsprechend Brei und Gemüse beigefüttert, wie auch für genügende Vitaminzufuhr gesorgt wurde.

Die *Herstellung der Pegninmilch* ist sehr einfach:

Die abgekochte mit Nährzucker (5—8—10—12%) angereicherte Kuhmilch wird im Wasserbad auf 40° erwärmt und Pegnin zugesetzt (auf 200 g Milch 2 g Pegnin = einem Maßlöffel der Packung). Sodann wird kräftig umgeschüttelt. Nach 2—3 Minuten tritt eine feine Gerinnung ein. Bis zum Gebrauch wird die gelabte Milch kühl gestellt. Vor Gebrauch muß sie kurz umgeschüttelt werden. Will man die Pegninmilch verdünnt geben, so setzt man zu der gelabten gezuckerten Vollmilch die entsprechenden Mengen abgekochten Wassers hinzu und schüttelt gut um.

Wir haben uns auch davon überzeugt, daß man auch die gewöhnlichen $^1/_2$ bzw. $^2/_3$ Milch-Grießschleimmischungen selbst mit 2% Larosanzusatz laben kann, und daß die so vorgelabte Nahrung oft dieselben Erfolge wie die vorher beschriebene Pegninmilch zeigte.

Wir kommen nun zu der Frage, bei welchen Säuglingen ist die Anwendung gelabter Kuhmilch angezeigt, wie wird sie verwendet, und welche Wirkung sahen wir klinisch davon.

Die 130 Säuglinge, bei denen wir die Pegninmilch angewendet haben, wurden in folgende Gruppen eingeteilt:

1. Dystrophie und Atrophie.
2. Pylorospasmus.

3. Fieberhafte Erkrankungen ohne Ernährungsstörungen.
4. Verschiedene andere Erkrankungen.
5. Akute Dyspepsie (parenterale und alimentäre).

I. Dystrophie und Atrophie.

Es handelt sich um 41 dystrophische und 18 atrophische Säuglinge im Alter von 4 Wochen bis 12 Monaten. Das Durchschnittsalter betrug 4—5 Monate. Ein gleichmäßiges, ungestörtes Gedeihen wurde 36mal bei den Dystrophen und 12mal bei den Atrophen erreicht. Unter den dystrophischen Kindern kann bei 2 nur von einem bedingten Erfolg gesprochen werden, da das eine vorzeitig aus der Klinik abgeholt wurde und das andere wegen schwerer Cystitis und parenteraler Ernährungsstörung auf Frauenmilch umgesetzt werden mußte. Bei 2 weiteren Fällen, dieser Gruppe mit chronischer Dyspepsie mußte wegen wiederholten schweren Infekten die gelabte Kuhmilch vorübergehend abgesetzt werden. Auch unter den atrophischen Kindern finden sich 2 Fälle, deren Gedeihen nicht genügte. Das eine Kind hatte eine schwere eitrige Cystopyelitis und Furunkulose, das andere kam nach vorübergehendem Gedeihen an Bronchopneumonie ad exitum. Einen glatten Mißerfolg hatten wir unter den Dystrophen einmal. Es handelte sich um ein Kind von 3 Monaten (Fall 46) mit Lues congenita. Bei diesem Säugling wurde ein Gedeihen erst nach Einleiten einer spezifischen Kur erreicht, wie man dies bei Lues häufiger sehen kann. Bei den Atrophen sahen wir 4 Mißerfolge: Bei Fall 2 wurde die gelabte Vollmilch fehlerhaft angewandt. Es war einer unserer ersten Fälle, bei dem noch ungezuckerte gelabte Vollmilch und diese schon im Alter von 10 Wochen ausschließlich gegeben wurde. Bei den 3 übrigen Fällen trat nach Breizulage und gleichzeitig auftretendem Infekte eine Verschlechterung der Stühle auf, so daß auf Eiweißmilch übergegangen werden mußte.

In der überwiegenden Mehrzahl der Fälle sahen wir von der Verwendung der Pegninmilch nur günstiges. Ganz auffallend wurde der Appetit der Säuglinge beeinflußt. Man kann sagen, alle Kinder tranken die gelabte Kuhmilch lieber als jede andere Nahrung. Es ging dies soweit, daß wir oft Schwierigkeiten hatten, auf ungelabte Milch überzugehen. Durch erneutes Laben wurde jedes Mal die Trinklust wieder angeregt (vgl. Austauschversuche). Wenn neben Frauenmilch gelabte Kuhmilch beigegeben wurde, so bevorzugten die Kinder häufig die gelabte Milch so sehr, daß sie die Frauenmilch verweigerten und sie erst nach Mischen mit gelabter Kuhmilch trinken wollten. Die Haut der Säuglinge blieb gesund, Turgor und Tonus wurden gut. Kinder, die zu Intertrigo neigten, zeigten oft eine Besserung der Hauterscheinungen, insbesondere des Wundseins am Gesäß. Letzteres ist darauf zurückzuführen, daß die Stühle bei gelabter Milch meist trocken und geformt werden, wenn sie vorher zerfahren und schleimig waren. Saure Stühle wurden alkalisch.

Wie schon oben erwähnt, wurde die Pegninmilch in unverdünnter wie verdünnter Form gezuckert gegeben. Als unverdünnte gezuckerte Vollmilch hat sie sich als einfache konzentrierte Nahrung bewährt bei Spinckern und bei Keuchhustenkindern, also bei Zuständen, wo wir eine konzentrierte Nahrung brauchen. In dieser Gruppe finden sich 18 derartige Fälle und ein Ruminant. Gerade bei diesen Säuglingen wurde hier in Köln Fettnahrung nicht vertragen.

Die Pegninmilch wurde gewöhnlich mehrere Wochen, bei Keuchhustensäuglingen sogar mehrere Monate gegeben.

Wir betonen, daß wir hauptsächlich bei Dystrophen und Atrophen die Pegninmilch angewendet wissen wollen.

An 3 Beispielen möge die Art der Anwendung gelabter Kuhmilch beim Atrophen und Dystrophen gezeigt werden.

1. Fall. Nr. 17. E. Fl., 7 Wochen alt. Aufnahmegewicht 3030 g. Säugling kommt wegen Dystrophie bei Zwiemilchernährung in die Klinik. Bei der Aufnahme besteht Pharyngitis und Bronchitis. Ernährung: Beginn mit Frauenmilch allein, am 3. Tage 4 × 60 g Frauenmilch und 4 × 60 g gelabte ½-Milch mit 5% Nährzucker. Am 6. Tage 4 × 75 g Frauenmilch und 4 × 75 g gelabte ½-Milch mit 7% Nährzucker. Die Stühle sind immer gebunden. Sehr gute gleichmäßige Gewichtszunahme. Nach 26 Tagen Entlassung mit einer Zunahme von 1030 g.

2. Fall. Nr. 68. Kl. Gr., 3 Monate alt. Aufnahmegewicht 3600 g. Kind kommt wegen sehr schweren Keuchhustens in die Klinik. Täglich 12—15 Hustenanfälle. Subconjunctivale Blutungen. Behandlung mit Lobelin-Cardiazol, Sauerstoff und Stronchin. Ernährung zuerst Frauenmilch und gelabte Vollmilch mit 8% Nährzucker. Dabei bekommt das Kind nach 10 Tagen parenteral eine Störung. Die Vollmilch wird sofort abgesetzt. Es wird Tee mit Ringer gegeben und mit 10 × 30 g Frauenmilch und 2% Plasmon begonnen. Nach weiteren 14 Tagen sind die Stühle zwar gebessert, aber noch zahlreich. Kind hat noch nicht zugenommen. Der Keuchhusten ist unverändert schwer. Jetzt wird das Kind von 8 × 80 g Frauenmilch flaschenweise auf 8 × 80 g gelabte ½-Milch mit 5% Nährzucker umgesetzt. Nach völligem Umsetzen nimmt das Kind regelmäßig und gut zu. Die Stühle werden rasch spärlicher und bleiben gebunden.

3. Fall. Nr. 107. I. B., 5 Monate alt. Aufnahmegewicht 2450 g. Typischer Ruminant. Kommt in sehr schlechtem Allgemeinzustand in die Klinik. Hat bei der Aufnahme Untertemperatur und erbricht schwarz. Erhält zunächst Frauenmilch in kleinen Mahlzeiten. Vom 6. Tage an 8 × 50 g Frauenmilch und 1 × 40 g gelabte Vollmilch mit 8% Nährzucker. Gleichzeitig wird die *Siegert*sche Ballonsonde 12 Tage lang angewendet. Am 11. Tage 3 × 90 g Frauenmilch und 3 × 85 g gelabte Vollmilch mit 8% Nährzucker. Trotz Nasopharyngitis und Bronchitis ungestörte steile Gewichtszunahme bei guten Stühlen. Allmählich wird die Frauenmilch weiter durch gelabte Vollmilch ersetzt bis 6 × 100 g gelabte Vollmilch und 8% Nährzucker. Nach 6 Wochen Entlassung aus der Klinik mit einer Gewichtszunahme von 1400 g. Zu Hause bei ungelabter ¾-Milch, Brei und Gemüse zunächst kein Gedeihen. Erst nachdem erneut die Milch mit Pegnin gelabt wurde nimmt das Kind gleichmäßig zu.

II. *Pylorospasmus.*

Aus der Arbeit „Gelabte Kuhmilch und Pylorospasmus" sei kurz berichtet, daß von 14 Säuglingen mit Pylorospasmus 2 nur mit gelabter

Vollmilch (mit und ohne Zucker) behandelt wurden. Die 12 übrigen Fälle erhielten

4	in der	1.	Woche
3	„ „	2.	„
2	„ „	3.	„
2	„ „	6.	„
1	„ „	7.	„

der Behandlung mit Zucker angereicherte gelabte Vollmilch zur Frauenmilch als konzentrierte Beinahrung hinzu. Inzwischen wurde ein weiterer schwerer Fall von Pylorospasmus, der von der 3. Woche an gelabte Vollmilch mit 5 und 8% Nährzucker erhielt, geheilt entlassen. Ein anderer Säugling, bei dem schon in der 2. Woche der Behandlung gelabte Vollmilch mit 5% Nährzucker zur Frauenmilch beigegeben wurde, befindet sich noch bei gutem Gedeihen in klinischer Behandlung. Auf die Einzelheiten der Behandlung des Pylorospasmus mit gelabter Vollmilch möchten wir an dieser Stelle nicht nochmals eingehen. Wir verweisen auf die zitierte Arbeit. Hier sei nur erneut die einfache Form konzentrierter Ernährung hervorgehoben, die auch das Erbrechen günstig beeinflußt.

III. *Fieberhafte Erkrankungen ohne Ernährungsstörungen.*

Bei dieser Gruppe handelt es sich um 8 Säuglinge im Alter von 1—9 Monaten. Sie waren durchweg schwer krank und es kam uns darauf an, eine möglichst konzentrierte und gut verträgliche Nahrung zu geben, die gern genommen wurde. Wir hatten 7mal einen guten Erfolg zu verzeichnen. Ein Säugling starb an Bronchopneumonie. Sehr günstiges sahen wir mit der Anwendung der gelabten Milch bei Pertussis-Bronchopneumonien, wenn auch häufiger sondiert werden mußte. Die Dauer der Anwendung betrug mehrere Wochen.

IV. *Verschiedene andere Erkrankungen ohne Ernährungsstörungen.*

Um über die Verträglichkeit der Pegninmilch bei ernährungsgesunden Säuglingen ein Urteil zu gewinnen, haben wir bei 25 Säuglingen im Alter von 1—8 Monaten die Pegninmilch angewandt. Es befanden sich darunter Säuglinge von nur 2000 g (Frühgeburten). Auch bei diesen Kindern sahen wir in allen Fällen gute Verträglichkeit und gutes Gedeihen.

V. *Akute Dyspepsie (alimentäre und parenterale).*

Ein besonderes Kapitel bleibt die Verwendung der Pegninmilch bei den akuten Ernährungsstörungen der Säuglinge.

Nachdem wir bei den dystrophischen Säuglingen gesehen hatten, daß oft zerfahrene und auch schleimige Stühle nach Übergang auf gelabte Milch trocken und geformt wurden, lag es nahe, bei akuten Ernährungsstörungen, die in der Reparation mit Eiweißmilch oder Buttermilch selbst bei höheren Kohlehydratzusätzen in der 3. bis 4. Woche noch keine wesentliche Besserung der Stühle und mangelndes

Gedeihen zeigten, die Pegninmilch zu versuchen. So wurden 20 Säuglinge mit akuter parenteraler Dyspepsie im Alter von 6 Wochen bis 18 Monate und 2 mit alimentärer im Alter von 2 und $8\frac{1}{2}$ Monaten so behandelt. Bei den 2 letzteren handelte es sich um leichtere Störungen eine Dyspepsie nach Fettnahrung und eine medikamentöse. Durch sofortigen Übergang auf gelabte Halbmilch konnten die Störungen behoben werden. Bei den 20 Säuglingen mit akuter parenteraler Dyspepsie hatten wir 18mal Erfolg: die Stühle wurden gebunden, und die Kinder gediehen. Auffallend war wieder, wie gern die gelabte Halbmilch getrunken wurde. 5mal wurde die gelabte Halbmilch sofort bei Auftreten der Störung als Heilnahrung gegeben. 1mal erfolgreich, 1mal ohne Erfolg. 2mal wurde nach der üblichen Reisschleimdiät auf gelabte Halbmilch mit Erfolg übergegangen. Einen 2. Mißerfolg sahen wir bei einem $3\frac{1}{2}$ Monate alten Säugling, bei dem wir bei noch nicht beendigter Reparation die Pegninhalbmilch als Verträglichkeitsprobe gaben. Sonst wurden die Fälle gewöhnlich erst dann auf Pegninmilch umgesetzt, wenn nach der üblichen Behandlung mit Eiweiß oder Buttermilch nach 3—4 Wochen keine Besserung eintrat.

Einen zufriedenstellenden Erfolg sieht man bei leichten parenteralen Dyspepsien. Wenn hier sofort nach Auftreten der Störung mit einer 12–24stündigen Teepause und Übergang auf gelabte Halbmilch zunächst in reduzierter Menge eingegriffen wird, kann man Erfolg erwarten.

Wir sind aber weit davon entfernt, die gelabte Halbmilch im allgemeinen als Heilnahrung der akuten Dyspepsien empfehlen zu wollen. Unsere Fälle waren wohl ausgesucht und klinisch genau bekannt. Sonst hatten wir bei der Anwendung der Pegninmilch bei dieser Gruppe von Säuglingen denselben günstigen klinischen Eindruck wie bei den dystrophischen und atrophischen.

Auch bei dieser Gruppe soll die Anwendung der Pegninmilch an 3 Beispielen gezeigt werden.

1. Fall. Nr. 24. A. R., 1 Monat alt. Aufnahmegewicht 3150 g. Kommt wegen akuter parenteraler Dyspepsie in mäßigem Ernährungszustand in die Klinik. Bei der Aufnahme besteht Pharyngitis und Bronchitis. Reparation mit 1 Liter milch und Buttermilch. Erhält vom 10. Tage an nur Buttermilch. Zunächst gute Gewichtszunahme. In der 4. Woche der Reparation bekommt Kind bei einer fieberhaften Bronchitis erneut Durchfall. Bleibt im Gewicht stehen und wird wund. Nach kurzer Teepause Übergang auf 6 Tage gelabte ½ Milch mit 5% Nahrzucker. Die Stühle werden sofort seltener, das Wundsein wird besser. Nach weiteren 2 Tagen wird der Zuckerzusatz auf 8% erhöht. Die Stühle sind nach 8 Tagen normal. Kind nimmt gut zu.

2. Fall. Nr. 80. A. Gl., 2 Monate alt. Aufnahmegewicht 4200 g. Nach Reparation einer leichten Abstillungsdyspepsie mit Buttermilch und nach Umsetzen auf Larosan-½-Milch bekommt der Säugling in der 4. Woche einen akuten Infekt mit zahlreichen dünnschleimigen Stühlen und starkem Wundsein am Gesäß. Nach 12stündiger Teepause wird zunächst auf 6 Tage gelabte ½ Milch mit 5% Nahrzucker übergegangen. Dabei werden die Stühle sofort [illegible], der Infekt trägt heilt. Ungestörter Heilungsverlauf bei weiterer Steigerung der Pegninmilch

2. Fall. Nr. 89. C. R., 3 Monate alt. Aufnahmegewicht 5300 g. Kommt wegen Pertussis und Dyspepsie in die Klinik. Kräftiges Kind mit starker Bronchitis. Hat 3—4mal täglich dünn-schleimigen Stuhl und ist sehr wund. Typische Eiweißmilchkur. Der sehr schwere Keuchhusten wird mit Lobelin-Cardiazol, Stronchin und Transpulmin behandelt. Nach 3 Wochen noch keine Gewichtszunahme bei noch vermehrten dünn-breiigen Stühlen. Die Eiweißmilch wird schlecht getrunken. Deshalb wird flaschenweise auf gelabte ¾-Milch mit 5% Nahrzucker übergegangen. Etwas Brei war schon vorher beigefüttert worden. Die Stühle werden jetzt sofort einwandfrei und das Kind nimmt trotz schwerem Keuchhusten gut zu.

Zusammenfassung.

1. Die vorwiegend an *poliklinischem* Material ausgeführten Ernährungsversuche früherer Autoren mit Pegninmilch werden besprochen.

2. Es wird versucht, die günstige Wirkung der Vorlabung der Kuhmilch auf Grund der heutigen Kenntnisse der Physiologie der Verdauung im Säuglingsalter zu erklären.

3. An Austauschversuchen mit gelabter und ungelabter Kuhmilch wird die günstige Wirkung der Pegninmilch auf das Gedeihen der Säuglinge gezeigt.

4. Die Technik der Anwendung der Pegninmilch im Säuglingsalter wird beschrieben.

5. Auf Grund der Erfahrungen an einem Material von 130 *klinisch* beobachteten Säuglingen ergeben sich folgende Indikationen für die Anwendung der Pegninmilch im Säuglingsalter:

a) Dystrophie und Atrophie.

b) Appetitlosigkeit bei fiebernden Säuglingen ohne Ernährungsstörungen.

c) als konzentrierte Nahrung beim Pylorospasmus und anderen mit Erbrechen verbundenen Krankheitsbildern (Pertussis, Spucken und Rumination).

d) gewisse Formen leichter parenteraler Dyspepsien.

6. Die Einfachheit und Billigkeit der Herstellung der Pegninmilch wird den Erfahrenen bei den oben angegebenen Indikationen ihr vor anderen dabei in Frage kommenden Ernährungsarten den Vorzug geben lassen.

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STUDIES ON THE ACTION OF RENNET ON CASEIN AND THE NATURE OF CLOTTING

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Summary

Casein and paracasein preparations have been compared by various methods, including titration techniques, solubility, and ultraviolet spectra of the proteins and their azo-derivatives. It is suggested that rennet may release imidazole or phenolic groups from intramolecular linkage. The liberation of proteoses was confirmed but their presence does not appear to be essential for clotting. Support is given to the view that α-casein is more affected by rennet than are other casein components. The stability of the clots and the effect of electrolytes on the clotting process have been examined.

I. Introduction

The mechanism of milk clotting can be resolved into two stages: the conversion of casein into paracasein under the influence of the rennet, and the coagulation of the paracasein in the presence of calcium ions. Linderstrøm-Lang (1929), who first demonstrated the inhomogeneity of casein, advanced the hypothesis that rennet destroys, or inactivates, a "protective colloid" component, thus rendering unstable the calcium caseinates corresponding to the other components, and leading to precipitation or clotting. Alexander (1912) had previously put forward a somewhat similar hypothesis, in which lactalbumin was considered as the protective colloid. The work of Cherbuliez and Meyer (1933), Cherbuliez and Jeannerat (1939), and Cherbuliez and Baudet (1950) has indicated that α-casein may be regarded as the protective colloid in Linderstrøm-Lang's theory and that the calcium salts of β- and γ-casein, which are themselves insoluble, are kept in solution by the soluble calcium salt of α-casein with which they are associated. Nitschmann and Lehmann (1947) have provided further support for Linderstrøm-Lang's hypothesis by showing that calcium ions cannot differentiate between casein and paracasein particles in a mixed solution.

