The Subunit Structure of Jack-Bean Urease

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1. Urease of specific activity 160–180 Sumner units/g. (Sumner, 1951) was purified from jack-bean meal. The preparation was pure on the basis of polyacrylamide-gel electrophoresis and *N*-terminal studies. 2. By using both the 1-fluoro-2,4-dinitrobenzene method and the phenyl isothiocyanate method a single *N*-terminal methionine residue was found. 3. A single *C*-terminal sequence -Tyr-Leu-Phe was found by studies with carboxypeptidase A, carboxypeptidase B and hydrazinolysis. 4. *N*-Bromosuccinimide cleavage showed that five unique tryptophan sequences were present: Trp-Ala, Trp-Glu, Trp-Gly, Trp-Met and Trp-Arg. 5. Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate showed that urease had a subunit molecular weight of 76 000. 6. The yield of *N*- and *C*-terminal amino acids, the number of tryptic peptides and tryptophan sequences and the above polyacrylamide-gel electrophoretic measurement all suggest that urease contains a single structural subunit of molecular weight 75 000.

Although urease was the first enzyme to be crystallized (Sumner, 1926), there are few reports of its physical and chemical substructure.

The initial ultracentrifugal examination (Sumner, Gralen & Eriksson-Quensel, 1938) showed the presence of a large and variable number of components in all the preparations they examined. The predominance of the 19s species suggested that this was the active enzyme molecule, and its molecular weight was measured as 483 000. This value has been confirmed by Reithel & Robbins (1967), who give a best value of $488\,000 \pm 5000$.

A number of the components found originally in urease preparations are now known to be active; the large 28s, 36s and 46s molecules (Kuff, Hogeboom & Striebig, 1955) are active and dissociate to the 19s species in the presence of reducing agents (Creeth & Nichol, 1960; Reithel & Robbins, 1967). More recently it has been shown that the 19s unit itself can be reversibly halved to give an active 10–12s species of molecular weight 190 000 (Gorin, Chin & Wang, 1968), 236 000 (Blattler, Contaxis & Reithel, 1967) or 262 000 (Tanis & Naylor, 1968).

The size of the structural unit(s) giving rise to these active polymers remains uncertain. The only direct evidence is that of Reithel, Robbins & Gorin (1964), who claimed that urease dissociated in 6M-guanidine hydrochloride has a molecular weight of 83 300. A number of indirect measurements of structural and active subunit size were reviewed by Varner (1960), who suggested that the evidence favours a subunit of about 100 000.

More recent work has attempted to estimate the

active subunit size from the number of active sites. Gorin & Chin (1965) estimated that there are eight sites/mol. of mol. wt. 473 000 by inactivation with Ag^+ , and Kobashi, Hase & Komai (1966) arrived at a value of two sites/mol. of mol. wt. 473 000 in the inactive urease-hydroxamic acid complex.

The aim of the present investigation was to examine the substructure of the enzyme by chemical means.

MATERIALS

Jack-bean meal from the Sigma Chemical Co., St Louis, Mo., U.S.A. (lot nos. 46B-4080 and 56B-5210), was used as the source of the enzyme.

Carboxypeptidase A (crystalline suspension in water), carboxypeptidase B-DFP (frozen aqueous solution of the di-isopropyl phosphorofluoridate-treated enzyme), cytochrome *c* (horse heart, type III) and alkaline phosphatase (*Escherichia coli*, type IIIS) were from the Sigma Chemical Co. Bovine serum albumin (fraction V) was from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex. Myoglobin (crystalline horse heart preparation) was from British Drug Houses Ltd., Poole, Dorset. Catalase (crystalline aqueous suspension) was from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Pepsin (twice recrystallized) and trypsin (salt-free, twice recrystallized) were from the Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Trypsin was assayed for tryptic and chymotryptic activity against toluene-*p*-sulphonyl-L-arginine methyl ester and benzoyl-L-tyrosine ethyl ester respectively by the spectrophotometric methods of Hummel (1959). Tryptic activity was 330 units/mg. at 35°; chymotryptic activity was undetectable (<0.4%).

Urea was recrystallized from 95% (v/v) ethanol and used as a freshly prepared solution; guanidine hydrochloride

solutions were prepared by dissolving guanidine carbonate (A.R.) in conc. HCl (A.R.); *N*-bromosuccinimide, iodoacetamide, and *p*-anisidine were recrystallized from hot water before use.

Other chemicals used were of the highest purity readily available.

METHODS

Urease purification. Jack-bean urease was prepared from 1 kg. lots of jack-bean meal by the acetone fractionation method (Sumner, 1926), as modified by Mamiya & Gorin (1965). All solutions were made up in 0.5% (v/v) 2-mercaptoethanol. After two recrystallizations the final solution was stored at 2° and either used within 1 month of preparation or freeze-dried. Loss of activity during storage of the final solution at 2° was less than 5%.

Urease assay. Activity was measured at $22 \pm 1^\circ$ by the differential titrimetric method (Reithel *et al.* 1964), with Bromocresol Green indicator in place of Alka-ver indicator. The activity is expressed in Sumner units (Sumner, 1951).

Determination of protein in solution. For estimation of the specific activity of urease preparations a specific extinction coefficient of 7.7 at 277 nm. was used (Gorin, Fuchs, Butler, Chopra & Hersh, 1962). After drying for 3 days *in vacuo* over P_2O_5 , $E_{1\%}^{1\text{cm}}$ of CM-urease* was measured as 7.2.

