PURIFICATION AND PROPERTIES OF BACTERIAL UREASE[1]

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Various groups of ureolytic organisms have been investigated from the point of view of taxonomic considerations (Viehover, 1913) or with special emphasis on nutritional requirements (Gibson, 1934; Knight and Proom, 1950). However, less attention has been paid to the characterization of bacterial urease despite the fact that the enzyme has been reported to occur in 200 species of bacteria (Sumner and Somers, 1947). Sizer (1941) determined the kinetics of bacterial urease, and in an earlier study Passmore and Yudkin (1937) investigated the relationship of growth on diverse substrates to the final level of urease in the harvested organisms. In both of these studies, intact cells of *Proteus vulgaris* were used as a source of enzyme.

So far as we are aware, no report has ever appeared on the purification and properties of urease of bacteria. The present work is a report on such a purification and a comparison of the properties of highly active bacterial urease with urease from other sources.

MATERIALS AND METHODS

Numerous strains of ureolytic bacilli were examined, and since *Bacillus pasteurii* (U.S.D.A. 673, Gibson no. 22) possessed relatively high enzymatic activity, it was employed throughout this study. The urea incorporated into all media was sterilized by filtration or intermittent steaming and added to the autoclaved media. Organisms were subcultured on two per cent urea meat infusion slants at room temperature. For growing cells in quantity 40 liters of medium (yeast extract, 0.7 per cent; polypeptone, 1.0 per cent; urea, 2 per cent) were distributed in 12 liter carboys and inoculated with the 24 hour growth from the agar surface of a Roux flask containing the same medium listed above. Growth was profuse under vigorous aeration, and from 250 to 300 grams of cells (wet weight) were recovered from each batch of 40 liters. In order to offset the cooling effect of aeration, growth was carried on in a 37 C incubator. Under these conditions the temperature of the medium could be maintained at 32 C. Foaming was prevented by using a silicone base, antifoam emulsion (Dow-Corning "antifoam AF emulsion"). Cells were harvested after 28 hours of growth and without washing were stored at −17 C under which conditions they could be held for periods of up to three months without appreciable loss in urease activity.

For extraction of the enzyme, desired quantities of frozen cells were thawed and suspended in distilled water and subjected to sonic vibration in a Raytheon 9 kc magnetostriction oscillator for 30 minutes. The cellular debris from the lysates was removed at 4 C in an Aminco high speed centrifuge.

The ammonium sulfate solution used for precipitation of the protein was saturated at 4 C and contained K_2HPO_4 to a final concentration of 0.05 M. The pH was adjusted to 7.2 with NH_4OH.

Protein was determined by the quantitative biuret method of Gornall *et al.* (1949). The method was standardized with a well dialyzed preparation of *B. pasteurii* protein, devoid of nucleic acids, the nitrogen content of which had been determined previously by the micro-Kjeldahl method of Hiller *et al.* (1948). Nucleic acids were removed essentially as described by Korkes *et al.* (1950). Calcium phosphate gel was prepared according to Singer and Kearney (1950).

The activity of urease was measured by the colorimetric method of Van Slyke and Archibald (1944) and by utilizing the unit system suggested by Sumner and Graham (1925) in which a unit of urease activity is defined as that amount of enzyme capable of producing one mg of ammonia nitrogen in 5 minutes at 20 C in a solution of

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urea buffered with phosphate. This assay was intended originally for urease activities of a lower order than those found in the present study, but experimentation established that the length of time for the pH of the buffer solution to change from 6.7 to 7.7 was strictly proportional to the amount of enzyme used. By increasing the volume of buffer and decreasing the amount of enzyme used, it was possible to measure very high activities. The speed and simplicity of the method were distinct advantages since bacterial urease is inactivated rapidly during the purification procedure. All activities subsequently referred to are in terms of Sumner units per gram of protein.

