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SOME PROPERTIES AND PURIFICATIONS OF UREASE*

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SUMMARY

1. The kinetics of urease (urea amidohydrolase, EC 3.5.1.5) catalysed ureolysis in maleic acid–NaOH and Tris–H_2SO_4 buffers have been examined over a range of pH from 5.0 to 9.0 using 10 different preparations of the enzyme, substrate concentrations of between 1 and 10 mM urea and temperature ranging from 1 to 29°. Michaelis–Menten kinetics were followed, but each preparation of urease employed displayed a unique pair of kinetic parameters.

2. Parallel responses by the Michaelis constants to changes of pH were found and suggested that histidine, α-ammonium and sulphydryl groups are of importance in the reaction.

3. Temperature dependances of the Michaelis constants were functions of the preparations of enzyme. It is concluded that urease as commonly prepared is not a homogeneous catalytic entity.

4. Ion-exchange chromatography on XE-64 yielded enzyme with maximum specific activity of 3000 Sumner units per mg.

5. Urease with specific activity of 5300 Sumner units per mg has been consistently obtained by passage of crude enzyme through molecular sieves of polyacrylamide gel and of Sephadex G-200.

INTRODUCTION

In recent publications[1,2] describing measurements of ^{13}C-isotope effects in the urease (urea amidohydrolase, EC 3.5.1.5) catalysed hydrolysis of urea, unique behaviour of each preparation of urease was observed even when the enzyme was crystallized by the method of DOUNCE[3] 2 or 3 times. Analysis of the data obtained showed[1,2] that none of the commonly recognized forms of enzyme inhibition would explain all of the results collected: a combination of partially competitive and partially non-competitive inhibition would explain only some of them. A re-examination of the conventional kinetics of urease-catalysed ureolysis was thus of interest.

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The discovery of the complex inhibitory and activating effects of phosphate buffers on the enzyme[4,5] vitiated the results of the early studies of the kinetics of urease catalysis which were made in those buffers. As neither maleate–NaOH (ref. 6 and 7) nor Tris–H_2SO_4 (ref. 8) buffer has any apparent effect on urease, both have been employed in the ureolysis experiments described here.

KISTIAKOWSKY and co-workers[6,7] used several preparations of urease in the maleate buffer system at varying pH and ionic strength. In this work only relative rates were measured. Such a procedure is not sufficiently sensitive to distinguish changes in the parameters of urease which may be displayed by measurements of K_m and v_{max} as may readily be demonstrated. However, from these studies it was concluded[6,7] that there were divergences from strict Michaelis-Menten kinetics which, while not great, led to suggestion of two alternative mechanisms for urease catalysis. Complete support for either hypothesis could not be found[11–13].

Tris–H_2SO_4 buffer was used by WALL AND LAIDLER[8–10] in a study in which accord with the Michaelis-Menten formulation was established for urease catalysis. However, direct measurements of the parameters K_m, v_{max} for different preparations of urease were not made.

The kinetic data reported below show that urease as commonly prepared is not a homogeneous catalytic entity, but rather demonstrates characteristics which are related to the particular preparation of the enzyme employed. Because of these data, a search for methods of purifying the enzyme was undertaken.

SUMNER[14] first reported that urease prepared by the method he had developed[15] was not homogeneous, and his observation has been confirmed by sedimentation[16–21], electrophoretic[22] and ion-exchange chromatographic[23,24] techniques. GORIN and co-workers[20,25–27] have isolated urease with consistently higher specific activity (175 Sumner units per mg) than the most active material obtained previously (130 Sumner units per mg; ref. 14). However, this preparation was not considered to be homogeneous[27] but to consist of polymers of the enzyme. Using a more elaborate extraction procedure, SEHGAL AND NAYLOR[28] obtained, from jack-bean meal, urease with specific activity varying from 570 to 1030 units per mg. The enzyme of highest specific activity was considered pure though it was not homogeneous in sedimentation measurements[28].

Extensive purification of urease on the Amberlite ion exchange resin XE-64 is here reported: more efficient purification, yielding enzyme with specific activity of 5300 Sumner units per mg, is shown to occur during gel filtration on polyacrylamide gel and on Sephadex G-200.

EXPERIMENTAL

Reagents

The water used throughout these experiments was de-ionized and then distilled in glass apparatus.

All buffer solutions were prepared from analytical grade reagents and contained 0.1 mM EDTA. Citrate buffer (pH 6.0, $I = 2.9$ M) was prepared as recommended by DOUNCE[3], maleate buffer (pH 6.5) was prepared from NaOH and maleic anhydride, the latter being initially present at a concentration of 0.2 M. The ionic strength of the maleate buffer was adjusted to 0.3 M with Na_2SO_4.