Preparation of CM-urease. Freeze-dried urease (150 mg.) was dissolved in 10 ml. of 2M-tris-HCl buffer, pH 8.5, made 6M with respect to guanidine hydrochloride. The mixture was flushed with N_2 before addition of 2-mercaptoethanol (0.1 ml.) and EDTA (30 mg.). Reduction was allowed to take place in a sealed flask for 4 hr. at room temperature. Recrystallized iodoacetamide (0.5 g.) was then added. After 30 min. at room temperature in the absence of light, the reaction mixture gave a negative nitroprusside test. It was dialysed overnight first against tap water and then against distilled water, freeze-dried and stored at -20° .

Preparation of AE-urease. Reduced urease prepared in the above manner was alkylated by addition of ethylenimine (0.25 ml.). Reaction was complete after 2 hr. at room temperature. The product was isolated by dialysis and freeze-drying.

Maleylation of urease preparations. The protein dissolved or suspended in water at 2° was adjusted to the phenolphthalein end-point by addition of aq. NaOH solution. Maleic anhydride (twice the weight of protein) was added with vigorous stirring. Aq. NaOH was added to maintain the pH at the phenolphthalein end point. The resulting maleyl-protein was recovered by freeze-drying after dialysis against tap water and distilled water. Maleyl content was determined by u.v. spectrophotometry (B. S. Hartley, personal communication).

Polyacrylamide-gel electrophoresis. For the examination of urease samples the method of Ornstein & Davis (1962) was used with occasional slight modifications (see the Results section).

N-Terminal-group identification. (1) DNP method. Samples of CM-urease (10 mg.) were dinitrophenylated by the method of Phillips (1958) and hydrolysed with 6M-HCl (0.5 ml.) at 105° in evacuated sealed tubes. The ether-soluble DNP-amino acids were separated and chromatographed by t.l.c. by the method of Brenner, Niederwieser & Pataki (1965).

graphed by t.l.c. by the method of Brenner, Niederwieser & Pataki (1965). For quantitative determination relevant areas of the silica layer were scraped off the plate, extracted with 1% (w/v) $NaHCO_3$ and centrifuged at 1000g for 5 min. The extracts were made to 5 ml. and their extinctions at 360 nm. determined. Blanks prepared from suitable areas of the plates had extinctions against 1% (w/v) $NaHCO_3$ of about 0.01 (cf. Spencer & Begg, 1966). Recovery experiments were carried out by the method of Fraenkel-Conrat, Harris & Levy (1955).

Examination of the water-soluble DNP-amino acid fraction for arginine was difficult because of large quantities of ϵ -DNP-lysine. Ether-extracted hydrolysates were therefore examined separately for ϵ -DNP-lysine and DNP-arginine by the following methods.

To determine ϵ -DNP-lysine the hydrolysate was dried repeatedly by rotary evaporation and dissolved in electrophoresis buffer, pH 6.5. The extract was analysed on Whatman 3MM paper by electrophoresis at pH 6.5 followed by chromatography in the second dimension with butan-1-ol-acetic acid-water (4:1:5, by vol.; upper phase) solvent (Sanger & Tuppy, 1951).

DNP-arginine was determined in hydrolysates after removal of ϵ -DNP-lysine as follows. The dried hydrolysate (10 mg.) was dissolved in water, neutralized with $NaHCO_3$, and buffered by a further addition of $NaHCO_3$ (150 mg.). Succinic anhydride (100 mg.) was then added with vigorous stirring until the reaction was complete. The succinylated hydrolysate was extracted four times with ethyl acetate-butan-1-ol (1:1, v/v). This extract was rotary evaporated to dryness, dissolved in M-HCl and extracted four times with ether and three times with ethyl acetate-butan-1-ol (1:1, v/v). The final extract was concentrated and examined for DNP-arginine by t.l.c. by the method of Brenner *et al.* (1965).

The identification of the DNP-amino acids was occasionally confirmed by paper electrophoresis at pH 2 of the $Ba(OH)_2$ -regenerated amino acids (Mills, 1950).

(2) Phenyl isothiocyanate method. The paper-strip method of Fraenkel-Conrat *et al.* (1955) was used. Samples of CM-urease (10 mg.) were applied to the strips in 70% (v/v) formic acid. The papers were washed for 2 days before cyclization of the phenylthiocarbamoyl derivatives of the amino acids. PTH-amino acids were identified by t.l.c. by the method of Brenner *et al.* (1965) and detected by spraying with iodine-azide (Sjoquist, 1954). This reagent gave excellent results provided that non-volatile chromatographic solvents were previously removed from the plate by heating at 130° for $1\frac{1}{2}$ hr.

C-Terminal investigation. For quantitative investigation digestion of CM-urease by carboxypeptidase A was carried out at 22° in 0.2M-phosphate buffer (NaH_2PO_4 - Na_2HPO_4), pH 7.8, made 1.5% (w/v) with respect to sodium dodecyl sulphate. Carboxypeptidase A (0.5 mg.), previously washed in water, was centrifuged, dissolved in 2M- NH_4HCO_3 (5 μ l.) and added to the protein (30 mg.), which was at a concentration of 6 mg./ml. Samples (0.5 ml.) were removed and quenched by the addition of 1 ml. of 0.5M-citric acid. The final solution was stored at -20° before analysis on a Technicon AutoAnalyzer fitted with a scale expander. Separate carboxypeptidase A and urease solutions were used as controls.

Qualitative and semi-quantitative analyses were carried out on digests of native urease and maleyl-CM-urease by

* Abbreviations: CM-urease, *S*-carboxamidomethyl-urease; AE-urease, *S*-aminoethyl-urease; PTH-, phenylthiohydantoin derivative of the specified amino acid.

paper electrophoresis at pH2. Digestion was carried out in 0.2M-NH₄HCO₃ (Potts, Berger, Cooke & Anfinsen, 1962) at enzyme/substrate ratios in the range 1:30 to 1:2000. Samples were removed at intervals and maleylated and native urease preparations acidified with acetic acid to pH3.5 and with picric acid (Efron, 1959) respectively. The de-proteinated solutions were analysed by paper electrophoresis at pH2.

