The Purification and Crystallization of Rennin

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The crystallization of urease by Sumner (1926) has been followed in the past few years by successful attempts to bring other enzymes into the crystalline condition. There has been some tendency to regard the crystalline form as an indication of a high degree of purity in the preparations concerned, but, as shown by Pirie (1940), this is theoretically unsound. Langmuir (1938) has pointed out that colloids may form coacervates having regular molecular orientation in one, two, or three dimensions. He regards some protein crystals as solid tactoids belonging to one of these three classes and holding a position intermediate between solutions and true crystals. Tactoids will thus tend to be purer than the solutions from which they are formed, but the purifying effect of precipitation will be less than in the formation of true crystals. Nevertheless, though crystalline form is not of itself complete and final evidence of either purity or true crystallinity, it is a matter of experience that unpurified enzymes cannot be crystallized and that quite small quantities of some impurities prevent crystallization (e.g. 1% of mucin prevents the crystallization of pepsin) (Northrop, 1939). It is therefore safe to regard an enzyme preparation which can be crystallized as likely to be purer than one which cannot.

The crystallization of rennin has previously been attempted by Tauber & Kleiner (1932) and by Hankinson (1943). The former obtained a small quantity of minute spheres by a method which they consider impracticable. The latter claims greater success in the crystallization and shows a photograph. Hankinson states that photography was difficult owing to Brownian movement of the particles, and to their gradual disappearance when placed on the microscope slide.

The present communication describes, after summarizing the experience gained in the preliminary experiments, the preparation and some properties of the rennin crystals shown in Pl. 1, Figs. 1, 2.

METHODS

(1) *Measurement of activity.* Throughout the preparations the activities of the various fractions were determined by measuring the time required for 1 ml. of the diluted enzyme to clot 10 ml. of standard substrate at 30 (±0·1)°. The substrate was made by dissolving 12 g. of good-quality spray-dried skim milk powder in 100 ml. of 0·02 N-$CaCl_2$. All the minor details of the technique of measurement, e.g. time of keeping milk at 30° before adding rennin, method of determining end-point, etc., were rigidly standardized. The end-point was observed by allowing a thin film of the milk to flow from a glass rod down the side of the test-tube containing the milk. When clotting occurred, the almost invisible film broke into a number of white particles. Reproducible results were obtainable as long as clotting times were kept within 4–6 min. The activity of the solution was then obtained from the formula

$$\text{Rennin activity} = \frac{100d}{t} \text{ Rennin units (R.u.)/ml.},$$

where d = dilution of the rennin before adding to the milk, and t = clotting time in sec.

(2) *Determination of purity.* Towards the end of the first preparation, figures for the comparative purity of the fractions were obtained by determining the activity per unit of nitrogen, but later, activities per unit of organic dry weight were used since they indicated also comparative freedom from non-nitrogenous impurities.

The dry-weight measurements were made by weighing on the microbalance the residue remaining after 16–20 hr. drying at 98° of 0·1 ml. of the rennin solution, containing NaCl as the only added inorganic material, in a small weighed platinum dish. The solutions were subjected to a preliminary evaporation on a steam-heated platinum plate before placing in the oven at 98°. The NaCl content was determined on the same sample by careful ashing with a drop of concentrated H_2SO_4 and calculating back to chloride. It was easy to obtain the enzyme free from other electrolytes when it was in the crystalline or granular state merely by washing the filtered solid with saturated NaCl solution. Five rapid washings at the pump sufficed for the complete removal of sulphate (as judged by the addition of $BaCl_2$ to a solution of the washed precipitate) from a preparation crystallized in half-saturated $MgSO_4$. Although only empirical, since the precautions mentioned by Chibnall, Rees & Williams (1943) concerning the drying of proteins were not carried out, the figures obtained were reproducible, usually to within ±1%, and were thus of sufficient accuracy for following the preparation. The purity of the fraction could then be expressed as the number of units of rennin activity per mg. of organic dry weight.

(3) *Method of obtaining granular precipitates.* Granular precipitates could be obtained before the rennin was pure enough to crystallize by adding the salting-out agent sufficiently slowly. Saturated solutions of salts were used, the rate of addition being controlled by displacement by a weight descending into the salt solution from a shaft connected to the winding spindle of an ordinary clock. The rate of displacement could be varied by varying either the diameter of the weight or that of the rotating shaft.

