PURIFICATION AND CHEMICAL NATURE OF RENNIN

BY

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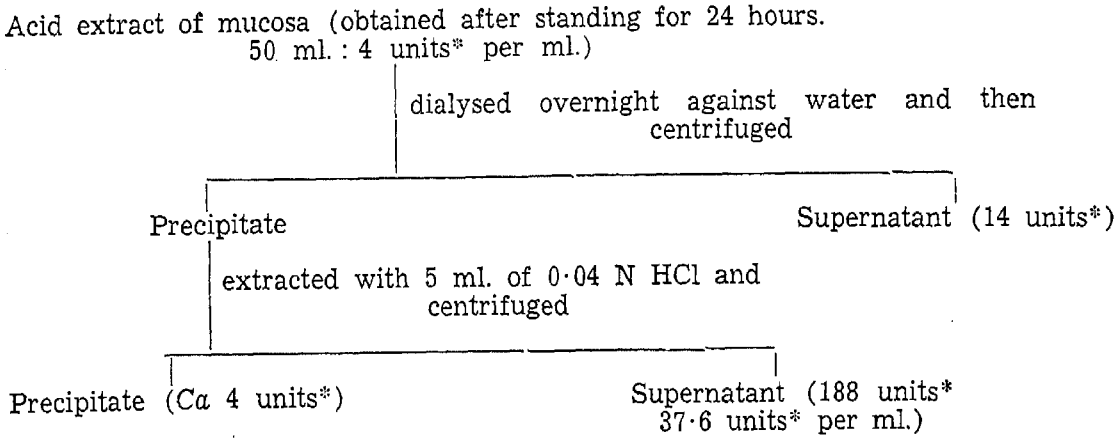
IN recent years, a number of attempts have been made to prepare integrally pure rennin and to establish its chemical nature. Among these, special mention should be made of the work of Fenger,[1] who obtained a preparation which was nearly free from pepsin and contained 14·0 per cent. nitrogen and 0·7 per cent. phosphorus; Lüers and Bader[2] whose product was over seven times as active as that of Fenger, but showed distinct peptic activity and contained only 0·68 per cent. nitrogen; Tauber and Kleiner[3] whose best preparation contained no pepsin and gave the following percentage composition: carbon, 61·3; hydrogen, 7·02; nitrogen, 14·4; phosphorus, nil; chlorine, nil; sulphur, 1·19; and ash, 0·4. The last authors admit the possibility of their preparation containing an impurity of high nitrogen content but conclude, tentatively, that the enzyme is a thioproteose.

The present enquiry was undertaken with the object of throwing some light on the nitrogen status of the enzyme and to obtain some fresh evidence regarding its chemical nature.

The procedure adopted by us for the initial purification of the enzyme from calf stomach mucosa is based on the following findings:—