Urea-phosphate solutions used in activity assays were prepared as described in ref. 29.

All apparatus used in the extractions and storage of urease, and in the assay of the activity, was washed with detergent and acid baths, liberally rinsed with distilled water, and, immediately before use, rinsed again with the buffer solution to be employed.

Extraction

Urease was commonly extracted from 200 g of jack-bean meal; when other weights were used the volume of aqueous acetone employed was varied proportionately. Acetone (analytical grade, 316 ml) and water (684 ml) were mixed and to this solution, at 26–28°, meal (200 g) was added with gentle swirling to disperse the powder. Filtration under gravity was begun through 2 layers of Whatman No. 1 paper within 5 min of beginning the extraction, and proceeded in a refrigerator at 5–10°. The extraction mixture was maintained at that temperature for about 70 h, after which the filtrate was centrifuged at 8000 × *g*, 4°, for 20 min. The precipitate was triturated with 20 ml of the citrate buffer (pH 6.0) for 24 h at 5–10°, and this mixture centrifuged (8000 × *g*, 4°) for 20 min, after which the supernatant, referred to as 'stock solution', was stored at 5° until required. Such solutions were commonly found to be stable over a period of several months.

When mercaptoethanol was employed in the extraction of urease[27] a 0.23-M solution in the aqueous acetone solvent was used; the procedure described above was then again followed.

Urease assay

The stock solutions of the urease were diluted to a convenient extent in maleate buffer (pH 6.5, $I = 0.3$ M) which is neither activating nor inhibitory for this enzyme[17]. Equal volumes (1 ml) of these dilutions and of urea–phosphate solution were mixed, and after 5 min at 20° ureolysis was stopped with 1 ml of 1 M HCl. The reaction mixture was diluted to a convenient volume and treated with Nessler's solution (Anachemia). Absorption at 4100 Å was measured on a spectrophotometer (Beckman Model DB) and the activity calculated from a standard ammonia/Nessler absorption curve using the relation between ammonia production and units of ureolytic activity suggested by SUMNER[29].

Protein determinations were made by the ninhydrin method of MOORE[30] which was standardized by comparison with nitrogen determinations of urease[31]. The assay procedure was checked with commercially available urease of known activity and protein content.

Jack-bean meal from 3 different suppliers (Nutritional Biochemicals Corporation; Hartman–Leddon Company; General Biochemicals) was used comprising six different manufacturer's 'Batch' numbers. These sources of meal will be designated N 93, N 45, N 77, H 50, H 51 and G 54 respectively. Furthermore, three different lots of N 77 were purchased at approx. 6 monthly intervals and will be distinguished, in chronological order of purchase, as N 77F, N 77S and N 77T.

From each of the batches of meal, various numbers of extractions were performed over periods of up to a year. In Table I are collected average specific activities of the enzymes obtained from these samples.

TABLE I

EXTRACTS FROM JACK-BEAN MEAL USING SUMNER'S PROCEDURE[15]

Jack-bean meal source	*Av. specific activity (Sumner units per mg) ± av. deviation*	*Average yield*		*No. of Expts.*
		Sumner units per g jack-bean meal	*mg extraction per g jack-bean meal*	
N 93	26 ± 5	6.5	0.25	5
N 45	79 ± 40	23.7	0.30	21
N 77F	877 ± 190	307	0.35	38
N 77S	617 ± 204	185	0.30	20
N 77T	423 ± 150	127	0.30	3
H 50	166 ± 61	58	0.35	4
H 51	184 ± 25	115	0.67	2
G 54	48 ± 4	43.2	0.09	3
N 77S*	1570 ± 380	695	0.44	9
N 77T*	1248 ± 247	562	0.45	12

* Extraction in presence of 0.23 M mercaptoethanol.

It is clear from the data of Table I that although there are fluctuations in the specific activity of urease prepared from a given batch of meal, the quality of the enzyme isolated depends primarily upon the quality of the meal used, (*cf.* refs. 27 and 32). Also, variations in specific activities found for extracted urease are attributable largely to variations in the amount of activity extracted, rather than to the yield of protein obtained.

Kinetic measurements

The preparations of urease employed are listed in Table II.