(4) *Proteolytic activity.* This was determined by a method similar to that of Anson (1938), a photoelectric absorptiometer being used for the colour readings. The pH values of the haemoglobin solutions were adjusted by adding previously determined quantities of HCl, and finally measured with the glass electrode after allowing 1 or 2 days at 2° for the establishment of equilibrium.

PREPARATIONS

Small scale (preliminary). A crude rennin solution was obtained by extracting eight dried salted calf abomasa with 10% NaCl for about 20 hr. with continuous stirring. The rennin from just over 3 l. of the extract was adsorbed on alumina precipitated *in situ* by the addition of a solution of 60 g. of potash alum followed by enough NaOH solution to bring the pH back to 6. After the alumina complex had been filtered and washed with water, the rennin was eluted with 1 l. of 0·2 M-sodium phosphate, pH 6·6. This process gave a considerable increase in purity without the necessity of preparing the special alumina usually employed for the adsorption of enzymes. [See, for example, Lüers & Bader (1927).] (It is possible to elute some rennin with 5–10% NaCl at a pH of 6·8. This appears to give a greater increase in purity, but the losses due to uneluted rennin may be very high.) The eluate was precipitated by saturating with NaCl, redissolved in 240 ml. of distilled water, and again salted out. A repetition of the adsorption and elution procedure and another salting out finally gave 10 ml. of a suspension with a total activity equivalent to slightly less than that of a normal dried abomasum. Alkali was added to this suspension until solution occurred (pH below 7), followed by sufficient acid to produce a faint turbidity. On warming, this turbidity increased, and therefore the suspension was cooled to about $-5°$, when a clear solution was obtained; as the temperature rose slowly an active slightly granular precipitate was formed. A little more acid was added and the process repeated twice. A fourth precipitate, obtained in the same way but with gentle agitation during the warming period, consisted of larger granules. In all, granular precipitates with an activity amounting to about a thirtieth of that of a dried abomasum were obtained.

The above process was repeated, except for minor differences, on approximately four times the previous quantities, until the 'crystallizing' stage was reached, when precipitation by temperature change was replaced by salting out through a rotating semipermeable membrane as suggested by McMeekin (1939). This resulted in a granular precipitate containing a proportion of spheroids. Recrystallization by the slow addition of $MgSO_4$ (saturated solution), gave a highly active precipitate consisting almost entirely of spheroids. Northrop (1939) has pointed out that enzymes are precipitated as spheres either when they are not quite pure enough to crystallize properly, or when the conditions are not suitable for crystallization. The above results therefore justified further experimentation.

Large scale. The first of these has been briefly reported (Berridge, 1943). Commercial rennet equivalent to about 80 l. of the usual cheese-making rennet was used as the starting material. The early part of the preparation was similar to that already described. The later part consisted in fractional precipitation by the slow addition of saturated magnesium or ammonium sulphate solutions, repeating the fractionations, and recombining the various fractions according to the usual fractional crystallization technique. The fate of the rennin at each stage of the process was followed at first by activity and nitrogen determinations, later by activity and organic dry-weight determinations. Finally, microscopic plates of crystalline appearance were obtained. It is unnecessary to describe this preparation in detail since the final one was simpler and more successful. During the first preparation, however, a number of interesting observations were made. (1) At the commencement of fractionation the least soluble, i.e. most easily salted-out fraction, was of low activity, and dissolved in very little water to give an extremely viscous solution. This was not examined but was presumably mucin. Since the mixture from which it was obtained was already considerably purified, it is possible that the preparations of earlier workers have contained this substance, which, if it be mucin, might account both for the high sulphur content reported [e.g. 1·19% by Tauber & Kleiner (1932) and 1·46% by Hankinson (1943)] and for the difficulty in crystallization. (2) Towards the end of the preparation only a comparatively slight further increase in purity could be effected by fractional adsorption on to alumina precipitated *in situ*, followed by complete elution with phosphate buffer. Thus the activity of the rennin adsorbed by 0·1% of alumina from a solution in about 400 ml., eluted and precipitated, was 130 units/mg. dry weight (R.u./mg.). That adsorbed by 1·0% of alumina from the filtrate, eluted and precipitated, had a purity of 141 R.u./mg., while that remaining in solution was 142 R.u./mg. (3) Treatment with commercial adsorption and decolorizing carbons* was without effect on crystallizability. (4) Adjusting a solution of partly purified rennet to pH 3·2 caused precipitation and a loss of 30% of the total activity in a day. The crystallizability of the remainder was not improved by this treatment. Fractionation by acid precipitation was of no value at this stage. (5) There was some indication that previous precipitation in the amorphous condition favoured subsequent crystalliza-