Hammarsten (1872) postulated that the action of rennet on casein resulted in an insoluble curd and a soluble whey-protein (usually known in the literature as "Hammarsten's proteose"). Cherbuliez and Baudet (1950) regarded their soluble δ-fraction as this proteose. Earlier Bosworth (1913) had claimed that the appearance of the proteose was not due to the action of the rennet and in view of some confusion in the literature clarification of the following points seemed desirable: whether the enzyme is really responsible for the liberation

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of the proteose, and, if this is so, whether the reaction or the proteose itself are essential for the clotting process.

The assumption sometimes made that the rupture of peptide bonds is the basic feature of the casein-paracasein transformation probably arises from the observation that various proteolytic enzymes can coagulate milk, but it has actually received little experimental support. Wright (1924) had concluded from the similarity of the racemization curves for casein and paracasein that rennet does not cause proteolytic cleavage of the protein, and Holter (1932) was able to obtain, without apparent increase in amino or carboxyl groups, paracasein which could be completely precipitated by calcium ions. More recently Nitschmann and Varin (1951) have concluded that clotting can take place with negligible proteolysis, amounting to the rupture of only one peptide link in 10,000. Berridge (1945) demonstrated that commercial rennet is a mixture of pepsin and rennin, and that rennin itself also exhibits considerable proteolytic activity. The relevant question therefore is not whether proteolysis of the casein occurs, but whether it is essential for coagulation. Nitschmann and Lehmann (1947) suggested that the essential chemical change in the transformation of casein into paracasein might not lie in a primary valence reaction, but that the rennet might act as a specific "denaturase" for one of the casein components.

Some clarification of these questions was sought in the experiments described below.

II. Materials and Methods

(a) *Protein Preparations*

Casein and α-casein for the experiment described in Section III(*e*) were prepared from the same batch of milk according to the methods of Warner (1944), except for minor deviations.

Casein and paracasein for comparative studies were prepared under strictly parallel conditions, except for the rennet treatment, as described by Higgins and Hayes (1951). Two pairs of preparations were used.

(b) *Acid Hydrolysates*

These were prepared by refluxing 0.5 g air-dry protein with 10 ml 6N hydrochloric acid for 20 hr.

(c) *Rennet*

A commercial preparation (New Zealand cheese rennet) was used.

(d) *Electrometric Titration*

The pH values were measured by means of a glass electrode system. The proteins (2.0 g) were dissolved in 40 ml N/20 sodium hydroxide and titrated with 1N hydrochloric acid. Acid groups were calculated from the equivalent between pH 6 and pH 2. Controls were run in the absence of protein and appropriate corrections applied.

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(*e*) *Formol Titration*

The method of Kekwick and Cannan (1936) was adopted. The formaldehyde was neutralized, the protein solutions (1 g in 50 ml N/50 sodium hydroxide) were adjusted to pH 9, and were titrated back to this point after the addition of the formaldehyde.

(*f*) *Titration in Organic Solvents*

The methods of Linderstrøm-Lang and Jacobsen (1940) were applied, the titrations in ethanol being carried out with N/10 sodium hydroxide in 90 per cent. ethanol to the thymolphthalein end-point (normally pH 9.3-10.5), and those in acetone with N/10 hydrochloric acid in 90 per cent. ethanol to the naphthyl red end-point (normally pH 3.7-5.0). The original solutions were made up by dissolving 0.2 g protein, with agitation, in 20 ml N/20 sodium hydroxide and adjusting to pH 6, following Lea and Hannan (1950), with 1N hydrochloric acid (*c.* 0.8 ml). Titrations were carried out after the addition of (i) 15 ml ethanol plus 2.5 ml thymolphthalein (0.5 per cent.), (ii) 50 ml ethanol plus 2 ml thymolphthalein, (iii) 10 ml acetone plus 0.5 ml naphthyl red (0.01 per cent.), (iv) 30 ml acetone plus 1 ml naphthyl red. Controls were run on volumes of water equal to those of the protein solutions, adjusted to pH 6 and with the solvent and indicator added.

(*g*) *Total Nitrogen*

The Kjeldahl method was used, with parallel duplicates of each preparation.

(*h*) *Amino Nitrogen*

Determinations were made by the Van Slyke nitrous acid procedure in the manometric apparatus (Peters and Van Slyke 1932).

(*i*) *Optical Absorption*

Extinctions and absorption spectra were measured with a Unicam S.P. 500 spectrophotometer, using 1-cm cells and water as the reference medium. No transverse measurements of the scattered radiation were made, but high optical clarity was assumed in dilute protein solutions which had been filtered through sintered glass.

(*j*) *Solubility*

The proteins (0.05 g) were equilibrated at 25°C with 5 ml solvent and centrifuged to remove the undissolved protein. Extinctions were measured at 277 mμ on the supernatants.

(*k*) *Coupling with Diazotized Sulphanilic Acid*

Coupling was carried out as described previously (Higgins and Fraser 1952). The proteins (0.01 g) were first dissolved in *c.* 5 ml N/20 sodium hydroxide for 5 min and the solutions acidified with 1N hydrochloric acid.

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III. Procedure and Results

(a) *Proteolysis. Acid and Basic Groups*

The various titration techniques were used to determine the state of the acid and basic groups in casein and paracasein preparations. As these procedures involved dissolving the protein in sodium hydroxide, an examination was made of the rate of hydrolysis of casein under the conditions used. The rate of rupture of peptide bonds was found, by titrations in alcoholic sodium hydroxide, to be about 2 per hour per 10^5 g casein in the initial stages, which is not great enough to introduce serious errors during the periods used to dissolve the casein (usually about ½ hr).

The pH values obtained upon dissolving 1.0 g protein in 50 ml N/50 sodium hydroxide were, for paracasein: 8.34, 8.32, and for casein: 9.55, 9.40. After adjustment to pH 9 and addition of formaldehyde for the formol titration the pH values were respectively 6.47 and 6.44.

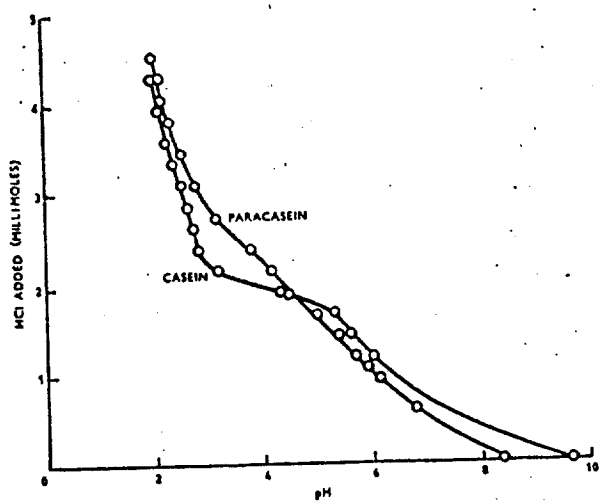

Fig. 1.—Electrometric titration of casein and paracasein. Amount of 1N HCl added to 2.0 g protein dissolved in 40 ml 0.05N NaOH.

Typical electrometric titration curves for casein and paracasein are shown in Figure 1. The titration equivalent between pH 2 and pH 6 has been used as a measure of free carboxyl groups by, for example, Herriott (1947), who drew attention to the uncertainty arising from the overlapping of the carboxyl and histidine titration regions. Although the method has been used for casein by Lea and Hanman (1950), a further objection arises from the presence of phosphoric acid groups. These have been taken into account by Hipp, Groves, and McMeekin (1952) on the assumption that the phosphoric acid affects the titration curve of casein as it does that of egg albumin (Cannan, Kibrick, and Palmer 1941). The total acid groups titratable between pH 2 and pH 6 have been calculated and are given in Table 1. The data have been corrected for

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the controls, but as these are the same for each preparation, the magnitude of the increase upon rennet treatment is not affected.

TABLE 1

COMPARISON OF CASEIN AND PARACASEIN FROM TITRATION DATA

Titration (see text)	No. of Groups per 10^6 g Air-dried Protein			
	Casein	Paracasein	Increase	
Electrometric	131	152	21	
Formol	73	69	−4	
Base in ethanol (15 ml)	58·6	80·1	21·5	Mean 21
Base in ethanol (50 ml)	90·4	110·0	19·6	
Acid in acetone (10 ml)	77·6	92·4	14·8	Mean 15
Acid in acetone (30 ml)	88·8	103·0	14·2	

The ε-amino groups of lysine were calculated from the formol titration equivalents. The apparent small decrease upon rennet treatment (Table 1) may be due to the difference in ionic strength consequent upon adjusting to pH 9; Kekwick and Canman (1936) drew attention to the large effect of salt upon the ionization of the protein.

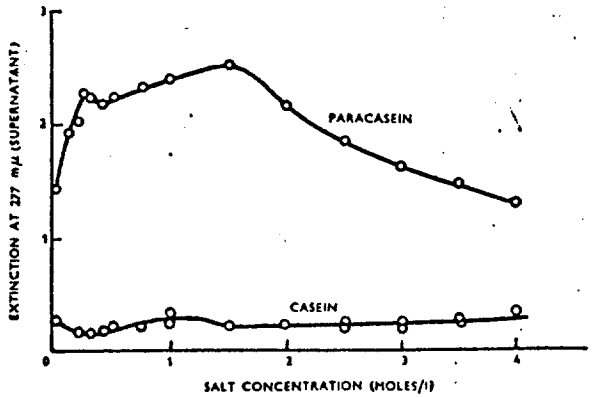

Fig. 2.—Solubility of casein and paracasein in the absence of calcium.

The interpretation of the ethanol and acetone titration data (Table 1) varies according to whether or not preliminary neutralization of the protein solution is effected (Richardson 1934), and there is also some disagreement between various authors (Richardson 1934; Linderstrøm-Lang and Jacobsen 1940; Lea and Hannan 1950).

The liberation of proteose by rennet was confirmed experimentally by examination, against appropriate controls, of the supernatants from the clotted

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sodium caseinate solutions of concentration 3.1 per cent. and pH 6.7, after adjustment to pH 4.2–4.3. The tests, which are described in detail in an unpublished report by the authors, showed that the material liberated from the protein by rennet action was inhomogeneous and could be fractionated by conventional methods. It was observed that paracasein after removal of the proteose-containing supernatant and re-dissolving (3 per cent. solution) could still be clotted with calcium chloride.

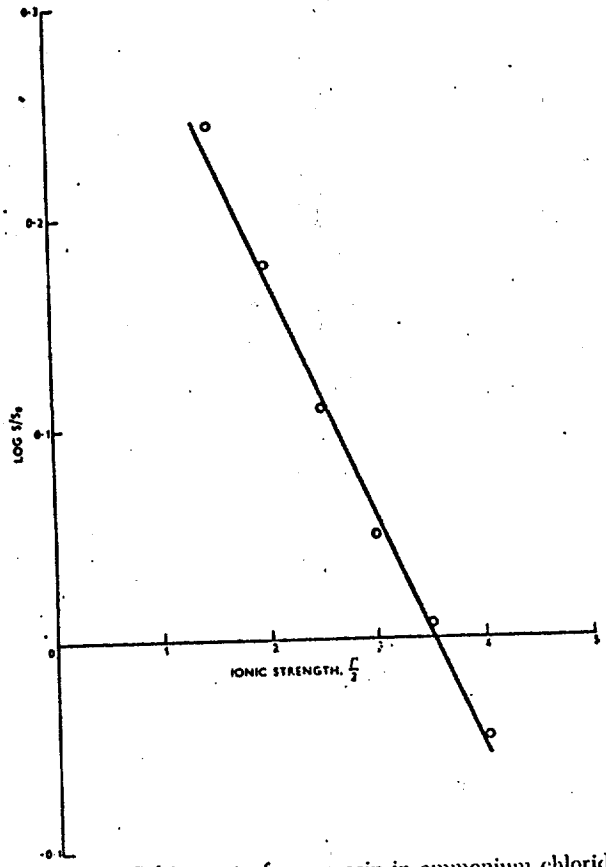

Fig. 3.—Salting-out of paracasein in ammonium chloride.

(*b*) *Solubility of Casein and Paracasein*

The solubility of casein in solutions of neutral salts is very considerably increased upon treatment with rennet, as shown in Figure 2 for ammonium chloride solutions. Apart from its greater solubility, paracasein exhibits very much greater sensitivity towards the concentration of salt. The subsidiary maximum at *c.* 0.25 mol/l suggests the presence of a component or group of components which can be salted out at concentrations much lower than that at which the main component reaches its maximum solubility (*c.* 1.5 mol/l). In Figure 3 the salting-out characteristics of paracasein are shown above this concentration. It is not even possible to derive such a curve from the data for

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casein for which log S/S_0 is practically zero. From the slope of the line in Figure 3 the salting-out constant for paracasein can be calculated (Cohn and Edsall 1943) as 0.114.

Despite its greater solubility in salt solutions at protein concentrations not greatly in excess of the solubility, paracasein shows a lower solubility at high protein concentrations. Thus when 16 g protein were equilibrated with 100 ml M/15 phosphate buffer of pH 6.9 by vigorous shaking at 25°C, the apparent solubilities were:

Casein 11.1 g/100 ml;
Paracasein 3.6 g/100 ml.

TABLE 2
ULTRAVIOLET ABSORPTION OF CASEIN AND PARACASEIN

Protein	Solvent	Extinction (1 g/l)	
		277, 280 mμ	320 mμ
Casein (12·15% N*)	M/15 phosphate buffer pH 6·8	1·02 (277)	0·231
	M/20 borax buffer pH 9·2	0·98 (277)	0·222
	N/10 sodium hydroxide	1·07 (280)	0·214
Paracasein (12·23% N*)	M/15 phosphate buffer pH 6·8	1·06 (277)	0·240
	M/20 borax buffer pH 9·2	1·03 (277)	0·230
	N/10 sodium hydroxide	1·10 (280)	0·222

* On an air-dry basis. Correction for moisture would increase these figures considerably.

(c) *Ultraviolet Absorption of Casein and Paracasein*

Porcher, Volkringer, and Brigando (1934) demonstrated the similarity of the ultraviolet absorption spectra of casein and paracasein. Spectra observed on one pair of the present preparations in phosphate buffer of pH 6.9 showed a maximum at *c.* 277 mμ, but the extinction coefficient at this wavelength was nearly 4 per cent. higher for paracasein after correcting for haze by subtraction of the extinction at 320 mμ (Beaven and Holiday 1952). This effect was examined, in relation to total nitrogen and at different pH values, with casein and paracasein derived from another batch of milk. The extinctions for paracasein corrected for haze and for nitrogen were higher in each case than those for casein by amounts in the vicinity of 3 per cent., as shown in Table 2.

Both casein and paracasein solutions were found to conform closely to Beer's Law at 280 mμ up to concentrations of at least 1 g/l when dissolved in phosphate buffer of pH 6.8.

(d) *Reaction of Casein and Paracasein with Diazonium Compounds*

The results of previous studies on the reaction of amino acids with diazonium compounds have been applied to the problem of the casein-paracasein

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transformation. The spectra shown in Figures 4 and 5 refer to different series of intact casein and paracasein preparations, made from two batches of milk. Similar experiments were made with acid hydrolysates prepared from the same protein preparations used in obtaining the spectra shown in Figure 4. The

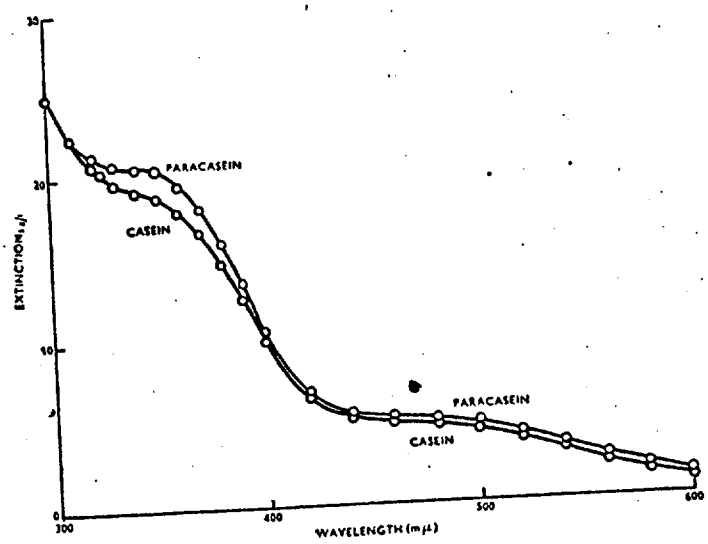

Fig. 4.—Absorption spectra of azo-derivatives of casein and paracasein (0.01 g/25 ml).

hydrolysates were first neutralized with sodium hydroxide, and ethanol was omitted from the coupling media because it increases the instability of azo-amino derivatives. The spectra obtained are shown in Figure 6 for a concentration at which all the potentially reactive groups in the hydrolysates can react with the diazonium salt. The data of Figures 4, 5, and 6 have been converted to a protein concentration of 1 g/l, but no correction has been made for other absorbing components, such as excess reactants. The only appreciable error thus introduced will result from the presence of unreacted *p*-diazobenzenesulphonic acid.

As the interpretation of these data involves the amino content of the proteins and their hydrolysates, the amino groups were determined, with the following results (expressed as equivalents $NH_2/10^5$ g air-dry protein): intact casein 43.7; intact paracasein 47.0; casein hydrolysate 665; paracasein hydrolysate 690.

(*e*) *Clotting of α-Casein*

The following experiment was carried out to determine the relative responses of whole casein and α-casein towards rennet treatment. To 5-ml aliquots of 1 per cent. solutions of each (on an air-dry basis), adjusted to pH 6.64, were added 0.05 ml rennet solution and 0.2 ml 1M calcium chloride solution. Clots formed after *c.* 10 min at 25°C. The supernatants were filtered off through

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sintered glass and their extinctions measured against water at 277 mμ. They were:

Whole casein 1.99;
α-Casein 1.52.

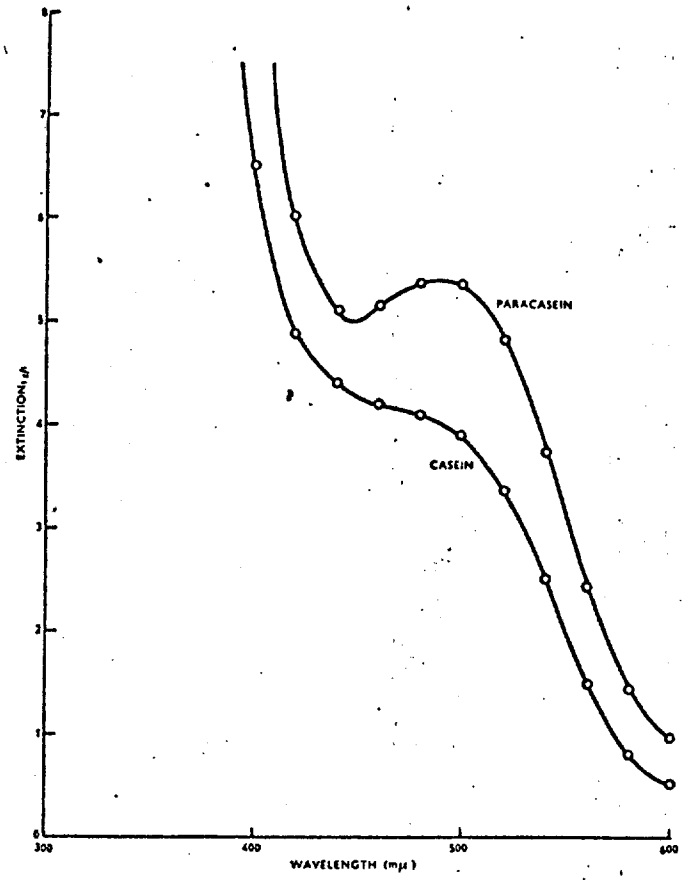

Fig. 5.—Absorption spectra of azo-derivatives of casein and paracasein (0.01 g/25 ml).