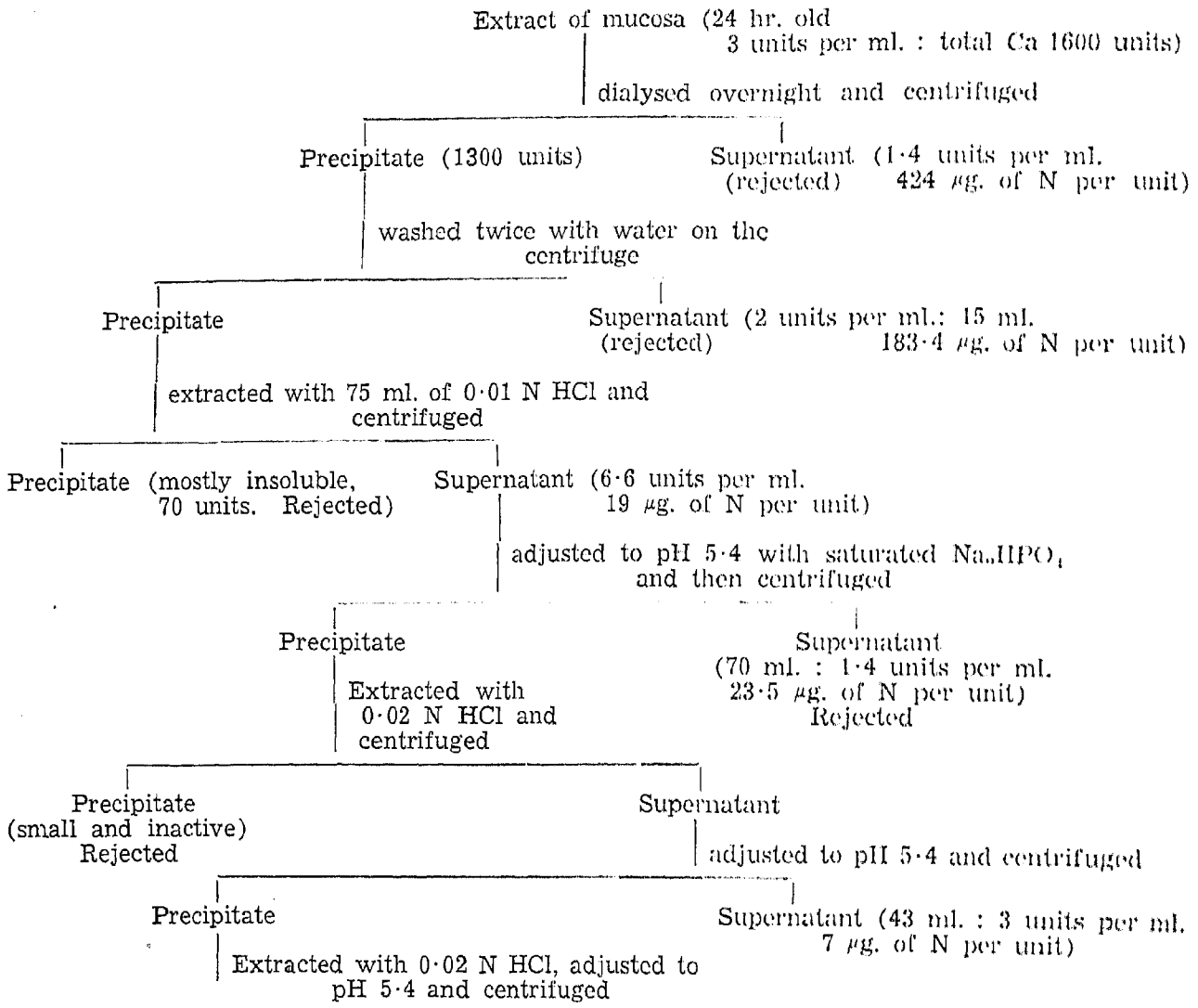
(1) On allowing ground mucosa to stand with 0·04 N HCl for 18 to 24 hours, an active extract is obtained which can be dialysed overnight without any appreciable loss of activity. During this dialysis, there is the separation of mucilaginous precipitate which carries down the major part of the enzyme. From this precipitate, the enzyme can be easily extracted in a concentrated form. The following is an example:



By using more dilute acid than the above, the enzyme can be extracted out of the mucilage in a number of small fractions of varying degrees of purity.

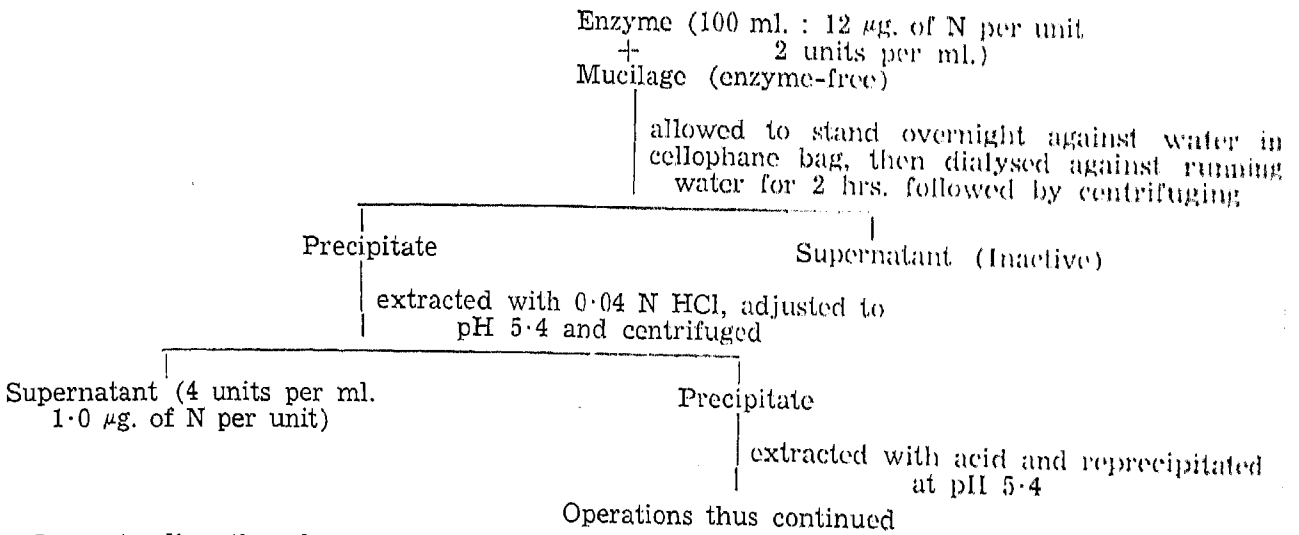
(2) On adjusting the acid extracts as obtained in (1) to pH 5·4 (when a fine precipitate forms) and centrifuging the resulting suspensions, clear supernatants at higher levels of purity (as determined by the ratio of nitrogen to activity) than the starting materials can be obtained. The following is an example:

