it appears unlikely that the pH-dependence of T_{11} (Fig. 5) is due to side-chain hydrogen bonding; rather, this behavior is probably the result of electrostatic effects.

If we accept the models proposed for native and denatured STI, the optical rotatory data indicate that a combination of STI and trypsin leads to essentially no change in the conformation of the backbone of either protein. It is not possible to discuss the possible involvement of side-chain tyrosyl or tryptophanyl groups in the association because of the autolysis of trypsin during the measurement of the difference spectrum.

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Some Properties of Urease*

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Samples of urease with reproducible specific activity, 153,000 Sumner units/g, and absorption spectrum, $E^{1\%}_{1cm}$ 7.71 at 272 mμ, have been obtained. Ultracentrifugal analysis indicates a high degree of purity. The molecule (M.W. 473,000) contains about 77 methionyl, 29 cystinyl, and 47 cysteinyl residues. Some of the mercapto groups are very reactive and may be involved in exchange reactions with disulfide groups, causing polymerization. The mercapto groups essential for enzymatic activity are less reactive.

Urease is a very interesting enzyme because of the high efficiency and specificity of its action. It deserves attention also because of its historical importance, as it was the first enzyme to be isolated in crystalline form. Despite this point of special interest, comparatively little progress has been made in the intervening period of time toward elucidation of its chemical nature (Varner, 1960). This paper describes some contributions toward the solution of this problem.

Experimental Procedures

Materials and Apparatus.—Commercially available reagent-grade chemicals were generally used without further purification, but samples were selected which, according to the manufacturer's analyses, had low heavy metal content. Cysteine hydrochloride hydrate, grade B, was obtained from the California Corporation for Biochemical Research, Los Angeles 63; crystalline bovine serum albumin from the Armour Laboratories, Kankakee, Ill.; *p*-chloromercuribenzoate from the Sigma Chemical Company, Inc., St. Louis, Mo.; *N*-ethylmaleimide from the Delta Chemical Works, New York; "Alka-ver" indicator from the Hach Chemical Company, Ames, Iowa. Guanidine hydrochloride, initially of "Aero" (technical) grade from the American Cyanamid Company, Bound Brook, N. J., was purified as described elsewhere (Leslie *et al.*, 1962). The water used was purified by ion exchange and distilled once through an all-glass still, except when otherwise specified.

The jack-bean meal was prepared from beans grown in 1959 by Mr. Ernest Nelson, Route 1, Waldron, Ark. The beans were first ground to pea-sized pieces in a motor-driven stainless-steel

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food chopper (Model 4222, Hobart Mfg. Co., Troy, Ohio) and then ground to fine powder in a stainless-steel hammer mill (Micropulverizer Type CF, Metal Disintegrating Co., Pulverizing Machinery Division, Summit, N. J.).

The composition of buffers used in this work was as follows (except when otherwise specified, quantities were dissolved in enough water to give 1 liter of solution, *p*H 7.0 ± 0.1). Phosphate (0.75 M): 28.0 g KH_2PO_4, 128.4 g $Na_2HPO_4 \cdot 7H_2O$ (same as 9.8% phosphate buffer prescribed by Sumner, 1955). Phosphate (0.02 M): 1.150 g $NaH_2PO_4 \cdot H_2O$, 3.128 g $Na_2HPO_4 \cdot 7H_2O$. Phosphate-chloride: 0.924 g $NaH_2PO_4 \cdot H_2O$, 3.570 g $Na_2HPO_4 \cdot 7H_2O$, 5.845 g NaCl. Phosphate-cysteine: 5.362 g $Na_2HPO_4 \cdot 7H_2O$; 1.176 g cysteine hydrochloride hydrate; 5.453 g NaCl. Phosphate-sulfite: 0.924 g $NaH_2PO_4 \cdot H_2O$, 3.570 g $Na_2HPO_4 \cdot 7H_2O$, 1.008 g Na_2SO_3, 0.208 g $NaHSO_3$, 4.092 g NaCl. Citrate (*p*H 6.1): 950 ml 1 M trisodium citrate, 50 ml 1 M citric acid.