* I am indebted to Sutcliffe Speakman and Co. Ltd. for kindly supplying samples of carbons.

tion. (6) Crystallization by temperature change was always less successful than that by the slow addition of saturated salt solutions. (7) Successful crystallization was dependent on a number of factors not completely understood. For example, on dissolving rennin consisting of approximately 50% crystalline plates and 50% spheres (prepared by the slow addition of saturated $MgSO_4$ to a rennin solution), and precipitating by salting out through a rotating membrane, only spheroids were obtained. Reversion to the previous method gave about 3% plates. The more concentrated the rennin solution the smaller the proportion of crystals. The most important factor subsequently discovered was the very low solubility of the crystals. The solutions used at this stage were highly supersaturated.

A second large-scale preparation, in which wasteful adsorption processes were not used, and in which an attempt was made to keep the less pure fractions as small as possible, gave no improvement in yield and little in crystallinity. At the later stages destruction occurred as rapidly as purification so that increase in purity could only be effected by discarding large quantities of material. Even when this was done the requisite conditions for crystallization could not be found—the results of the previous preparation could not be repeated. Finally, a very few apparently perfectly crystalline rectangular plates, large enough to be visible to the naked eye, were obtained by filtering a saturated rennin solution into a vessel seeded with a very little solid $MgSO_4$ and rennin spheroids. There was too little of these crystals to be of any use, and after a few days the sample set aside for photography dissolved and reprecipitated as spheroids, while the sample used to seed a crystallization became covered with spheroids. Attempts to copy the conditions of filtration into a seeded vessel on a larger scale gave an increase in quantity and size of crystals, but the latter were very poor in quality, containing many rounded angles, and were mixed with numerous spheroids.

It seems probable that the conditions for crystallization depend partly on the impurities present, and that although this preparation was comparatively pure only the peculiar conditions described sufficed to prevent co-precipitation of the rennin and impurities.

Final preparation

In contrast to the earlier preparations this one was carried out with coarser fractionation so that larger impure fractions were discarded. The material was divided into four portions according to solubility in NaCl, the two middle portions combined, impurities adsorbed on to alumina, and the remaining rennin crystallized.

The preparation is summarized in the accompanying scheme (p. 182), and a detailed description is given below. Thymol, used in conjunction with the high salt concentrations and low temperatures, proved an effective preservative.

Precipitated commercial rennet* (equivalent to about 100 gal. of ordinary cheese-making rennet), specially prepared from fresh abomasa, was the starting material. Half of it was dissolved in ~40 l. of tap water, giving a solution of 540 R.u./ml. The solution was saturated with NaCl; the resulting flocculent precipitate was easily filtered off. The filtrate, containing 17 R.u./ml., was discarded. When redissolved in 27·5 l. the precipitate gave a solution of only 666 R.u./ml. Thus, of the original $21{\cdot}6 \times 10^6$ units only $18{\cdot}5 \times 10^6$ remained. NaCl was added to this solution until the filtrate from a small portion showed only about half the activity of the original, i.e. ~330 R.u./ml. After filtration, the second half of the rennin was precipitated by raising the NaCl concentration to saturation; the filtrate containing 30 R.u./ml. was discarded. Thus, that portion of the original material insoluble in saturated NaCl was divided into a more soluble and a less soluble portion. The rest of the raw material was similarly divided, but the division was carried out first, and each portion then precipitated with NaCl. The result was the same by this method, but the difficulties of filtration were very great. The first precipitate could be filtered off only by making the whole of the mixture into paper pulp and then extracting the filter cake with water to recover the rennin, whilst the second precipitate was removed with the aid of about 2% (w/v) aluminium phosphate formed *in situ*, and extraction of the cake with water as before. The scheme below regards the two processes as identical and the former process as being applied to the whole material.