The Michaelis-Menten constants were derived by the method of Lineweaver and Burke (1934). The reaction mixtures, composed of phosphate to a final concentration of 0.2 M, urea at the desired concentration, and the enzyme, were incubated under carefully controlled conditions in a water bath at 20 C. At zero time and at other time intervals, aliquots were removed and added to HCl to stop the reaction. Ammonia, which had formed as a result of urease action, was aerated into boric acid and subsequently titrated with standard acid prepared from constant boiling HCl. Velocities of hydrolysis of urea were calculated then from graphs on which the ammonia formed was plotted against time.

Temperature coefficients between 10 and 50 C were determined in a well controlled water bath with a reaction mixture composed of 0.2 M phosphate buffer (pH 6.7) containing 3 per cent urea and desired quantities of enzyme. Prior to the experiment in each case the components of the reaction mixtures were carefully prewarmed to the temperature involved, then the reaction mixture was made up rapidly, and at the end of a five minute incubation period the urease was inactivated by the addition of HCl. Activation energies were calculated using the Arrhenius equation:

$$\mu = \frac{2.303\ R \log \frac{k_2}{k_1}}{\frac{1}{T_1} - \frac{1}{T_2}}$$

Purification procedure for bacterial urease. Crude lysates of *B. pasteurii* routinely displayed activities of about 2,500 Sumner units per gram dry weight. After removal of the cellular debris by centrifugation, the activity increased to 4,500 units per gram dry weight. Since the supernatant fluid contained considerable nonprotein material, the activities expressed in terms of dry weight and in terms of protein do not agree until the bulk of the nonprotein material has been removed by a preliminary ammonium sulfate fractionation and by removal of nucleic acids. Following the latter step, activities expressed on a dry weight basis or in terms of protein measured by the biuret method correspond fairly well. Activities noted, however, are all in terms of Sumner units per gram of protein. Stages numbered in the procedure given below correspond to those shown in table 1 which was prepared by averaging eight successive runs.

Fractionation with ammonium sulfate (Stage 1). The cell lysate was diluted to 2.3 mg of protein N per ml, an equal volume of saturated ammonium sulfate solution was added, and the protein was allowed to flocculate for two hours at 4 C, following which the precipitate was removed by centrifugation in the cold and discarded. To the

TABLE 1

Stages in the purification of urease from Bacillus pasteurii

STAGE OF PURIFICATION	PROTEIN	TOTAL PROTEIN	TOTAL UREASE UNITS	SUMNER UNITS PER G PROTEIN	UREASE PER CENT OF TOTAL
	mg/ml	*mg*			
Lysed bacteria	—	—	—	2,500	—
Centrifuged lysate	14.4	4,425	37,600	8,500	100
1. 1st $(NH_4)_2SO_4$ fraction	9.2	1,490	25,400	19,200	67
2. Protamine—SO_4 supernate	6.2	1,270	22,200	19,800	59
3. 1st gel supernate	4.5	946	18,600	22,400	51
4. Adsorbed and eluted enzyme	5.8	248	10,800	44,100	29
5. 2nd $(NH_4)_2SO_4$ fraction	8.7	74	6,200	85,200	16
6. Acetone fraction	4.1	18	3,400	165,000	9

supernatant an equal volume of saturated ammonium sulfate was added, and precipitation was allowed to proceed for three hours. At the end of this time the flocculated protein was recovered by centrifugation, and the supernatant was discarded. A minimum of water was used to redissolve the precipitate after which the enzyme solution was dialyzed for three hours in the cold against three liters of distilled water containing 0.003 M H_2S. At this point activities were generally between 17,000 and 23,000 units per gram of protein.

Precipitation of the nucleic acids (Stage 2). The dialyzed protein solution was titrated to a pH of 6.0 with 0.1 M acetic acid. The solution was stirred constantly to avoid excessive inactivation of the protein. For each 100 mg of protein, 30 mg of protamine sulfate, as a one per cent solution, were added with a pipette immersed under the surface of the mixture while it was stirred mechanically. The precipitate was removed by centrifugation and discarded. The ultraviolet absorption ratio (280 mμ/260 mμ) could not be raised above values of 0.90 to 0.93 without incurring inordinate losses in urease, indicating that the process did not remove all the nucleic acids. The activities of the enzymatic preparations following the protamine treatment were usually about 500 units higher than in the preceding stage of purification. Nevertheless, despite the fact that all the nucleic acids were not removed and the activities showed only a small increase, this step was essential since it was established that in the presence of the bulk of nucleic acids the adsorption of the enzyme on calcium phosphate gel in later stages was very poor. Also many early failures to so adsorb the urease in this study have been traced to the presence of nucleic acids.