Assay of Urease.—The test solution (Nelson, 1955) was prepared by dissolving 25.0 mg crystalline bovine serum albumin in 30–40 ml 0.75 M phosphate buffer, adding 1.5 g urea, and diluting with buffer to 50 ml. This solution was prepared fresh as needed, and stored with refrigeration no longer than 24 hours. One milliliter of test solution was mixed with 1 ml of 0.02 M phosphate buffer, 2 drops of "Alka-ver" indicator were added, and the mixture was titrated with 0.1 M hydrochloric acid to the first purple color; A = volume of acid used in ml. For the assay, 1 ml each of test solution and sample were mixed and allowed to stand *exactly* 5 minutes at 20 ± 0.5°; then the indicator and A ml of acid were added within 10 seconds, and titration was continued to the purple end-point; B = volume of acid used, in ml. The activity of the sample in Sumner units (S.U.) was taken as equal to the number of milligrams of ammonia nitrogen liberated in the conditions as a result of enzyme action: S.U. = $(B - A) \times M \times 14.0$, where M is the molarity of the acid. The accuracy of the determination was checked with samples of known ammonia content. The samples to be tested contained 4 S. U./ml or less. Phosphate buffer, 0.02 M, was used to dilute the samples. The dilution was made only a few minutes before the assay.

The jack-bean meal was assayed by stirring 1.00 g of it for 10 minutes with 100 ml of water at 25 ± 1°, filtering a small portion by gravity through Whatman No. 41 paper, and assaying promptly. The aqueous solutions obtained in this way contained 2.2 S.U./ml, corresponding to 220 S.U./g of meal; clearly, this may not represent the total urease content, but only the amount of active enzyme extracted in the conditions.

Preparation of Urease.—The first attempts to prepare crystalline urease by the procedure of Sumner (1951, 1955) were unsatisfactory, and it became apparent that rigid control of all experimental conditions was necessary to obtain reproducible results. A detailed account of the problems encountered and the procedure employed have been given in another paper (Gorin *et al.*, 1960).

TABLE I
TYPICAL ISOLATION PROCEDURE

Material	Quantity	Activity (S.U.)
Jack-bean meal	1 kg	220,000
Filtrate	4000 ml	76,000
Solution of 1× crystd. urease	40 ml	32,000
Solution of 2× crystd. urease	25 ml	20,000
Solution of 3× crystd. urease	23 ml	18,500
Solution of 4× crystd. urease	20 ml	16,800

In the present work, some twenty large-scale preparations were executed, each using 800–1600 g of meal. Table I gives the results obtained in a representative case. Several preparations gave substantially greater yields of enzyme, and a few gave smaller yields; in all cases, a crystalline product of high activity was obtained.

Ultracentrifugation.—The ultracentrifugal analyses were conducted in a Spinco Analytical Ultracentrifuge, Model E, equipped with temperature control and a phase plate in the schlieren optical system. The speed of the rotor was generally 52,640 rpm; its temperature was maintained constant and usually near 4°. The observed sedimentation coefficients were corrected to standard conditions, water medium and 20°. Relative amounts of the observed components were determined by measuring the areas with a Gaertner Toolmaker's Microscope. The observed areas were corrected by the inverse square law of dilution. No corrections were made for the Johnston-Ogston effect.

Analyses.—Crystalline urease was taken up in water, and the nitrogen content of aliquot portions was determined by Kjeldahl analysis (Association of Official Agricultural Chemists, 1960). Other portions were evaporated to dryness at 95° and the residues allowed to stand *in vacuo* over phosphorus pentoxide to constant weight. Kjeldahl analyses were also done on many solutions of urease in water and in 0.02 M phosphate buffer.

Sulfur was determined by the methods of Carius or of Schöniger (1956). The sulfate obtained by either method was determined according to the directions of Munger *et al.* (1950).

Methionine was determined by the procedure of Horn *et al.* (1946) after hydrolysis of the urease in 20% hydrochloric acid at 115° for 18 hours.

Cystine and cysteine were determined by the methods of Akabori and Fujiwara (1958) or of Bandemer and Evans (1960).