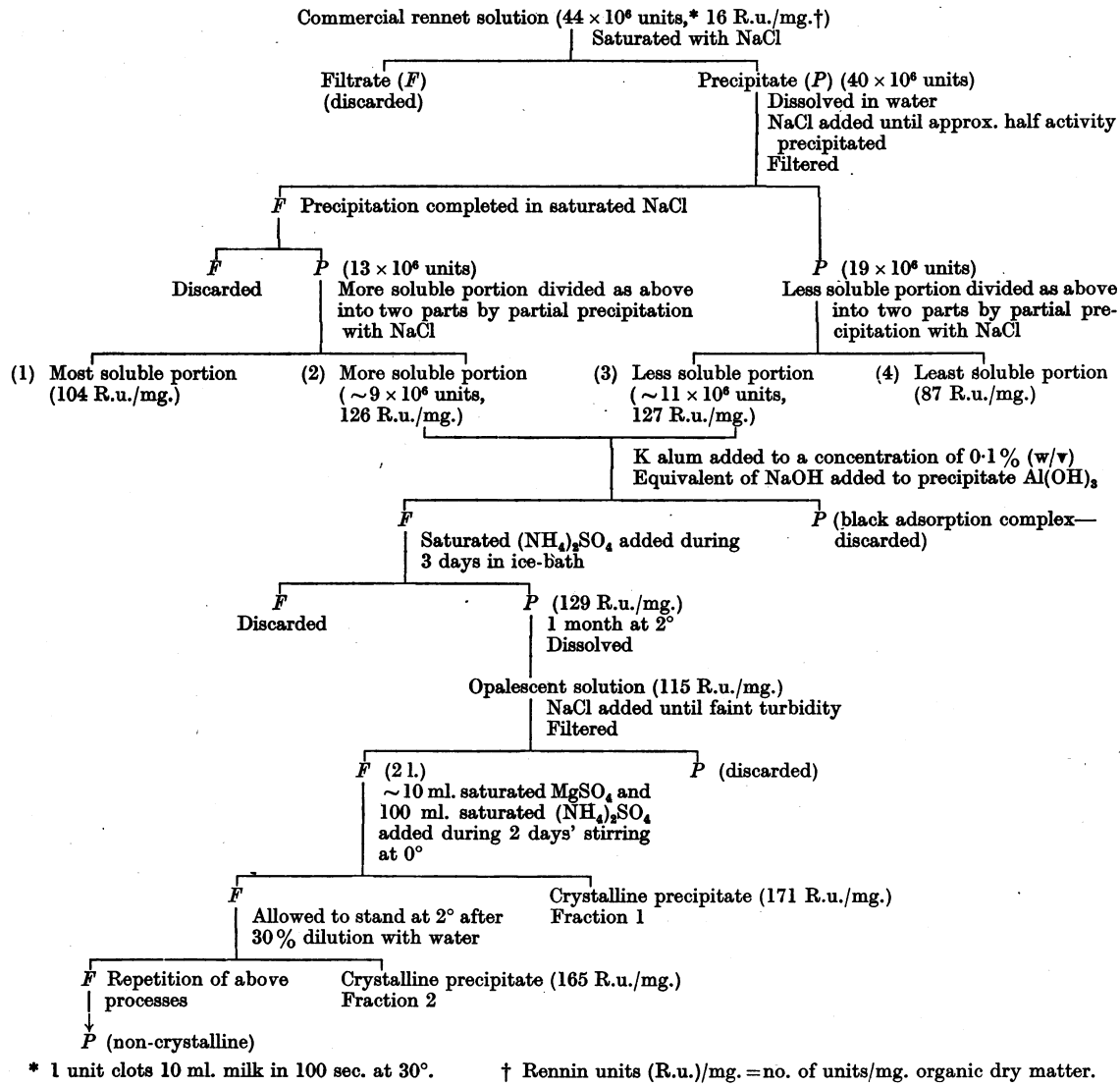
The united less soluble precipitates were dissolved in 14 l. and divided into a more and a less soluble portion by fractional precipitation with NaCl as already described. The first precipitate was flocculent and readily filterable. The second precipitate, which was formed slowly by adding ~100 g. of NaCl every 15 min. with continuous stirring until saturation, was sufficiently granular to be filtered easily on one paper. Thus the first less soluble portion was again divided into two portions according to solubility.

The second portion, consisting of the united more soluble precipitates, was similarly further subdivided into two portions. Here the first of these was granular and was readily collected on one filter. The attempted precipitation of the second resulted in an almost colloidal suspension which could not be filtered. Various methods of treating it were tried, such as re-solution and adsorption on alumina, but it was so unstable that by the time it had passed through these processes much of the activity had been lost. The remainder of this fraction, except the small amount of precipitate which had with difficulty been filtered off, was therefore discarded.