(f) *Effect of Ionic Strength and Anion Valency on Rate of Clotting*

Experiments based on the investigations of Lovelock and Porterfield (1952) on the clotting of blood plasma were carried out with paracasein. The solutions, of final concentration 3 g/100 ml adjusted to pH 6.4 with sodium hydroxide, were maintained at 25°C under the various conditions shown in Table 3, which indicates whether or not a clot formed 30 min after the addition of the calcium chloride. It was observed that, where clots formed in the presence of potassium ferricyanide, the process was almost instantaneous and they were more rigid and coherent than those formed in the presence of electrolytes of lower anion valency.

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(g) *Stability of the Clots*

In order to obtain further information on the nature of the intermolecular bonds within the rennet-sponsored clots, the stability of the clots was examined under various conditions. To three 10-ml aliquots of a 3 per cent. solution of paracasein of pH 6.7, 1 ml 1M calcium chloride was added, the clots were allowed to form, and the supernatants were removed. Five ml of the reagents shown in Table 4 were added, one to each clot, with the results indicated.

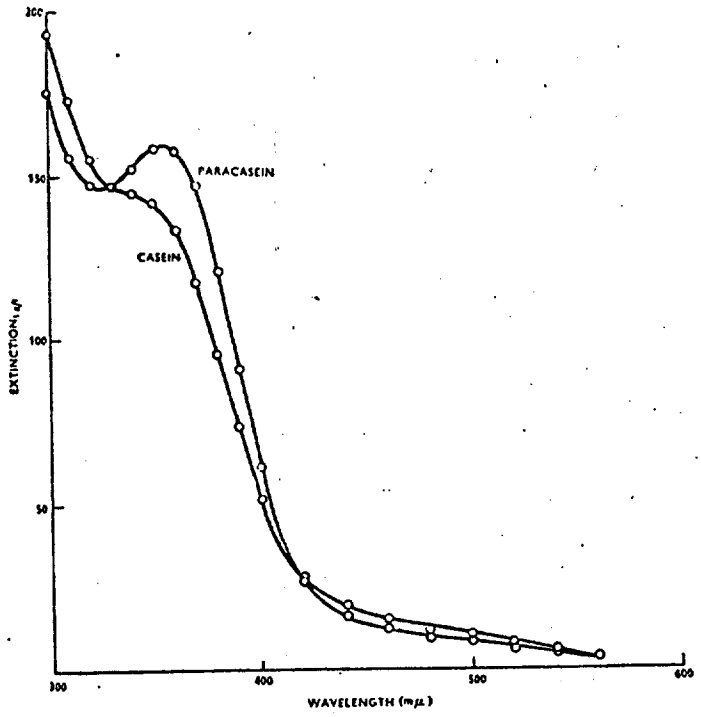

Fig. 6.—Absorption spectra of acid hydrolysates of casein and paracasein, after reaction with diazotized sulphanilic acid (0.01 g intact protein/25 ml).

IV. Discussion

The interpretation of the titration data is by no means unequivocal, but they assist in evaluating the change brought about in the casein upon rennet treatment. The electrometric titration curve shown in Figure 1 shows the greater binding capacity of the paracasein above pH 6.8 (also shown by its lower equilibrium pH value in the presence of a given quantity of sodium hydroxide) and between pH 5.2 and pH 3.2.

If the ethanol and acetone titrations are interpreted according to Lea and Hannan (1950), who were working with casein, it can be concluded from Table 2 that the increase in basic groups upon rennet treatment is greater by six than that in the acid groups. This cannot be ascribed to α-amino (since

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the appearance of each group of this type would be accompanied by a corresponding carboxyl), to ε-amino (from the formol titration), or to guanidino (since arginine is not determined) and so attention can be directed to the

TABLE 3

EFFECT OF IONIC STRENGTH AND ANION VALENCY ON CALCIUM CHLORIDE CONCENTRATION REQUIRED FOR CLOTTING

Crosses indicate clotting; dashes no clotting

Electrolyte		Sodium Chloride				Potassium Ferricyanide			
Molarity		0·1	0·2	0·3	0·4	0·1	0·2	0·3	0·4
Ionic Strength*		0·1	0·2	0·3	0·4	0·6	1·2	1·8	2·4
Final molarity of calcium chloride	0·018	+	−	−	−				
	0·045	+	−	−	−				
	0·072	+	+	+	−				
	0·090	+	+	+	−	−	−		
	0·18					−	−	−	−
	0·27					−	−	−	−
	0·38					−			+
	0·45	+	+	+	+	+	+	+	+
	0·91					+	+	+	+

* Taking molarity as molality and assuming complete ionization.

imidazole group of histidine. Richardson (1934) maintained that the scission of ester bonds will be estimated by the alkaline titration but not by the acid, and this could also account for the observed differences if his interpretation is correct.

TABLE 4

STABILITY OF CLOTS

Reagent	Result
1N NaOH	Clot dissolved rapidly
1N HCl	Clot did not dissolve
Saturated NH_4Cl	Clot dissolved slowly

The observation that paracasein, after removal of the proteose-containing supernatant and re-solution, could still be coagulated suggests that the liberated proteose is not essential for clotting, a view also favoured by Cherbuliez and Baudet (1950), although the possibility remains that a substance in the proteose is strongly adsorbed on the paracasein. The possibility that liberation of the

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proteose exposes functional groups which can then react with calcium ions is also not precluded.

The relatively low solubility of paracasein at high protein concentration may be due to salting out by a small component or to complex formation between components of the protein mixture.

The differences in the ultraviolet absorption of casein and paracasein suggest that rennet may affect the absorbing capacity of the tyrosine groups, perhaps by rupturing intermolecular hydrogen bond structures involving the phenolic hydroxyl, and a detailed spectrophotometric study of this question might well be warranted. Paracasein exhibits greater coupling capacity with diazonium salts than casein, as shown by the higher extinctions of the azo-derivatives (Figs. 4 and 5). The absorption in the region 480-500 mμ can be related to the imidazole or phenolic hydroxyl groups (Higgins and Fraser 1952) and that at *c.* 360 mμ to amino groups (from unpublished data of the authors). The spectra of the azo-derivatives of the hydrolysates (Fig. 6) do not reveal a higher histidine or tyrosine content in the paracasein, as shown by extinctions at 480-500 mμ and at 325 mμ, and differences in the intact protein thus appear to be related to the accessibility of the groups concerned.

The suggestion that rennet may release some of the imidazole rings from intramolecular linkages is in accordance with the indirect indications of the ethanol and acetone titrations. D'yachenko (1950) also refers to the release of the imidazole ring during rennet fermentation, on the basis of potentiometric titration data, although he does not assign to this reaction a subsequent role in the clotting process.

According to the definition of denaturation given by Neurath *et al.* (1944), paracasein may perhaps be considered as a denatured protein in that the reactivity of certain side chains is apparently increased by intramolecular modification. On the other hand, the striking increase in solubility (in the absence of calcium ions) and the maintenance of molecular symmetry, as determined by viscosity measurements (D'yachenko 1950; Higgins and Hayes 1951), upon rennet treatment of casein are changes in the opposite sense to those usually encountered in denaturation processes. Like casein, paracasein also yields negative sulphydryl tests.

The data on the clotting of α-casein and whole casein suggest that the α-casein enters more fully into the structure of the clot, which supports the idea that the α-casein is more affected by the rennet than the other components. This result is in accordance with those of Pyne (1951), who found that, after rennet treatment, whole casein required a higher calcium ion concentration than α-casein to effect the same rate of coagulation.

The variation with ionic strength of the amount of calcium required for coagulation in the presence of sodium chloride shows that a certain electrolyte balance is necessary for clotting, as for blood plasma (Lovelock and Porterfield 1952). Whether, as these authors consider is the case for plasma, the function of the calcium is to maintain the surface charge of one or more of the colloidal components at a value suitable for interaction with the others, or whether D'yachenko and Nitschmann and Lehmann are correct in regarding the calcium ions as acting as bridges between paracaseinate molecules, is still open to doubt.

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Although potassium ferricyanide requires a higher calcium ion concentration for clotting than sodium chloride, on a molar basis, the clots form almost instantaneously when the electrolyte balance is suitable and are more rigid and coherent. A possible explanation of this behaviour is that the trivalent anion facilitates three-dimensional cross-linking through calcium bridges attached to acid groups in different protein molecules.

The observations on the stability of the clots, considered in conjunction with the data discussed above, seem to be compatible with the formation of intermolecular bonds of a predominantly ionic character.

V. Acknowledgments

Experimental assistance was received from Mr. K. J. Harrington. The continued interest of Mr. G. Loftus Hills has been greatly appreciated, as have discussions with Mr. J. W. Legge and Mr. K. F. Plomley.

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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 κ-casein. Rennin did not attack other peptides from this sequence not containing serine, and it is suggested that, in both κ-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 κ-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 thioglycolic 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 pentapeptide 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 dicyclohexylurea. 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 amino 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 propan-2-ol-ammonia (7:3). Controls were similarly assayed and chromatographed.

The reaction between rennin and the phenyl sulphite ester or the acetyl imidazole compounds was followed either in a pH stat (Radiometer Titrat TTT 1C and Titrigraph SBR 21c) 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 for pH stat experiments were unbuffered in order to obtain [illegible] sensitivi

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.

RESULTS

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 $1{\cdot}3 \times 10^{-3}$ M to $1{\cdot}7 \times 10^{-4}$ 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 ± 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

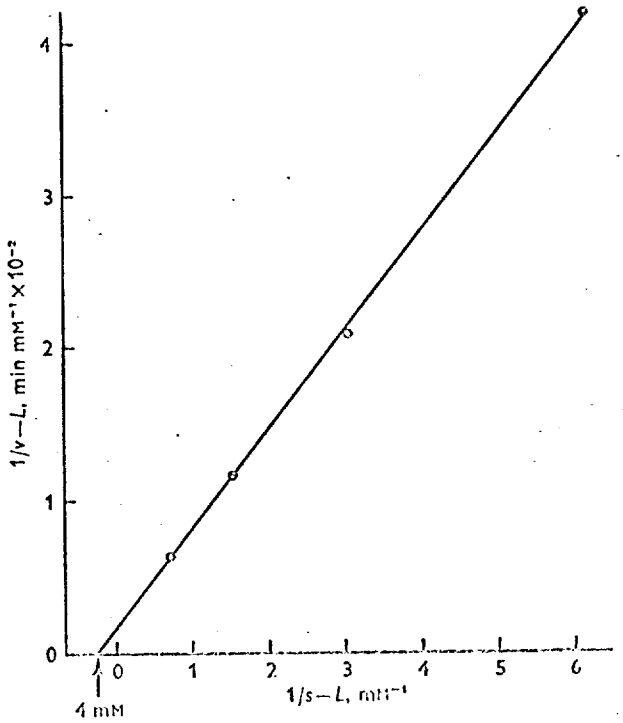

Fig. 1. Lineweaver-Burk plot for the action of rennin on ser-leu-phe-met-ala-gly. Conditions—rennin 10^{-6} M, in 0·01 N sodium acetate at pH 4·7 and 30 °C.

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 $7{\cdot}4 \times 10^{-6}$ moles/h. On adding rennin (0·25 mg) the proton uptake was 20×10^{-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 & Delaney (1957) 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, and those tested, *viz.* phenyl, *o*-chlorophenyl, *o*-bromophenyl and *p*-

bromophenyl sulphite [illegible] bromophenyl ester formed the best substrate for rennin, as it was for pepsin [illegible] Fahrney, 1967).

However, the phenyl sulphite ester was a more convenient substrate than the *p*-bromophenyl sulphite, [illegible] soluble and less prone to form micelles at concentrations near 10^{-4} M. Fig. 2 shows the course of the reaction at pH 3·7 and 25 °C between rennin and the [illegible] together with curves showing the hydrolysis in control solutions. The rate of the [illegible] 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 $0{\cdot}25$–1×10^{-6} M, and to substrate concentration in the range $0{\cdot}2$–$1{\cdot}2 \times 10^{-4}$ M. As with pepsin, the failure to observe Michaelis and Menten kinetics is no doubt due to the value of K_m for the

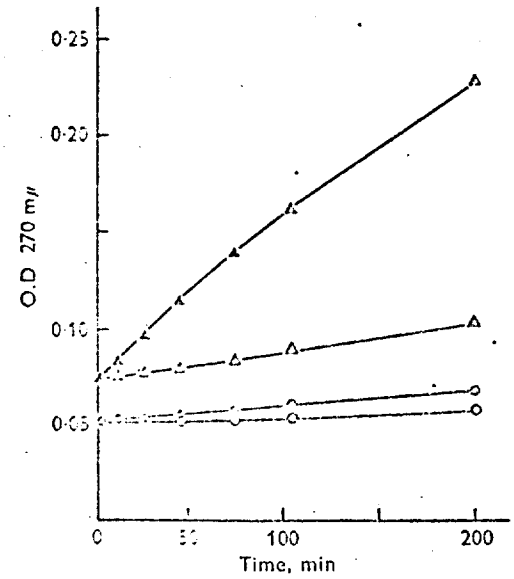

Fig. 2. Action of rennin on phenyl sulphite ester at pH 3·7 and 25 °C. ○, Control adjusted to pH 3·7 with HCl only; ●, [illegible] to pH 3·7 with 0·032 N glycine-HCl; △, as for ○, but containing $0{\cdot}85 \times 10^{-6}$ M [illegible] inactivated by boiling 1 m; ▲, as for ○, but containing 0·85 $\times 10^{-6}$ M active rennin. Concentration of phenyl sulphite ester $1{\cdot}3 \times 10^{-4}$ 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 [illegible] proteolytic activity also is shown (Fish, 1957). The over-all rate constant [illegible] conditions is 35–40×10^2 l. mole^{-1}.min^{-1} and is about one third of that found [illegible] (Reid & Fahrney, 1967). At pH 4·7 the value of the rate constant dropped [illegible], to 8×10^2 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 [illegible] 10^{-2} M can therefore cause a rate of hydrolysis comparable to that caused by [illegible] rennin.

DISCUSSION

The work described in this paper was mainly concerned with the [illegible] of the rennin-sensitive bond in casein. There were, however, other points of [illegible] the desirability of providing a practical synthetic substrate for use in [illegible] of rennin activity. At present, these assays are made using reconstituted [illegible] 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 κ-casein, attack by rennin upon the phe-met bond in κ-casein is very much ($\simeq 10^5$) faster th

[illegible] the similar bond in the pentapeptide. The effect of [illegible] must [illegible] be regarded as a part only of the reason for the rapidity of the [illegible] on the [illegible] 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 [illegible] in 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 κ-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.

It is a pleasure to acknowledge the helpful discussions on peptide chemistry with Dr F. Stewart, of the C.S.I.R.O. Protein Research Division, Melbourne, and on phenyl sulphite esters with Dr T. P. Stein and Dr D. Fahrney of the University of California, Los Angeles.

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STUDIES ON A PROTEOLYTIC ENZYME SYSTEM IN SCHIZOPHRENIA

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DURING our investigations on the protein metabolism of schizophrenic patients a number have been found who exhibited a specific, pathological pattern of emotional response to stress and abnormally high concentrations of a proteolytic enzyme inhibitor in the serum. The studies of West and associates [1] have demonstrated a relationship between the concentrations of this substance, rennin (human pepsin) inhibitor, and those of another, chymotrypsin inhibitor, and the rate of growth of cancer. The higher the titer of rennin inhibitor and the lower that of chymotrypsin inhibitor, the slower is the growth of the neoplasm. On the other hand, if the concentration of chymotrypsin inhibitor approaches or exceeds that of rennin inhibitor, the rate of growth is proportionately increased. Comparable relationships have been found between the relative concentrations of these enzyme inhibitors in active and in arrested pulmonary tuberculosis.[2]

Acute infections, physical traumas, and burns are accompanied by high chymotrypsin- and low rennin-inhibitor titers. Anxiety states, as reactions to immediate acute stresses, also follow this pattern when unaccompanied by hysterical defenses. On the contrary, high rennin- and low chymotryspin-inhibitor titers occur in a variety of chronic diseases. Pregnancy, hyperthyroidism, and, in some cases, hypertension are associated with significantly elevated rennin-inhibitor titers with a somewhat increased chymotrypsin-inhibitor titer.[4] West and associates, in establishing the normal values for these proteolytic-enzyme inhibitors, noted the consistent occurrence of high rennin- and normal chymotrypsin-inhibitor titers in childhood.[4]

The observation by one of the authors (C. E. T.) that the high concentrations of rennin inhibitor in the sera of schizophrenic patients occurred most frequently in the catatonic group suggested the importance of defining precisely the psychiatric

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Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the author are the result of his own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

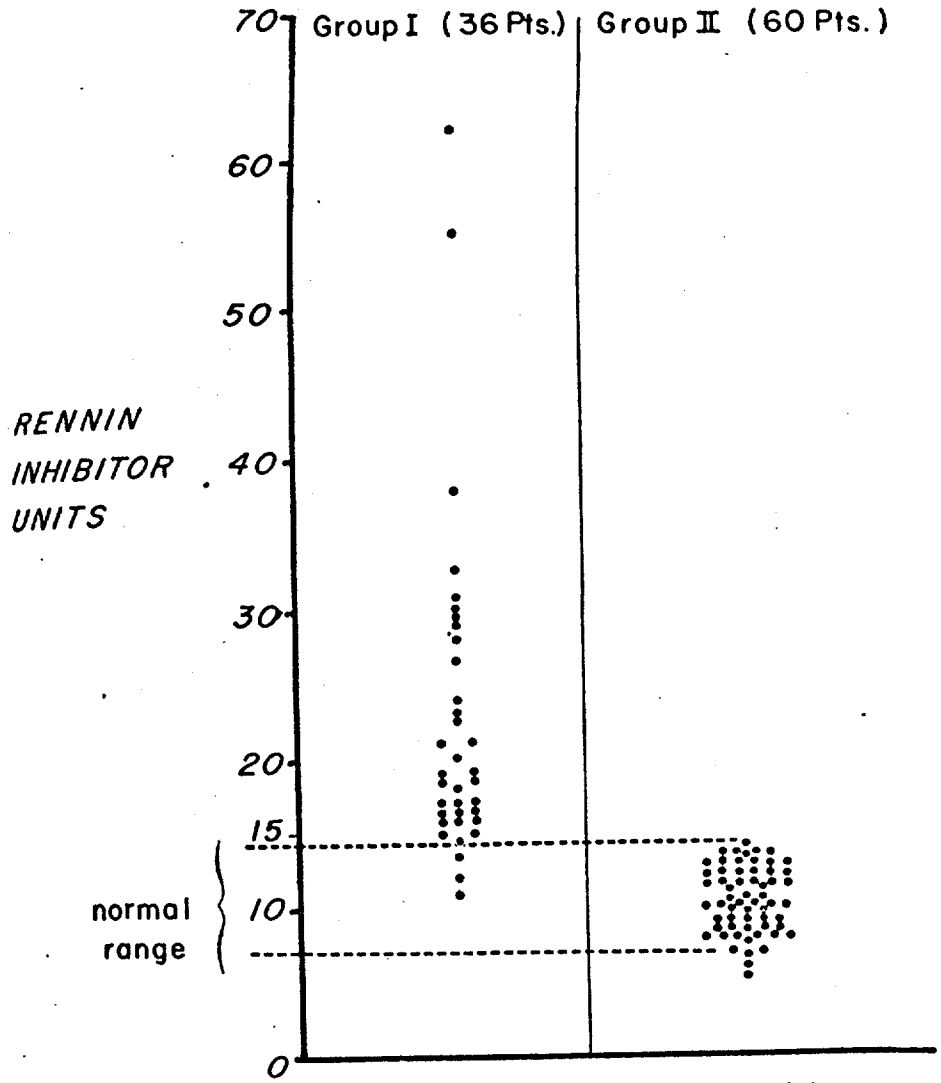
1. West, P. M., and Hilliard, J.: (*a*) Ann. West. Med. & Surg. **3**:227, 1949; (*b*) Proc. Soc. Exper. Biol. & Med. **71**:169, 1949. (*c*) West, P. M.; Rapaport, S. I., and Tempereau, C. E.: Cancer **4**:177, 1951.