Reaction with p-Chloromercuribenzoate (Boyer, 1954).—In a representative experiment, the mercurial was dissolved in 1 ml 0.1 M sodium hydroxide and diluted to 50 ml with 0.05 M phosphate buffer to give an approximately 10^{-4} M solution; the exact concentration was determined spectrophotometrically, with the value 1.69 ×

10^4 being used for the molar absorbancy coefficient at 232 mμ. The urease solution, in 0.02 M phosphate, was about 10^{-5} M. Varying amounts of mercurial were mixed with 0.200-ml aliquots of urease solution to give molar ratios between 10 and 250 and sufficient 0.05 M phosphate buffer was added to make the volume 5.00 ml. The absorbance of the reaction mixtures was determined after appropriate intervals of time, and the difference between the experimental value and that calculated for the unreacted components of the mixture was computed; the final readings were taken after 3 hours.

The hydrolytic activity of the reaction mixtures was determined in the manner described except that the test solution did not contain bovine serum albumin; the activity is expressed as a percentage of that found in the solution containing no mercurial.

Some experiments were done in the presence of 4 M guanidine hydrochloride. Solutions of this denaturant were made up in 0.05 M phosphate buffer, the *p*H was readjusted to 7.0, and the solutions were then mixed with buffered solutions of urease and mercurial. The absorbance of the mercurial reagent is altered by guanidine hydrochloride, and the "blank" absorbance values were therefore computed from measurements upon appropriate mixtures of these reagents.

Reaction with N-Ethylmaleimide.—The amount of *N*-ethylmaleimide which reacts with urease was determined by the procedure of Leslie *et al.* (1962). In a representative experiment, 0.5 ml of 0.004 M *N*-ethylmaleimide was mixed with 0.75 ml of 7×10^{-5} M urease and 4 ml 5 M guanidine hydrochloride. The final absorbance reading was taken after 30 minutes.

Reaction with Ferricyanide.—Solutions of potassium ferricyanide were made up quantitatively in 0.05 M phosphate buffer, *p*H 7.0, from weighed samples of solid reagent, protected from light, and used the same day. Guanidine hydrochloride, 6 M, was made up in 0.05 M phosphate buffer and adjusted to *p*H 7.0 with base. In a typical experiment, there were mixed 0.250 ml of approximately 1.3×10^{-5} M urease, 0.250 ml of 0.001 M ferricyanide, 2 ml of guanidine hydrochloride, and enough buffer to make 3 ml. The absorbance at 410 mμ was determined at intervals and compared with that of an appropriate blank.

Results

The isolation procedure of Sumner (1951, 1955) does not afford a good recovery of the enzymatic activity from the starting material, and some attempts were made to modify the first step in the procedure, extraction with 32% acetone, since the greatest loss was sustained at this point. This was due, at least in part, to incomplete extraction, since considerable enzymatic activity was left in the residue. However, it was found, in general, that the modifications effective in extracting more enzyme would also extract more inactive material, which interfered with the subsequent purification. The crystallization procedure, originated by Dounce (1941) and recommended by Sumner (1951, 1955), was found quite satisfactory, and could be repeated several times with only moderate loss of activity. Usually, the enzyme was crystallized four times, but fewer or more crystallizations were executed in some cases, as noted.

The nitrogen content of two four-times-crystallized preparations was found to be 15.8 ± 0.2%. The ultraviolet absorption spectrum had a maximum at 272 mμ. The absorbance and specific activity of several preparations are given in Table II. These data are based on the nitrogen content determined by analysis; many other preparations, which were not analyzed for nitrogen, had activity/absorbance ratios in accordance with the values tabulated. The activity and absorbance determinations were done in 0.02 M phosphate buffer within one day from the last crystallization; however, it was found that the activity suffered little or no change during a week's storage in the refrigerator.