Thus, four precipitates differing in solubility and purity were obtained. The first contained most of the less soluble mucilaginous substances, the last,

* Kindly supplied by Benger's Ltd.

Preparation of crystalline rennin



* 1 unit clots 10 ml. milk in 100 sec. at 30°. † Rennin units (R.u.)/mg. = no. of units/mg. organic dry matter.

most of the more soluble proteins and peptones which had been adsorbed on the rennin last precipitated; the middle two contained least of both types of impurity. Activity and dry wt. of organic matter determinations were carried out on each of these fractions by the methods described, so that their purities could be expressed as the number of R.u./mg. of dry organic material present. The values obtained were 87, 127, 126 and 104 R.u./mg. respectively, in order of increasing solubility. The second and third fractions, containing 9×10^6 and 11×10^6 R.u. respectively out of the original 44×10^6 R.u., are thus considerably purer than the original material, which was about 16 R.u./mg.

These two precipitates were therefore combined. They dissolved readily when a little water was added. The resulting viscous solution set to a firm gel when left overnight at 2°. More water was therefore added and a permanent solution was obtained at a volume of ~3·5 l. All subsequent operations were carried out as far as possible in a refrigerator, or ice-bath. The very dark, turbid solution was clarified by adding 3·5 g. of potash alum crystals (in solution), followed by its equivalent of caustic soda solution (22·1 ml. N-NaOH), and filtering off the almost black adsorption complex. Although three large filters were used the filtration required nearly 4 days. About four-fifths

of the activity of the filtrate was precipitated by the drop-wise addition of 1·5 l. of saturated $(NH_4)_2SO_4$ during 3 days. The precipitate, in the form of microscopic masses of spheres, had a purity of 129 R.u./mg. which decreased, during storage for about a month at 2°, to 115 R.u./mg. The precipitate then dissolved only very slowly in 1 l. of water to give a turbid solution. This was purified by the addition of sufficient NaCl to cause a slight precipitate, and filtration. Overnight a small quantity of saturated $MgSO_4$ was added very slowly, through a capillary, to the stirred solution in an ice-bath. A precipitate of microscopic, rectangular prisms crystallized out. A further 100 ml. of saturated $(NH_4)_2SO_4$ were slowly added during the day and stirring continued overnight. An attempt was then made to separate the larger crystals by a short centrifuging. This necessitated a 30 % dilution with water to prevent the precipitation of amorphous rennin as the temperature of the solution rose. The precipitate was washed at the centrifuge with (*a*) half-saturated NaCl, (*b*) three-quarters-saturated NaCl, and finally suspended in saturated NaCl. A portion of this suspension was filtered at the pump and resuspended in distilled water to give a mixture containing per ml. 11·6 mg. dry organic matter, 5·8 mg. NaCl, and an activity of 1980 R.u., giving for the organic matter a purity of 171 R.u./mg. Thus the crystals were certainly rennin. The appearance of the suspension in saturated NaCl is shown in Pl. 1, fig. 2. The cracks are due to damage while centrifuging and washing. Thus the first crop, consisting of approximately 10 g. moist weight of rennin crystals, was obtained.

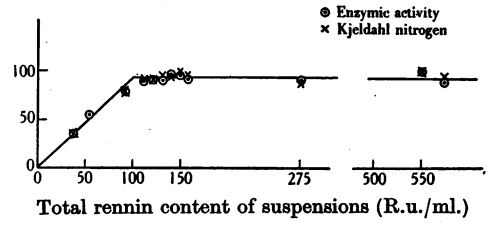
In spite of the addition of water, a second and larger crop of crystals separated from the mother liquor of the first crop during 3 days' standing in the refrigerator. These were photographed before washing (Pl. 1, fig. 1). They were washed as before, but at the pump, and stored in saturated NaCl with thymol added. Their purity was 165 R.u./mg.

By further slow additions of saturated $(NH_4)_2SO_4$ two more precipitates were obtained. They were granular but amorphous. Their purities were 119 and 112 R.u./mg.

PURITY OF CRYSTALLINE RENNIN

As a measure of the purity of their preparations earlier workers have used the activity per unit of dry weight or per unit of N content, and have been able to report large increases in these ratios, and thus in purity. Without an absolute measure of purity, however, these results are of limited value. The high activities reported are difficult to interpret and to compare, as differences of pH, calcium content and temperature, in addition to the natural variations in protein make-up of the milk, cause wide variations in susceptibility to rennet. It is thus very desirable to carry out absolute measurements of purity. Of these, solubility determinations in the presence of increasing quantities of solid phase give the best single criterion of purity.