* The unit of enzyme as adopted by us may be defined as that required to clot 10 ml. of a 30 per cent. solution of 'Klim' whole milk powder in 0·3 M acetate buffer of pH 4·6 in one minute at 37°. This unit is roughly 15 times and in some cases 30 times, that adopted by earlier workers and was necessitated by the high activity of our preparations and the need for obtaining quick, reproducible and sharp clots. Where comparisions with earlier observations are necessary, the results have all been computed on the same basis.



Fractionation continued in the above manner until a level of 2–3 µg. per unit was attained.

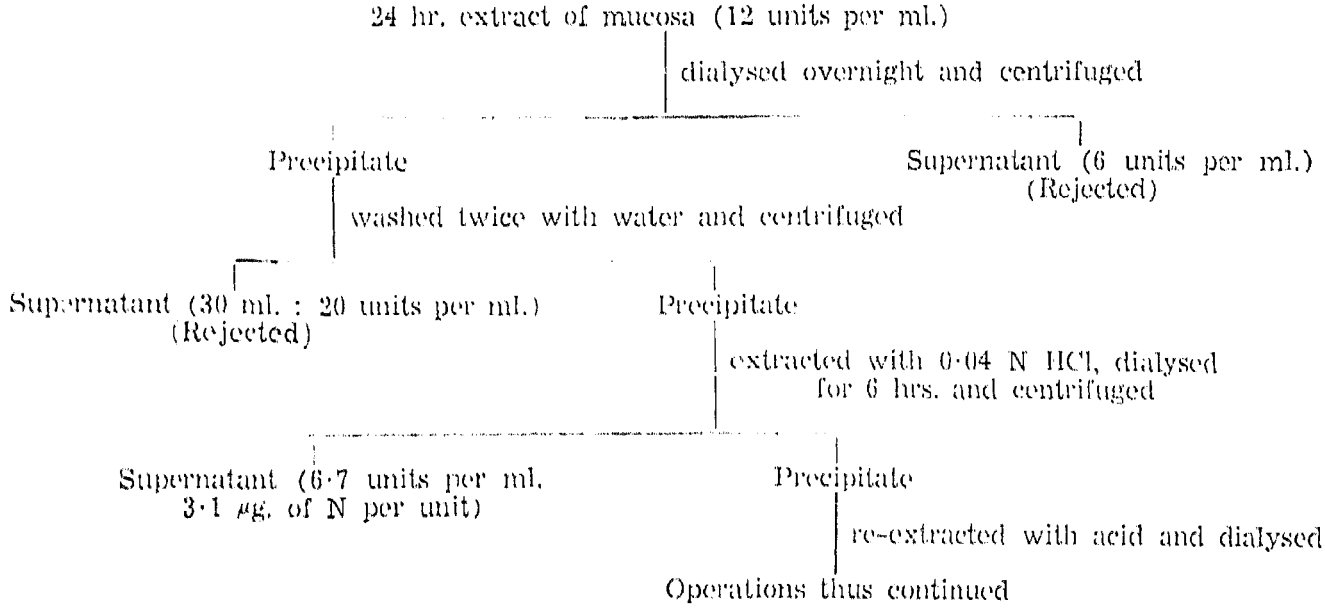
The next step in the purification was based on the observation that the mucilage (obtained on dialysis) can be repeatedly extracted with acid and reprecipitated at pH 5·4 until it is free from enzyme. It can then be used for adsorbing the enzyme, preferentially, from the associated nitrogenous impurities. Example:



Operations thus continued

By extending the above operations, fractions containing even less than 1 µg. of N per unit were obtained, but the yields in such cases were generally small, totalling only 5 to 10 units in each case.

Considerable amount of purification can be effected by merely following a process of successive acid extraction followed by dialysis. Example:



By continuing the above series of operations, enzyme preparations at as low a level as 1·4 μg. of N per unit have been obtained.

The preparation keeps tolerably well when maintained out of contact with air and in the cold. At the ordinary temperature (25 to 35°), the activity is rapidly lost. At this level of purity the enzyme does not lend itself to concentration by the usual freezing and desiccation methods. The major part of the enzyme is lost during concentration. The preparation can be dialysed against water for a short period without appreciable loss of activity.

It may be mentioned, however, that the above procedure is slow and tedious. It involves considerable wastage of enzyme. It is not therefore recommended except for certain types of preparatory work wherein phosphate-free enzyme is required.

Properties of the enzyme at 1·0 μg. (per unit of N) level.—The enzyme at this level gives a water clear solution which does not form a precipitate either on heating or on addition of trichloracetic acid. On drying, it gives a pale yellow solid corresponding 30 μg. per unit. Calculated on the same basis of activity, this would be approximately 9 to 10 times as pure as Tauber's in regard to nitrogen and nearly twice as pure in regard to dry weight.

There is a peculiar feature in regard to the ratio of dry weight to nitrogen. Even assuming that the nitrogen formed a part of the enzyme, there is a strong suggestion that the preparation contains a non-nitrogenous impurity. This can be confirmed both after short period dialysis and on allowing the preparation to stand for some time, when some of the non-nitrogenous impurity is deposited without appreciably altering the activity of the preparation.

The preparation does not respond to most protein tests, while, in a few cases, the colouration is faintly positive.

Tests for sulphur, pepsin and carbonic anhydrase were negative.

Relation of nitrogen to enzyme.—There was considerable amount of indirect evidence to suggest that the nitrogen associated with the enzyme at different stages was an impurity:

(1) When the mucilage containing the enzyme was extracted with successive portions of dilute acid of the same strength (*e.g.*, 0·01 N HCl) and centrifuged after adjusting to pH 5·4, the quantities of nitrogen present in the different extracts were more or less the same and practically independent of the amount of enzyme (as shown by activity) present in each fraction.

(2) On mixing the enzyme at a high level of purity (1–2 μg. of N per unit) with a small amount of enzyme-free mucilage and centrifuging the suspension, all the nitrogen was left in the supernatant, while practically all the activity passed into the precipitate. This could not have occurred if the nitrogen were a part of the enzyme molecule.