2. Escovitz, W. E.: Personal communications to the authors.

3. Jacobs, J. S. L.; West, P. M., and Tempereau, C. E.: Proc. Soc. Exper. Biol. & Med. **78**:410, 1951.

4. West, P. M.; Hilliard, J., and Mietus, A. C.: Surg., Gynec. & Obst. **92**:209, 1951.

syndrome accompanying this biochemical abnormality. A direct association was found to exist between an increased rennin-inhibitor titer and the following emotional reaction pattern: (1) chronically low tolerance to frustration; and (2) the rapid or immediate appearance of confusion, obnubilation, amnesia, fugue, syncope, or stupor, as dissociative symptoms, in response to the type of external stress



Division of 96 male schizophrenic patients into two groups on the basis of the presence or absence of the formulated reaction pattern.

which would be conducive to the production of intensely hostile feelings, but which in this syndrome evokes transitory, slight, or no recognition of hostility.

METHOD AND MATERIAL

Ninety-six male schizophrenic patients were chosen consecutively from patients admitted to the psychiatric wards. Only patients were included who had received no physical psychiatric treatment in the past and who exhibited no evidence of physical illness. Corroborative diagnoses

were obtained by two or more psychiatrists. Confused patients, (four) were included only when unequivocal substantiating data were obtained from their families. Psychoneurotic patients and patients with latent and incipient schizophrenia were excluded from this study. Eight or more daily morning determinations of rennin and chymotrypsin inhibitors were obtained prior to any therapy or physical procedures on 92 of the schizophrenic patients. In the remaining subjects fewer determinations had revealed increased rennin-inhibitor titers. All biochemical studies were performed in the laboratory of Dr. P. M. West, at this hospital, according to methods described by him in 1949.[5b] Normal titers of rennin inhibitor in the male range from 7 to 14 units per 100 cc. The method is reproducible to within 1 unit.

RESULTS

The 96 schizophrenic patients were divided into two groups: (1) 36 who satisfied both criteria of the formulation described, and (2) 60 who did not satisfy both criteria.

The accompanying Figure demonstrates division of the patients into these two groups. When their maximum rennin-inhibitor titers are plotted, a significant correlation is found between an abnormally high concentration of this enzyme inhibitor and the fulfilment of both criteria of the formulation. However, the presence of dissociative symptoms or a low tolerance to frustration alone was unaccompanied by this biochemical abnormality. Two patients of the second group who had responded with intense anger and dissociative symptoms to delusions of reference and infidelity, but who had not demonstrated this reaction to external stimuli, exhibited normal rennin-inhibitor titers.

In general, the highest titers were obtained in patients with catatonic schizophrenia who displayed classic states of stupor or excitement. The distribution in both groups bore no relation to the remaining classifications of schizophrenia. No correlation was found between an abnormally high concentration of rennin inhibitor and the following signs and symptoms, singly or in combination: impulsiveness, loss of control over hostile impulses, assaultiveness, destructiveness, overwhelming hostile feelings, anxiety, delusions, and hallucinations. Similarly, the mood, the affective and intellectual status, changes in weight or libido, and the chronicity of the illness did not correlate with this enzymatic disturbance. No specific neurotic defenses appeared to be associated with it except so far as the formulation denoted.

COMMENT

It should be stressed that the schizophrenic patients who were studied were all men, between 22 and 44 years of age. Seventy-six had been psychotic for 5 to 10 years; 24 showed evidence of deterioration. Precipitating factors were often operative up to the time of admission. It is interesting in this respect that the highest rennin-inhibitor titers were usually found during the first few days of hospitalization. Thereafter, the rennin-inhibitor values sometimes decreased temporarily into the normal range, only to become elevated again in the presence of stress.

Within the hospital these patients were exposed to two general types of stress. In the first category were electric convulsion therapy, sedation and insulin coma therapy, and the intravenous administration of glucose, corticotropin, or epinephrine. The second category comprised psychiatric and psychodiagnostic examinations,

transfer from open to closed wards, and the anticipation of undergoing electric convulsion or insulin coma therapy or of taking a trial visit at home. This second group of stresses was sometimes accompanied by elevations in rennin-inhibitor titers, but only in patients who satisfied the complete formulation. Many patients, notwithstanding their group, exhibited this enzymatic change when subjected to any of the physical stresses mentioned, but this elevation was much more frequent and pronounced among patients within the formulated reaction pattern. Unquestionably, in some cases, the administration of physical procedures or therapies constituted threats which provoked hostility. Under controlled conditions, all patients subjected to psychological stresses conducive to the production of intensely hostile feelings who exhibited elevations of rennin-inhibitor levels belonged to the group of 36 who satisfied both criteria of the formulation.

Earlier attempts[5] to relate enzyme kinetics to mental status provided results which were suggestive of abnormalities, but not definitive. The metabolic significance of rennin inhibitor is unknown. It may be increased in a variety of chronic illnesses. High titers occur with a significant degree of consistency in patients with arrested cancer or inactive pulmonary tuberculosis. Under these circumstances the stress is prolonged and relatively constant. In childhood high titers of rennin inhibitor are the rule. The transition from the immature to the mature pattern occurs between the ages of 16 and 21.[4] It is generally agreed that the characteristic total biological response to chronic stress is the adoption of relatively undifferentiated, immature, regressive defenses. The satisfaction of both criteria of the formulated reaction pattern defines a most primitive form of ego defense. Psychopathologically, this pattern of behavior appears in infancy. The findings in these schizophrenic patients indicate a high correlation of immature, weak ego defenses and an immature pattern of enzyme kinetics. It will be pertinent to investigate psychiatrically the emotional status of those patients with chronic illness who exhibit elevated rennin-inhibitor titers. Studies are now being conducted with a group of psychoneurotic patients. These, and investigations of the effects of corticotropin, epinephrine, and glucose, will be reported in subsequent communications.

Over three-fourths of the schizophrenic patients studied belonged to the so-called ambulatory group. The remission rate following therapy was high. Most of them were able to resume social and occupational responsibilities. It is recognized that persons such as these comprise a significant proportion of the general population. Examination of the life histories of the patients who satisfied both criteria of the formulation revealed a high frequency of antisocial acts which were associated with confusion, amnesias, and fugues. Assaultiveness, destructiveness, sexual offences, and military transgressions, accompanied by such dissociative symptoms, were often recounted by patients in this group. It seems reasonable to assume that should the findings reported in this paper be confirmed for large numbers of persons, they may be of sociologic and forensic value.

SUMMARY

In a study of 96 physically healthy, schizophrenic male patients, abnormally high rennin-inhibitor titers were encountered only in those who exhibited the following

5. Langfeldt, G.: The Endocrine Glands and Autonomic Systems in Dementia Praecox: Clinical and Experimental Investigations, Bergen, J. W. Eide, 1926.

emotional reaction pattern: (1) chronically low tolerance to frustration; and (2) the rapid or immediate appearance of confusion, obnubilation, amnesia, fugue, syncope, or stupor, as dissociative symptoms, in response to the type of external stress which would be conducive to the production of intensely hostile feelings, which in this syndrome evokes transitory, slight, or no recognition of hostility.

It is concluded that these findings indicate a high correlation of immature, dissociative ego defenses and an immature pattern of enzyme kinetics in schizophrenia.

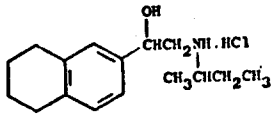
James Eatinger, Virginia Hansen, Willard Keye, Bronnetta Scott, Reed Severin, and Ruth Ruderman gave technical assistance.

Table 3 (*continued*)

No.	Trade name, source	Producer's code name[a]	Principal and additional enzymes	Primary action on substrate	Utilization	Recommended values: For pH	Recommended values: For temperature, °C
65	Pancreatin 6XNF	A	α-amylase, protease, lipase	digests food	pharmaceuticals, digestive aids		37
66	Pancreatin, pancreas glands	M	α-amylase, protease, lipase	liquefies starch, proteins, fat	leather, pharmaceuticals, textile desizing	6.0–8.0	20–50
67	Pancreatic lipase, hog pancreas	M	lipase	hydrolyzes fats to glycerin and fatty acids	pharmaceuticals, digestive aids, improves whipping of egg whites		37
68	Pancreatin, animals: hog, ox	Cud	amylase, protease, lipase	hydrolyzes starch, (also proteins, fat)		7.4–8.2	40
69	Pancreatin NF, defatted port pancreas	Wil[b]	protease, diastase, lipase	hydrolyzes proteins, starch, fat	digestive aid	7.0	37
70	Papain, papaya plant	M	protease, phosphatase, peroxidase	hydrolyzes proteins	brewing, meat, pharmaceuticals	6.0–8.0	30–75
71	Pectinol 5-B, fungal	R&H	pectic enzymes	hydrolyzes pectins	production of wines, production of concentrates, clarification of fruit juices	3.0–5.5	50
72	Penicillinase, bacterial	M	penicillinase	hydrolyzes penicillin	pharmaceuticals, research, analytical	7.0	40
73	Pepsin, hog stomach mucosa	M	protease	hydrolyzes proteins	animal feed, cereals, pharmaceuticals	1.5–3.0	30–50
74	Pepsin NF, glandular portion of hog stomach	Wil[b]	protease, cathepsin	hydrolyzes proteins, also milk curdling activity	digestive aid, pharmaceuticals	1.5–3.0	37
75	Pepsin, 1:3000, hog stomach	Cud[b]	protease	hydrolyzes proteins	digestive aids, formulations, pharmaceuticals	1.8–2.2	40
76	P/L Ficin, tropical fig tree	PL	protease	hydrolyzes proteins	chillproofing beer, tenderizing meat, dehairing hides	4.0–8.0 (optimal 5.0)	up to 50
77	P/L Liquid Cerevase	PL	protease	hydrolyzes proteins	chillproofing beer	4.0–4.5	
78	P/L Papain, papaya plant	PL	protease	hydrolyzes proteins	chillproofing beer, tenderizing meat, dehairing hides	4.0–8.5	70
79	P/L Rennet Ext, Liquid, P/L Rennet Powder, stomach of calves, lambs	PL[b]	protease	milk clotting	cheese manufacturing		37
80	P/L 219 Urease, jack	PL	urease	decomposes urea	research, clinical tests in measuring urea in blood		

Swiss. pat. 234,886 (1945 to F. Hoffmann-La Roche & Co., AG).
Crystals from abs ethanol, mp 194-195°.
MED USE: X-ray contrast medium.

Recetan. *α-[(sec-Butylamino)methyl]-5,6,7,8-tetrahydro-2-naphthalenemethanol hydrochloride.* $C_{16}H_{25}NO.HCl$; mol wt 283.86. C 67.70%, H 9.23%, Cl 12.49%, N 4.94%, O 5.64%. Prepn: Ferrari *et al.*, *Boll. Chim. Farm.* 103(1), 32 (1964), *C.A.* 61, 5580d (1964); Fr. pat. 1,390,056 (1965 to Holding Ceresia S.A.), *C.A.* 62, 16162d (1965).

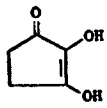


Crystals, mp 129-130°.
MED USE: Adrenergic blocking agent.

Reddingite. $3(Mn,Fe)O.P_2O_5.3H_2O$—manganese iron phosphate trihydrate.

Redergam. A brand of dihydroergotoxin ethanesulfonate. Used like Hydergin(e).

Reductic Acid. *2,3-Dihydroxy-2-cyclopenten-1-one;* 2-cyclopenten-2,3-diol-1-one. $C_5H_6O_3$; mol wt 114.10. C 52.63%, H 5.30%, O 42.07%. Obtained from pectic substances: Reichstein, Oppenauer, *Helv. Chim. Acta* 17, 390 (1934); Goldstein, U.S. pat. 2,854,484 (1958). From glutaric acid: Hesse, Bücking *Ann.* 563, 31 (1949); from cyclopentanone: Hesse *et al.*, *ibid.* 592, 137 (1955).



Pale yellow crystals (has been obtained colorless) from ethyl acetate, dec 213-213.5° (also reported as 207.5° and 211°). Soluble in water, methanol, ethanol. Sparingly sol in ether, ethyl acetate, acetone. Insoluble in benzene.
USE: As antioxidant, like isoascorbic or ascorbic acid. Has no vitamin C activity. Its reducing power is surpassed by that of *5-methylreductic acid*, mp 71°.

Reinecke Salt. *Ammonium reineckate;* ammonium tetrathiocyanodiammonochromate. $NH_4[Cr(NH_3)_2(SCN)_4].H_2O$; mol wt 354.47. $C_4H_{10}CrN_7S_4.H_2O$; anhydr salt 94.92%, H_2O 5.08%, SCN 65.55%, Cr 14.67%, NH_3 14.41%. For the anhydr: C 14.28%, H 3.00%, Cr 15.46%, N 29.14%, S 38.12%. Made by fusing ammonium thiocyanate with ammonium dichromate. Prepn: Dakin, *Org. Syn.*, coll. vol. II, 555 (1943).
Dark red crystals or red cryst powder. Sparingly sol in cold water; sol in hot water, alcohol. Dec in aq soln with formation of a blue color and free HCN; decompn occurs in about 2 weeks at room temp, rapidly above 65°. A similar decompn takes place in boiling alcohol.
USE: As a precipitant for primary and secondary amines, proline, hydroxyproline, and certain amino acids; also as a reagent for mercury with which it gives a red color or precipitate.
Note: The K-salt also is known as Reinecke salt.

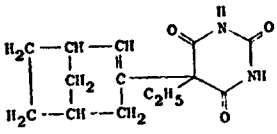
Reissert Compounds. A term used to designate 2-acyl-1,2-dihydroisoquinaldonitriles and 1-acyl-1,2-dihydroquinaldonitriles: McEwen, Cobb, *Chem. Revs.* 55, 511 (1955); Reissert, *Ber.* 38, 1603, 3415 (1905).
Human Toxicity: See Hydrogen Cyanide.

Relaxin. Releasin; Cervilaxin. Mol wt 10,000-12,000. Nitrogen 13.9-14.8%. A polypeptide hormone secreted by the corpus luteum during pregnancy. It facilitates relaxation of the pelvic ligaments of mammals, but has no estrogenic or progestational effect: Hisaw, *Proc. Soc. Exptl. Biol. Med.* 23, 661 (1926). Extraction from corpora lutea: Fevold *et al.*, *J. Am. Chem. Soc.* 52, 3340 (1930); Albert *et al.*, *Endocrinology* 40, 370 (1947); from pregnant rabbit serum: Abramowitz *et al.*, *Anat. Rec.* 84, 456 (1942); *cf.* Hall, Newton, *J. Physiol.* 106, 18 (1947). *Review:* Frieden, Hisaw in *Recent Progress in Hormone Research* 8, 333-378 (1953). Obtained commercially from pregnant sows' ovaries. Isoln procedure and purification by resin chromatography: Lehrman *et al.*, *J. Am. Pharm. Assoc.* 44, 206 (1955); Kroc, Phillips, U.S. pat. 2,852,431; Phillips, U.S. pat. 2,852,432 (1958 to Warner-Lambert). Isoln from ovarian tissue using glacial acetic acid: Cohen, U.S. pat. 2,930,737 (1960 to Princeton Labs.). Alternate method: Keck, U.S. pat. 3,008,878 (1961 to Dr. Karl Thomae GmbH). Prepn from hog ovaries: Doezi, U.S. pat. 3,096,246 (1963 to Warner-Lambert). Contains alanine, aspartic acid, cysteic acid (representing cystine or cysteine), glutamic acid, glycine, histidine, lysine, serine, tyrosine, valine, guanidine residues. No unique structural features have been found.
Amorphous powder. Diffusion constant, $cm^2/sec = 12.6 \times 10^{-7}$. Sedimentation constant, $sec = 1.6 \times 10^{-13}$. Isoelectric point around pH 7.0. Absorption max 277.5 mμ, $E^{1\%}_{1cm}$ 7.20 (H_2O at 7.0 pH). Slightly sol in water and 95% alcohol. Soluble in acid or alkaline solns. Dec above pH 9. Neutral or acid solns which are free from oxidizing agents, are stable. Ampuled solns at pH 7.0 and 3.2 have been kept in refrigerator for one year without loss of activity. Insolubie in abs alcohol, ether, acetone, petr ether, benzene.
MED USE: Has been used as uterine relaxant; to soften cervix. *Side Effects:* Allergic reactions may occur.

Renin. Highly specific proteolytic enzyme released by the kidney under conditions of ischemia. Present in human amniotic fluid. Mol wt determn: Kemp, Rubin, *Acta Chem. Scand.* 18, 2403 (1964). Prepn from hog kidneys: Haas *et al.*, *Arch. Biochem. Biophys.* 42, 368 (1953); from kidneys of various species: Haas *et al.*, *ibid.* 48, 256 (1954). Prepn of human renin: Haas *et al.*, *ibid.* 110, 534 (1965). Exists in α, β and γ configurations: Haas *et al.*, *ibid.* 44, 79 (1953). Purification on DEAE cellulose columns: Passananti, *Biochim. Biophys. Acta* 34, 246 (1959); Maier, Morgan, *ibid.* 128, 193 (1966). Renin is secreted by juxtaglomerular cells and acts as a trophic hormone on zona glomerulosa of the adrenal cortex thereby regulating the production and liberation of aldosterone: Hartroft, Hartroft, *Fed. Proc.* 20, 845 (1961). *Review:* W. S. Peart, "The Renin-Angiotensin System" in *Pharmacol. Rev.* 17, 143 (1965).

Rennin. Chymosin; rennase; lab; abomasal enzyme. German: Lab Ferment. Proteolytic enzyme from the true stomach or abomasum of the suckling calf: Tauber, Kleiner, *J. Biol. Chem.* 96, 745 (1932). A typical protein, mol wt 40,000; isoelectric point at pH 4.5. Obtained by extracting dried strips of calf stomach with 5 to 10% NaCl soln contg boric acid for 5 days; improved process: Keil, U.S. pat 2,339,931 (1942). Amino acid composition: Schwander *et al.*, *Helv. Chim. Acta* 35, 553 (1952). *Review:* N. J Berridge, "Rennin and the Clotting of Milk" in *Advances in Enzymology* 15, 423-448 (1954).
Yellowish powder, grains or scales. Peculiar, not unpleasant odor. Characteristic, slightly salty taste. Slightly hygroscopic. Can be purified enough to crystallize. The dry substance (sometimes diluted with NaCl or lactose) is stable aq solns are not. Partially sol in water, dil alcohol. pH of a soln 5.8 (acceptable range 5.3-6.3). Strongly affected by ultraviolet (sunlight). Causes milk to coagulate, optimum temp 37-43°, optimum pH of milk 5.8. Not active below 15° nor above 55°. When a commercial prepn is labeled 1:10,000 it means that one gram will curdle 10 liters of milk at 35° within 40 minutes. Other strengths are 1:100,000 to 1:7,600,000.
USE: *Rennet* (a dried extract contg rennin) is used in the manuf of cheese and rennet casein, also sold retail for the making of junket or rennet custards.
MED USE: Sometimes used as digestant.

Reposal. *5-Bicyclo[3.2.1]octen-2-yl-5-ethylbarbituric acid;* 5-ethyl-5-(bicyclo[3.2.1]octenyl)barbituric acid; WT 16 Reposamal. $C_{14}H_{18}N_2O_3$; mol wt 262.30. C 64.10% H 6.92%, N 10.68%, O 18.30%. Prepn: Taub, U.S. pat 2,808,408 (1956 to Calanda Stiftung).



Needles from isopropanol, mp 213°. pKa 7.4-7.5. LD i.p. in mice: 175 mg/kg.
MED USE: Short acting hypnotic.

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The Progress of the Reaction between some Antigens and their Respective Antibodies*

by

Hideo Moriyama

Department of Bacteriology
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I. When Ricin or Urease is used as Antigen.

I[1] have previously ascertained that the haemoagglutinating action of ricin promoted by adding antiricin serum gradually diminishes with time, and that the haemoagglutinating action of an urease-antiurease compound also diminishes with time. This specific action of an antibody against its respective antigen can be imitated by tannic acid, whose action

TABLE 1

The relation between the time elapsing between the mixing of ricin and tannin and the addition of the blood corpuscles, and the influence of tannic acid upon the haemoagglutination.

