JOURNAL
OF THE
Tennessee Academy of Science

VOL. XXXIX JULY, 1964 No. 3

AN EXTRACELLULAR RENNIN-LIKE ENZYME OF STREPTOCOCCUS FAECALIS VAR. LIQUEFACIENS[1]

L. HOWARD MOSS III,[2] J. O. MUNDT, AND L. BEISHIR [3]
Department of Bacteriology, University of Tennessee
Knoxville, Tennessee

ABSTRACT

A study was made of some of the extracellular proteolytic properties of a rennin-proteolytic variant of *Streptococcus faecalis* var. *liquefaciens*. The crude enzyme preparation was derived as a by-product of growth of the organism in sterilized skim milk. The activity of the enzyme upon casein was estimated by determining colorimetrically the amount of tyrosine in protein-free filtrates of enzymatic hydrolysates. A pilot fractionation of the crude enzyme preparation with $(NH_4)_2SO_4$ revealed a single active fraction containing 146% of the original enzyme activity. Thermal inactivation in the presence of casein occurred between 60 C and 65 C. Maximum enzyme activity was defined clearly at pH 7.0-7.5. A Michaelis-Menten constant of 0.1% casein was derived. The substrate dependence curve of enzyme action deviated from the normal by exhibiting a decrease in velocity at high substrate concentrations. A linear response to increasing enzyme concentration occurred when the substrate concentration was optimal. The peptide bond of the synthetic dipeptide, L-tyrosyl-glycine, was hydrolyzed by the enzyme at pH 7.2. The enzyme derived from a milk substrate to which free arginine had been added displayed a 35% increase in enzyme activity.

INTRODUCTION

Several strains of bacteria are known to produce a rennin-like enzyme in milk, but only in the last decade has attention turned to this property as found in a variant of *Streptococcus faecalis* var. *liquefaciens*. Typical *Streptococcus faecalis* var. *liquefaciens* is known to bring about coagulation and hydrolysis of milk proteins, characteristically acting at an acid pH of 4.8 in a reaction termed "acidoproteolytic." In contrast, the sweet curdling of milk at pH 6.4 by an extracellular rennin-like enzyme of this organism and the resulting rapid proteolysis of casein has been reported only rarely. Dudani (1950), in studying the coagulating and proteolytic properties of the latter enzyme, attempted unsuccessfully to separate the two activities, thus indicating that they were manifestations of the same enzyme system. This report deals with the isolation and partial purification of the extracellular rennin-like enzyme of this variant and includes a basic kinetic study of its proteolytic properties and a limited investigation of certain factors affecting its coagulating activity.

MATERIALS AND METHODS

Organisms. The strain of *Streptococcus faecalis* var. *liquefaciens* employed in these experiments was isolated from a gray squirrel by Mundt (1961, *unpublished investigations*) and was used previously in an enzyme study by Beishir (1961). The organism produces a rennin-like enzyme and fails to produce a strong acid condition (terminal pH of 5.8) in litmus milk.

Preparation of enzyme. Stock cultures of the test organism were maintained at 10 C in an azide-dextrose broth containing 0.5% $CaCO_3$. Prior to their experimental use, actively-growing cultures of the microorganism were prepared by transferring them twice at 24-hr intervals in azide-dextrose broth free of $CaCO_3$. These were incubated at 37 C. The extracellular enzyme was derived as a by-product of growth in 11% reconstituted skim milk. Two ml of a 24-hr culture of the organism were inoculated into 200 ml of sterilized skim milk and incubated at 37 C for 48 hr. The clear whey then was decanted from the coagulated casein, clarified by centrifugation at low speed for 20 min, and the supernatant fluid stored at 10 C until used. This preparation of whey constituted the crude enzyme. The active fraction of the crude enzyme, derived by precipitation with $(NH_4)_2SO_4$, was used in all experiments. After treatment of the bulk of the crude enzyme with $(NH_4)_2SO_4$, the protein precipitate was separated at 30,000 x *g* for 10 min. The precipitated protein was resuspended in a volume of distilled water equal to that of the crude preparation and then dialyzed overnight at 4 C against 100 volumes of distilled water.