Only one solubility experiment has, so far, been concluded. The graph shown in Text-fig. 1 was obtained. While far from perfect it indicates that the crystals are probably almost pure. It seems fairly safe to assume that the deviations from the ideal are due to experimental errors, except the slope of the first portion of this graph, which is < 1, probably owing to the presence of a small quantity of an impurity of very low solubility which prevents complete solution of the rennin, as suggested for pepsin by Steinhardt (1938). Indeed, a little insoluble residue was observed even in the most dilute sample. The extremely low solubility of the crystals indicated the need for a strongly salting-in electrolyte in the solvent. $MgCl_2$ was chosen; 50 g. of $MgCl_2.6H_2O$ and 15 g. of $CH_3COONa.3H_2O$ were dissolved in 1 l. and the solution adjusted to pH 5·4 with 10N-H_2SO_4. Crystals of crop 1 were recrystal-

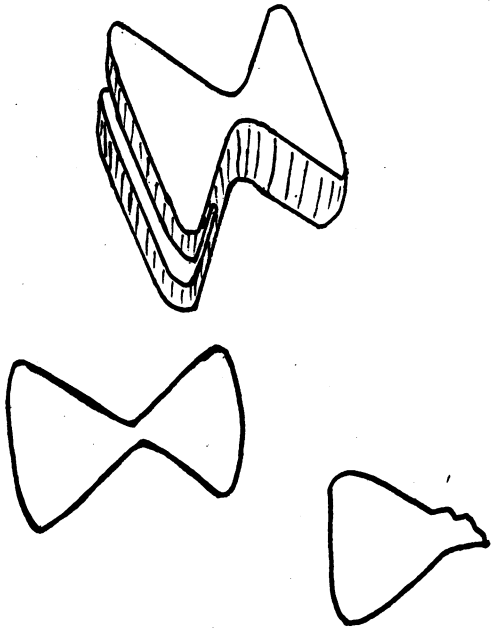


Text-fig. 1. Solubility of crystalline rennin with increasing quantities of solid phase. Solvent: 5 g. $MgCl_2.6H_2O$, 3 g. sodium acetate ($CH_3COONa.3H_2O$)/100 ml.; pH, 5·4. Ordinate: Activity of supernatant (R.u./ml.) = nitrogen of supernatant (mg./ml. × 1170).

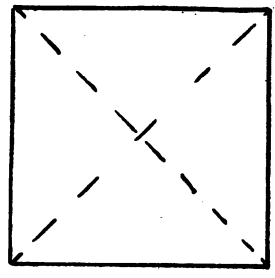
lized twice from this solvent (as described below, in detail, under 'Properties'), washed five times with small portions of solvent, allowing several minutes in each change of solvent, and resuspended to give a mixture containing 5500 R.u./ml. Dilutions of this were equilibrated with solvent in 6 ml. test-tubes in a container rotated in a vertical plane, an air bubble in each tube acting as stirrer. No traces of denaturation at the solvent-air interface were noticed. The container of tubes was immersed in a bath at 1–2°. After 5 days' equilibration, each tube was centrifuged for 7 min. in ice (packed in a 50 ml. metal cup of the International Centrifuge supported in cotton-wool in a 250 ml. metal cup), and the supernatant solution analyzed for activity and total nitrogen. Special care was necessary when removing the bung to avoid contaminating the solution with crystals collected on the side of the tube above the level of the liquid.

PROPERTIES OF CRYSTALLINE RENNIN

(*a*) *Solubility.* While crystalline proteins are generally less soluble than their amorphous forms the difference with rennin is very striking. Amorphous rennin precipitated by salting-out, whether finely divided or in the form of spheroids, cannot be



2*a*.



2*b*.

Text-fig. 2. *a*, crystal fragments remaining after dissolving most of the rennin. *b*, structure of some original crystals showing diagonal markings (faint) related to shape of fragments shown in *a*.

washed with distilled water. As soon as the excess of salt has been removed, the rennin dissolves in the dilute saline remaining, to form a gummy mass; it is apparently miscible with dilute salt solutions in almost all proportions. The crystals, however, may be washed with water, and however slow the washing, the loss is quite low. Even in the strongly salting-in solution used for the solubility experiment the solubility is very low, being approximately 0·06 g./100 ml. The solubility in distilled water has not yet been determined. If it is correspondingly lower, rennin may, like lactoglobulin, be another useful protein for solubility work in general, since it will not appreciably affect the dielectric constant of its own solution.