TABLE II

SUMMARY OF UREASE PREPARATIONS EMPLOYED IN KINETIC MEASUREMENTS

Prep. No. N 45	*Specific activity* (Sumner units per mg)*	*Prep. No. N 77F*	*Specific activity* (Sumner units per mg)*
I**	210/1.2 = 175	I	6100/5.0 = 1220
II	270/5.0 = 54	II	6100/5.0 = 1220
III**	115/1.0 = 115	III	3330/3.0 = 1110
IV	455/3.5 = 130	IV	2200/2.2 = 1000
V	412/7.5 = 55		
VI	120/3.0 = 40		

* Ratio (activity per ml stock soln./mg protein per ml.
** Recrystallized once[3].

Method. In maleate buffers measurements have shown[6] that variation of the ionic strength of the reaction medium will affect the enzyme kinetics at pH's above 7.0. For that reason, all buffer solutions employed in this work were maintained at an ionic strength of 0.3 M, adjustment to this being made with Na_2SO_4 (ref. 33).

Flasks were charged with solutions of urea in buffer to provide reaction mixtures which, on addition of urease in buffer, were 1, 1.33, 2.0, 3.0, 5.0 and 10.0 mM in urea. These solutions were allowed to attain temperature equilibrium in a bath

with control to $\pm$ 0.01°, as was one of urease. A constant volume of the latter, such as to produce ureolysis of convenient velocity, was added to each reaction vessel at time zero, and aliquots removed at measured intervals. Each experiment consisted of the range of urea concentrations listed above, each concentration being present in duplicate (that is, each experiment consisted of 12 reaction mixtures).

The aliquots from the ureolysis solutions were quenched in 1 M HCl. These quenched samples were immediately diluted to convenient concentrations and treated with Nessler's solution. Absorption at 4100 Å was measured against standard samples in a Beckman DB spectrophotometer.

The reaction was not followed beyond 20% of completion, and only with the more dilute urea solutions was that extent of hydrolysis necessary. Initial rates were estimated from plots of absorption *versus* time. Averages of the values obtained from the duplicate experiments were used in estimating K_m and v_{max}, Lineweaver–Burk plots being prepared for that purpose.

Purification methods

Throughout these experiments glass chromatographic columns of 0.9 cm internal diameter and 50–70 cm height were used. The eluants employed were the maleate and citrate buffers described above, and throughout any one chromatographic run no change in the eluant was made. When, in some experiments with XE-64, changes of ionic strength of maleate buffer were required, either Na_2SO_4 (ref. 33) was used or the concentrations of the buffer constituents were increased. The results obtained were independent of the way in which the ionic strength was varied. Flow rates were changed from 2 to 7 ml/h without affecting the separations achieved.

Commonly 1 or 2 ml of the stock solution of urease containing 5–10 mg of protein were applied to the column; after absorption there-on elution was begun and 1-ml fractions collected on a drop-counting fraction collector. Activity recovered was measured immediately following chromatography as experience showed that the purified enzyme was unstable.

XE-64 ion-exchange resin. The resin was prepared from Amberlite IRC-50 (Mallinckrodt) in the manner described by HIRS[34] except that the sodium salt was converted to the free acid form with H_2SO_4 rather than HCl. 2 different preparations of XE-64 were used in the separations described here with no difference discernible in the results obtained.

Polyacrylamide gel. This medium was prepared for molecular sieving by a modification of the method reported by HJERTEN[35]. Gel with total monomer concentration of 5% and 3% cross linking was used. After formation[35] it was broken up with a glass rod and washed repeatedly with distilled, de-ionized water. It was then washed with acetone (Mallinckrodt, A.R. grade) and coarsely chopped into lumps of about 1 cm^3. These were discrete entities, as the washing with acetone hardened the outer surfaces of the gel. The lumps of gel were then macerated in a Waring blender while suspended in acetone, a coarsely granular powder being obtained which was air dried and sieved, the fraction between mesh numbers 100 and 200 being retained and coarser material macerated further in acetone. The gel was then equilibrated with buffer.

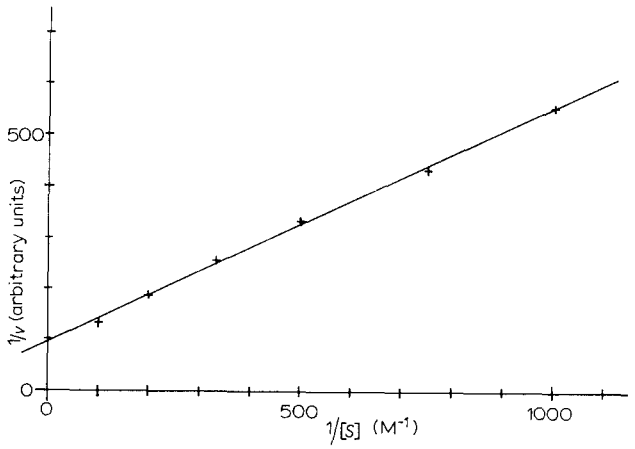


Fig. 1. Typical Lineweaver–Burk plot from initial rates measured in maleate buffer (pH 6.5; I = 0.3 M, 21°).