Digestion with carboxypeptidase B was as follows. AE-urease (3mg./ml.) in 0.2M-sodium phosphate buffer, pH7.8, made 1.5% (w/v) with respect to sodium dodecyl sulphate was incubated at 35° with carboxypeptidase B, at an enzyme/substrate ratio of 1 EC unit/20mg. of protein. Samples (1ml.) were removed at various times up to 4hr. and added to 0.2g. of Zeo-Karb 225 (8% crosslinked; 100 mesh; H⁺ form). The resin was centrifuged, washed with water and extracted three times with 5M-NH₃ solution. The extracts were spotted for electrophoresis at pH2 and analysed as described above.

For investigation of the C-terminal amino acids by hydrazinolysis (Akabori, Ohno & Narita, 1952) samples of CM-urease (20mg.) were dried *in vacuo* over P₂O₅. Hydrazine (0.2ml.) was added and reaction was allowed to take place *in vacuo* for 24hr. at 80° or 6hr. at 100°. The hydrazinolysate was shaken with benzaldehyde (0.3ml.) for 1hr. and extracted with ethyl acetate. The aqueous fraction was dinitrophenylated and DNP-amino acids were extracted by the procedure of Kawanishi, Iwai & Ando (1964). The ether-soluble DNP-amino acids were examined by t.l.c. as described above.

N-Bromosuccinimide cleavage of CM-urease. The reaction was carried out in urea-acetate solutions by the method of Funatsu, Green & Witkop (1964); the concentration of the reagent was determined from E₂₇₅. The reaction was terminated by the addition of 2-mercaptoethanol (1μl./mg. of protein), and then the mixture was adjusted to pH8-8.5 with N-ethylmorpholine. 1-Fluoro-2,4-dinitrobenzene (0.1ml.) was added and allowed to react overnight with shaking. The dinitrophenylated peptides were precipitated by dilution of the mixture with acidified acetone, and washed in acetone and ether. The N-terminal residues were isolated and chromatographed by the procedures described above.

Tryptic digest of AE-urease. AE-urease (10mg.) in 8M-urea (0.5ml.) was adjusted to pH8.2 on a Radiometer TTT 1c autotitrator. The solution was diluted to 2M-urea and trypsin sufficient to give an enzyme/substrate ratio of 1:100 by weight was added in 10μl. of 1mM-HCl. The digestion at 37° was followed on the autotitrator and terminated by absorption on a column of Dowex 50 (X2; 200 mesh). After removal of all urea by washing with water, the peptides were recovered by elution in 4M-NH₃ solution. The eluate was dried *in vacuo* at 2° over conc. H₂SO₄ and NaOH.

Peptide 'maps'. The following method, a modification of that of Katz, Dreyer & Anfinsen (1959), gave the best results of a number tried.

The peptide mixture (2.5mg.) in electrophoresis buffer, pH3.5 (50μl.), was streaked along a 2cm. origin on 60cm. × 58cm. sheet of Whatman no. 3 paper. Chromatography was carried out overnight with butan-1-ol-acetic acid-water-pyridine (15:3:12:10, by vol.) solvent (Waley & Watson, 1953), until a Methyl Red marker had moved a distance of 35cm. The sheet was dried in an air draught for 4hr. and chromatographed again. After drying overnight the sheet

was cut to 60cm. × 45cm., and electrophoresis in the second dimension was carried out at pH3.5 at 3kv for 80min.

Paper-electrophoretic techniques. High-voltage paper electrophoresis was carried out by the method of Ryle, Sanger, Smith & Kitai (1955) in tanks (Michl, 1951). The buffers used were: pH6.5 (pyridine-acetic acid-water, 25:1:225, by vol.); pH3.5 (pyridine-acetic acid-water, 1:10:289, by vol.); pH2.0 (acetic acid-formic acid-water, 4:1:45, by vol.).

The carboxypeptidase A 'diagonal' technique was as follows (cf. Naughton & Hagiopian, 1962). A tryptic digest of AE-urease was loaded on to Whatman 3MM paper at a concentration of 1mg./cm. Electrophoresis was performed at pH2 with a potential gradient of 50 v/cm. for 50min. A strip 2cm. wide was cut from the electrophoretogram and sprayed lightly with a fresh solution of washed carboxypeptidase A (10μg./ml.) in 0.2M-NH₄HCO₃. Spots of Phenol Red indicator were incorporated at the ends of the strip and spraying was continued until the end point was reached. The damp strip mounted on a spiral support (Brown & Hartley, 1966), was incubated for 20min. at room temperature in a desiccator containing filter paper wetted with 0.2M-NH₄HCO₃. Digestion was terminated by evacuation, which was continued until the Phenol Red marker indicated that the buffer had been removed. The strip was then sewn on to a further sheet of Whatman 3MM paper for electrophoresis in the second dimension.

Location of amino acids and peptides on paper. The cadmium-ninhydrin reagent of Heilman, Barollier & Watzke (1957), was used as a routine. Other reagents and sequential application methods were those recommended by Easley (1965) with the exception of the Pauly reagent; in this case the Sanger & Tuppy (1951) dip reagent was preferred, although it was not suitable for sequential methods.