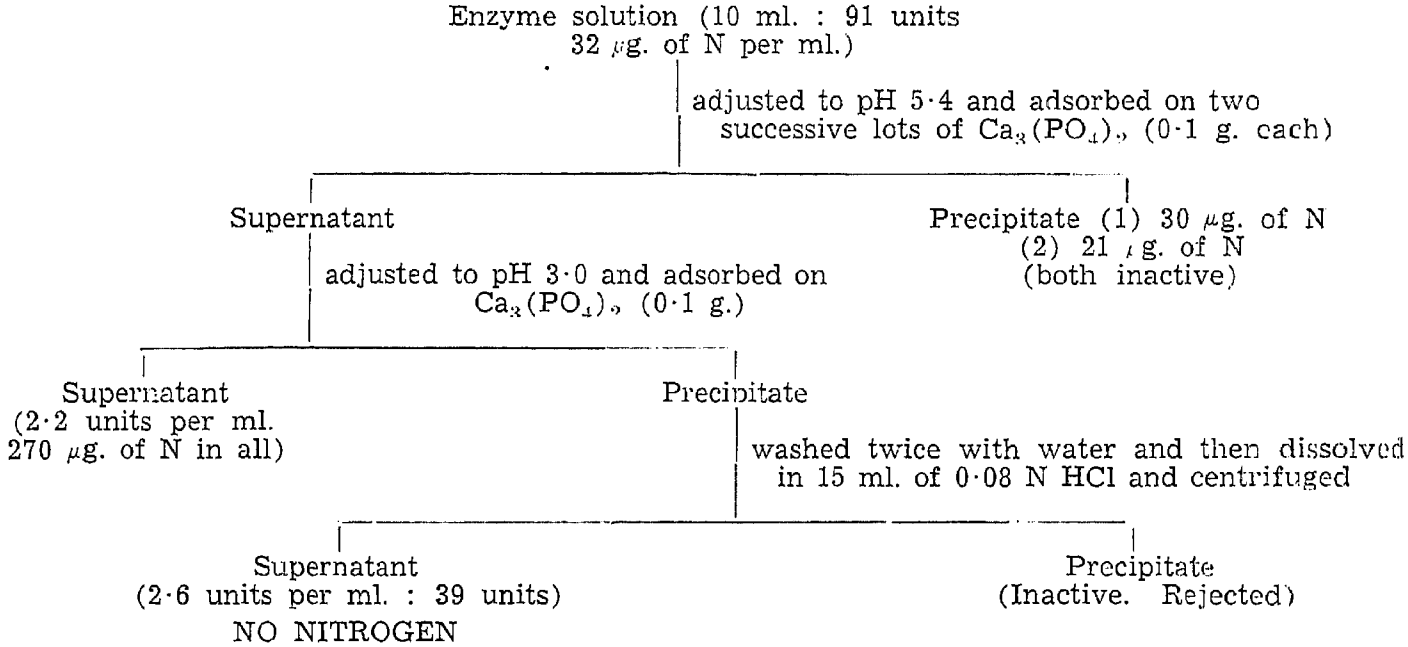
(3) Filter aids (*e.g.*, Celite 501) adsorbed practically the whole of the enzyme leaving the nitrogen in the supernatant.

(4) Alumina C_r adsorbed the enzyme

preferentially (25 : 1 when mixed with a preparation at 12·5 μg. of N per unit level) leaving the major part of nitrogen in the supernatant. Adsorption on kaolin also gave a similar result.

In none of the above cases was it possible to elute the enzyme successfully. Thus, the muco-protein of the mucilage always tended to dissolve to some extent (1·5 — 5·0 μg. of N per ml. depending on concentration of reagents) when extracted with dilute acid and precipitated at pH 5·4; the enzyme could not be eluted from celite and kaolin; phosphate buffer (pH 7·0 to 7·2—a more alkaline buffer could not be used because of its adverse effect on the enzyme) eluted the nitrogen preferentially from alumina C_γ leaving the major part of the enzyme in an unextractable condition.

Tricalcium phosphate proved a more promising material. It adsorbed both the enzyme and nitrogen, when used as aqueous suspension, but showed considerable preference for the enzyme when used in the partially dried, solid condition (the reason for this is still not clear). Elution with buffers proved inefficient, so the entire adsorbate was dissolved in acid with satisfactory results. Example:



The determinations of nitrogen were ordinarily carried out by a micro-kjeldahl method followed by titration, but in cases like the above, each figure was checked, in replicate, by colorimetric estimation, the values being correct to 0·1 μg. The results show conclusively that the adsorbate obtained at pH 3·0 contained enzyme which was completely free from nitrogen.

The above observations have since been repeated a number of times and on a large scale. An essential condition for success seems to be the presence of an electrolyte (*e.g.*, phosphate in the present case) which apparently checks the adsorption of nitrogen by the tricalcium phosphate.

There has been some difficulty in estimating the true dry weight of the nitrogen-free enzyme. This is partly due to the difficulty in removing the last traces of tricalcium phosphate. Indirect evidence already obtained would suggest that the true dry weight is less than 10 μg. per unit.

The enzyme at the highest level of purity is extremely labile. The activity drops by about 75 per cent. in the course of a day. Experiments on the stabilisation of the pure enzyme and the study of its various properties and kinetics of reaction are in progress.