Sephadex G-200. This dextran gel (Pharmacia Inc.) was equilibrated with buffer as recommended by the manufacturer.

RESULTS AND DISCUSSION

Kinetic measurements

Fig. 1 is a representative Lineweaver–Burk plot prepared from initial rate measurements of ureolysis in maleate buffer (pH 6.5; I = 0.3 M; 21°). It is apparent that under the conditions employed in these experiments the Michaelis–Menten formulation for the enzyme kinetics is applicable. In agreement with the results of WALL AND LAIDLER[8] urease-catalysed hydrolysis of urea in Tris–H_2SO_4 buffers was also found to proceed in accordance with Michaelis–Menten kinetics over the range of urea concentrations from 10^{-3} to 10^{-2} M. Preliminary experiments showed that urease was stable in maleate buffer at pH's between 5.0 and 7.5, and in Tris–H_2SO_4 at pH's between 6.5 and 9.0.

TABLE III

EFFECTS OF pH, ON K_m AND ON v_{max}

Experiments at 21.0°, I = 0.3 M. K_m expressed in M and v_{max} in arbitrary units.

pH	*Prep. N 45-I in*				*Prep. N 45-III in maleate*		*Prep. N 45-V in*			
	Maleate		*Tris*				*Maleate*		*Tris*	
	K_m	v_{max}	K_m	v_{max}	K_m	v_{max}	K_m	v_{max}	K_m	v_{max}
5.0	0.0040	0.0025					0.0060	0.0010		
5.5	0.0045	0.0050			0.0055	0.0050	0.0065	0.0041		
6.0	0.0047	0.0059					0.0070	0.0067		
6.5	0.0046	0.0100	0.0034	0.0080	0.0056	0.0080	0.0064	0.0100	0.0036	0.0080
7.0	0.0049	0.0067	0.0051	0.0084			0.0072	0.0066	0.0068	0.0118
7.5			0.0064	0.0100	0.0061	0.0042	0.0080	0.0046	0.0071	0.0135
8.0			0.0036	0.0067					0.0060	0.0059
8.5			0.0024	0.0028					0.0024	0.0044
9.0			0.0027	0.0017					0.0019	0.0019

The values of K_m and v_{max} determined in this work are summarized in Table III. Comparison of values of v_{max} cannot be made directly between results from different preparations of the enzyme, as this parameter is dependant on the enzyme concentration which cannot be accurately determined at present [18,19]. If all preparations of urease were kinetically identical, K_m under any given set of reaction variables should be constant, assuming the Michaelis–Menten formulation to be applicable. It will be seen in Table III that, for example in maleate buffer at pH 6.5, such constancy is not found.

The data of Table III do show, however, that each enzyme preparation responds to changes of pH, in the two buffer systems used, in similar ways. It is apparent, for example, that the pH of maximal catalytic activity in maleate is about 6.5 units, as has been reported by KISTIAKOWSKY[6,7], and that the maximum in Tris is reached at about pH 7.5 (refs. 8–10). The nature of the buffer medium is known[36] to exercise a profound effect on urease as previous work[1,2] and the above results confirm for the two reaction media employed.

The relationship between pH and both K_m and v_{max} has been discussed, on the basis of a simplified model of protein amino acid dissociation constants, by DIXON AND WEBB[37] and by WALEY[38]. These concepts were applied in the interpretation of the data reported here and the results of this analysis are collected in Table IV.

TABLE IV

SUMMARY OF ANALYSES OF pH-DEPENDANCE CURVES

Medium	*pK identified*	*Curves analysed*	*Amino acids as ionizing groups implicated*[37]	*Role in reaction*
Maleate buffer	5.8	log v_{max}/pH	Histidine	Reaction of E–S complex
	7.0	log v_{max}/pH	α-Amonium	Reaction of E–S complex
Tris buffer	7.0	pK_m/pH: log v_{max}/pH	α-Amonium	Formation of E–S complex
	8.0	pK_m/pH: log v_{max}/pH	α-Amonium	Reaction of E–S complex
	8.3	log v_{max}/pH	Sulphydryl	E–S equilibrium

It is of interest that in both buffer systems investigated ionization of a group with pK 7.0 is apparently relevant to the catalysis by urease. MYRBÄCK[39–41] has also suggested that a group with similar pK (6.6) is implicated in urease catalysis in phosphate buffer. The group at pK 5.8 tentatively identified with histidine may be compared with that of pK about 6.1 reported by LAIDLER[42] as involved in reaction of the urease–substrate complex. The importance of the sulphydryl groups to urease catalysis has long been recognized from inhibition studies. The data of Table IV suggest that it is in the establishment of the enzyme–substrate complex that these groups are relevant.