Determination of molecular weight by polyacrylamide-gel electrophoresis. The method of Schapiro, Viñuela & Maizel (1967) was used with the following modifications. Proteins were dissolved in 0.1M-sodium phosphate buffer, pH7.1, made 1% (w/v) with respect to sodium dodecyl sulphate and 1% (v/v) with respect to 2-mercaptoethanol. Duplicate samples (20-50μg.) of each protein, to one of which cytochrome c was added as a marker, were run on 10% gels. Samples were added in a solution containing 0.1% (w/v) sodium dodecyl sulphate, 0.1% (v/v) 2-mercaptoethanol and 25% (w/v) glycerol. Electrophoresis was carried out until the cytochrome c band had migrated 4cm. The gels were stained for 30min. in 0.25% (w/v) Coomassie Blue in methanol-water-acetic acid (5:5:1, by vol.) and washed overnight in 7% (v/v) acetic acid. The fixed stained gels were scanned on the Joyce-Loebl Chromoscan.

RESULTS

Purification of urease. The purification of the enzyme was followed by determining the specific activity. The crude crystals had specific activities of about 30 Sumner units/mg. After one recrystallization specific activities in the range 80-170 Sumner units/mg. were recorded. After two recrystallizations the specific activities were measured as 160-180 Sumner units/mg. Continued recrystallization produced no further increase in specific activity.

The activity in the meal, measured by the method of Gorin, Butler, Katyal & Buckley (1960), was about 240 Sumner units/g. On this basis the overall recovery of activity was about 10%, i.e. about 150 mg./kg. of starting material.

Polyacrylamide-gel electrophoresis. The twice-recrystallized enzyme was shown to be pure on polyacrylamide-gel electrophoresis by a modification of the procedure of Ornstein & Davis (1962). The sample was applied in a solution containing 10 mM-thioglycollate and 15 mM-tris and run in a buffer incorporating 1 mM-thioglycollate and 1 mM-tris. The result is shown in Fig. 1. In addition to the main urease band there were traces of two other components, although scanning of the stained gel in the Joyce-Loebl Chromoscan showed that 97% of the stain was in the main band.

N-Terminal-group identification. The only DNP-amino acid found in the ether-soluble fraction was methionine. Occasionally the product was partially oxidized to the sulfoxide and sulphone, presumably during chromatography, and therefore for quantitative determinations the ether extracts were oxidized with performic acid before chromatography. The oxidation was performed after evaporation of the ether, and excess of performic acid was removed *in vacuo* over sodium hydroxide pellets. After 16.5 hr. hydrolysis a reproducible quantity of 0.44 mole of DNP-methionine sulphone/10⁵ g. of CM-urease was obtained, whereas control experiments yielded a variable recovery of 25–50% of DNP-methionine sulphone. After 8 hr. hydrolysis 0.36 mole of DNP-methionine sulphone/10⁵ g. of CM-urease was obtained with an overall recovery of 70%.

Large amounts of ϵ -DNP-lysine were found in all hydrolysates. An 8 hr. hydrolysate contained an uncorrected amount of 50 moles/10⁵ g. of CM-urease.

DNP-arginine could not be detected (<0.02 mole/10⁵ g. of urease) in hydrolysates of DNP-CM-urease.

Edman degradation yielded 1.32–1.41 moles of PTH product/10⁵ g. of CM-urease, calculated from

the u.v.-absorption spectrum. Satisfactory extinction ratios ($E_{260}/E_{270.5}$) in the range 0.84–0.86 were obtained. Chromatography identified PTH-methionine as the major product, but small quantities of PTH-methionine sulfoxide and traces of monophenylthiourea and an unidentified compound remaining at or near the origin were also found. Further extraction of the paper yielded only the last compound, which was, according to the spectrum, not PTH material.

C-Terminal investigation. Preliminary paper-electrophoretic tests showed that leucine, tyrosine and phenylalanine were rapidly released by the action of carboxypeptidase A.

In one experiment recrystallized urease (9 mg./ml.) was incubated at room temperature with carboxypeptidase A at an enzyme/substrate ratio of 1:50 by weight. Samples (0.8 ml.) of the digest were removed at times between 1 hr. and 26 hr. and analysed. Inspection of the amino acid products after electrophoresis showed that about equimolar amounts of tyrosine, phenylalanine and leucine were present in all cases, i.e. release of these three amino acids was complete within 1 hr. During this period there was no significant loss of urease activity in the digest compared with a control containing no carboxypeptidase A.

The same three amino acids were released by the action of carboxypeptidase A on maleyl-CM-urease. Release was complete within 5 min. after incubation at room temperature at an enzyme/substrate ratio of 1:250 by weight. Distinction between the release rates of these amino acids was only observed during digestion at 2° with an enzyme/substrate ratio of 1:2000 by weight. After 15 sec. digestion leucine and phenylalanine had been released, but not tyrosine.

The quantitative release of tyrosine, phenylalanine and leucine from CM-urease is shown in Table 1.

The relationship between the amino acids released by carboxypeptidase A was also investigated by a 'diagonal' method. The result is shown in Fig. 2. The newly released amino acids all belong to the

Fig. 1. Polyacrylamide-gel electrophoresis of twice-recrystallized urease in tris-glycine buffer (Ornstein & Davis, 1962), containing 1 mM-thioglycollate and 1 mM-tris, pH 8.5.

Table 1. Release of amino acids from CM-urease by carboxypeptidase A

Full experimental details are given in the text.