The low solubility of the crystalline form may account in some degree for the difficulty first experienced in obtaining crystals. The solutions used in the early preparations were so highly supersaturated that they could not be expected to crystallize readily.

The experiment already described has shown that the solubility of the crystals is independent of the amount of saturating body, but that a small proportion of the rennin did not dissolve even in the most dilute sample. The residue remaining after most of the rennin had dissolved contained a few fragments of characteristic shape (shown in Text-fig. 2*a*), apparently related to the structure of the crystals as indicated in Text-fig. 2*b*. The diagonal lines occur on only a few of the original crystals and are too faint to photograph in the ordinary way; they can be seen by only very careful examination. They may indicate planes in the crystal where the impurity of low solubility, believed to exist, has been deposited and has later prevented solution of the rennin.

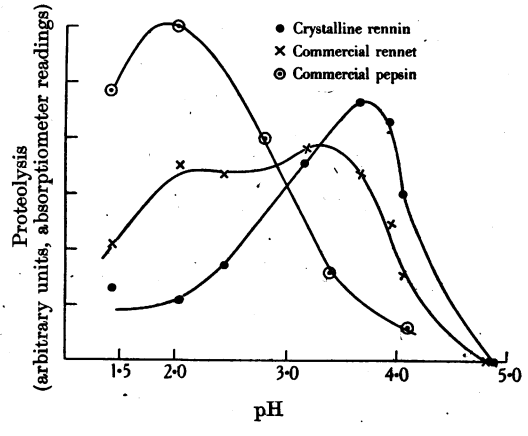
(*b*) *Recrystallization.* The difficulty of dissolving the crystals has already been described. This was partially overcome by raising the pH of the mixture to 6·8. In this way, 10 g. of filter cake of crystals moist with saturated NaCl could be dissolved in 800 ml. of approximately 0·06M-NaH_2PO_4. After solution and filtration, the pH was adjusted to between 5·4 and 5·5. The very slow addition of saturated $(NH_4)_2SO_4$ to such a solution, followed by storage in the refrigerator as soon as crystals began to appear, led to the formation of a thick layer of crystals on the bottom of the vessel. Subsequent attempts to crystallize from this type of solution by the continual very slow addition of saturated $(NH_4)_2SO_4$, with continuous stirring, led to the precipitation of spheroids, in the centre of which the cubic crystals used for seeding could be seen. Precipitates of this type dissolved very rapidly in the $MgCl_2$-sodium acetate buffer used for the solubility experiment, to give a solution from which good crystals began to be formed almost at once. The recrystallization procedure finally adopted was, therefore: solution at pH 6·8, precipitation in the amorphous condition with $MgSO_4$ or $(NH_4)_2SO_4$ at pH 5·4, and solution in $MgCl_2$-sodium acetate buffer at pH 5·4, from which crystallization readily occurred.

(*c*) *Effect of desiccation.* A small quantity of filtered crystals was kept for several weeks at room

temperature in a desiccator over P_2O_5, without loss of crystalline form. The activity was difficult to measure owing to the extreme slowness of solution even in a large excess of distilled water. A suspension of 3·5 mg. of crystals in 1 l. of distilled water reached its maximum activity after 4 hr. although still not completely dissolved. According to the figure then obtained the purity of the sample was 136 R.u./mg., indicating that not more than a quarter of the rennin had decomposed.

The stability of dried rennin crystals suggests that rennin might be useful for studies in which water usually interferes, as, for example, examination by infra-red rays for the detection of hydrogen bonds.

(*d*) *Proteolytic activity.* The purified and partly crystalline material from one of the preliminary preparations was used for these experiments. It has been shown that commercial rennin will digest haemoglobin (Hilton, 1941), and since this has already been adopted as a suitable substrate for pepsin it was chosen for rennin also. The results suggest that, had the earlier workers been able to control the pH of their enzyme-protein mixtures, the controversy over the identity or otherwise of pepsin and rennin would not have arisen. Text-fig. 3 indicates clearly that pepsin has been largely or entirely removed during the purification of the rennin, and that commercial rennet is a mixture of rennin, having an optimum pH of about 3·7 for its action on haemoglobin, and pepsin, with an optimum at pH 1·8. Text-fig. 3 also confirms the



Text-fig. 3. Proteolytic activity of rennin, commercial rennet and commercial pepsin on haemoglobin at various pH values.

generally held, though not previously proven, view that rennin is a protease. The optimum pH is rather different from that of pH 4·7 given by Davis & Davies (1934) for the protease of commercial rennet. This is probably due partly to their use of casein as a substrate, although the type of rennet they used and the conditions chosen for proteolysis (42 hr.–18 days as against 20 min.) may have exerted some effect.