Assays of enzymatic activity. A colorimetric estimation of soluble tyrosine present in protein-free filtrates of casein hydrolysates was used as a measure of proteolytic activity. This colorimetric method was first described by Gerngross, Voss, and Herfield (1933), and later modified by Ottaway (1957). A 1% phosphate-buffered casein solution incubated at 37 C was used routinely as the test substrate. The reaction was initiated by addition of the enzyme preparation to the substrate and was terminated by addition of hot 12% trichloroacetic acid. The extent of enzyme activity was determined with a

[1] Portion of a thesis submitted in partial fulfillment of the requirements for the Master of Science degree at the University of Tennessee. This study was presented in part at the annual meeting of the Kentucky-Tennessee branch of the American Society for Microbiology, Lexington, Kentucky, 1961.
[2] Present address: Department of Microbiology, University of Tennessee Medical Units, Memphis.
[3] Present address: Department of Infectious Diseases, University of California at Los Angeles.

Klett-Summerson colorimeter at a wavelength of 540 mu. Several of the experiments were duplicated utilizing nitrogen determinations as a measure of degradation of the casein molecule. The protein concentration of the enzyme solution was measured from the optical density of the active fraction in a Beckman DB spectrophotometer at wavelengths of 260 and 280 mu, after the procedure of Warburg and Christian (1941). The rennin clotting activity of the enzyme was determined by the Conn (1922) coagulation test.

Results

The enzymic activities of the crude extract and of the active $(NH_4)_2SO_4$ fractions were quite stable when stored at 10 C, losing less than 1% of their activity over a 4-week test period. The enzyme was rapidly inactivated between 60 C and 65 C, as shown in Fig. 1. An apparent energy of activation at varying temperature intervals, calculated from the integrated form of the Arrhenius equation, had a value of approximately 15,600 calories. The coagulating activity of the enzyme was found to be most rapid at 37 C, and maximum clotting activity appeared after 48 hr of incubation of the milk culture.

Pilot fractionation of the enzyme preparation with $(NH_4)_2SO_4$ revealed that the greatest quantity of enzymatic activity was precipitated at a salt molarity of 2.57 and an ionic strength of 7.71 (Fig. 2.). The yield of the original enzyme activity in active fractions so prepared was 146%, suggesting that some dissociable inhibitor was lost. Protein turbidity increased almost linearly to a maximum of 86% of the original enzyme solution. Spectrophotometric analysis of the active fraction indicated that the enzyme-bearing protein concentration was 4.8 mg/ml of solution, but constant volume of the enzyme rather than its concentration was used in all experiments because of lack of enzyme purity.

Fig. 3 indicates the relationship of pH to the activity of the enzyme. The pH optimum using a casein substrate is defined clearly at pH 7.0-7.5. The effect of pH on the thermostability of the enzyme alone at 50 C and 60 C did not yield pH-dependent curves significantly different from that of Fig. 3. When nitrogen determinations were used as a measure of enzyme activity an identical pH-dependent curve was derived.

The effect of increasing substrate concentration on the activity of the enzyme is illustrated in Fig. 4. The curve is typically diphasic, although a diminution of rate was observed at substrate concentrations beyond the optimal. This rate was found to decrease to as low as 25% of the optimum rate at a casein concentration of 5%. These results are commonly encountered with complex protein substrates. The Michaelis-Menten con-