Time (min.)	Amino acid yield (moles/10 ⁵ g.)		
	Phe	Leu	Tyr
15	0.91	1.42	0.85
45	1.1	1.50	1.22
100	1.72	1.35	1.24

Fig. 2. 'Diagonal' electrophoretic demonstration of C-terminal sequence of urease. First dimension: separation of tryptic digest of AE-urease by electrophoresis at pH 2. Second dimension: repeat of first dimension after carboxypeptidase A digestion. DNS-Arg ('dansyl'-arginine) and DNS-OH (1-dimethylaminonaphthalene-5-sulphonic acid) are the spots in the 'dansyl' marker mixture of Brown & Hartley (1966). The amino acid marker mixture was run only in the second dimension. (T) indicates a positive tyrosine test. Released tyrosine and phenylalanine could not be separated in these experiments.

same peptide. In a control experiment without carboxypeptidase A digestion all the peptides located lay along the diagonal.

No basic amino acids were released from AE-urease by carboxypeptidase B. However, tyrosine, phenylalanine and leucine were again released in equimolar amounts of about 1 mole/ 10^5 g. Release of three amino acids was complete after 30 min. At 4 min. only leucine and phenylalanine were detected, confirming that tyrosine is released more slowly than the other two amino acids.

Phenylalanine was the only amino acid released in significant quantity after hydrazinolysis for 6 hr. at 100° ; the yield was about 0.1 mole/ 10^5 g. of CM-urease. Trace quantities of glutamic acid, serine, alanine and lysine or tyrosine were also found, together with larger quantities of the DNP-glycine-like artifact described by Kawanishi *et al.* (1964). Hydrazinolysis for 24 hr. at 80° also produced DNP-phenylalanine in a yield of 0.3 mole/ 10^5 g. The identity of this compound was confirmed by regeneration of phenylalanine. One other product

Fig. 3. *N*-Bromosuccinimide titration of CM-urease in 8M-urea. The top curve is the spectrum of CM-urease in 8M-urea-0.2M-acetate buffer, pH 3.5, at a concentration of 1.3 mg./ml. The successive lower curves show the effect of adding 0.05 ml. portions of 3 mM-*N*-bromosuccinimide in 8M-urea to 2 ml. of the urease solution. Further addition after 0.40 ml. produced no further decrease in u.v. absorption.

was present on the chromatogram in a position close to that expected for DNP-valine. The compound had the typical DNP-amino acid u.v.-absorption spectrum with a peak at 360 nm. corresponding to a yield of 0.6 mole/ 10^5 g. However, it could not be partitioned into ether from 1M-hydrochloric acid, and therefore was not a DNP-amino acid. After extraction into ethyl acetate-butan-1-ol (1:1, v/v), it was isolated and hydrolysed in 6M-hydrochloric acid. DNP-aspartic acid was found in the ether-soluble layer. The hydrolysate contained a number of amino acids, which were not accurately characterized because of the small amounts available.

***N*-Bromosuccinimide cleavage of CM-urease.** Reaction of the CM-urease with *N*-bromosuccinimide took place instantaneously after an initial lag phase until the reagent concentration reached about 1μ mole of *N*-bromosuccinimide/mg. of urease. A linear relationship between the destruction of tryptophan and consumption of *N*-bromosuccinimide was observed; about 6 moles of the reagent were required to react with 1 mole of tryptophan. A typical experiment is shown in Fig. 3.

Five new end groups were found in the DNP-peptide hydrolysate as summarized in Table 2.

The identity of the glutamic acid, lysine, alanine and glycine end groups was confirmed by paper electrophoresis of the barium hydroxide hydrolysate of the DNP-amino acid ether extract.

Fig. 4. Peptide 'map' of tryptic digest of AE-urease. Eleven spots were histidine-positive and twelve were sulphur-positive.

Fig. 5. Molecular-weight estimation by polyacrylamide-gel electrophoresis. The marker proteins used were as follows (in order of increasing mobility with the molecular weight indicated in parentheses): alkaline phosphatase dimer (80000), bovine serum albumin (67000), catalase (61000), pepsin (35000), cytochrome c dimer (24500), myoglobin (17000), cytochrome c (12300).

Peptide 'map'. Base uptake during trypsin digestion of AE-urease corresponded to 95 bonds/ 10^5 g. of urease, assuming an average pK 7.5 for the newly released amino groups. This is equivalent to 85% cleavage of the total AE-cysteine, lysine and arginine bonds. The peptide pattern of the digest is shown in Fig. 4. The total number of resolved peptide spots observed was 74, but three of these were not found in all digests. No 'core' material remained at the origin.

Molecular weight by polyacrylamide-gel electrophoresis. The results of the investigation are summarized in Fig. 5. The standard proteins had mobilities inversely related to their molecular weights. The logarithmic relationship between

Table 2. Yield of DNP-amino acid end groups of the *N*-bromosuccinimide cleavage mixture

The hydrolytic-loss corrections were: glycine, none; methionine, from CM-urease values; all other amino acids, as given by Porter (1957).

DNP-amino acid	Yield (mole/ 10^5 g.)
Ala	0.3
Glu	0.3
Gly	0.03
Lys	0.15
Met	0.8
Arg	0.15

molecular weight and mobility (Schapiro *et al.* 1967) fits the results very well. Samples of AE-urease, CM-urease and native urease were all tested; all ran as one band of the same mobility with respect to the cytochrome c dimer, namely 0.33. This corresponds to a molecular weight of 76000.

DISCUSSION

The urease preparations described in this paper had similar specific activities to those given by Mamiya & Gorin (1965), although our yields never approached theirs. The main reason for this appeared to be the inefficiency of the initial extraction. Tests showed that about 28000 Sumner units/kg. of meal were initially extracted and about 24000 Sumner units could be recovered in the twice-recrystallized product. In some cases loss was incurred by saturation of the recrystallizing media. This was discovered when solutions of the recrystallized enzyme were found to have a constant activity of 1120 ± 20 Sumner units/ml. Mamiya & Gorin (1965) indicate that the enzyme is soluble to a concentration of at least 1500 Sumner units/ml.