Throughout the immediately preceding discussion of the effects of pH on K_m and v_{max}, the data obtained from the various enzyme preparations employed

have been considered as a whole. Clearly the responses of the preparations to the changes of pH imposed are parallel, but they are not identical, (Table III). In confirmation of that conclusion the temperature dependances of both K_m and v_{max} have been determined over the range from 1° to 29° in both maleate and Tris–H_2SO_4 buffers. In the former solvent the temperature dependances were investigated with 4 different preparations of urease at pH 6.5 and, with one preparation, at pH's 5.5 and 7.5 also. In Tris–H_2SO_4 2 preparations of urease were employed and temperature dependance measured at pH 7.5.

In Table V are summarized the results obtained, expressed where possible as Arrhenius energies of activation. $E_{a(K_m)}$ in maleate buffer (pH 6.5) ranges from 4.6 to 9.5 kcal/mole; $E_{a(v_{max})}$ in the same buffer ranges from 7.9 to 11.1 kcal/mole.

TABLE V

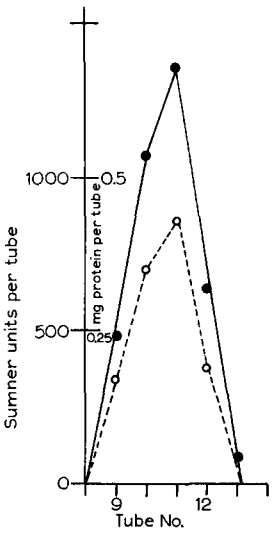
ARRHENIUS ENERGIES OF ACTIVATION OF K_m, v_{max}

Medium	*Enzyme Prep.*	*Buffer pH*	$E_{a(K_m)}$ *(kcal/mole)*	$E_{a(V_{max})}$ *(kcal/mole)*
Maleate buffer, $I = 0.3$ M	N 45-I	6.5	4.0	8.7
	N 45-II	6.5	7.5	10.8
	N 45-III	5.5	0	9.0
	N 45-III	6.5	4.6	11.1
	N 45-III	7.5	6.8	7.5
	N 45-IV	6.5	9.5	7.9
Tris buffer, $I = 0.3$ M	N 45-I	7.5	0*	0**
	N 45-II	7.5	0	—***

* Over temp. range 4.5° to 25.7°.
** Over temp. range 9.0° to 25.7°.
*** log v_{max} *vs.* $1/T$, curved line.

Of the data from investigation of the temperature dependance of urease-catalysed ureolysis in Tris–H_2SO_4 only that for K_m, Prep. N 45-II, yielded a straight line in an Arrhenius plot over the full temperature range investigated and gave a value for $E_{a(K_m)}$ of zero. From the same preparation, log v_{max} *versus* $1/T$ described a curve with maximum v_{max} at 29°, descending to become asymptotic to the $(1/T)$ axis. For Prep. N 45-I, which was studied more intensively in the Tris buffer, plots of log K_m and log v_{max} *versus* $1/T$ gave parallel complex curves with a wide range of temperatures over which there was no measureable response to that reaction variable (4.5° to 25.7°, and 9.0° to 25.7°, respectively). At both extremes of the temperature range examined the apparent Arrhenius energies of activation are large: approx. 23 kcal/mole at the upper, approx. 35 kcal/mole at the lower. Unambiguous interpretation of these data is not possible (see ref. 37 for discussion) until urease homogeneous in kinetic studies has been obtained.