Tannic acid: Kahlbaum; laboratory temperature

Time elapsing between the mixing of ricin and tannin and the addition of the corpuscles	Dilution of ricin (before the addition of 1/10 volume tannic acid (0.001%) and the same volume of 5% rabbit's blood corpuscle suspension)						
	1/20000	1/40000	1/80000	1/160000	1/320000	1/640000	1/1280000
Immediately after	++	++	++	++	++	+	±
1 hour	++	++	+	+	±	−	−
2 hours	++	++	+	±	−	−	−
3 hours	++	++	+	±	−	−	−
Without the addition of tannin	++	++	±	−	−	−	−

+, − represent occurence or non-occurence of haemoagglutination after 24 hours in an ice chamber.

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TABLE 2

The relation between the time elapsing between the mixing of urease and tannin and the addition of the blood corpuscles and the influence of tannic acid upon the haemoagglutination.

Urease: Squibb; laboratory temperature

Time elapsing between the mixing of urease and tannin and the addition of the corpuscles	Dilution of urease (before the addition of 1/10 volume tannic acid (0.001%) and the same volume of 5% horse blood corpuscles suspension)				
	1/20000	1/40000	1/80000	1/160000	1/320000
Immediately after	++	++	++	+	−
1 hour	++	++	±	−	−
2 hours	++	+	−	−	−
3 hours	++	+	−	−	−
Without the addition of tannin	++	++	++	+	−

+, − represent occurence or non-occurence of haemoagglunation.

against the haemoagglutinating property of ricin is as shown in Table 1, and against urease in Table 2.

Thus, it may be clear that the behaviour of antiricin serum or of antiurease serum towards their respective antigen are identical with that of tannic acid towards the agglutinins. That is, in the case of ricin both tannic acid and antiserum, immediately after their addition to ricin, promote its agglutinating action, which afterwards gradually diminishes. On the other hand, against urease both tannic acid and the antiserum display an inhibiting action, which increases with time.

TABLE 3

Decrease in progress in urease action due to antiurease.

Urease: Squibb; antiserum: antiurease rabbit serum

Time elapsing between the addition of antiserum (1:2) to the same volume of 0.5% urease solution and the addition of urea (laboratory temp.)	Quantity of NH_3 produced in 30 minutes at laboratory temperature, mg.
5 minutes	3.23
10 minutes	3.01
20 minutes	2.89
40 minutes	2.84
Without the addition of antiserum	4.08

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Against the urea-decomposing property of urease, the antiurease action appears gradually with time as indicated in Table 3. In Table 4 is shown an instance when tannic acid was used instead of antiurease. I have already reported that tannic acid in a high concentration inhibits the urease action, and in a low concentration rather promotes it, in the same manner as antiurease serum acts upon urease; as regards the progress of the reaction, tannic acid shows the same behaviour towards urease as antiurease does.

TABLE 4

Decrease in progress in urease action due to tannic acid.

0.2% tannic acid solution is added to the same volume of 1% urease solution

Time elapsing between the addition of tannin to the same volume of urease solution and the addition of urea	Quantity of NH_3 produced in 30 minutes at laboratory temp., mg.
Immediately after	10.2
1 hour	4.33
2 hours	2.83
3 hours	2.00

As mentioned above, the progress of the reaction between ricin or urease and their respective antibodies is similar to that between the antigens and tannic acid. Since between the action of the antigen and its quantity there is no simple relation as in the case of urease, or if such a relation exists, the action of the antigen can not be measured quantitatively as with haemoagglutination, it is, therefore, impossible to study the progress of the reaction minutely with these substances.

II. When Rennet is used as Antigen.

Rennet is very convenient for use in such experiments, for, as shown in Table 5, the product of the quantity of rennet and the time required for the coagulation of milk by it is a constant. Hence, its quantity can be calculated easily from its action. While the action of rennet is inhibited manifestly by NaCl, as will be understood by comparing A with B in Fig. 5, the relation just mentioned can exist entirely irrespective of the presence or non-presence of NaCl.

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TABLE 5

Relation between the quantity of rennet and the time required by it for the coagulation of cow's milk.

Rennet: Dr. Fraenkel & Dr. Landau; laboratory temperature (ca. 31°C)

A: rennet solution, 1 cc+water, 1 cc+cow's milk, 1 cc (1% $CaCl_2$ added)

Rennet concentration, (%)	Time required for milk coagulation, (sec.)	conc. × time
0.02	14	0.28
0.02×2/3	20	0.27
0.02×(2/3)^2	29	0.26
0.02×(2/3)^3	45	0.26
0.02×(2/3)^4	67	0.27
0.02×(2/3)^5	101	0.27
0.02×(2/3)^6	151	0.27
0.02×(2/3)^7	228	0.27

B: rennet solution, 1 cc+0.9% NaCl, 1 cc+cow's milk, 1 cc (1% $CaCl_2$ added)

Rennet conc., (%)	Time required for milk coagulation, (sec.)	conc. × time
0.01	51	0.51
0.005	106	0.53
0.0025	208	0.52
0.00125	412	0.52

The diminution of rate in the action of rennet added with antirennet serum is shown in Table 6–7, and Fig. 1–2. Antirennet serum was obtained from rabbits injected with 5 cc of 1% rennet solution (Dr. Fraenkel & Dr. Landau) once a week during 4 weeks.

In the above Tables and Figures the experimental results when carried out in a medium of 0.45% NaCl solution are shown, while, when the experiment is carried out in a medium containing no NaCl, different reaction curves are obtained as indicated in Table 8–9 and Fig. 3–4. In the former case, although both the velocity constants KI and KII tend to decrease as the reaction progresses, the decreasing grade of KII is smaller than that of KI, and even KII tends to become a constant when the same amount of antiserum is acted upon. However in the medium

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TABLE 6

Decrease in progress in the action of rennet due to antiserum.

Antiserum: No. 1; laboratory temp. (ca. 30°C)

Rennet solution (0.01%), 1 cc+antiserum 0.9% NaCl solution, 1 cc+cow's milk, 1 cc (1% $CaCl_2$ added)

Dilution of anti-serum	Time elapsing from the addition of antiserum to rennet until mixing with milk															
	5 minutes				10 minutes				15 minutes				20 minutes			
	Time required for the coagulation (sec.)	Rennet remaining (%)	K I	K II	Time required for the coagulation (sec.)	Rennet remaining (%)	K I	K II	Time required for the coagulation (sec.)	Rennet remaining (%)	K I	K II	Time required for the coagulation (sec.)	Rennet remaining (%)	K I	K II
1:10	229	17	0.35	0.0098	415	9.4	0.23	0.0096	530	7.4	0.18	0.0083	720	5.4	0.15	0.0089
1:20	89	41	0.16	0.0025	128	30	0.12	0.0023	173	23	0.097	0.0022	231	17	0.037	0.0024
1:40	54	72	0.067	0.00078	65	60	0.051	0.00067	76	51	0.041	0.00061	85	46	0.039	0.00059
1:80	45	87	0.028	0.00030	50	78	0.025	0.00028	56	70	0.023	0.00029	58	67	0.020	0.00025
1:160	43	91	0.019	0.00020	48	81	0.021	0.00024	49	80	0.015	0.00017	50	78	0.012	0.00016

Coagulation time when 0.9% NaCl solution is added instead of antiserum: 39 sec.

Quantity of rennet remaining: 39 sec./sec. required for coagulation.

$$KI = \frac{1}{0.4343\,t} \log \frac{a}{a-x}$$

$$KII = \frac{x}{t(a-x)a}$$

t: time, a: quantity of rennet at the beginning, x: quantity of rennet deprived of its action within time t.

TABLE 7

Decrease in progress in the action of rennet due to antiserum.

Antiserum: No. II; laboratory temp. (ca. 30°C)

Rennet solution (0.01%). 1 cc+antiserum 0.9% NaCl solution, 1 cc+cow's milk, 1 cc (1% $CaCl_2$ added)

Dilution of anti-serum	Time elapsing from the addition of antiserum to rennet until mixing with milk															
	5 minutes				10 minutes				15 minutes				20 minutes			
	Time required for the coagulation (sec.)	Rennet remaining (%)	K I	K II	Time required for the coagulation (sec.)	Rennet remaining (%)	K I	K II	Time required for the coagulation (sec.)	Rennet remaining (%)	K I	K II	Time required for the coagulation (sec.)	Rennet remaining (%)	K I	K II
1:10	660	5.3	0.59	0.036	1140	3.1	0.35	0.031	1860	1.9	0.28	0.034	2520	1.1	0.24	0.045
1:20	165	21	0.31	0.0095	316	11	0.22	0.0081	570	6.1	0.19	0.0103	960	3.7	0.17	0.013
1:40	77	46	0.16	0.0023	103	34	0.11	0.0019	135	26	0.093	0.0019	175	20	0.084	0.0020
1:80	41	85	0.032	0.00035	41	80	0.022	0.00025	50	70	0.025	0.00029	52	67	0.021	0.00025
1:160	38	92	0.017	0.00017	40	88	0.013	0.00014	43	81	0.015	0.00016	44	79	0.012	0.00013

Coagulation time when 0.9% NaCl solution is added instead of antiserum: 35 sec.

Quantity of rennet remaining: 35 sec./sec. required for coagulation.

$$KI = \frac{1}{0.4343\,t} \log \frac{a}{a-x}$$

$$KII = \frac{x}{t(a-x)a}$$

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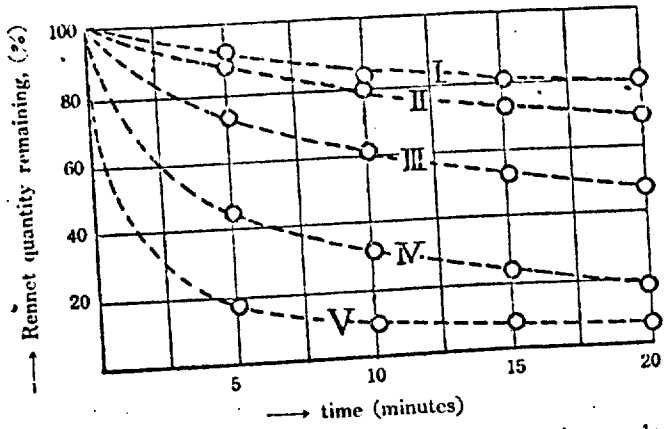
Fig. 1

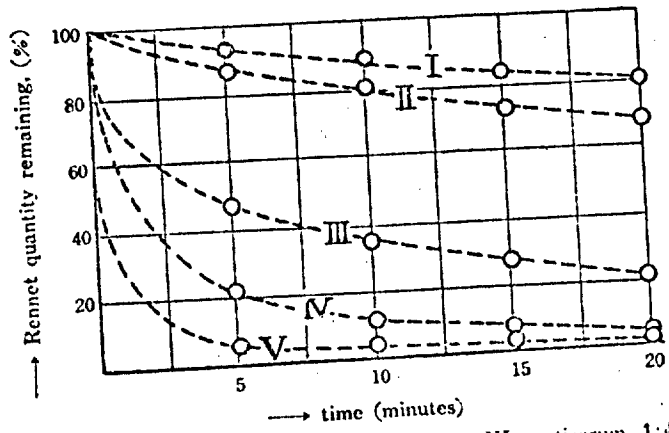
Decrease in progress in the action of rennet due to antiserum (Table 6).
Antiserum: No. I; laboratory temp. (ca. 30°). Rennet solution (0.01%), 1 cc+ antiserum 0.9% NaCl solution, 1 cc+cow's milk, 1 cc (1% $CaCl_2$ added)



I: antiserum, 1:160; II: antiserum, 1:80; III: antiserum, 1:40; IV: antiserum, 1:20; V: antiserum, 1:10.

Fig. 2

Decrease in progress in the action of rennet due to antiserum (Table 7).
Antiserum: No. II; laboratory temp. (ca. 30°C). Rennet solution (0.01%), 1 cc+ antiserum 0.9% NaCl solution, 1 cc+cow's milk, 1 cc (1% $CaCl_2$ added)



I: antiserum, 1:160; II: antiserum, 1:80; III: antiserum, 1:40; IV: antiserum, 1:20; V: antiserum, 1:10.

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in which there is no NaCl even KII as well as KI has a tendency to decrease distinctly as the reaction progresses.

When the NaCl concentration is increased, both KI and KII tend to become constants, and especially so in the case of KII, as shown in Table 10 and Fig. 5. In the other words, with no NaCl the reaction progresses rapidly at first and afterwards the velocity markedly decreases. On the contrary, when NaCl exists, the rate tends to become constant, therefore, the difference in the diminution in the action of rennet in the two cases becomes greater with time.

The diminution in the action of rennet due to antirennet continues for a long time. It never comes to an end even after 7 hours as shown in Table 11 and Fig. 6.

When tannic acid is used instead of antirennet serum, the same diminution in the rate of the action of rennet is observable, and with a medium containing no NaCl, the velocity of the reaction tends to decrease more manifestly than with a medium containing NaCl as with antirennet. (Tables 12–16 and Fig. 7–11)

A strange point in Fig. 9 and Fig. 10 is that the curves of diminution of the action of rennet are wavy owing to the reappearance of the action after it has disappeared. Such a disturbance in the curve can sometimes be observed with the antiserum as shown in Fig. 12, although not so manifestly as with tannic acid.

Moreover, a similar phenomenon can be found when the grade of turbidity produced by the addition of tannic acid to protein solution, is measured from time to time. In this case the disturbance occurs without any relation to the presence or non-presence of NaCl. (Fig. 13–14)

The action of rennet is affected conspiquously also by $HgCl_2$, but the diminution is finished instantaeously and never continues for a long time as in the both cases of tannic acid and antiserum. Furthermore, a linear relation exists between the quantity of $HgCl_2$ and its action upon rennet, while the relation is far from linear either with tannic acid or with the antiserum. (Fig. 15–19) By comparing Fig. 16 and Fig. 17 with Fig. 18 and Fig. 19, it will become clear that the relation between the quantity of tannic acid and its action upon rennet is very like that between the quantity of antiserum and its action upon rennet.

TABLE 8

Decrease in progress in the action of rennet due to antiserum.

Antiserum: No. I, laboratory temp. Rennet solution (0.01%), 1 cc+antiserum water solution, 1 cc+cow's milk, 1 cc (1% $CaCl_2$ added)

Dilution of anti-serum	Time elapsing from the addition of antiserum to rennet until mixing with milk															
	5 minutes				10 minutes				15 minutes				20 minutes			
	Time required for the coagulation (sec.)	Rennet remaining (%)	K I	K II	Time required for the coagulation (sec.)	Rennet remaining (%)	K I	K II	Time required for the coagulation (sec.)	Rennet remaining (%)	K I	K II	Time required for the coagulation (sec.)	Rennet remaining (%)	K I	K II
1:10	65	26	0.28	0.0057	84	20	0.16	0.0040	106	16	0.12	0.0035	128	13	0.10	0.0033
1:20	32	53	0.13	0.0018	38	45	0.081	0.0012	47	36	0.069	0.0012	49	35	0.053	0.00093
1:40	24	71	0.069	0.00082	26	65	0.041	0.00054	30	57	0.037	0.00050	30	57	0.028	0.00038
1:80	22	77	0.053	0.00060	20	85	0.016	0.00018	22	77	0.017	0.00020	22	77	0.013	0.00015
1:160	19	89	0.023	0.00028	21	81	0.021	0.00024	20	85	0.011	0.00012	21	81	0.011	0.00012

Coagulation time when water is added instead of antiserum: 17 sec.

Quantity of rennet remaining: 17 sec./sec. required for coagulation.

$$KI = \frac{1}{0.4343\,t} \log \frac{a}{a-x}$$

$$KII = \frac{x}{t(a-x)a}$$

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TABLE 9

Decrease in progress in the action of rennet due to antiserum.

Antiserum: No. II, temperature: laboratory temp. Rennet solution (0.01%), 1 cc+antiserum water solution, 1 cc+cow's milk, 1 cc (1% $CaCl_2$ added)

Dilution of anti-serum	Time elapsing from the addition of antiserum to rennet until mixing with milk															
	5 minutes				10 minutes				15 minutes				20 minutes			
	Time required for the coagulation (sec.)	Rennet remaining (%)	K I	K II	Time required for the coagulation (sec.)	Rennet remaining (%)	K I	K II	Time required for the coagulation (sec.)	Rennet remaining (%)	K I	K II	Time required for the coagulation (sec.)	Rennet remaining (%)	K I	K II
1:10	79	23	0.29	0.0067	98	18	0.17	0.0046	125	14	0.14	0.0041	152	12	0.11	0.0037
1:20	36	50	0.14	0.0020	43	42	0.086	0.0014	47	38	0.037	0.0011	51	35	0.055	0.00093
1:40	27	67	0.080	0.0012	29	62	0.048	0.00061	31	58	0.038	0.00018	34	53	0.33	0.00044
1:80	22	82	0.040	0.00041	23	78	0.025	0.00028	23	78	0.017	0.00019	24	75	0.015	0.00017
1:160	21	86	0.030	0.00033	22	82	0.020	0.00022	22	82	0.014	0.00015	22	82	0.010	0.00011

Coagulation time when water is added instead of antiserum: 18 sec.

Quantity of rennet remaining: 18 sec./sec. required for coagulation.

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TABLE 10

Influence of NaCl upon the decrease in progress in the action of rennet due to antiserum.

Antiserum: No. I; temperature: laboratory temp. Rennet solution (0.01%), 1 cc+antiserum solution, 1 cc+cow's milk (1% $CaCl_2$ added), 1 cc

Concentration of NaCl			0.9%			0.45%			0.%		
Dilution of antirennet serum			1:20	1:40	1:80	1:20	1:40	1:80	1:20	1:40	1:80
Time elapsing between the mixing of rennet and antiserum until the addition of milk	5 minutes	Rennet remaining, %	44	74	78	44	72	87	53	71	77
		K I	0.16	0.060	0.028	0.16	0.067	0.028	0.13	0.069	0.053
		K II	0.0025	0.00070	0.00030	0.0025	0.00078	0.00030	0.0018	0.00082	0.00060
	10 minutes	Rennet remaining, %	29	58	77	30	60	78	45	65	85
		K I	0.12	0.055	0.026	0.12	0.051	0.025	0.81	0.041	0.016
		K II	0.0024	0.00072	0.00029	0.0023	0.00067	0.00028	0.0012	0.00054	0.00018
	15 minutes	Rennet remaining, %	21	49	70	23	51	70	36	57	77
		K I	0.10	0.048	0.024	0.097	0.044	0.023	0.069	0.037	0.017
		K II	0.0025	0.00069	0.00029	0.0022	0.00064	0.00029	0.0012	0.00050	0.00020
	20 minutes	Rennet remaining, %	16	42	63	17	46	67	35	57	77
		K I	0.092	0.043	0.023	0.087	0.039	0.020	0.057	0.028	0.013
		K II	0.0026	0.00069	0.00029	0.0024	0.00059	0.00025	0.00093	0.00038	0.00015

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TABLE 11

Decrease in progress in the action of rennet due to antirennet serum.

Antiserum: No. II; temperature: laboratory temp.

Rennet solution (0.01%), 1 cc+antiserem 0.9% NaCl solution, 1 cc+milk (1% $CaCl_2$), 1 cc.