Oppenheimer (1936) has pointed out the possibility that rennin consists largely of a cathepsin liberated by the breakdown of the cells of the mucosa during extraction. That it is actually secreted is shown by the results obtained by Berridge, Davis, Kon, Kon & Spratling (1943) on stomach juice removed through a fistula from a living calf (cf. Fomin, 1939). The activated juice had a proteolytic activity on haemoglobin, and an optimum pH and ratio to milk-clotting activity similar to that of purified rennin. It is possible that rennin, acting at pH 3·7, corresponds to the human cathepsin with an activity at pH 4·7 reported by Freudenberg & Buchs (1940). Unlike the cathepsins, it is not activated by H_2S (Hilton, 1941), and, according to Moriyama & Ōhasi (1941), is rather inhibited by H_2S and traces of heavy metals.

DISCUSSION

As already mentioned, the properties of crystalline rennin indicate that it may be an extremely useful member of the protein group for the study of certain properties. Its low solubility and its stability on drying should facilitate the solution of solubility and structural problems, while an examination of its hydrolytic activity towards synthetic substrates (cf. Bergmann & Fruton, 1941) would probably provide further interesting examples of enzyme specificity.

It is interesting to note how the history of work on rennin is similar to that of work on pepsin. Just as in the case of pepsin we have evidence for the existence of both an enzymically active protein and a non-protein enzyme, so with rennin; the results reported here, as well as those of other workers (e.g. Hankinson, 1943; Tauber & Kleiner, 1932), support the theory that rennin is a nitrogenous substance, while Lüers & Bader (1927), and Rao, Rao, Ramaswamy & Subrahmanyan (1941) present evidence to the contrary. The solution of the apparent contradiction necessitates further work, possibly along lines similar to those of Kraut & Tria (1937), who prepared the two types of pepsin from the same source, going further, if possible, with the preparation of the nitrogen-free enzyme from the pure nitrogenous material. If this is impossible, evidence could perhaps be obtained to show whether both substances were originally present in calf gastric fluid, or whether the non-nitrogenous material is present in the cells of the mucosa as a precursor of the zymogen which is actually secreted. This might lead to important discoveries concerning the synthesis of enzymes by glandular tissue.

SUMMARY

1. Experiments culminating in the crystallization of rennin are described.

2. Evidence that the crystals are pure rennin is presented.

3. Rennin crystals are of very low solubility in water and can be dried without decomposition.

4. Rennin is probably a protease, acting optimally on haemoglobin at pH 3·7.

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Physical Properties of Bovine Serum after Heating with Formaldehyde

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Several workers have tried bovine serum as a substitute for human plasma for transfusion, and in most cases serious reactions were observed (Edwards, 1944). Untreated bovine serum is antigenic and contains agglutinins for all groups of human red cells up to a dilution of 1/16. Cohn (1942) found that human and bovine serum albumins have similar crystal form and physical properties. The transfusion of bovine albumin, however, causes violent reactions though all agglutinins have been removed (Edwards, 1944). Any satisfactory substitute for human plasma must be non-toxic, non-antigenic, free from agglutinins and must have a colloid osmotic pressure similar to that of human plasma.

Since bovine serum is a convenient starting material, attempts were made to modify it so as to make it suitable for transfusion; this was done by heating it with formaldehyde and ammonia. The clinical results obtained with this material have been reported by Edwards (1944),* and an account is now given of its physical properties. It may be recalled that Van der Scheer, Wyckoff & Clarke (1941) found the formation in heated serum of a complex of large molecular weight migrating electrophoretically with a velocity intermediate between those of albumin and β-globulin. Davis, Hollander & Greenstein (1942) showed that the effects of ultra-violet light are similar to those of heating, but more slowly produced. Kleczkowski (1941*a*, *b*, *c*) discusses the antigenic properties of complexes formed by heating mixtures of proteins and the effect of heat on antibodies.

* The reporting, in this paper, of the ultracentrifuge results obtained by one of the present authors at Oxford was done without his knowledge or approval.