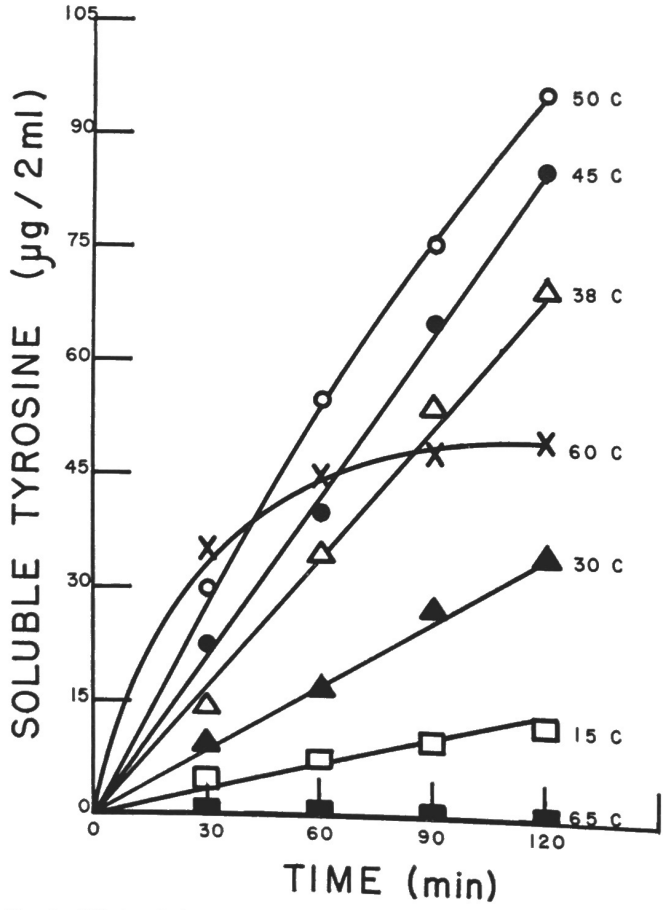

Fig. 1. Effect of temperature on the activity of the enzyme. The enzyme solution contained 4.8 mg of protein per ml and was buffered at pH 7.2 with appropriate mixture of 0.15M K_2HPO_4 and KH_2PO_4. Assays performed at temperatures indicated. Enzyme activity expressed as ug of tyrosine released from a 1% casein substrate per 2 ml of enzyme solution per unit of time.

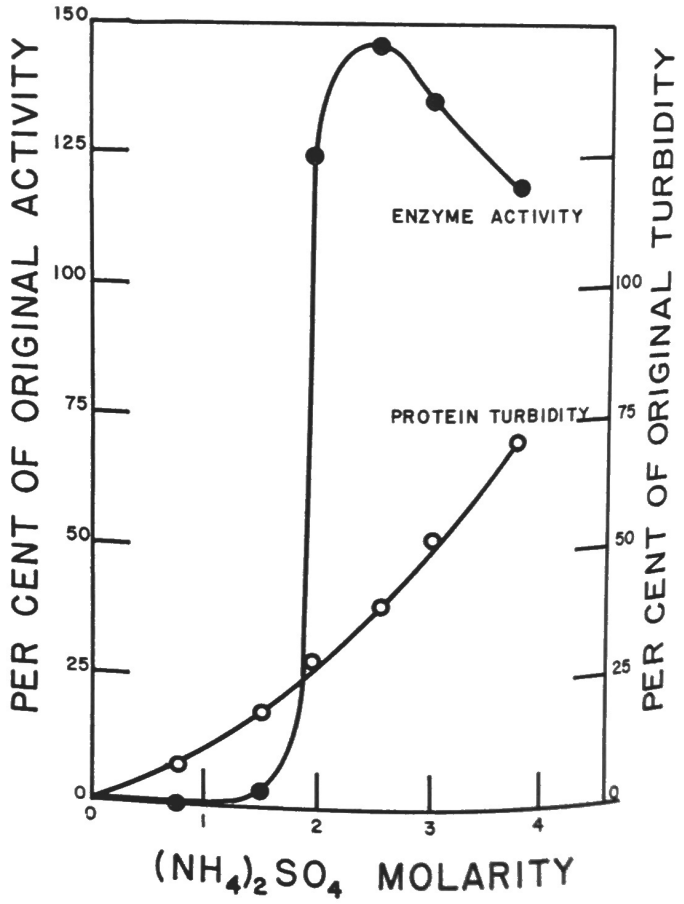

Fig. 2. Plot of enzyme activity and protein turbidity in protein precipitates prepared by salt fractionation with increasing concentration of $(NH_4)_2SO_4$.