Table II
Properties of Some Urease Preparations

Preparation No.	Times Crystd.	Specific Activity (S.U./mg N)	$E^{1\%}_{1\,cm}$ (15.8% N)
1	4	965	(8.66)
	6	970	7.76
2	4	1060	7.65
3	4	970	7.77
4	4	954	7.95
	6	984	7.77
5	3	850	7.34
6	4	987	7.58
7	4	890	7.58
8	3	1100	7.96
	Av.	973 ± 48	7.71 ± 0.15

Samples 1, 2, and 3 were subjected to ultracentrifugal analysis. In phosphate-sulfite buffer, the first two samples showed only one component, of sedimentation coefficient 19 *S*, while sample 3 also contained a small amount, about 7%, of a component of 3.4 *S*. Aliquot portions of the same preparations dissolved in plain or phosphate-cysteine buffer showed more complex sedimentation patterns that indicated the presence of aggregated products. Thus, sample 1 dissolved in phosphate-cysteine contained about 3% of a component of 29 *S*, and sample 3 about 2%. Sample 2 in plain phosphate buffer was even more aggregated, with 16% of 29 *S* and 1% 38 *S* components; after standing 4 weeks in the refrigerator, the solution showed a decrease in 19 *S* component and an increase in 29 *S* and 38 *S* as well as the appearance of a 44 *S* component (71%, 21%, 5%, and trace, respectively).

TABLE III
ANALYTICAL RESULTS

Element or Group	Method	Determinations	No./473,000
S	Carius or Schöniger	4	177 ± 5
	Akabori and Fujiwara	9	106 ± 8
Cysteinyl + $^1/_2$ Cystinyl	Bandemer and Evans	2	110 ± 2
Methionyl	Horn *et al.*	10	79 ± 7
Cysteinyl	Boyer	6	48 ± 4
	Leslie *et al.*	3	47 ± 4

Table III summarizes the analytical results obtained in this work with 3- and 4-times crystallized samples of urease. The determination of mercapto groups by the method of Boyer (1954) (Fig. 1) was less than fully satisfactory because the points corresponding to excess *p*-chloromercuribenzoate often showed considerable scatter, as illustrated by one of the cases represented. That the technique of measurement is not directly at fault is indicated by the fact that the points corresponding to lower mercurial/protein ratios all conformed closely to a straight line; it is suspected that the difficulty may be caused by the occurrence of association and the consequent scattering of light. Fortunately, the final result is not very sensitive to the location of the segment delineated by the scattered points, and fairly concordant results were obtained from several determinations. Aliquot portions of the reaction mixture were assayed and the results are shown in Figure 1. From this (and other determinations) it can be seen that 10–15 mercapto groups reacted with little diminution of the activity, and that about 75% of the activity was left after one-half of the mercapto groups had combined with the mercurial.

Urease treated with 4 M guanidine hydrochloride reacted with *p*-chloromercuribenzoate at a much faster rate than the native enzyme, but the amount of mercurial bound was the same. The same number of mercapto groups was found in denatured urease with *N*-ethylmaleimide.

Ferricyanide did not react with native urease in the conditions employed in this work. In 4 M guanidine hydrochloride, a rapid reaction took place, which was complete in less than 10 minutes; this was followed by a very slow further consumption of reagent, that came to no definite end. The amount of ferricyanide consumed in the first rapid reaction increased gradually as the reagent/protein was increased. Figure 2 shows some representative results.

DISCUSSION

The specific activity of enzyme preparations is an important criterion for establishing purity, but in the case of urease considerable difficulties attach to determining this property and establishing its significance. The values reported by various investigators vary rather widely (Hellerman *et al.*, 1943; Desnuelle and Rovery, 1949; Ambrose *et al.*, 1951; Sumner, 1951; Creeth and Nichol, 1960). Because of its high activity, the enzyme is assayed in exceedingly dilute solution, about 10^{-9} M, and it is very difficult to exclude the possibility of partial inhibition by some impurity at this concentration level. It has been shown in this work that 0.5% solutions of the enzyme in 0.02 M phosphate buffer retain their activity during several days' storage in the refrigerator, but solutions in other media may lose activity more quickly, and this is generally true for more dilute solutions. The work of Hofstee (1948–1949) provides some examples of how the activity of urease solutions may be decreased or increased by the conditions of storage. In the present state of knowledge concerning this enzyme and its action, the *absolute* value of its specific activity cannot be used as an accurate measure of purity.

In the present work, precautions were taken to insure that the different samples of urease would be tested in identical conditions; the final solutions contained about the same concentration of urease, were stored only a short time before testing, were diluted in a reproducible manner, and were assayed with the same reagents. In this way, the measured values of the specific activity would be strictly comparable. It was then possible to demonstrate that different preparations of urease had essentially the same activity, and that the activity of some preparations did not change on further crystallization.