Preliminary treatment with calcium phosphate gel (Stage 3). For each 100 mg protein in the supernate of the previous stage, 50 mg dry weight of calcium phosphate gel were added with constant stirring. Following a mixing period of five minutes the gel was removed by centrifugation and discarded. The supernatant solution at this point generally displayed activities of between 45,000 and 60,000 units. Adsorption at pH 6.0 under the conditions given did not remove appreciable amounts of urease from solution, but the step was essential because it apparently removed nonurease protein which interfered somewhat with the adsorption of urease in the next purification step.

Adsorption of urease with calcium phosphate gel (Stage 4). The supernatant solution from the previous step was adjusted to a pH of 5.6 with 0.1 N acetic acid, and while the mixture was stirred mechanically, 100 mg of calcium phosphate gel were added for each 100 mg of protein in the solution. Addition of the gel to the solution was carried out by gradually adding the suspension of gel to the solution by means of a pipette immersed beneath the surface of the solution. Agitation was continued for ten minutes after the addition of the gel, after which the gel was recovered by conventional means. The supernatant liquid showed little urease activity and was discarded. Elution was accomplished by vigorous mixing of the gel with two 15 ml portions of 0.2 M phosphate buffer (pH 7.2) for ten minutes each, after which the eluates were pooled and displayed activities of between 45,000 and 60,000 units per gram.

Second ammonium sulfate fractionation (Stage 5). The eluate from Stage 4 was made up to 50 per cent saturation with ammonium sulfate. After a two hour flocculation period, the precipitate was discarded and the supernate was made up to 75 per cent saturation. A precipitation period of 12 hours in the cold was allowed at this point prior to separating the precipitated proteins which were redissolved then in distilled water and dialyzed for three hours against H_2S-water in the cold. Activity: 75,000 to 90,000 units per gram protein.

Acetone fractionation (Stage 6). A 50 per cent (v/v) solution of acetone in water was precooled to about 4 C, and the enzymatic solution from Stage 5 was placed in a dialyzing tube and dropped into the acetone-water solution. The entire system then was placed in the deep-freeze at −17 C for 24 hours. This procedure apparently removed (by precipitation) considerable inactive protein and also served to concentrate the proteins remaining in solution. The inactive precipitate was removed at −15 C in a refrigerated centrifuge and discarded. Using acetone precooled to −17 C, the supernatant solution was adjusted to a final concentration of 70 per cent (v/v) acetone. Care was exercised not to allow significant rises in temperature since this invariably led to inordinate and rapid precipitation coupled with irreversible inactiva-

tion of the urease. The precipitation in the acetone solution was allowed to proceed for 24 hours, after which the precipitate was collected carefully by centrifugation in a refrigerated centrifuge and redissolved in 5 ml of ice cold water. Some inactive protein did not dissolve at this stage, and final clarification by centrifugation was usually necessary. Urease obtained at this stage was the most active material obtained displaying activities of between 150,000 and 190,000 units per gram of protein. Further attempts to increase the specific activity or to crystallize the enzyme met with consistent failure. It is of interest to recall that crystalline jackbean urease assayed by the same method and utilizing the same unit system has an activity of 133,000 units per gram (Sumner and Somers, 1947).