Reversible inactivation of rennin and the evidence for the existence of a thermostable component in the enzyme.—Extensive series of experiments were carried out on this aspect of the problem and the more important findings may be summarised as follows:

(1) Although rennin is rapidly and irreversibly inactivated at pH 8·0 and above, it does, nevertheless, undergo a slow and reversible type of inactivation in the region of pH 7·0 to 7·4. The latter type of inactivation can be arrested by adding sufficient acid to adjust the reaction to pH 2·0.

(2) On adding a small portion of boiled enzyme or autolysate of mucosa to partially inactivated enzyme as obtained in (1), a

considerable part of the original activity is restored. This would suggest the presence of a thermostable component in the enzyme.

(3) A number of known substances were examined with a view to determining whether any of them can replace the thermostable component, but, so far, only zinc salts have been found to possess that property.

The following results will illustrate the above:

	Substrates 20% milk	30% milk
1. Partially inactivated enzyme alone	4′ 30″	6′ 20″
„ + boiled enzyme	2′ 40″	4′ 15″

Original enzyme clotted in 1′ 20″. Boiled enzyme alone had no clotting effect.

	30% milk
2. Partially inactivated enzyme alone	7′ 30″
„ „ + autolysate from mucosa (boiled)	2′ 35″

Original enzyme clotted in 1′ 30″. The autolysate (boiled) had no clotting effect.

	30% milk
3. Partially inactivated enzyme alone	6′ 30″
„ + $CaCl_2$ (1 drop 1%)	> 7′
„ + $CdSO_4$ „	> 7′
„ + $MgSO_4$ „	> 7′
„ + $SnCl_2$ „	> 7′
„ + $ZnCl_2$ „	2′ 30″

Original enzyme clotted in 1′ 20″. None of the salts (at the concentration used) had any direct effect on the milk. It may be further added that contrary to the report of Andreitchewa,[4] zinc at the concentration used has no effect on fresh rennin (before inactivation) and that the restoration is observed only in the case of the partially inactivated enzyme.

The above and similar experiments (which were repeated dozens of times) were carried out under identical conditions and with the necessary controls. These will be described in a detailed paper.

Some of the properties of the thermostable component.—It is stable in moderately acid or alkaline media; withstands prolonged boiling but is fairly rapidly lost on dialysis; present in the enzyme preparations at all levels of purity so far tested; mostly lost or otherwise transformed during prolonged evaporation, aeration or distillation under reduced pressure; inhibited in its action by sodium chloride and other salts in high concentration. It behaves generally like a co-enzyme.

Possible relation of ascorbic acid (vitamin C) to rennin.—Some of the properties of the enzyme, *e.g.*, instability in alkaline media, and sensitiveness to air and mild oxidising agents (*e.g.*, iodine), suggested some resemblance to ascorbic acid, hence attempts were made to determine whether it contained any of that vitamin. This was, in fact, found to be the case, the vitamin being present exclusively in some combined condition in all the preparations (including the nitrogen-free enzyme) in the proportion of about 0·10 μg. per unit.

It is not yet possible to state whether the vitamin is present as a part of the enzyme molecule or is associated with an impurity, still present in the final stages, but in view of its possibly great practical importance, the above observation is now being carefully followed up.

Chemical nature of rennin.—The foregoing observations suggest that rennin is probably a less complex substance than has hitherto been assumed. The absence of nitrogen, sulphur or phosphorus greatly restricts the scope of the enquiry. The purest enzyme is now being prepared on a sufficiently large scale to facilitate the study of its elementary chemical composition, which is probably made up of only carbon, hydrogen, oxygen together with some mineral constituents. The properties and behaviour of the thermostable component would suggest that it is something very similar, if not, identical with zinc. (Zinc has already been found at various levels of purity, though the quantitative data require confirmation). If the presence of combined ascorbic acid is conclusively established, it would point to a definite rôle for that vitamin not only in rennin but also in other similar enzyme systems.

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[1] Fenger, F., *J.A.C.S.*, 1923, **45**, 249.

[2] Lüers, H., and Bader, J., *Biochem. Zeit.*, 1927, **190**, 122.

[3] Tauber, H. and Kleiner, I. S., *J.B.C.*, 1932, **96**, 745.

[4] Andreitchewa, M., *Bull. Soc. Chim. Biol.*, 1930, **12**, 44.