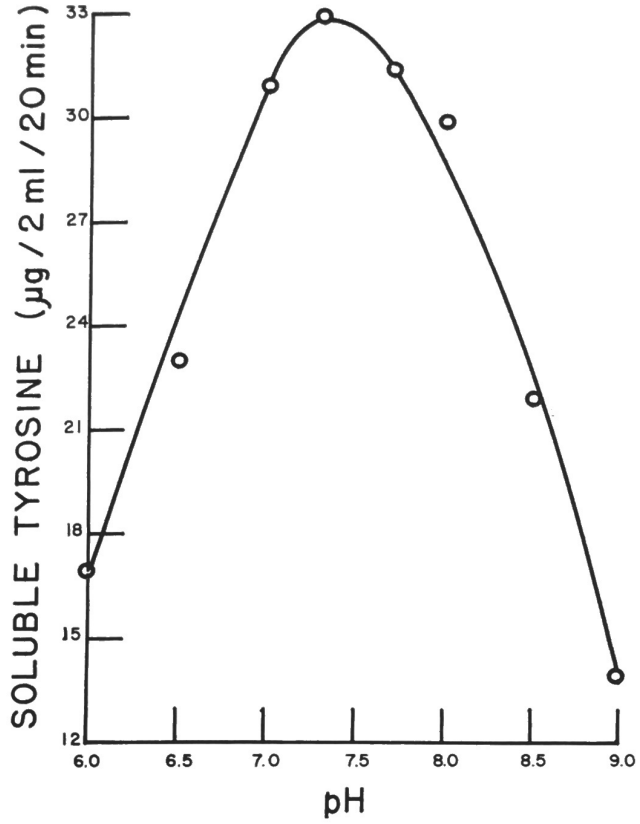

Fig. 3. Effect of pH on the activity of the enzyme. Assays performed at 40 C. At each pH value aliquots were removed at 10, 20, and 30 min and tested for proteolytic activity. Reaction velocities were estimated from the linear portion of each progress curve.

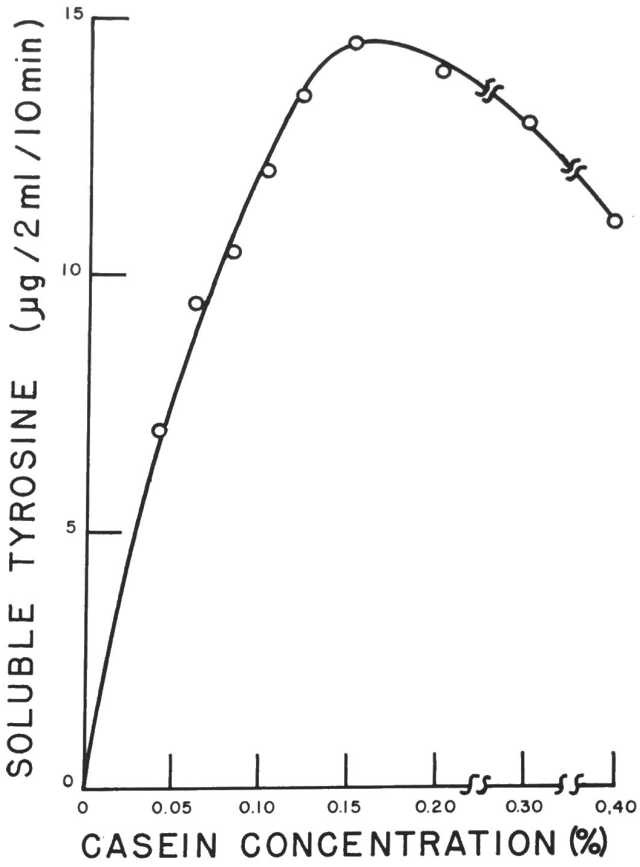

Fig. 4. Effect of substrate concentration on the activity of the enzyme. Assays performed at 40 C and pH 7.2. Reaction velocities estimated as in Fig. 3.

stant, determined graphically (Fig. 5 and Table 1) according to the method of Lineweaver and Burk (1934), had a value of 0.1% casein. The linear response of initial velocity to increasing enzyme concentration is illustrated in Fig. 6.