These observations suggest that proof of purity by the solubility method (Northrop, Kunitz & Herriott, 1948) may well be possible. However, the more sensitive technique of polyacrylamide-gel electrophoresis was used, and the results, together with those of the DNP *N*-terminal investigation, show that the preparation is essentially pure, thus confirming the recent results of other workers.

Reithel *et al.* (1964), Sehgal & Naylor (1966) and we in the present work have isolated urease of specific activity in the range 160–180 Sumner units/mg. at 22–25°; the purity of the preparations has been demonstrated by ultracentrifugation (Reithel *et al.* 1964), polyacrylamide-gel electrophoresis (Blattler *et al.* 1967; Tanis & Naylor, 1968), and confirmed by the latter technique and *N*-terminal studies in the present work. The specific activity of these preparations is but little changed from the 129 Sumner units/mg. of the original

crystalline preparations (Sumner & Hand, 1928). In view of the fact that the latter preparations were assayed at 20° and considering the difficulties then involved in obtaining heavy-metal-free solutions, it seems likely that the preparations of Reithel *et al.* (1964) and Sehgal & Naylor (1966) are identical in covalent purity to those of Sumner (1926).

There are, however, reports suggesting that jack-bean urease of a much greater specific activity can be obtained. Lynn (1967) obtained impure preparations with a specific activity of 5300 Sumner units/mg., and even after a tenfold decrease (K. R. Lynn, personal communication) this activity is much greater than has been found for apparently pure preparations. Hill & Elliott (1966) also claimed an occasional specific activity as high as 300 Sumner units/mg. in fractions from CM-cellulose chromatography. It is not clear what relationship these preparations with higher specific activities bear to those quoted here with specific activity of about 180 Sumner units/mg., which are essentially pure.

In considerations of the purity of urease preparations, much confusion has undoubtedly been caused by the fact that the enzyme exists in many polymeric forms. At least five are known to be active: a 12s form (mol.wt. 236 000) (Blattler *et al.* 1967), an 18s form (mol.wt. 490 000) (Reithel & Robbins, 1967) and 28s, 36s and 46s forms (Kuff *et al.* 1955). We have also observed a number of components, some of which showed enzyme activity, after gel electrophoresis of our preparations. However, when electrophoresis was carried out in 1 mM-thioglycollate at pH 8.5 one major band was found (Fig. 1). With the usual 'disc' conditions without thioglycollate a small amount of faster material of mobility 2.3 relative to the main band was also present; the proportional amount of this increased with the age of the preparation, and it was also present after electrophoresis in tris-glycine buffer adjusted to pH 6.5 with 5 mM-thioglycollic acid. This polymer is almost certainly the α -urease described by Blattler *et al.* (1967).

The discovery of a single *N*-terminal amino acid suggests that urease is made up of a single type of subunit. However, it is possible that an acetylated or other blocked *N*-terminal group is present, and hence the *C*-terminus was studied.

The apparently simultaneous release of the three amino acids by carboxypeptidase A provided an ambiguous answer to the problem of the number of types of subunit, and therefore the 'diagonal' technique was developed. As the only spots that moved out of the diagonal in this technique came from the same position, only one peptide had been digested. The new spots corresponded in position to leucine, phenylalanine and tyrosine, although the last two amino acids could not be separated. This is probably because of the two-stage nature of the

process leading to lower resolution and the fact that the parent peptide runs imperfectly owing to its high molecular weight (the measured mobility of 0.32 relative to serine corresponded to a molecular weight of 3700). The experiment also indicated the specificity of the tryptic digest. From these results it may be concluded that urease has a *C*-terminal sequence of -Tyr-(Leu,Phe), since further work with carboxypeptidase B showed no sign of a basic terminal chain. Hydrazinolysis confirmed these results and allowed deduction of the sequence -Tyr-Leu-Phe. The yield of phenylalanine in these experiments was not high. However, a large part of the *C*-terminus appeared to be in the peptide found in the 80° hydrazinolysate. Insufficient material was available to deduce the sequence of this blocked peptide. However, it was shown that carboxypeptidase A released leucine, phenylalanine, tyrosine, valine, serine, glycine and alanine, and acid hydrolysis indicated that the peptide contained these amino acids plus proline, isoleucine, glutamic acid and lysine. Clearly these results support the suggestion that the peptide is a partial hydrazinolysis product of the *C*-terminal sequence, as does the fact that the *N*-terminal residue was determined to be aspartic acid, which is a preferred hydrazine cleavage point (Haruna & Akabori, 1960).

Taken together the terminal amino acid studies therefore rule out the possibility of more than one chain, unless there are chains with both the *N*- and *C*-termini blocked.

The size of the single polypeptide subunit was also studied by these end-group methods. As is commonly observed (e.g. Ambler, 1963), the recovery of the DNP derivative was low and allowed only the deduction that the subunit size is about 100 000. The recovery of PTH-methionine was greater and allowed a more accurate assessment of the *N*-terminal group. The molar yield was in excellent agreement with the yield of leucine from the *C*-terminus.

Further evidence was sought by the use of specific cleavage methods. Experience with the cyanogen bromide cleavage method (Gross & Witkop, 1962) made clear the fact that the methods used would have to produce either a small number of large peptides to be examined by end-group methods or a large number of small peptides to be examined by peptide 'mapping'.

Therefore tryptophan cleavage was chosen as the preferred end-group method and tryptic digestion was the peptide 'mapping' method.