Dilution of antiserum			1:10	1:20	1:40	1:80	1:160
Time elapsing between mixing rennet and antiserum and the addition of milk	1/2 hour	Rennet remaining, %	2.1	4.2	18	60	72
		K I	7.7	6.3	3.4	1.0	0.64
		K II	0.93	0.46	0.091	0.013	0.0078
	1 hour	Rennet remaining, %	1.5	2.7	8.5	47	67
		K I	4.2	3.6	2.5	0.75	0.39
		K II	0.66	0.036	0.011	0.0011	0.0049
	2 hours	Rennet remaining, %	1.4	1.8	4.1	39	59
		K I	2.1	2.0	1.6	0.47	0.26
		K II	0.035	0.027	0.012	0.0078	0.0035
	3 hours	Rennet remaining, %	1.3	1.5	2.7	28	51
		K I	1.5	1.4	1.2	0.42	0.22
		K II	0.025	0.022	0.012	0.0086	0.0032
	4 hours	Rennet remaining, %	1.3	1.4	2.2	23	46
		K I	1.1	1.1	0.95	0.37	0.19
		K II	0.019	0.018	0.011	0.0034	0.0029
	5 hours	Rennet remaining, %	1.2	1.2	1.9	19	43
		K I	0.88	0.88	0.79	0.33	0.17
		K II	0.017	0.016	0.010	0.0085	0.0027
	6 hours	Rennet remaining, %	1.2	1.2	1.8	14	43
		K I	0.74	0.74	0.67	0.33	0.14
		K II	0.014	0.014	0.0091	0.010	0.022
	7 hours	Rennet remaining, %	1.1	1.2	1.7	8.0	41
		K I	0.64	0.63	0.58	0.36	0.13
		K II	0.013	0.012	0.0083	0.016	0.020

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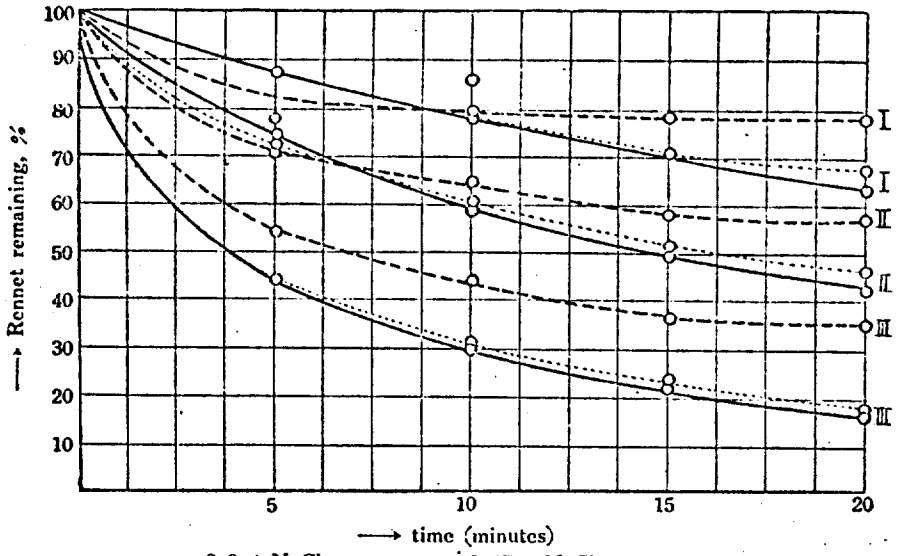
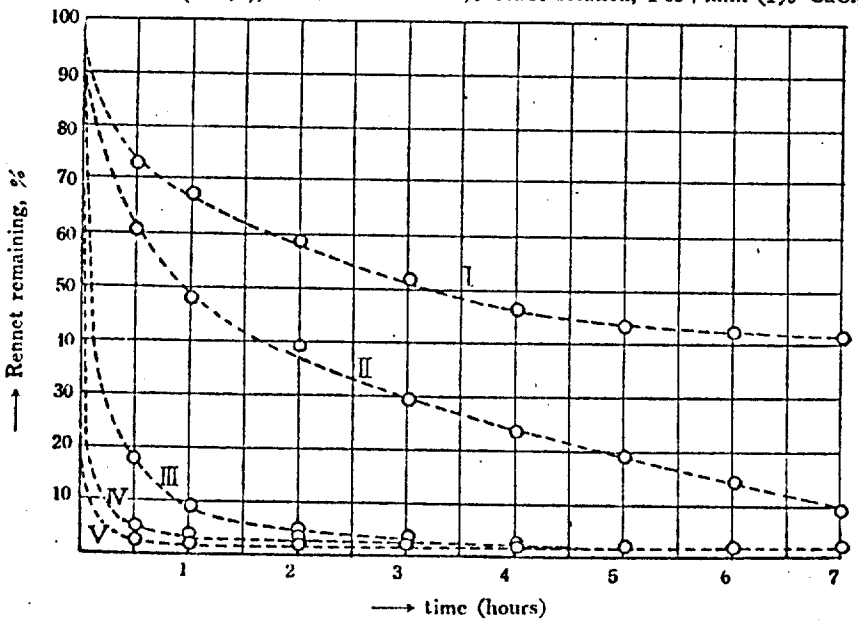
Fig. 5
Influence of NaCl upon the decrease in progress in the action of rennet due to antiserum (Table 10).



———: 0.9% NaCl, - - - - - -: 0.45% NaCl, – – – – –: 0% NaCl
I: antiserum 1:80, II: antiserum 1:40, III: antiserum 1:20

Fig. 6
Decrease in progress in the action of rennet due to antirennet serum (Table 11).
Antiserum: No. II; temperature: laboratory temp.
Rennet solution (0.01%), 1 cc+antiserum 0.9% NaCl solution, 1 cc+milk (1% $CaCl_2$) 1 cc



Dilution of antiserum: I: 1:160, II: 1:80, III: 1:40, IV: 1:20, V: 1:10

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TABLE 12

Decrease in progress in the action of rennet due to tannic acid.
Tannic acid: Kahlbaum, used immediately after being dissolved.
temperature: laboratory temp. (ca. 27°C)
Rennet solution (0.01%) 1 cc+tannic acid 0.9% NaCl solution, 1 cc+milk (1% $CaCl_2$), 1 cc

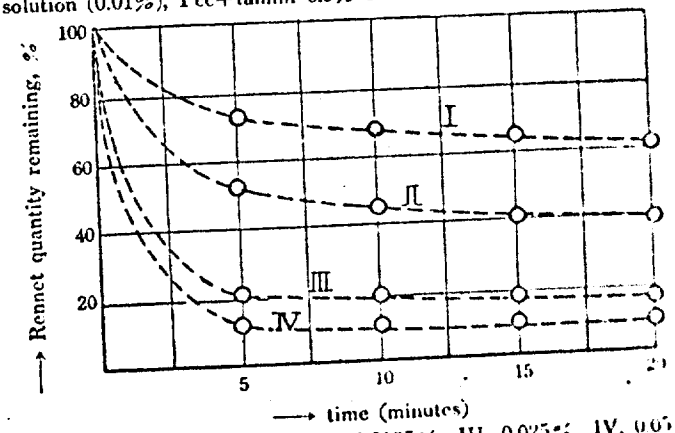
Concentration of tannic acid, %	Time elapsing between mixing tannin and rennet and the addition of milk											
	5 minutes			10 minutes			15 minutes			20 minutes		
	Time required for the coagulation (sec.)	Rennet remaining (%)	K I	Time required for the coagulation (sec.)	Rennet remaining (%)	K I	Time required for the coagulation (sec.)	Rennet remaining (%)	K I	Time required for the coagulation (sec.)	Rennet remaining (%)	K I
0.1	348	11	0.44	412	9.6	0.28	438	9.0	0.17	471	8.4	0.13
0.05	323	12	0.42	383	10	0.23	411	9.6	0.16	453	8.7	0.11
0.025	197	20	0.32	213	19	0.17	237	17	0.12	277	14	0.10
0.0125	76	52	0.13	86	46	0.78	99	40	0.069	101	39	0.04
0.00625	56	71	0.068	57	69	0.037	62	64	0.031	65	61	0.021

Coagulation time when 0.9% NaCl solution is added instead of tannic acid: 39.5 sec.
Quantity of rennet remaining: 39.5 sec./sec. required for the coagulation.

$$K\ I = \frac{1}{0.4343\,t} \log \frac{a}{a-x}$$

Fig. 7

Decrease in progress in the action of rennet due to tannic acid (Table 12).
Tannic acid: Kahlbaum, used immediately after being dissolved; temperature: laboratory temp. (ca. 27°C)
Rennet solution (0.01%), 1 cc+tannin 0.9% NaCl solution, 1 cc+milk (1% $CaCl_2$), 1 cc



I, tannic acid: 0.00625% II, 0.0125% III, 0.025% IV, 0.05%

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TABLE 13

Decrease in progress in the action of rennet due to tannic acid.

Tannic acid: Kahlbaum, used about a month after its dissolving; temperature: laboratory temp.

Rennet solution (0.01%), 1 cc+tannin 0.9% NaCl solution, 1 cc+milk (1% $CaCl_2$), 1 cc

Concentration of tannic acid, %	Time elapsing between mixing tannin and rennet and the addition of milk											
	5 minutes			10 minutes			15 minutes			20 minutes		
	Time required for the coagulation (sec.)	Rennet remaining (%)	K I	Time required for the coagulation (sec.)	Rennet remaining (%)	K I	Time required for the coagulation (sec.)	Rennet remaining (%)	K I	Time required for the coagulation (sec.)	Rennet remaining (%)	K I
0.025	414	17	0.35	490	14	0.20	564	12	0.14	583	12	0.11
0.0125	149	46	0.16	171	40	0.092	181	38	0.064	197	36	0.041
0.00625	93	74	0.060	95	72	0.033	102	68	0.026	106	65	0.022
0.003125	74	93	0.015	79	87	0.014	92	84	0.012	91	85	0.0081

Coagulation time when 0.9% NaCl solution is added instead of tannic acid: 69 sec.

Quantity of rennet remaining: 69 sec./sec. required for coagulation.

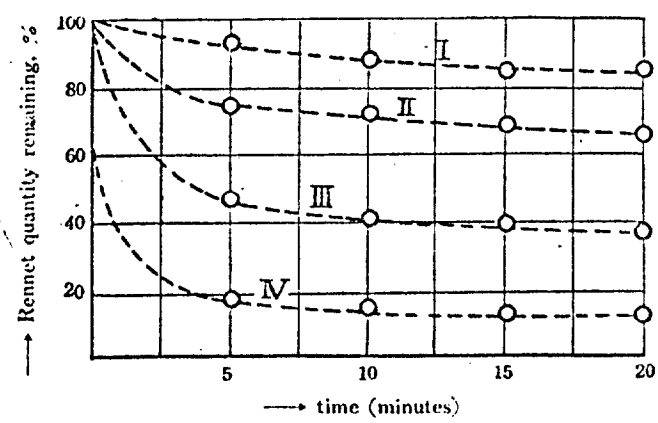
$$K\ I = \frac{1}{0.4343\,t} \log \frac{a}{a-x}$$

Fig. 8

Decrease in progress in the action rennet due to tannic acid (Table 13).

Tannic acid: Kahlbaum, used about a month after its dissolving

Rennet solution (0.01%), 1 cc+tannin 0.9% NaCl solution, 1 cc+milk (1% $CaCl_2$), 1 cc



Concentration of tannic acid: I: 0.003125%, II: 0.00625%, III: 0.0125%, IV: 0.025%

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TABLE 14

Decrease in progress in the action of rennet due to tannic acid.

Tannic acid: Kahlbaum, used immediately after being dissolved; temperature: laboratory temp.

Rennet solution (0.01%), 1 cc+tannic acid solution, 1 cc+milk (1% $CaCl_2$), 1 cc

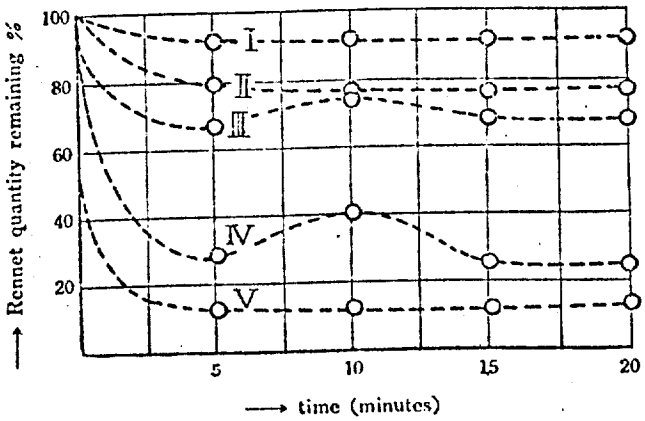
Concentration of tannic acid, %	Time elapsing between the mixing of tannin and rennet and the addition of milk							
	5 minutes		10 minutes		15 minutes		20 minutes	
	Time required for the coagulation (sec.)	Rennet remaining (%)	Time required for the coagulation (sec.)	Rennet remaining (%)	Time required for the coagulation (sec.)	Rennet remaining (%)	Time required for the coagulation (sec.)	Rennet remaining (%)
0.1	161	12	169	11	180	11	162	12
0.05	68	28	47	40	77	25	78	24
0.025	29	66	26	73	28	68	29	66
0.0125	24	79	25	76	25	76	25	76
0.00625	21	91	21	91	21	91	21	91

Coagulation time when water is added instead of tannic acid: 19 sec.
Quantity of rennet remaining: 19 sec./sec. required for the coagulation.

Fig. 9

Decrease in progress in the action of rennet due to tannic acid (Table 14).

Tannic acid: Kahlbaum, used immediately after being dissolved; rennet solution (0.01%), 1 cc+tannic acid solution, 1 cc+milk (1% $CaCl_2$), 1 cc



I, 0.00625% tannic acid II, 0.0125% tannic acid III, 0.025% tannic acid
IV, 0.05% tannic acid V, 0.1% tannic acid

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TABLE 15

Decrease in progress in the action of rennet due to tannic acid.

Tannic acid: Kahlbaum, used a month after its dissolving.

Rennet solution (0.01%), 1 cc+tannic acid solution, 1 cc+milk (1% $CaCl_2$), 1 cc

Concentration of tannic acid, %	Time elapsing between mixing tannin and rennet and the addition of milk							
	5 minutes		10 minutes		15 minutes		20 minutes	
	Time required for the coagulation (sec.)	Rennet remaining (%)	Time required for the coagulation (sec.)	Rennet remaining (%)	Time required for the coagulation (sec.)	Rennet remaining (%)	Time required for the coagulation (sec.)	Rennet remaining (%)
0.1	391	6.9	390	6.9	—	—	—	—
0.05	209	13	364	7.4	304	8.9	293	9.2
0.025	112	24	174	16	144	19	137	20
0.0125	62	44	59	46	57	47	77	35
0.00625	43	63	34	79	33	82	36	75
0.003125	36	75	35	77	30	90	34	79

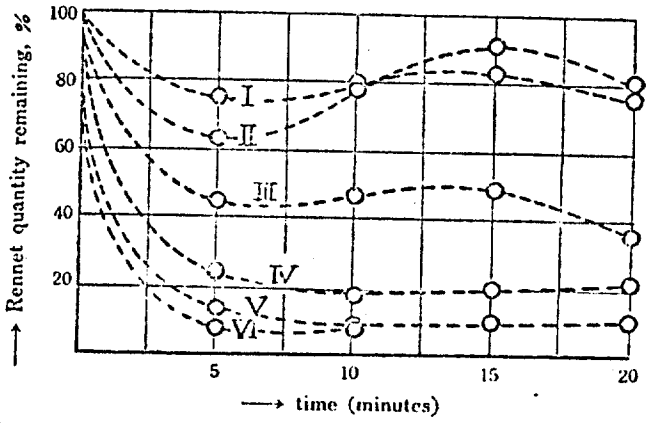
Coagulation time when water is added instead of tannic acid: 27 sec.

Quantity of rennet remaining: 27 sec./sec. required for the coagulation.

Fig. 10

Decrease in progress in the action of rennet due to tannic acid (Table 15).

Tannic acid: Kahlbaum, used a month after its dissolving; rennet solution (0.01%), 1 cc+tannic acid solution, 1 cc+milk (1% $CaCl_2$), 1 cc



I, 0.003125% tannic acid II, 0.00625% tannic acid III, 0.0125% tannic acid
IV, 0.025% tannic acid V, 0.05% tannic acid VI, 0.1% tannic acid

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TABLE 16

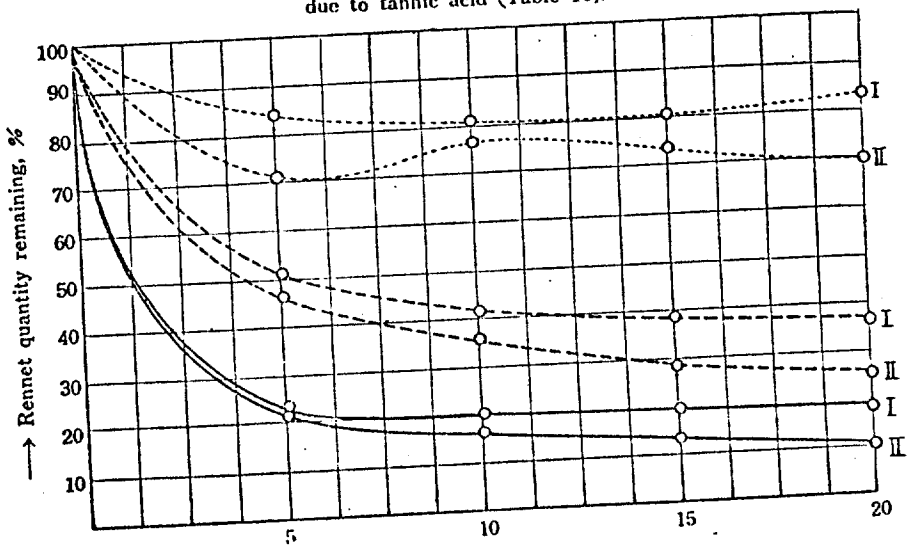
Influence of NaCl upon the decrease in progress in the action of rennet due to tannic acid. Tannic acid: Kahlbaum, used immediately after its dissolving; rennet solution (0.01%), 1 cc+tannic acid solution, 1 cc+milk (1% $CaCl_2$), 1 cc

NaCl Concentration			0.9%		0.45%		0%	
Tannic acid concentration			0.01%	0.02%	0.01%	0.02%	0.01%	0.02%
Time elapsing between mixing rennet and tannin and the addition of milk	5 minutes	Rennet remaining, %	45	21	50	22	83	70
		K I	0.16	0.32	0.14	0.30	0.037	0.071
	10 minutes	Rennet remaining, %	36	16	41	20	80	76
		K I	0.10	0.18	0.089	0.16	0.022	0.027
	15 minutes	Rennet remaining, %	28	13	38	19	80	73
		K I	0.088	0.13	0.065	0.12	0.016	0.022
	20 minutes	Rennet remaining, %	25	9.4	36	18	83	70
		K I	0.072	0.12	0.053	0.089	0.0097	0.019

$$K\,I = \frac{1}{0.4343\,t} \log \frac{a}{a-x}$$

Fig. 11

Influence of NaCl upon the decrease in progress in the action of rennet due to tannic acid (Table 16).