The active fraction of the enzyme was tested on two synthetic dipedtides, utilizing a modified photometric ninhydrin method suggested by Troll and Cannan (1952), in an effort to gain some knowledge concerning its specific activity. Carbobenzoxy-L-glutamyl-L-tyrosine was not hydrolyzed at pH 4.0 by the enzyme preparation over the 19-hr test period. L-tyrosyl-glycine, however, displayed a striking and regular increase in color-producing free amino groups during the same test period at pH 7.2.

When the test organism was propagated in the skim milk growth medium to which free arginine had been added in the amount (460 u moles/50 ml) suggested by Hartman and Zimmerman (1960) a 35% increase in the proteolytic activity of the crude whey preparation resulted. The amino acid had no effect in either dextrose or lactose broth upon biosynthesis of the enzyme by the organism, indicating the absence of some essential factor in these media.

In confirmation of the presence of rennin, skim milk was decalcified by the addition of oxalic acid; no clot was formed upon subsequent addition of the enzyme. If calcium chloride was added to this preparation, however, the development of a firm curd ensued. Neither the ratio of surface area to volume, anaerobiosis, nor increased reduction potential of liquid cultures were critical in rennin production. If liquid cultures were agitated during incubation, however, rennin activity was destroyed.

The enzyme was found to be active on a variety of protein substrates containing tyrosine. Some of those on which it was most active were soy, hemoglobin, lactalbumin, gelatin, egg albumin, gluten, and blood albumin.

Discussion

Thermal destruction of the enzyme between 60 C and 65 C in the presence of casein substrate is in agreement with the findings of Dudani (1950). The enzyme is very thermostable at temperatures below 60 C and retains some measurable activity at 15 C. The value obtained for the apparent energy of activation is reasonable for this enzymatic reaction.

In the case of a complex protein substrate, such as casein, it is very difficult to maintain the rigid conditions necessary for the accurate measurement of a Michaelis-Menten constant. The quality of the substrate, the purity of the enzyme preparation, and the

Table 1

Relationship between Reciprocal of Casein Concentration and Reciprocal of its Velocity of Hydrolysis by the Proteinase

Concentration of Substrate (S) in Per Cent	Reciprocal of Concentration 1/(S)	Initial Velocity (ugm/2 ml/10 min) v_0	Reciprocal of Initial Velocity $1/v_0$
0.04	25.00	7.0	0.143
0.06	16.66	9.5	0.105
0.08	12.50	10.5	0.095
0.10	10.00	12.0	0.083
0.12	8.33	13.6	0.074
0.15	6.66	14.5	0.069
0.20	5.00	14.0	0.071
0.30	3.33	13.0	0.077
0.40	2.50	11.0	0.091

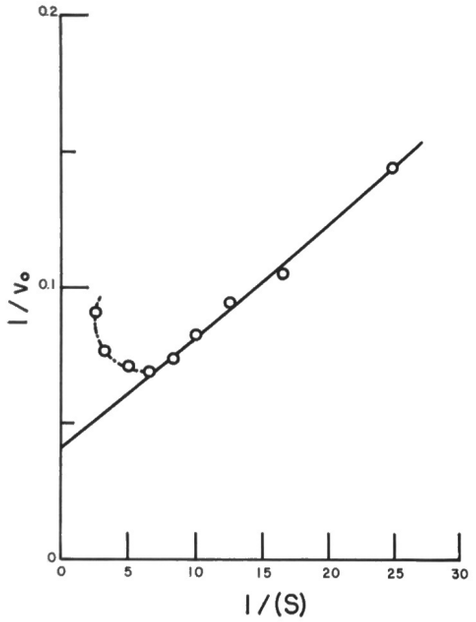

Fig. 5. Lineweaver-Burk plot of casein hydrolysis. Reciprocal of initial reaction velocity ($1/v_0$) plotted against reciprocal of substrate concentration (1/(S)). Dotted line represents diminution of reaction velocity at high substrate concentrations.

accuracy of the method for assaying the activity of the enzyme all play major roles in determining the validity of the constant obtained. The Km value for the enzyme on a casein substrate, as determined above, is regarded as an approximation.