The total number of tryptic peptides found, taken in conjunction with the amino acid analysis (Milton & Taylor, 1969), leads to a value of 68 000 for the molecular weight. This value must be taken as minimal as it is based on the assumption that all tryptic cleavages occurred and that all peptides

were resolved. No fragments other than tryptic peptides were found in the digest (see Fig. 2).

The five new end groups found after cleavage at the tryptophan sites lead to a value of the molecular weight in good agreement with that derived from other measurements. It should be noted, however, that one other DNP compound was always found on the final chromatograms in the position expected for DNP-asparagine and very close to that found for the DNP product of *S*-carboxymethylcysteine. The compound was also formed in the *N*-bromosuccinimide cleavage mixtures of AE-urease, bovine serum albumin and myoglobin. Bovine serum albumin released the amino acid found previously (Ramachandran & Witkop, 1959); horse heart myoglobin released glutamic acid and glycine, suggesting that glutamine occurs in position 8 and glycine in position 15 of the polypeptide chain (see Dayhoff & Eck, 1968). The compound could not be formed during a number of control experiments without protein and therefore appears to be an artifact associated with the reaction.

To complement the chemical determinations of number-average molecular weight, the physical method of polyacrylamide-gel electrophoresis was used.

The good agreement between the value obtained by this method and the chemical methods is a further proof that there is one homogeneous subunit.

The values for the subunit weight given by the methods used are collected in Table 3. Agreement between the methods used is generally good and leads to a value of 75 000 for the molecular weight of the single urease subunit species. The value obtained from the amino acid analysis in the Addendum to this paper (Milton & Taylor, 1969) is also included. It was based on an integral value of 19 moles of phenylalanine/mole of subunit of mol.wt. 75 000.

The best value of 75 000 is not in complete agreement with that of 83 300 determined by ultracentrifugal methods (Reithel *et al.* 1964). Neither is

our value an exact sub-multiple of the mol.wt. 490 000 (Reithel & Robbins, 1967), although it is clear that our evidence indicates that urease is made up of six equivalent subunits. The molecular weight of the trimeric form, 236 000, determined by Blattler *et al.* (1967), agrees rather more exactly with our value for the structural monomer.

The large molecular weight of the subunit has physiological implications that are discussed elsewhere (Bailey & Boulter, 1969), and also synthetic implications. There appear to be few reports of single polypeptide chains with such a high molecular weight. We have therefore examined our preparations for the presence of carbohydrate by using the phenol-sulphuric acid reagent (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). Undialysed preparations of urease contained 1-2% by weight of carbohydrate measured as glucose equivalents. A large part of this total could be removed by dialysis or trichloroacetic acid precipitation, but even after these treatments preparations still contained 0.5-1.0% of carbohydrate. Although small, this quantity is significant and further work is necessary to establish unequivocally whether or not urease is a glycoprotein.

Reithel (1963) pointed out the necessity to distinguish between the smallest structural subunit and the smallest active unit. The smallest active unit yet found has a molecular weight of about 240 000 (Blattler *et al.* 1967; Tanis & Naylor, 1968), and the data of Kobashi *et al.* (1966) suggest that this unit has only one active site. It might therefore be anticipated that further dissociation would result in inactivation, and this does in fact happen when the structural subunit is produced by dissolving polymeric urease in 6M-guanidine hydrochloride (Tanis & Naylor, 1968) or in sodium dodecyl sulphate (Gorin, Mamiya & Chin, 1967). However, both of these reagents inactivate many enzymes (Reithel, 1963), and in this case it is not possible to separate the effect of denaturation from that of dissociation. It has also been shown that urease dissociates into 3-4s units of mol.wt. 180 000-320 000 in acid solution (Gorin *et al.* 1968; Reithel & Robbins, 1967); their formation is also accompanied by inactivation.

An approach to the question of protein polymerization is by way of the Fisher (1964) limiting law. By applying this rule to urease it can be shown that the structural subunit is theoretically stable. Therefore the formation of urease polymers is not a non-specific process based on the necessity of protecting hydrophobic residues, and it is thus likely that association is a specific function of the enzyme.

The overall evidence thus favours the view that urease exists enzymically as a trimeric or hexameric polymer of six similar polypeptide chains. One fundamental difficulty with this model is that it is

Table 3. *Molecular weight of the urease subunit*

Method	Mol. wt.
DNP-methionine	100 000
PTH-methionine	74 000 ± 3000*
C-Terminal leucine	73 000 ± 1800†
Tryptic peptides	68 000
Tryptophan content	75 000 ± 3500*
Polyacrylamide-gel electrophoresis	76 000 ± 2000‡
Amino acid analysis	75 100

* Experimental limits of mean values.

† S.E.M. of five determinations.

‡ Graphically estimated limits.

theoretically impossible for three identical units to react with urea, a substrate of twofold symmetry. The possibility of some form of ultramicroheterogeneity merits further consideration. In this connexion it is notable that H. G. Kolmark (personal communication) has found two genetic loci for the urease of *Neurospora crassa*.