RESULTS AND DISCUSSION

Bacterial urease is inactivated rapidly on standing, even during refrigeration, by dialysis in the absence of reducing agents, by pH values lower than 5.2, and in the presence of organic solvents except at low temperatures. Owing to the untoward effects of low pH on the enzyme the ammonium sulfate solution was neutralized, and care was exercised in all pH adjustments by utilizing weakly acid solutions and efficient stirring during such manipulations. The time allotted to dialyzing was held to a minimum since some inactivation occurred even in the presence of H_2S. It was not possible to reactivate the enzyme by pH changes or the additions of reducing substances once inactivation had taken place. The purification of bacterial urease is attended with difficulties, not the least of which is the fact that the bulk of the purification procedure (through Stage 5) must be carried out within 24 hours.

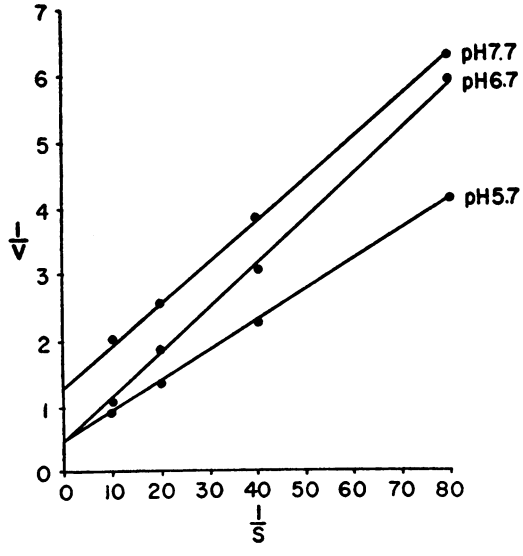

Figure 1. Lineweaver-Burke plots for purified bacterial urease. S = moles per liter. V = moles hydrolyzed per minute $\times 10^3$ at pH 5.7 and 6.7 and $\times 10^4$ at pH 7.7. 0.2 M phosphate buffer at 20 C. Urease activity: 165,000 units per gram protein.

At present the kinetics of the action of urease have not been resolved completely despite a formidable literature on the subject. Van Slyke and Cullen (1914) presented evidence for a two stage action of urease, and their data indicated an increase in the affinity of the enzyme for urea as the pH of the reaction medium became more alkaline. Support for the pH effect was afforded later by work with crystalline urease (Howell and Sumner, 1934) in which it was noted that the optimal pH of the enzyme depended upon the type of buffer used and the concentration of urea employed. Harmon and Nieman (1949) explained the pH effect as the result of competitive inhibition of urease by phosphate, but later a report from the same laboratory (Fasman and Nieman, 1951) rejected this hypothesis and in its stead put forth the view that the inhibition was the result of sodium and potassium ions and that phosphate actually served as an activating influence. In view of these experiments much early published work on the inhibitory effects of various ions on urease may well be questioned. In this connection the observed rise in specific activity of very dilute solutions of urease in hydrogen sulfide may be open to new interpretations as new data accumulate (Peterson *et al.*, 1948). Despite the difficulties attendant with interpretations of urease kinetics, it was felt that interesting comparative data might become available from such studies on bacterial urease, and a few investigations in this direction were carried out.

Figure 1 is the curve which results when 1/V is plotted against 1/S, where V is the quantity of urea hydrolyzed per minute and S is the concentration of substrate in moles per liter. From these data the K_m constants were derived and found to be as follows: 0.10 M at pH 5.7, 0.13 at

pH 6.7, and 0.04 at pH 7.7. The affinity of the enzyme for urea appears to be essentially the same at the two lower pH levels, but at pH 7.7 the affinity is increased. The K_m values found for bacterial urease are somewhat higher than corresponding values calculated with plant ureases. A considerable number of K_m values appear in the literature for urease; these vary rather considerably depending upon the buffer employed, pH, and source and concentration of the urease. Thus, the data of Van Slyke and Cullen (1914) yield a K_m of 0.025 M for soybean urease whereas Peterson *et al.* (1948) first reported a figure of 0.0105 M for crystalline jackbean urease in 0.1 M phosphate buffer at a pH of 7.0 at 25 C, but this figure was changed later to 0.035 M (Harmon and Nieman, 1949). If maleic acid buffer is used under identical conditions, the K_m for crystalline urease is 0.003 M. It would appear wise to accept all these values, including those reported for *B. pasteurii* urease, with some reservations until such time as the questions of urease inhibition and activation by various ions have been clarified.