The urease preparations which had the same specific activity also had the same ultraviolet absorption spectrum. Absorption at 272 mμ is shown by many proteins (Beaven and Holiday, 1952), and hence cannot be used for qualitative characterization. However, it follows that contamination of the enzyme by inactive protein would lower the activity/absorbance ratio; this is not the case for the samples under discussion. Based on a molecular weight of 473,000 (see below), the molar absorbancy coefficient calculated from our data is 365,000. This is much lower than the 798,000 previously reported by Landen (1940), even though that investigator reported that his preparation had a specific activity of 130,000 S.U./g.

The establishment of the degree of purity by still other methods is desirable. Sumner *et al.* (1938) subjected samples of urease to ultracentrifugal analysis, and found all of them heterogeneous to some extent; however, a component of sedimentation coefficient 19 *S* clearly predominated in the preparations, and the authors felt it could be unequivocally identified with the enzyme. McLaren *et al.* (1948) obtained results similar to those of Sumner. Finally, Creeth and Nichol (1960) have reported the analysis of five samples of urease, all of which contained components of

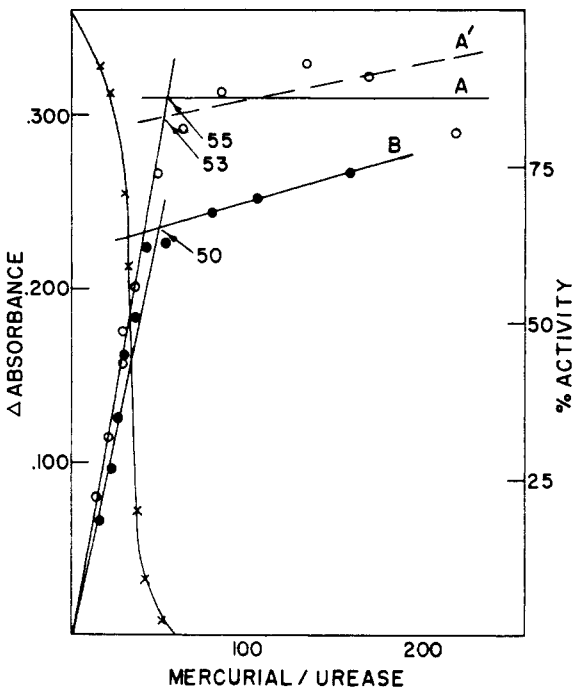

FIG. 1.—Reaction of urease with *p*-chloromercuribenzoate; increase in absorbance vs. mercurial/urease (molar) ratio. Open circles: Preparation 3; note that two quite differently placed segments (A and A') drawn through the points give about the same number of mercapto groups per molecule, 55 and 53, respectively. Full circles: Preparation 11. Crosses: Activity of preparation 11 after addition of mercurial compared to activity of uninhibited sample.

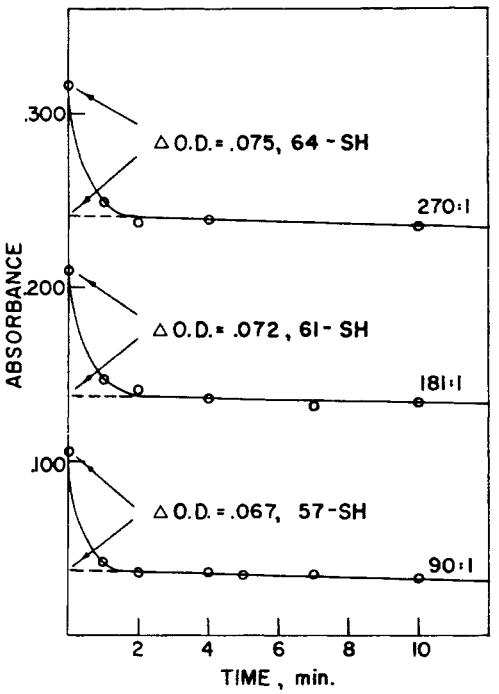

FIG. 2.—Reaction of urease with ferricyanide in 4 M guanidine hydrochloride at initial ferricyanide/urease molar ratios of 270, 181, and 90. Number of mercapto groups per molecule, calculated from decrease in absorbance (ΔO.D.) is 64, 61, and 57, respectively; value extrapolated to zero excess of ferricyanide, 55 groups per molecule.