The role of arginine in the biosynthesis of proteinase has not been completely clarified. Previous investigation by Oginsky and Gehrig (1952) showed that *Streptococcus faecalis* contains an enzyme which converts arginine to citrulline to ornithine by a process coupled to the phosphorylation of adenosine diphosphate to adenosine triphosphate. If the adenosine triphosphate derived from the arginine were an important source of energy for synthesis of proteinase, a large amount of arginine would be required for maximum formation of proteinase. This large requirement was demonstrated for the enzyme under study. Finally, Hartman and Zimmerman (1960) obtained data which indicate that it is the depletion of arginine alone in the medium that leads to cessation of proteinase synthesis, for when arginine is replenished, biosynthesis is resumed.

It is not uncommon to find that the velocity of an enzyme-catalyzed reaction will decrease at high substrate concentrations. The effect may be due to a number of different causes. Many enzymes are thought to have two or more groups, each combining with a particular part of the substrate molecule. In an effective enzyme-substrate complex one substrate molecule is combined through each of these groups, but the possibility exists that an ineffective complex could be formed in which the substrate molecule may combine with only one of these groups if the others are linked with other substrate molecules. At high substrate concentrations, where the substrate molecules tend to crowd on to the enzyme, the chance that an ineffective complex will be formed increases. Since all enzymes act in aqueous media, high substrate concentrations would reduce the relative concentration of water and thus lower the reaction velocity, especially if water is one of the reactants.

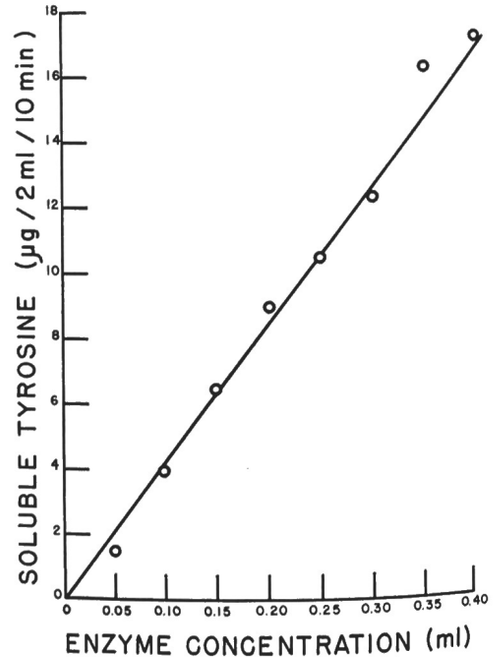

Fig. 6. Effect of enzyme concentration on the activity of the enzyme. Assays performed at 40 C and pH 7.2. Reaction velocities estimated as in Fig. 3.

Both pepsin and chymotrypsin are commonly known to clot milk and are classified as proteinases. Both of the enzymes act at peptide linkages and require the presence of an L-tyrosine residue for their maximum activity on synthetic substrates. Pepsin favors the hydrolysis of peptide linkages in which an aromatic amino acid provides the amino group for the sensitive peptide bond, while chymotrypsin readily hydrolyzes CO-NH linkages in which the carbonyl group is supplied by that amino acid. Although the two enzymes are similar in their ability to clot milk and their requirement of an aromatic amino acid residue for activity, their pH optima are decidedly different. The optimum pH for pepsin is near 2.0, while chymotrypsin acts at an alkaline pH of approximately 8.0. The proteinase under study has an optimum pH of approximately 7.2 and in the acid range demonstrated a limited activity that was rapidly destroyed at elevated temperatures. The failure of the test enzyme to hydrolyze carbobenzoxy-L-glutamyl-L-tyrosine may well be attributed, in part, to the pH chosen (4.0) for the test.