Figure 2 is a plot of the concentration of substrate against the velocity of the hydrolysis of the urea which, in accord with expectation, indicates that the action of bacterial urease is a zero order reaction. This corresponds to the activity of urease from plant sources.

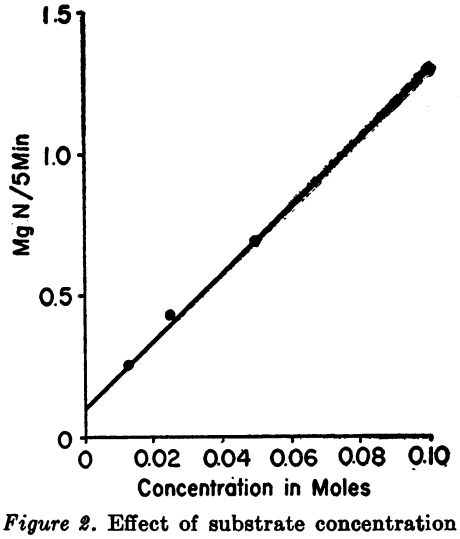

Figure 2. Effect of substrate concentration on rate of urea hydrolysis. Phosphate buffer, pH 6.7, at 20 C. Urease activity: 157,000 units per gram protein.

TABLE 2

Temperature coefficients of purified bacterial urease

TEMPERATURE	Q_{10} BACTERIAL UREASE	Q_{10} JACKBEAN UREASE*
C		
10–20	1.80	1.95
20–30	1.44	1.63
30–40	1.30	1.49
40–50	1.24	1.34

* Data of Sumner (1951).

Temperature coefficients for bacterial urease are somewhat lower than similar figures derived from work with the jackbean enzyme as may be seen in table 2 where the coefficients for both types of urease have been listed. Similarly the activation energies of the bacterial enzyme appear to be lower than those found for plant ureases. Data for activation energies are given in graphic form in figure 3 which is the result of plotting the log rate of the hydrolysis of urea against the reciprocal of temperature in the conventional fashion. Activation energies of 9,900 calories below and 4,400 calories above a critical temperature are the calculated values for purified bacterial urease. Sizer (1939, 1940, 1941) obtained similar data working with plant

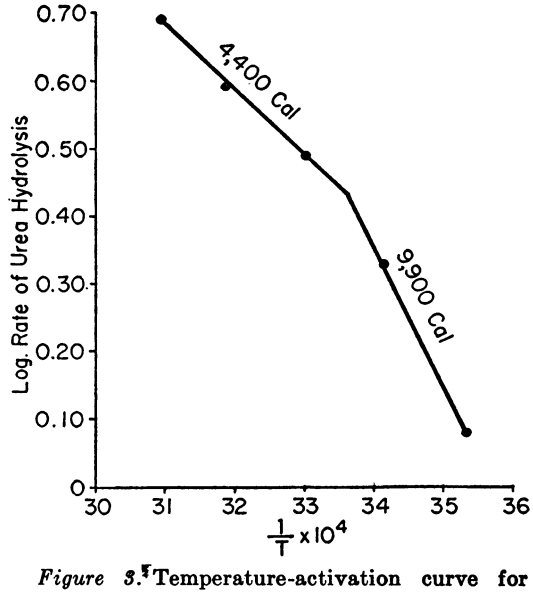

Figure 3. Temperature-activation curve for purified bacterial urease. 0.2 M phosphate buffer, urea 3 per cent. Urease activity: 160,000 units per gram protein.