———: 0.9% NaCl – – – –: 0.45% NaCl - - - - - -: 0% NaCl

I, tannic acid: 0.01% II, tannic acid: 0.02%

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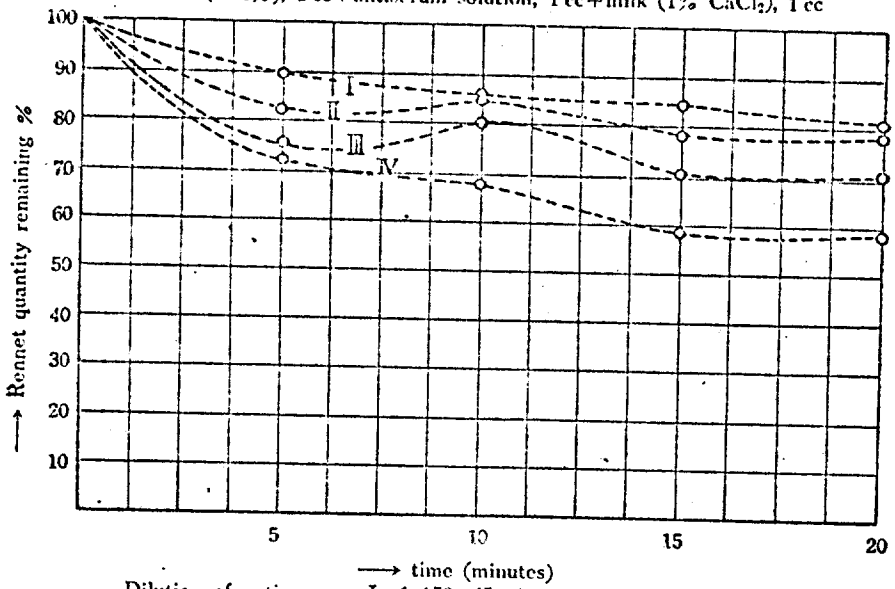
Fig. 12

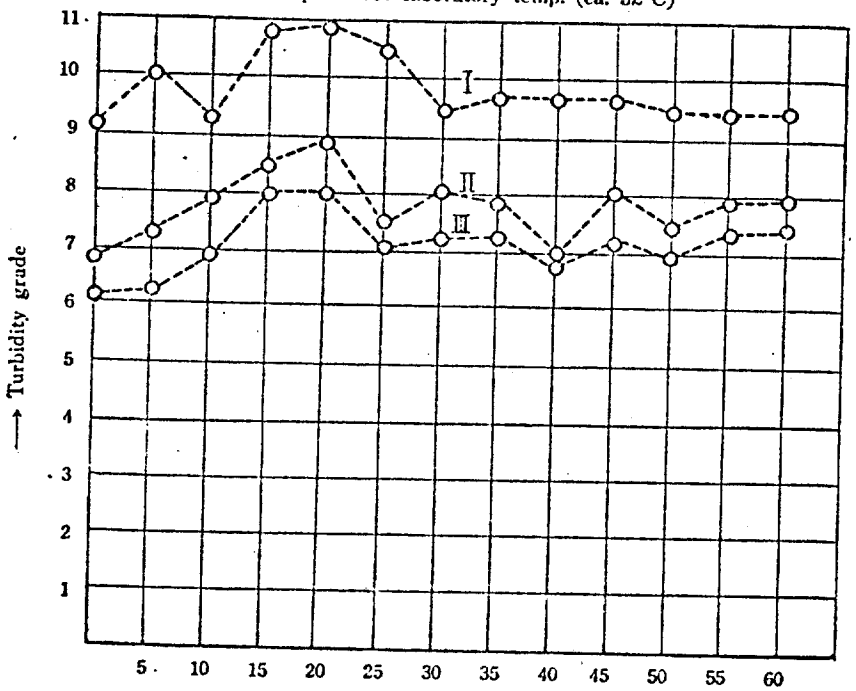
Decrease in progress in the action of rennet due to antirennet serum.
Antiserum: No. 1, temperature: laboratory temp. (ca. 31°C), medium: water; rennet solution (0.01%), 1 cc+antiserum solution, 1 cc+milk (1% $CaCl_2$), 1 cc



Dilution of antiserum: I; 1:150 II; 1:100 III; 1:75 IV; 1:50

Fig. 13

Change in progress in the turbidity grade of ovo-albumine due to tannic acid.
Medium: water, tannic acid: Kahlbaum, ovo-albumine: Merck, temperature: laboratory temp. (ca. 32°C)



time elapsing from the addition of tannic acid to the albumine (minutes)

I, 1% albumine solution: 1% tannic acid solution=3:2
II, " " =4:1
III, " " =9:1

Turbidity grade: that of 1/1000 mol. solution of $BaSO_4$ is taken as turbidity grade 10.

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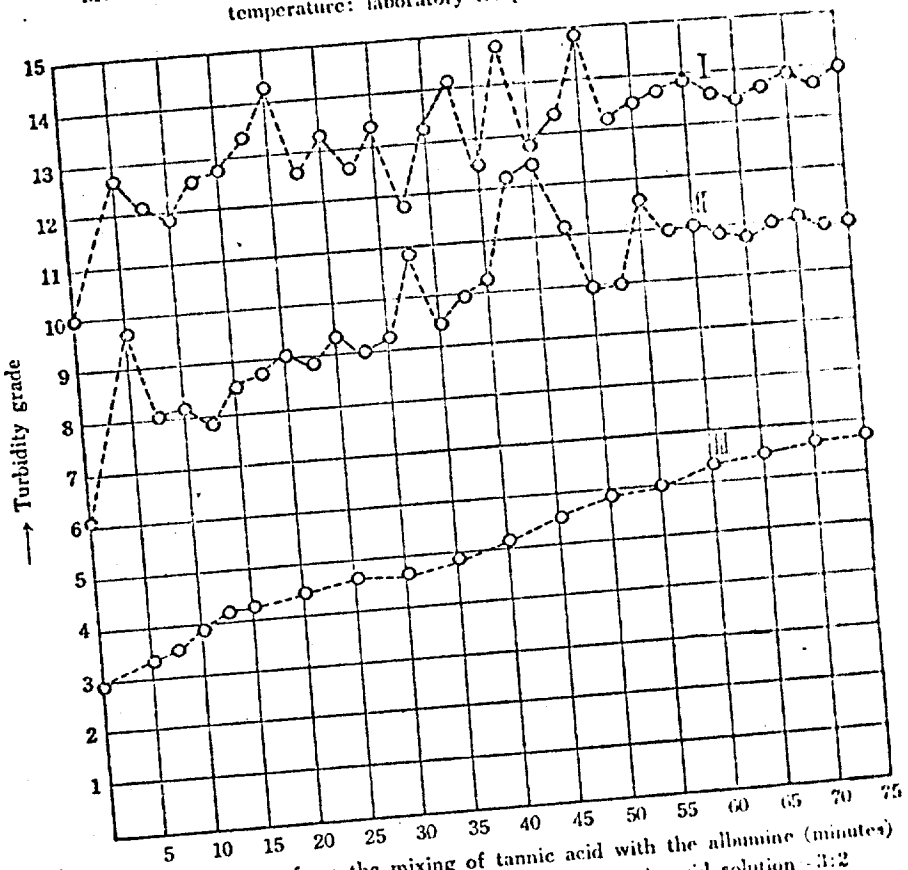
Fig. 14

Change in progress in the turbidity grade of ovo-albumine due to tannic acid. Medium: 0.45% NaCl, tannic acid: Kahlbaum, ovo-albumine: Merck, temperature: laboratory temp. (ca. 32°C)



→ time elapsing from the mixing of tannic acid with the albumine (minutes)

I, 1% albumine 0.9% NaCl solution: 0.01% tannic acid solution = 3:2

II, " " = 4:1

III, " " = 9:1

Turbidity grade: that of 1/1000 mol. solution of $BaSO_4$ is taken as turbidity grade 10.

Fig. 15

Relation between the amount of $HgCl_2$ and the quantity of rennet deprived of its action by it. Rennet solution (0.01%): $HgCl_2$ solution = 1:1

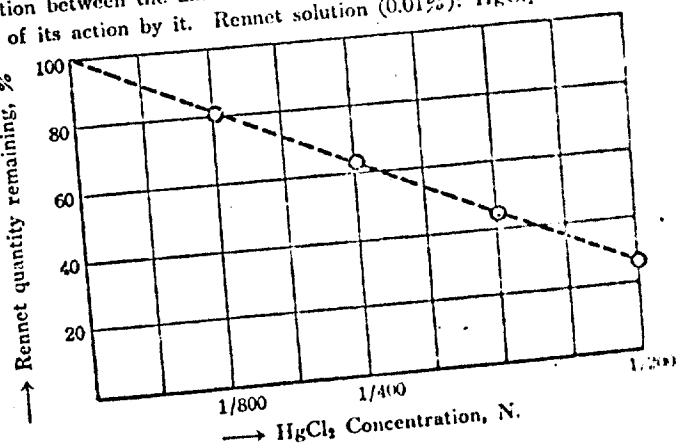


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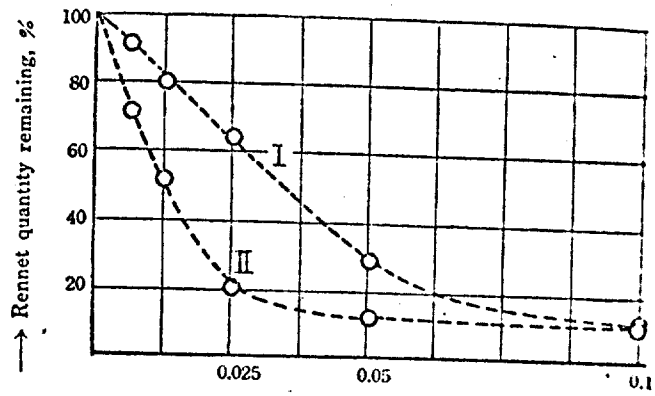
Fig. 16

Relation between the amount of tannic acid and the quantity of rennet deprived of its action by it (see Tables 12 & 14).

Tannic acid: I



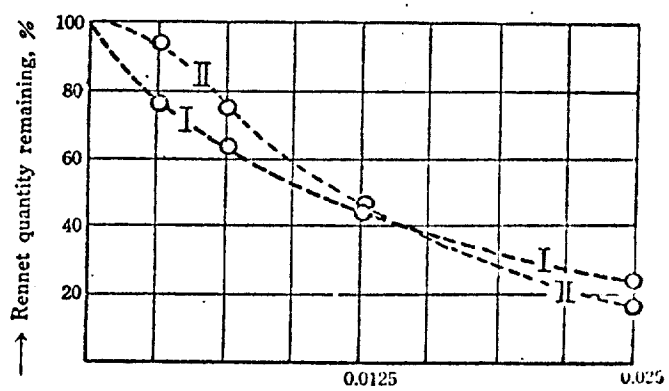
⟶ Concentration of tannic acid, %

I: 0% NaCl, 5 minutes after II: 0.45% NaCl, 5 minutes after

Fig. 17

Relation between the amount tannic acid and the quantity of rennet deprived of its action by it (see Tables 13 & 15)

Tannic acid: II



⟶ Concentration of tannic acid, %

I: 0% NaCl, 5 minutes after II: 0.45% NaCl, 5 minutes after

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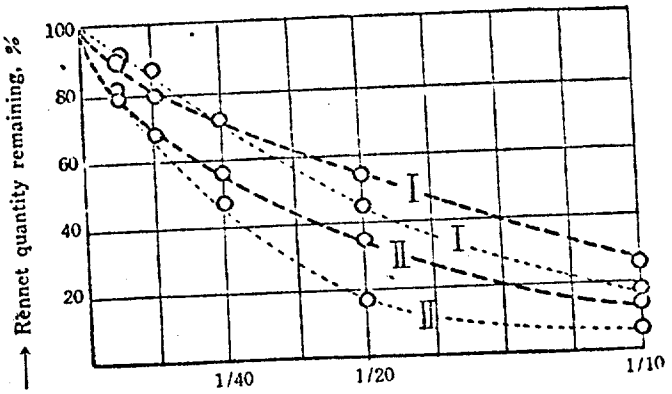
Fig. 18

Relation between the amount of antirennet serum and the quantity of rennet deprived of its action by it (see Tables 6 & 8).

Antiserum: I



——: medium is water

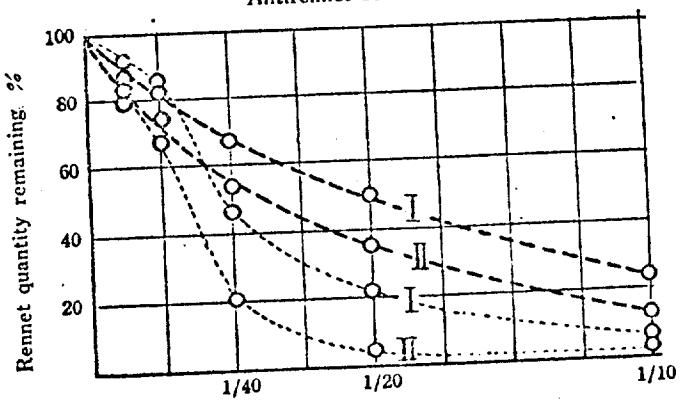
- - - - - - : medium is 0.45% NaCl solution

I: 5 minutes after II: 20 minutes after

Fig. 19

Relation between the amount of antiserum and the quantity of rennet deprived of its action by it (see Tables 7 & 9).

Antirennet serum: II



——: medium is water

- - - - - - : medium is 0.45% NaCl solution

I: 5 minutes after II: 20 minutes after

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Discussion

As I[2] reported previously, tannic acid can imitate the action of antibody such as the action of antiricin serum in promoting the haemoagglutinating property of ricin, and the specific influence of antiurease serum upon the haemoagglutinating property of urease, and, moreover, it can even act as a substitute for antiurease by sometimes promoting the urea-decomposing action of urease, and, on the other hand, sometimes inhibiting it. In this paper I was again able to ascertain that even in the progress of the reaction between antigens and their respective antibodies tannic acid can act as a substitute for an antibody.

By minutely examining the decrease in the action of rennet caused by antirennet serum, it was found that it, as shown in Table 6, tends to decrease constantly, and that the velocity constant, KI, is almost directly proportional to the amount of antibody. For instance, in Table 6, KI at t=5 is 0.35 when the dilution of antiserum is 1:10, 0.16 when the dilution is 1:20, 0.067 when 1:40, 0.028 when 1:80, and 0.019 when 1:160. This is also the case with tannic acid, for instance in Table 13, KI at t=5 is 0.35 when the cencentration of tannic acid is 0.025%, 0.16 when 0.0125%, 0.060 when 0.00625%, and 0.015 when 0.003125%.

Hence, it seems as if the antibody as well as tannic acid acted upon the antigen as a catalyser. It is, however, unreasonable to regard the antibody or tannic acid as a true catalyser in the strict sense. It might rather be permissible to assume that the antigen with which the antibody or tannic acid combined underwent a secondary change following the combination, which might be a denaturation of the protein, the colloidal carrier of the enzyme, and the velocity of such a secondary change could be assumed as increasing in direct proportion to the amount of antibody or tannic acid.

Now, I intend to discuss Fig. 6. In this example, with the addition of the antiserum to the same volume of 0.01% rennet, when the antiserum concentration is over 1:40, the reaction generally comes to an end as the quantity of rennet remaining diminishes to about 1–2%, although the reaction velocity is never the same. Such a remainder of a quantity of rennet can be regarded as due to the rennet action continuing unaffected even when the colloidal carrier undergoes a complete secondary change. On the other hand, however, when the antiserum concentration is 1/160, the reaction tends to finish after depriving the rennet of 60%

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of its action only. This may be regarded as a result of their being too small a quantity of antibody to be distributed to all the molecules of rennet present in order to cause the secondary change. It is, however, quite uncertain whether the number of molecules of the antibody required for causing the secondary change in a molecule of antigen is one or more than one.

At any rate, with too small a quantity of antibody, a part of the rennet molecules only can combine with the antibody so as to undergo the secondary change, the rest of the molecules either have no antibody to combine with or, if a small quantity of antibody remains it is never enough to cause the change in the antigen, and a part of the molecules therefore remain unchanged. When more than the quantity of antibody, sufficient to cause the secondary change in the antigen molecule, combines with the latter, the change is proportional to the antibody combining with the rennet molecule; because of this, antibody appears to be a catalyser, but it is reasonable to consider that it is by no means a true catalyser in the strict sense, as it may be regarded as being consumed by combining with the antigen in the same way as tannic acid.

The secondary change caused in such a manner may perhaps be in its nature a monomolecular reaction. When assumed to be bimolecular the velocity constant has a greater tendency to become a constant than when assumed a monomolecular. This may be, however, due to a decrease in the velocity with time, an effect which is uncertain. It is unreasonable to assume that the diminution in the action of rennet occurs through the combination of every two of the rennet molecules with each other. When observing such a decrease in the reaction velocity when invertase loses its action by heat, Euler and Laurin[23] claimed that it might be due to the dissimilarity in the resistance of invertase molecules.

The great influence of NaCl upon the reaction velocity might be causedt hrough a change in the colloidal state of the rennet brought about by the NaCl. Since, the decrease in progress in the action of rennet in a medium with no NaCl is irregular, a part of the rennet once deprived of its action regaining it later so as to make the reaction curve wavy, it may be possible to assume that the lessening of the action of rennet occurs not only because of a chemical but also owing to some physical factor. This may also be conjectured from the fact that the turbidity produced by adding tannic acid to a protein solution changes with time very markedly as shown in Fig. 13 & 14, since the change in the turbidity grade may be due to the change in the grade of dispersion.

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The argument discussed above may be applied generally to the influence of an antienzyme upon the enzyme action and also to that of antiricin or of antiurease upon the haemoagglutinating property of their respective antigens. Even when the action of antigen is promoted in its combination with the antibody, as in the case of ricin-antiricin, the antigen gradually loses its action with time through the secondary change caused by combination with the antibody. However, whether such an argument is also applicable to toxin-antitoxin or not, can be decided by further experiments.

Summary.

The progress of the reaction between antigen and the antibody was observed in using ricin, urease and rennet as antigens. The progress could be minutly observed, especially when the antigen was rennet. The same phenomena were observable when tannic acid was used instead of antibody. The course of the action of the antibody upon the antigen is also entirely the same as the action of tannic acid upon the antigen.

References.

1) **H. Moriyama:** Jap. Jour. of exp. Medicine, (14), 87, 1936.
2) **H. Moriyama:** J. Shanghai Sci. Inst., Section IV, (3), 93, 1937.
3) **H. v. Euler & I. Laurin:** Hoppe-Seyler's Z. (108), 64, 1919.

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TABLE 2 — USAGE LEVELS REPORTED FOR NAS APPENDIX A SUBSTANCES (GROUP I) USED IN REGULAR FOODS(R)

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO.	NAME	# FIRMS REPORTING	*** USUAL USE *** WTD MEAN, %	*** MAXIMUM USE *** WTD MEAN, %
PROPYL GALLATE (CONT.) NAS 0165 FEMA 2947	24	BEV TYPE II(R)	*	.00001	.00001
	25	NUT PRODS(R)	*	.00085	.00095
	27	GRAVIES(R)	*	.00010	.00010
	31	CHEWING GUM(R)	*	********	********
PROPYLPARABEN NAS 0166 FEMA 2951	01	BAKED GOODS(R)	5	.09697	.09700
	03	OTHER GRAIN(R)	*	.00020	.00030
	04	FATS OILS(R)	*	.03200	.10000
	06	CHEESE(R)	4	.00002	.00002
	07	FROZN DAIRY(R)	*	.00120	.00764
	08	PROCSD FRUT(R)	*	.01000	.01000
	14	PROCSD VEGS(R)	*	.10000	.10000
	16	SOFT CANDY(R)	*	.00610	.00432
	19	SWEET SAUCE(R)	*	.00000	.00005
	20	GELATIN PUD(R)	*	.00030	.00096
	23	BEV TYPE I(R)	*	.00006	.00115
	24	BEV TYPE II(R)	*	.00005	.00200
	33	SUGAR SUBS(R)	*	.02500	.02500
	48	SEAS FLAVRS(R)	*	.03100	.03100
PYRIDOXINE HYDROCHLORIDE NAS 0167	01	BAKED GOODS(R)	4	.00099	.00155
	02	BREAK CERLS(R)	8	.00286	.00482
	05	MILK PRODS(R)	5	.00277	.00360
	10	MEAT PRODS(R)	*	.00082	.00146
	22	SNACK FOODS(R)	*	.00029	.00029
	23	BEV TYPE I(R)	*	.00000	.00000
	26	RECONST VEG(R)	*	.00040	.00040
	28	IMIT DAIRY(R)	*	.00200	.00200
RENNET NAS 0168	05	MILK PRODS(R)	*	.00020	.00030
	06	CHEESE(R)	11	.02131	.03169
	07	FROZN DAIRY(R)	*	.00010	.00010
	20	GELATIN PUD(R)	*	.00270	.00400
RIBOFLAVIN NAS 0169	01	BAKED GOODS(R)	20	.00023	.00045
	02	BREAK CERLS(R)	11	.00206	.00542
	03	OTHER GRAIN(R)	14	.00030	.00043
	05	MILK PRODS(R)	7	.00050	.00074
	08	PROCSD FRUT(R)	4	.00029	.00029
	10	MEAT PRODS(R)	*	.00071	.00071
	11	POULTRY(R)	*	.00300	.00300
	13	FISH PRODS(R)	*	.00300	.00300
	15	CONDM RELSH(R)	*	.00016	.00017