The data reported above show that the kinetics of urease-catalysed ureolysis, in maleate and in Tris–H_2SO_4 buffer, are quantitatively dependant on the preparation of urease as extracted by the Sumner procedure, although displaying qualitative characteristics which are independent of the enzyme preparation employed. Such a conclusion is in agreement with data reported earlier[1,2]. Furthermore, LARSON AND KALLIO[43], in presenting some kinetic measurements for urease derived from



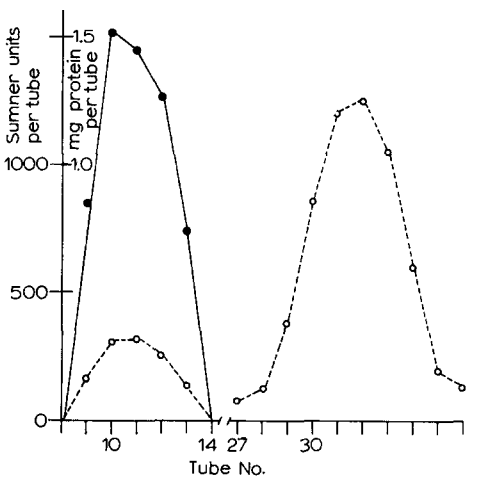


Fig. 2. Chromatography on XE-64: elution with maleate buffer (pH 6.5; I = 0.3 M; 1 ml/tube).

Fig. 3. Gel filtration on polyacrylamide gel: elution with maleate buffer (pH 6.5; I = 0.3 M; 1 ml/tube).

bacteria, imply an identical conclusion, as do PETERSON, HARMON AND NIEMANN[4] for jack-bean urease.

Purification on XE-64

The results of a typical experiment on an ion-exchange column of XE-64 equilibrated and eluted with maleate buffer (pH 6.5; I = 0.3 M) are presented in Fig. 2. The recovery of activity from the experiment illustrated there was 3656 Sumner units, and of protein, 1.14 mg: 3600 Sumner units and 5 mg respectively were applied. Thus, although there was recovery of all applied ureolytic activity, giving a product with specific activity 3200 Sumner units per mg, there was loss of 75% of the applied protein, which material was apparently firmly attached to the exchange resin, as it could not be eluted, even after prolonged washing with buffer at 3 M ionic strength. Results comparable with these were obtained also with Prep. N 77F-XXXVII (yielding urease with specific activity 3290 Sumner units per mg), and Prep. N 77F-XXXVIII (producing urease at 3000 Sumner units per mg), but in no case was there total recovery of the applied protein.

Although reproducible purification of urease on XE-64 could be obtained, as shown above, there was a variability in the performance of the resin which made it an unsatisfactory medium for enzyme purification[37]. Changing the buffer pH (maleate at pH's 7.5, 6.5 and 5.5, all at I = 0.3 M) type (citrate, pH 6.0, I = 2.9 M) or ionic strength (maleate, pH 6.5, ionic strength ranging from 0.3 to 1.6 M) neither increased the specific activity of the recovered urease above about 3200 Sumner units per mg, nor eliminated the variability in the performance of the exchange resin noted.

Purification on polyacrylamide gel

Results typical of those found after molecular sieving of crude urease solutions

TABLE VI

PURIFICATION OF UREASE ON POLYACRYLAMIDE-GEL FILTRATION

Experimental conditions			*Recovery*	
*Prep.**	*Applied (Sumner units per mg)*	*Buffer*	*Activity (Sumner units)*	*Specific activity (Sumner units per mg)*
II	5 000/5.0	Maleate (pH 7.5; I = 0.3 M)	5170	4720
II	6 500/6.5	Maleate (pH 7.5; I = 0.3 M)	6000	4840
II	5 000/5.0	Maleate (pH 6.5; I = 0.3 M)	5190	4950
II	10 000/10.0	Maleate (pH 6.5; I = 0.3 M)	9370	4860
III	8 000/7.0	Maleate (pH 6.5; I = 0.3 M)	8180	5300
III	6 860/6.0	Citrate (pH 6.0; I = 2.9 M)	6580	5080
XIV	5 800/9.0	Citrate (pH 6.0; I = 2.9 M)	5660	4700

* All from sample N 77F.

through columns of polyacrylamide gel equilibrated with maleate buffer (pH 6.5, I = 0.3 M) with the same buffer as the filtration medium, are shown in Fig. 3. There was complete separation of ureolytically active from inactive protein, the greater portion of the applied material being found in the latter peak. There was also complete recovery of both activity and of protein, as 5800 Sumner units and 7 mg respectively were applied and 5810 Sumner units (specific activity 4840 Sumner units per mg) and 7 mg were recovered in the experiment illustrated in Fig. 3.

15 different preparations of crude urease were examined after filtration on polyacrylamide gel, 2 different preparations of gel being used. The buffer systems employed and the results obtained are reported in Table VI, where it will be seen that none of the experimental variables examined markedly influenced the recovery of activity or the purity of the enzyme obtained. The purification attained was identical with that from filtration through columns of Sephadex G-200 (see Tables VI, VII, VIII).