ureases and the urease of *P. vulgaris* except that his figures are 11,000 calories and 8,700 calories, respectively. The sharp break in the curves of Sizer has been criticized by Kistiakowsky and Lumry (1949) who found that with jackbean urease as the experimental material the activation energy was 8,830 calories throughout the whole temperature range. Results obtained with the bacterial enzyme are similar to those obtained by Sizer, as inspection of figure 3 will demonstrate, although it may be possible that a larger number of experiments at various temperatures would result in data which would yield a smooth curve (indicating a gradual rise in the activation energy as the temperature is lowered) rather than the sharp break illustrated. The results graphed in figure 3 were repeated carefully a number of times with identical results so that considerable justification exists for presenting the findings in the form shown. With respect to the work of Sizer (1940) on the urease of *P. vulgaris*, it may be pointed out that intact cells were used, and the presence of the cell membrane may present some complications even with such a readily diffusible compound as urea. In the case of *B. pasteurii*, for example, resting cells hydrolyze urea only one-fifth as rapidly as an equivalent weight of lysed cells. It is possible that the lower temperature of activation displayed by bacterial urease accounts for its greater activity as compared with other ureases.

The specificity of bacterial urease appears to be as absolute as that of jackbean urease. The following urea derivatives were tested: Urea nitrate, semicarbazide, thiourea, biurea, biuret, guanidine, arginine, aminoguanidine, acetylurea, methylurea, 1-acetyl-2-thiourea, 1-*tert*-butylurea, phenylurea, *p*-phenetylurea, *tert*-amylurea, *m*-tolylurea. Only one of these compounds (urea nitrate) which spontaneously hydrolyzes to urea was attacked as measured by ammonia formation.

The finding that bacterial urease has greater activity than jackbean urease is paralleled by the behavior of bacterial catalase which was found to have activity of nearly twice that of catalases from other sources (Herbert and Pinsent, 1948). Furthermore, the higher activity of bacterial catalase could be attributed only to the protein moiety since the number of hematin groups was found to be the same as in other catalases.

It is of interest to speculate on the role of urease on the metabolism of *B. pasteurii*. If the activity of pure bacterial urease is 250,000 Sumner units per gram, then it may be calculated that the organisms on a dry weight basis would be one per cent urease, a figure close to the calculated catalase content of *Micrococcus lysodeikticus* (Herbert and Pinsent, 1948). *B. pasteurii* has been shown to require either ammonia or urea for growth by Gibson (1934) who presented evidence to show that urease is required only for the production of ammonia from urea. This peculiar nitrogen requirement for this organism has been confirmed in this laboratory and will be presented elsewhere in greater detail as part of a metabolic survey of ureolytic bacilli. If urease, then, is required only for the production of ammonia from urea, it is difficult to understand why the organism contains such an active enzyme in the quantity shown by crude cell lysates—clearly the organism is capable of producing prodigious quantities of ammonia from urea, considerably more than could be utilized for growth. An explanation may lie in one or a combination of the following factors: (1) Intact cells hydrolyze urea only one-fifth as rapidly as an equivalent weight of lysed cells, thus indicating that in the case of the breakdown of urea by resting or growing cells "permeability" to urea is an incumbent argument particularly at the optimal pH for growth. (2) The organism grows best at a pH range of 8.5 to 9.0 under which circumstances ammonium ions are released rapidly from the medium as free ammonia and require constant replacement. (3) The optimal pH for urea hydrolysis by either intact cells or the purified enzyme is between 6.5 and 7.0; thus, the activity of urease is reduced much at the optimal pH for multiplication, and this reduction in activity may be compensated for by the synthesis of more urease.

The procedure for purifying bacterial urease has been reported in some detail as it would seem of considerable comparative interest to determine the molecular weight as well as other physicochemical constants for this species of urease.

SUMMARY

A fractionation procedure for the purification of bacterial urease from *Bacillus pasteurii* has been developed. Enzymatic activities of 150,000 to 190,000 Sumner units per gram of protein have been described. Some kinetic studies have

been carried out on bacterial urease, and the results have been compared with corresponding results obtained with urease from other sources. The possible role of urease in the metabolism of *Bacillus pasteurii* in relation to the total content of urease in the bacterial cell has been discussed briefly.

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