Fruton and Bergmann (1939) described the hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine by crystalline pepsin at pH 4.0. Later workers, however, found that a pH near 2.0 was necessary for maximum activity of the enzyme. The optimum pH for rennin action is close to pH 5.0, and its identity for many years was confused with that of pepsin. This resulted in part from the lack of information concerning the specific linkages attacked by rennin. It is known, however, that rennin causes the coagulation of casein in the presence of calcium ions. Similar behavior of the enzyme under study was demonstrated by Beishir (1961).

L-Tyrosyl-glycine possesses the peptide linkage that is specific for chymotrypsin. Acylation of the α-amino group of the aromatic amino acid is known to increase its activity, as is true in the case of pepsin. The presence of a free carboxyl group is also known to deter maximum activity. L-tyrosyl-glycine, however, displayed a constant increase in color-producing free amino groups during the test period when mixed with the enzyme preparation.

The pH-stability curve of the activity of the enzyme on a casein substrate is in agreement with Dudani (1950) and Beishir (1961). The short incubation period of the enzyme with casein resulted in a narrow peak of maximum activity.

This enzyme is presently being quantitatively purified in the laboratory. These purification procedures and further information regarding the specific linkages attacked by the enzyme, as determined by its activity on synthetic substrates, will be dealt with in a future communication.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the advice of Dr. Raymond W. Beck. This study was supported by research grant G-9572 from the National Science Foundation.

LITERATURE CITED

Beishir, L. 1961. A characterization and enzyme study of a proteolytic enterococcus producing a rennin-like enzyme. Master's Thesis. University of Tennessee, Knoxville.

Conn, H. J. 1922. A method of detecting rennet production by bacteria. *J. Bacteriol.* 7:447-448.

Dudani, A. T. 1950. Proteolytic and coagulating enzymes of enterococci. Dissertation. Iowa State College of Agriculture and Mechanic Arts, Ames.

Fruton, J. S., and M. Bergmann. 1939. The specificity of pepsin. *J. Biol. Chem.* 127:627-642.

Gerngross, O., Voss, K., and T. Herfield. 1933. A highly sensitive, strictly tyrosine-specific color reaction of p-substituted phenols. The tyrosine content of different proteins, especially of collagen and gelation. *Ber.* 66:435.

Hartman, R. E., and L. N. Zimmerman. 1960. The effect of the arginine dihydrolase enzyme system on proteinase biosynthesis by *Streptococcus faecalis* var. *liquefaciens*. *J. Bacteriol.* 80: 753-761.

Knivett, V. A. 1952. Citrulline as an intermediate in the breakdown of arginine by *Streptococcus faecalis*. *Biochem. J.* 50:xxx.

Lineweaver, H., and D. Burk. 1934. The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* 56:658-666.

Oginsky, E. L., and R. F. Gehrig. 1952. The arginine dihydrolase system of *Streptococcus faecalis*. I. Identification of citrulline as an intermediate. *J. Biol. Chem.* 198:791-797.

Ottaway, J. H. 1957. A colorimetric method for the estimation of tyrosine. *Biochem. J.* 68:239-244.

Troll, W., and R. K. Cannan. 1952. A modified photometric ninhydrin method for the analysis of amino and imino acids. *J. Biol. Chem.* 200:803-811.

Warburg, O., and W. Christian. 1941. Isolation and crystallization of enolase. *Naturwiss.* 29:589-590.

NOTICE TO MEMBERS OF TAS

Have you paid your dues for the current year? Please make checks payable to the Tennessee Academy of Science and mail to Treasurer G. B. Pennebaker, Tennessee Polytechnic Institute, Cookeville, Tennessee.