Purification on Sephadex G-200

A representative set of data from one preparation of crude enzyme is shown in Table VII where it will be seen that the average recovery of activity was 100% and the average specific activity of urease collected was 5142 Sumner units per mg.

As was found in experiments with polyacrylamide gel, separation of a highly active from an ureolytically inactive component could equally well be effected using sodium citrate buffer (pH 6.0). A typical comparison is shown in Table VIII. Included there is the result from one experiment using the EDTA–phosphate buffer (pH 8.0) employed by SIEGEL AND MONTY[44]. On a column of Sephadex G-200 equilibrated with that buffer, those authors reported separation of a commercial sample of largely water-insoluble crystalline urease into 4 active components which were

TABLE VII

PURIFICATION OF STOCK SOLUTION N77S-I; GEL FILTRATION ON SEPHADEX G-200 WITH MALEATE BUFFER (pH 6.5; $I = 0.3$ M)

Three different filtration columns were used: for Expts. 1 and 2, 3, and 4, 5.

*Expt. No.**	*Applied (Sumner units)*	*mg*	*Recovered (Sumner units)*	*Specific activity (Sumner units per mg)*
1	2800	3.2	2450	4920
2**	2800	3.2	3030	5350
3	2400	3.2	2337	5000
4	6000	8.0	6400	5410
5	6600	8.8	6790	5030
				5142 ± 190 Sumner units

* Expts in chronological order over a period of 6 weeks.
** Elution buffer contained 0.03 M Na_2SO_3 and 0.013 M $NaHSO_3$.

categorised as polymers of urease. In our hands, no such separation was observed, the pattern of elution being identical with that of Fig. 3; one apparently homogeneous active component was separated from an inactive component.

The difference between the results reported here and those of SIEGEL AND MONTY[44] may be attributed to their use of an entirely different source of urease in which there was apparently extensive polymerization causing the low solubility noted. CREETH AND NICHOL[18,19] have shown that in sedimentation studies, where polymeric forms of enzyme are clearly distinguished, a single sedimenting entity is formed on treatment with sulphite ions, as presumably the intermolecular disulphide bonds are split[45]. Addition of this reagent (0.03 M) to the maleate buffer (pH 6.5, $I = 0.3$ M) used in this work effected no measurable change in the shape

TABLE VIII

EFFECTS OF ADDITIVES, BUFFER TYPES AND pH, GEL FILTRATION ON SEPHADEX G-200

Stock solutions	*Elution*	*No. of Expts.*	*Specific activity (Sumner units per mg)*
N 77F-XXVII	Maleate (pH 6.5; $I = 0.3$ M)	3	5490 ± 490
	Citrate (pH 6.0; $I = 2.9$ M)	3	5250 ± 210
	Maleate (pH 6.5; $I = 0.3$ M)*	1	5400
N 77F-XL	Maleate (pH 6.5; $I = 0.3$ M)	3	5270 ± 170
	EDTA–HPO_4^{2-}	1	5070
N 77S-I	Maleate (pH 6.5; $I = 0.3$ M)	4	5190 ± 300
	Maleate (pH 6.5; $I = 0.3$ M)**	1	5350

* Contained 0.001 M H_2S.
** Contained 0.03 M Na_2SO_3 plus 0.013 M $NaHSO_3$.

or position of the active urease peak, or on the specific activity of the purified urease (Table VII, VIII). Addition of H_2S (1 mM) to the same eluting medium likewise was without effect on the purification achieved (Table VIII).

A total of 120 purifications of 38 different preparations of crude urease from sources N 77F and N 77S on both polyacrylamide gel and Sephadex G-200 equilibrated with the buffers described above gave urease of specific activity 5300 (± 500) Sumner units per mg. Recycling the purified enzyme through the gel-filtration column failed to discover any inhomogeneity of the enzyme, if the recycling were performed within 22 h of the initial purification. However, there was appreciable loss of activity in this process. The active fractions from purification of crude urease by gel filtration were commonly stored in stoppered flasks at 5–10°. After such treatment for 25 days, and concentration with dried polyacrylamide gel[46], a sample of purified enzyme originally containing 5730 Sumner units activity and 1.06 mg protein was filtered through Sephadex G-200 in the presence of maleate buffer (pH 6.5; $I = 0.3$ M). 2 fractions were obtained, one of high molecular weight containing 3276 Sumner units activity and 0.77 mg protein (specific activity, 4254 Sumner units per mg), and another, inactive, consisting of 0.32 mg of protein with mol. wt. about 70 000 (estimated from the elution volume[47] by comparison with standards). Degradation of highly purified urease apparently occurs on storage, with simultaneous loss of ureolytic ability. RIETHEL, ROBBINS AND GORIN[48] have recently suggested, from sedimentation studies of urease in the presence of 6 M guanidine, that urease molecules are comprised of 6 unit polypeptides, mol. wt. 83 300. As no attempt to exclude O_2 from the storage solutions of purified urease was made in the work reported here, there may have been oxidative degradation of the enzyme to its component sub-units. This possibility is being examined.