19, 28, and 36 *S*. The first component predominated in all cases, but the proportion of heavier components was 10% to 40%. When these samples were treated with sulfite, the heavier components disappeared. On the basis of these results, Creeth and Nichol suggest that urease forms dimers and trimers in solution by a mercapto-disulfide exchange reaction; sulfite splits the disulfide bonds and thus reduces the teleomers to monomer. Creeth and Nichol do not specify the activity of the individual preparations used by them, but indicate it varied between 10,000 and 80,000 S.U./g.

The results of our ultracentrifugal analysis, performed in part before the paper of Creeth and Nichol had appeared, are in agreement with their findings, except that our preparations contained smaller proportions of polymeric material. The activity of our preparations leaves no doubt that the 19 *S* component is active enzyme and indicates that the preparations of Creeth and Nichol likely contained considerable amounts of an inhibitor, which, however, did not affect the ultracentrifugal behavior.

Our analytical results for the nitrogen and sulfur content of the enzyme are more precise than those previously reported, but agree with them within the precision of the respective measurements (Sumner, 1951). In the conversion of the analytical data into numbers of atoms or groups per molecule, 473,000 was used as the molecular weight (Sumner *et al.*, 1938), although it is now clear that this value may require some modification (Creeth and Nichol, 1960). The results of the calculations are listed in the last column of Table III. The total, cysteine + cystine + methionine, agrees with the sulfur analysis within experimental error, and this lends confidence to the results. A set of consistent values has been calculated by taking the average between the total sulfur analysis and the sum of cysteinyl-cystinyl-methionyl (182), and reducing the number of methionyl and cysteinyl plus cystinyl proportionally to conform with this value (77 and 105, respectively). Cystinyl has been calculated by difference.

The work presented in this paper was inspired in part by the very interesting study of Hellerman *et al.* (1943), which presented evidence for the existence of three types of mercapto groups of differing reactivity in urease. According to that study, a unit of 473,000 molecular weight contains 20–22 highly reactive groups (*a*), which are not concerned with enzymatic activity; a like number of groups (*b*), less reactive but still able to combine with *p*-chloromercuribenzoate, which are essential for enzymatic action; and possibly as many as 60 more groups, which react only after

denaturation of the protein in concentrated guanidine hydrochloride solutions.

In the present work the amount of *p*-chloromercuribenzoate bound to the native enzyme molecule was measured by Boyer's method, and the value obtained, 50 molecules of mercurial per molecule of enzyme, compares fairly well with the value 40–44 found by Hellerman *et al.* Our data on the inhibition of enzyme activity are, furthermore, in qualitative agreement with the suggestion that the mercurial is bound preferentially, but not exclusively, to the highly reactive, nonessential *a* groups.

Application of Boyer's method and of *N*-ethylmaleimide to the determination of the mercapto groups in denatured urease gave, within the somewhat limited precision of the data, the same number as found in native urease. Our results therefore do not support the estimate of Hellerman *et al.* regarding the existence of some 60 groups of low reactivity besides the *a* and *b* groups. This estimate was based on the reduction of porphyrindin and especially *o*-iodosobenzoate by denatured urease, and there are reasons to doubt that such oxidizing agents are generally reliable. A full discussion of the matter cannot be given here, and reference should be made to the review of Cecil and McPhee (1959) for the pertinent arguments.

It is interesting to consider in this connection the results obtained in the reaction of ferricyanide with denatured urease. These results show that the amount of ferricyanide consumed increased with the reagent/protein ratio. At low stoichiometric ratios the amount of oxidant consumed approaches the value expected for the oxidation of the mercapto groups to disulfide, but the stoichiometry observed at higher reagent/protein ratio bears no definite relation to the mercapto group content. Accordingly, we believe that the value for this quantity found with *p*-chloromercuribenzoate or *N*-ethylmaleimide should be preferred to that obtained with oxidizing reagents.

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