TABLE 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE - - - SEE EXPLANATORY NOTES IN EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	********** (AGE)	POSSIBLE DAILY INTAKE, MG. ********** AVERAGE	HIGH A	HIGH B
PYRIDOXINE HYDROCHLORIDE NAS 0167	10 MEAT PRODS(R)	*	0-5 MO.	.009020	.023780	.016880
			6-11 MO.	.169740	.457560	.302220
			12-23 MO.	.247640	.425580	.440920
			2-65+ YR.	.642880	1.066820	1.144540
PYRIDOXINE HYDROCHLORIDE NAS 0167	22 SNACK FOODS(R)	*	0-5 MO.	**********	.006270	**********
			6-11 MO.	.001160	.003190	.001160
			12-23 MO.	.003190	.009990	.003190
			2-65+ YR.	.003770	.010730	.003770
PYRIDOXINE HYDROCHLORIDE NAS 0167	23 BEV TYPE I(R)	*	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	.000000	.000000	.000000
			12-23 MO.	.000000	.000000	.000000
			2-65+ YR.	.000000	.000000	.000000
PYRIDOXINE HYDROCHLORIDE NAS 0167	26 RECONST VEG(R)	*	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	.000000	.000000	.000000
			12-23 MO.	.000000	.000000	.000000
			2-65+ YR.	.000800	.002400	.000800
PYRIDOXINE HYDROCHLORIDE NAS 0167	28 IMIT DAIRY(R)	*	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	.000000	.000000	.000000
			12-23 MO.	.010000	.010000	.010000
			2-65+ YR.	.010000	.010000	.010000
PYRIDOXINE HYDROCHLORIDE NAS 0167	31 BAKED GOODS(B)	*	0-5 MO.	.000000	.010000	.000000
			6-11 MO.	.015000	.010000	.017000
			12-23 MO.	**********	**********	**********
PYRIDOXINE HYDROCHLORIDE NAS 0167	82 CEREALS(B)	*	0-5 MO.	.024300	.041940	.074[illegible]
			6-11 MO.	.016560	.043200	.0[illegible]
			12-23 MO.	.003600	.016200	.011[illegible]
PYRIDOXINE HYDROCHLORIDE NAS 0167	33 FORMULAR(B)	7	0-5 MO.	.234990	.430500	16.[illegible]
			6-11 MO.	.047[illegible]	.22[illegible]	[illegible]
			12-23 MO.	.015400	.004300	1.[illegible]
PYRIDOXINE HYDROCHLORIDE NAS 0167	ALL CATEGORIES ********** ********** **********	22	0-5 MO.	.473710	.715400	16.452160
			6-11 MO.	2.896060	11.32[illegible]	7.[illegible]
			12-23 MO.	3.061490	7.66[illegible]	[illegible]
			2-65+ YR.	3.089860	7.9[illegible]	5.67[illegible]
PENNET NAS 0168	05 MILK PRODS(R)	*	0-5 MO.	.010800	.009000	.016200
			6-11 MO.	.124800	.600200	.107200
			12-23 MO.	.109000	.34[illegible]	.163[illegible]
			2-65+ YR.	.079000	.241200	.11[illegible]

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[illegible] 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL SURVEY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE - - - SEE EXPLANATORY NOTES IN [illegible]

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	********** POSSIBLE DAILY INTAKE, MG. ********** (AGE)	AVERAGE	HIGH A	HIGH B
RENNET NAS 0168	06 CHEESE(R)	11	0-5 MO.	**********	.021310	**********
			6-11 MO.	.578370	2.067070	[illegible]
			12-23 MO.	1.662180	4.73[illegible]	2.4[illegible]
			2-65+ YR.	2.[illegible]	[illegible]	[illegible]
RENNET NAS 0168	07 FROZN DAIRY(R)	*	0-5 MO.	.001000	.004100	[illegible]
			6-11 MO.	.009500	.026400	[illegible]
			12-23 MO.	.014400	.033000	.014400
			2-65+ YR.	.025600	.061700	.025600
RENNET NAS 0168	20 GELATIN PUD(R)	*	0-5 MO.	.054000	.072000	[illegible]
			6-11 MO.	.[illegible]	1.[illegible]	[illegible]
			12-23 MO.	.372600	.907200	[illegible]
			2-65+ YR.	.550820	1.417500	.816000
RENNET NAS 0168	ALL CATEGORIES ********** ********** **********	12	0-5 MO.	.065900	.[illegible]	[illegible]
			6-11 MO.	1.050270	3.741270	1.[illegible]
			12-23 MO.	2.150100	6.[illegible]	3.[illegible]
			2-65+ YR.	2.650540	6.[illegible]	3.[illegible]
RIBOFLAVIN NAS 0169	01 BAKED GOODS(R)	20	0-5 MO.	.007820	.01[illegible]	[illegible]
			6-11 MO.	.0[illegible]	.119140	.11[illegible]
			12-23 MO.	.12[illegible]	.20[illegible]	[illegible]
			2-65+ YR.	.313560	.460740	.617400
RIBOFLAVIN NAS 0169	02 BREAK CERLS(R)	11	0-5 MO.	.012360	.035000	.03[illegible]
			6-11 MO.	.4[illegible]	1.[illegible]	[illegible]
			12-23 MO.	.[illegible]	1.[illegible]	[illegible]
			2-65+ YR.	.412[illegible]	1.[illegible]	[illegible]
RIBOFLAVIN NAS 0169	03 OTHER GRAIN(R)	14	0-5 MO.	.001500	.00[illegible]	.002150
			6-11 MO.	.029100	.0[illegible]	.041[illegible]
			12-23 MO.	.046200	.118700	[illegible]
			2-65+ YR.	.08[illegible]	.1[illegible]	.11[illegible]
RIBOFLAVIN NAS 0169	05 MILK PRODS(R)	7	0-5 MO.	.027000	.0[illegible]	[illegible]
			6-11 MO.	.312000	1.500500	[illegible]
			12-23 MO.	.272500	.072000	[illegible]
			2-65+ YR.	.197000	.[illegible]	[illegible]
RIBOFLAVIN NAS 0169	08 PROCSD FRUT(R)	4	0-5 MO.	.013630	.0[illegible]	[illegible]
			6-11 MO.	.150220	.374100	.1[illegible]220
			12-23 MO.	.291740	.579130	.291740
			2-65+ YR.	.343070	.726740	.343070

TABLE 11, PART A -- ANNUAL POUNDAGE DATA FOR NAS APPENDIX A SUBSTANCES (GROUPS I & II)

SUBSTANCE NAME (SURVEY NO.)	# REPORTS TO NAS 1960/1970	POUNDAGE REPORTED TO NAS (MATCHING REPORTS FOR BOTH YEARS) 1960	1970	TOTAL 1970 POUNDAGE REPORTED TO NAS	# REPORTS TO FEMA	POUNDAGE REPORTED TO FEMA-- 1970 ONLY	TOTAL 1970 POUNDAGE NAS + FEMA
PROPIONIC ACID NAS 0163 FEMA 2924	*/ *	4,000	26,500	26,500	22	3,667	30,167
PROPYLENE GLYCOL NAS 0164 FEMA 2940	42/ 50	1,412,949	1,448,542	1,597,910	92	4,105,985	5,703,895
PROPYL GALLATE NAS 0165 FEMA 2947	13/ 15	80,901	96,420	97,020	7	40,126	137,146
PROPYLPARABEN NAS 0166 FEMA 2951	12/ 12	132	3,978	3,978	5	53	4,031
PYRIDOXINE HYDROCHLORIDE NAS 0167	19/ 20	2,400	16,834	17,314			17,314
RENNET NAS 0168	9/ 12	96,600	220,729	231,371			231,371
RIBOFLAVIN NAS 0169	39/ 45	73,800	113,970	121,105			121,105
RIBOFLAVIN 5-PHOSPHATE NAS 0170	*/ *	33	41	41			41
SACCHARIN NAS 0171 FEMA 3672	5/ 6	99	1,360	1,434	*	1,207	2,641
SILICA AEROGEL NAS 0174 FEMA 3506	14/ 16	66	23,277	29,627	*	6,666	36,293
SODIUM ACETATE NAS 0175 FEMA 3024	12/ 12	109,200	199,650	199,650	4	10,716	210,366
SODIUM ACID PYROPHOS NAS 0176	28/ 34	6,691,400	19,704,938	20,883,293			20,883,293
SODIUM ALGINATE NAS 0177 FEMA 2015	16/ 19	745,867	561,331	570,971	19	84,974	655,945
SODIUM ALUMINOSILICATE NAS 0178	17/ 20	3,305,600	4,352,536	4,591,182			4,591,182
SODIUM ALUMINUM PHOS NAS 0179 FEMA 3657	19/ 26	7,470,500	21,183,812	23,305,687	*	233	23,305,920

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-- ANNUAL POUNDAGE DATA FOR NAS APPENDIX A SUBSTANCES (GROUPS I & II)

SUBSTANCE NAME (SURVEY NO.)	# REPORTS TO NAS 1960/1970	POUNDAGE REPORTED TO NAS (MATCHING REPORTS FOR BOTH YEARS) 1960	1970	TOTAL 1970 POUNDAGE REPORTED TO NAS	# REPORTS TO FEMA	POUNDAGE REPORTED TO FEMA-- 1970 ONLY	TOTAL 1970 POUNDAGE NAS + FEMA
RENNET NAS 0168	9/ 12	96,600	220,729	281,371			281,371

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-- USAGE LEVELS REPORTED FOR NAS APPENDIX A SUBSTANCES (GROUP I) USED IN REGULAR FOODS(R)

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO.	NAME	# FIRMS REPORTING	*** USUAL USE *** WTD MEAN, %	*** MAXIMUM USE *** WTD MEAN, %
RENNET NAS 0168	05	MILK PRODC(R)	*	.00020	.00030
	06	CHEESE(R)	11	.02131	.02169
	07	FROZN DAIRY(R)	*	.00010	.00010
	20	GELATIN PUD(R)	*	.00270	.00400

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ON THE APPEARANCE OF RENNIN DURING THE FETAL LIFE

Ch. Procher, J. Voron and A. Tapernoux

In the previous two reports[1)], we showed that lactase on the one hand and pancreatic trypsin, amylase, and lipase, and pepsin and erepsin on the other hand appeared during the fetal life. Today we are concerned more particularly with rennin. It is not the moment to ask the question whether rennin and pepsin are two different enzymes or, on the contrary, whether it is a question of a single enzyme. This greatly discussed point will be the subject of a particular paper. Today we will deal with the appearance of rennin in the stomach of the fetus. As in our previous notes, we were concerned with the calf fetus taken in a perfectly fresh state from the slaughterhouse, but we could have also used the human fetus. We already pointed out the problem involved in determining exactly the age of the calf fetus. We were better informed as to the age of the human fetus.

The widely opened stomachs were macerated for 24 hours at the laboratory temperature in hydrochloric water at 4 per 1,000, which appeared to be the best solutions since hydrochloric water at 2 per 1,000 and hydrochloric water at 6 per 1,000 produce a less active maceration. We heated 10 cc of milk to 40° and then poured in a drop of the hydrochloric stomach maceration. After homogeneous mixing, using a chronometer computed in seconds, we measured how much time was required to curdle the milk. It was considered advisable to use normal droppers, without which the weights of the drops could vary a great deal from each other and, under these conditions, the measurements would run the risk of not being comparable.

Sixteen calf fetuses provided us with macerations which, under the conditions indicated above, always succeeded in curdling the milk – which proved that rennin was present in the mucosa of these stomachs:

	Ages	Weight of the rennet	Duration of the curdling
.....	Difficult to specify	30 centigr.	42 minutes
2	50 days	4 gr.	32 minutes
3	55 days	6 gr.	15 minutes
4	70 days	17 gr.	12 minutes
5	90 days	15 gr.	6 minutes
6	95 days	30 gr.	14 minutes
7	115 days	80 gr.	3 minutes
8	135 days	90 gr.	2 minutes

Another series of tests involving eight fetuses of different ages gave us similar results. The influence of the fetal age is regularly marked here, and curdling takes place more quickly and the curd is firmer as the age of the fetus increases.

The few human fetuses which were available to us ranged in age as follows: the first was 6 months old (two twins), the second 7 months (also two twins), the third 8 months, and the fourth was a term baby. The effect of the hydrochloric macerations gave results similar to those which we obtained with the calf. But in order to have a very distinct curdling, it is nevertheless necessary to increase the quantity of hydrochloric maceration. With 5 drops per 10 cc of milk, the maceration of the stomachs of the two 6-month-old twins caused curdling within 12 minutes; that of the 7-month-old twins required more than an hour. The 8-month-old fetus caused the milk to curdle, always with five drops, within one minute and fifty seconds. Finally, the term fetus gave a maceration which – always at the same dose – caused the milk to curdle within a few seconds. Except for the case of the 7-month-old fetus (the stomachs were hemorrhagic, which undoubtedly explains the failure), as with the calf fetus, we see that curdling is all the faster with the human fetus stomach maceration as the age increases.

The results summarized in this report, as well as the two preceding reports, corroborate those described in the recent book of Feldman[1]. In the area of

comparative physiology, similar investigations are capable of providing interesting data. We hope to continue such research towards the objective of clarifying certain points concerning the physiology of the intestinal tract in the newborn.

(Chemistry Laboratory of the Veterinary School of Lyon)

REFERENCE

1) Feldman: Ante-natal and post-natal Child Physiology pure and applied.

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prouver qu'il ne s'agit pas là d'une manifestation de l'activité cellulaire, mais plutôt de phénomènes de dégénérescence. Nous n'avons pas constaté comme Plato, un rapport inverse entre la graisse interstitielle et la graisse intra-tubulaire.

6° Nous n'avons pas trouvé de corps bi-réfringents vrais, mais dans un cas aux points où la graisse était très abondante des cristaux en aiguille ayant l'aspect des cristaux d'acides gras, qu'on rencontre dans le tissu adipeux normal et donnant de la biréfringence.

(Laboratoire d'histologie de la Faculté de médecine de Lyon).

Sur l'apparition de la présure pendant la vie fœtale,

par Ch. Porcher, J. Voron et A. Tapernoux.

Dans deux notes précédentes (1), nous avons montré, d'une part, que la lactase et, d'autre part, que la trypsine, l'amylase et la lipase pancréatiques, la pepsine et l'érepsine apparaissaient pendant la vie fœtale. Aujourd'hui nous allons nous occuper plus spécialement de la présure. Ce n'est pas le moment de poser la question de savoir si présure et pepsine sont deux ferments différents, ou, au contraire, s'il ne s'agit que d'un même ferment. Ce point très discuté fera ailleurs l'objet d'un travail particulier. Nous nous occuperons aujourd'hui de l'apparition de la présure dans l'estomac du fœtus. Nous nous sommes adressés, comme dans nos notes antérieures, aux fœtus de veau pris dans un état de parfaite fraîcheur à l'abattoir, mais nous avons pu disposer également de fœtus humains. Nous avons déjà signalé la difficulté qu'il y a d'être fixé exactement sur l'âge du fœtus de veau ; nous étions mieux renseignés sur l'âge du fœtus humain.

Les estomacs largement ouverts étaient mis à macérer pendant 24 heures à la température du laboratoire dans de l'eau chlorhydrique à 4 p. 1.000, qui paraît être la solution optima, car l'eau chlorhydrique à 2 p. 1.000 et l'eau chlorhydrique à 6 p. 1.000, donnent une macération moins active. Nous avons porté 10 c.c. de lait à 40°, puis nous y avons versé une goutte de la macération chlorhydrique stomacale ; après mélange homogène, nous avons mesuré avec le chronomètre à secondes, au bout de combien de temps, le lait faisait prise. Il est indiqué d'utiliser le compte-gouttes normal, faute de quoi les poids des gouttes pourraient être très différents les uns des autres et, dans ces conditions, les mesures risqueraient de ne pas être comparables.

(1) C. R. de la Soc. de biologie, t. XXXIII, p. 470 et 619, 1920.

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Dans deux notes précédentes (1), nous avons montré, d'une part, que la lactase et, d'autre part, que la trypsine, l'amylase et la lipase pancréatiques, la pepsine et l'érepsine apparaissaient pendant la vie fœtale. Aujourd'hui nous allons nous occuper plus spécialement de la présure. Ce n'est pas le moment de poser la question de savoir si présure et pepsine sont deux ferments différents, ou, au contraire, s'il ne s'agit que d'un même ferment. Ce point très discuté fera ailleurs l'objet d'un travail particulier. Nous nous occuperons aujourd'hui de l'apparition de la présure dans l'estomac du fœtus. Nous nous sommes adressés, comme dans nos notes antérieures, aux fœtus de veau pris dans un état de parfaite fraîcheur à l'abattoir, mais nous avons pu disposer également de fœtus humains. Nous avons déjà signalé la difficulté qu'il y a d'être fixé exactement sur l'âge du fœtus de veau ; nous étions mieux renseignés sur l'âge du fœtus humain.

Les estomacs largement ouverts étaient mis à macérer pendant 24 heures à la température du laboratoire dans de l'eau chlorhydrique à 4 p. 1.000, qui paraît être la solution optima, car l'eau chlorhydrique à 2 p. 1.000 et l'eau chlorhydrique à 6 p. 1.000, donnent une macération moins active. Nous avons porté 10 c.c. de lait à 40°, puis nous y avons versé une goutte de la macération chlorhydrique stomacale ; après mélange homogène, nous avons mesuré avec le chronomètre à secondes, au bout de combien de temps, le lait faisait prise. Il est indiqué d'utiliser le compte-gouttes normal, faute de quoi les poids des gouttes pourraient être très différents les uns des autres et, dans ces conditions, les mesures risqueraient de ne pas être comparables.

(1) C. R. de la Soc. de biologie, t. XXXIII, p. 430 et 619, 1920.

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16 fœtus de veaux nous ont donné des macérations qui, dans les conditions indiquées ci-dessus sont toujours parvenues à cailler le lait, ce qui était la preuve de la présence de la présure dans la muqueuse de ces estomacs :

	Ages	Poids de la caillette	Durée de la prise
1	Difficile à préciser	30 centigr.	42 minutes
2	50 jours	4 gr.	32 —
3	55 —	6 —	15 —
4	70 —	17 —	12 —
5	90 —	15 —	6 —
6	95 —	30 —	14 —
7	115 —	80 —	3 —
8	135 —	90 —	2 —

Une autre série de recherches portant sur 8 fœtus d'âges différents nous a donné des résultats analogues. L'influence de l'âge du fœtus est ici régulièrement marquée, et la prise est d'autant plus rapide, et le caillot également d'autant plus ferme, que le fœtus est d'un âge plus avancé.

Les quelques fœtus humains dont nous avons pu disposer étaient âgés, le premier de 6 mois (2 jumeaux); le second de 7 mois (2 jumeaux également), le troisième de 8 mois, et le quatrième était à terme. L'action des macérations chlorhydriques a donné des résultats analogues à ceux que nous avons obtenus avec le veau. Mais pour avoir une prise très nette, il faut toutefois augmenter la quantité de macération chlorhydrique. Avec 5 gouttes pour 10 c.c. de lait, la macération des estomacs des deux jumeaux de 6 mois a fait faire prise en 12 minutes ; celle des jumeaux de 7 mois n'avait pas fait prise après une heure. Le fœtus de 8 mois a fait cailler le lait, toujours avec 5 gouttes, en une minute 50 secondes ; enfin, le fœtus à terme a donné une macération qui, toujours à la même dose, a fait cailler le lait en quelques secondes. Le cas des fœtus de 7 mois mis à part (les estomacs étaient hémorragiques, ce qui nous explique sans doute l'insuccès obtenu), nous voyons, comme pour le fœtus bovin, que la prise est d'autant plus rapide avec la macération d'estomac de fœtus humain, que celui-ci est plus âgé.

Les résultats résumés dans cette note, ainsi que dans les deux précédentes, viennent corroborer ceux qui sont signalés dans le livre récent de Feldman (1). Sur le terrain de la physiologie comparée, de semblables recherches sont susceptibles de fournir des renseignements intéressants ; nous comptons les continuer dans le but d'élucider certains points de la physiologie du tractus intestinal du nourrisson.

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(1) Feldman. Antenatal and post-natal Child Physiology pure and applied.