As has been noticed above, there was invariably loss of activity by purified urease on recycling through Sephadex G-200 (or polyacrylamide gel). Such losses also occurred during storage of urease with the high specific activities obtained by the procedures reported here. This lack of stability of the purified enzyme (which occurred in the presence or absence of EDTA, and so could not be attributed to the presence of heavy-metal ions) may result from a general loss of secondary and tertiary structure following separation of the active urease from inactive proteinous material. Support for this explanation is found in the greater stability of preparations of enzyme with specific activities lower than the maximum obtained (see below); in the fact that stock solutions of crude urease were stable over periods of several months, and from the observation that addition of bovine plasma albumin (2 times crystallized) prolonged the half-life of the purified urease. Varying the buffer pH (6.0 to 8.0), ionic strength (0.3 to 2.9 M), or the type of buffer (maleate, citrate, EDTA–phosphate) failed to stabilize the purified enzyme. Similarly ineffective were H_2S (0.001 M), cysteine (0.1 M) and mercaptoethanol (0.1 M).

Several preparations of crude enzyme which differed in no discernible way from those previously used yielded, on gel filtration, purified enzyme with specific activity about 3000 Sumner units per mg. For example, with Prep. N 77F-XIII (specific activity 1200 Sumner units per mg) 4 different gel filtrations on polyacrylamide gel, using as solvents both maleate at pH 6.5 and citrate at pH 6.0, each yielded an active fraction with specific activities which averaged 3330 (± 390) Sumner units per mg. Separation qualitatively similar to that of Fig. 3 was achieved,

and there was complete recovery of both the applied activity and applied protein. Results similar to these were obtained with several other preparations of crude enzyme from the same batch of jack-bean meal.

The purification effected on both polyacrylamide gel and Sephadex G-200 is dependent on molecular sizes. Apparently whereas preparations such as those of Table VIII contain a proportionately small amount of a high molecular weight fraction associated with ureolytic activity, those such as N 77F-XIII contain a greater proportion of protein with molecular weight approximating that of urease, which may be inactive enzyme or protein unrelated to urease.

Separations qualitatively identical with that described in Fig. 3 were also obtained for samples of crude urease prepared from jack-bean meal from a number of different sources (Table I): the columns usually employed were loaded as in obtaining the results of Table VIII. However, the specific activities of the fractions of high molecular weight separated from inactive residue on Sephadex G-200 were invariably lower than those reported above. A selection of representative results is listed in Table IX.

TABLE IX

PURIFICATION OF UREASE FROM VARIOUS SOURCES, ON SEPHADEX G-200 WITH MALEATE BUFFER (pH 6.5; $I = 0.3$ M)

Jack-bean meal source	*Av. specific activity ± av. deviation (Sumner units per mg)*		*No. of Expts.*
	Applied	*Recovered*	
N 93	26 ± 5	1050 ± 120	2
H 50	223 ± 116	2910 ± 510	8
H 51	223 ± 116	1280 ± 180	2
N 77T	423 ± 150	2450 ± 220	4

The explanation adduced above for the isolation of urease with specific activity lower than maximal must here (Table IX) also be valid. In those former examples there had been, apparently, during the extraction of the urease from meal, occasional contamination of the enzyme with protein of high molecular weight, either inactive urease or some other protein. The reproducibility of the data for each preparation contained in Table IX shows that for these, contaminants in the active fractions from gel filtrations were consistently obtained, thus suggesting that they were readily extractable ingredients of the various samples of meal used and not occasional artifacts of the preparative method employed. These data confirm the observation above of the influence of the source of jack-bean meal on the nature of the urease extracted from it.

Although the reproducible isolation of urease with specific activity of about 5000 Sumner units per mg has been achieved, this has been found to depend on the quality of the jack-bean meal employed as a source of the enzyme. It has been shown that some samples of beans yield enzyme so mixed with inactive protein that gel filtration produces urease of lower than maximal specific activity: by contrast, it must be apparent that the most highly purified samples of enzyme here described

(approx. 5000 Sumner units per mg) are not necessarily pure urease. Studies of the characteristics of that material will be reported in a succeeding paper. Other methods of purifying urease are also being examined.

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