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REFERENCES

- Akabori, S., Ohno, K. & Narita, K. (1952). *Bull. Soc. Chem. Japan*, **25**, 214.
- Ambler, R. P. (1963). *Biochem. J.* **89**, 341.
- Bailey, C. J. & Boulter, D. (1969). In *Chemotaxonomy of the Leguminosae*, Ed. by Boulter, D., Harborne, J. B. & Turner, B. L. London: Academic Press (Inc.) Ltd. (in the Press).
- Blattler, D. P., Contaxis, C. C. & Reithel, F. J. (1967). *Nature, Lond.*, **216**, 275.
- Brenner, M., Niederwiesser, A. & Pataki, G. (1965). In *Thin-Layer Chromatography*, p. 414. Ed. by Stahl, E. New York: Academic Press Inc.
- Brown, J. R. & Hartley, B. S. (1966). *Biochem. J.* **101**, 214.
- Creeth, J. M. & Nichol, L. W. (1960). *Biochem. J.* **77**, 230.
- Dayhoff, M. O. & Eck, R. V. (1968). *Atlas of Protein Sequence and Structure*. Silver Spring, Md.: National Biomedical Research Foundation.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). *Analyt. Chem.* **28**, 350.
- Easley, C. W. (1965). *Biochim. biophys. Acta*, **107**, 386.
- Efron, M. L. (1959). *Biochem. J.* **72**, 691.
- Fisher, H. F. (1964). *Proc. nat. Acad. Sci., Wash.*, **51**, 1285.
- Fraenkel-Conrat, H., Harris, J. I. & Levy, A. L. (1955). *Meth. biochem. Anal.* **2**, 359.
- Funatsu, M., Green, N. M. & Witkop, B. (1964). *J. Amer. chem. Soc.* **86**, 1846.
- Gorin, G., Butler, M. F., Katyal, J. M. & Buckley, J. E. (1960). *Proc. Okla. Acad. Sci.* **40**, 62.
- Gorin, G. & Chin, C.-C. (1965). *Biochim. biophys. Acta*, **99**, 418.
- Gorin, G., Chin, C.-C. & Wang, S. F. (1968). *Experientia*, **24**, 685.
- Gorin, G., Fuchs, E., Butler, L. G., Chopra, S. L. & Hersh, R. T. (1962). *Biochemistry*, **1**, 911.
- Gorin, G., Mamiya, G. & Chin, C.-C. (1967). *Experientia*, **23**, 443.
- Gross, E. & Witkop, B. (1962). *J. biol. Chem.* **237**, 1856.
- Haruna, I. & Akabori, S. (1960). *J. Biochem., Tokyo*, **47**, 513.
- Heilman, J., Barollier, J. & Watzke, E. (1957). *Hoppe-Seyl. Z.* **309**, 219.
- Hill, R. M. & Elliott, A. (1966). *Arch. Biochem. Biophys.* **113**, 405.
- Hummel, B. C. W. (1959). *Canad. J. Biochem. Physiol.* **37**, 1393.
- Katz, A. M., Dreyer, W. J. & Anfinsen, C. B. (1959). *J. biol. Chem.* **234**, 2897.
- Kawanishi, Y., Iwai, K. & Ando, T. (1964). *J. Biochem., Tokyo*, **56**, 314.
- Kobashi, K., Hase, J. & Komai, T. (1966). *Biochem. biophys. Res. Commun.* **23**, 34.
- Kuff, E. L., Hogeboom, G. H. & Striebach, M. J. (1955). *J. biol. Chem.* **212**, 439.
- Lynn, K. R. (1967). *Biochim. biophys. Acta*, **146**, 205.
- Mamiya, G. & Gorin, G. (1965). *Biochim. biophys. Acta*, **105**, 382.
- Michl, H. (1951). *Mh. Chem.* **82**, 489.
- Milton, J. M. & Taylor, I. E. P. (1969). *Biochem. J.* **113**, 678.
- Mills, G. L. (1950). *Nature, Lond.*, **165**, 407.
- Naughton, M. A. & Hagiopian, H. (1962). *Analyt. Biochem.* **3**, 276.
- Northrop, J. H., Kunitz, M. & Herriott, R. M. (1948). *Crystalline Enzymes*, 2nd ed., p. 29. New York: Columbia University Press.
- Ornstein, L. & Davis, B. J. (1962). *Disc Electrophoresis* (Preprint). Rochester, N.Y.: Distillation Product Industries (Eastman Kodak Co.).
- Phillips, D. M. P. (1958). *Biochem. J.* **68**, 35.
- Porter, R. R. (1957). In *Methods in Enzymology*, vol. 4, p. 221. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Potts, J. T., Berger, A., Cooke, J. & Anfinsen, C. B. (1962). *J. biol. Chem.* **237**, 1851.
- Ramachandran, L. K. & Witkop, B. (1959). *J. Amer. chem. Soc.* **81**, 4028.
- Reithel, F. J. (1963). *Advanc. Protein Chem.* **18**, 124.
- Reithel, F. J. & Robbins, J. E. (1967). *Arch. Biochem. Biophys.* **120**, 158.
- Reithel, F. J., Robbins, J. E. & Gorin, G. (1964). *Arch. Biochem. Biophys.* **108**, 409.
- Ryle, A. P., Sanger, F., Smith, L. F. & Kitai, R. (1955). *Biochem. J.* **60**, 451.
- Sanger, F. & Tuppy, H. (1951). *Biochem. J.* **49**, 463.
- Schapiro, A. L., Viñuela, E. & Maizel, J. V. (1967). *Biochem. biophys. Res. Commun.* **28**, 815.
- Sehgal, P. P. & Naylor, A. W. (1966). *Plant Physiol.* **41**, 567.
- Sjoquist, J. (1954). *Acta chem. scand.* **7**, 447.
- Spencer, R. D. & Begg, B. H. (1966). *J. Chromat.* **21**, 52.
- Sumner, J. B. (1926). *J. biol. Chem.* **69**, 435.
- Sumner, J. B. (1951). In *The Enzymes*, vol. 1, part 2, p. 873. Ed. by Sumner, J. B. & Myrback, K. New York: Academic Press Inc.
- Sumner, J. B., Gralen, N. & Eriksson-Quensel, I. B. (1938). *J. biol. Chem.* **125**, 37.
- Sumner, J. B. & Hand, D. B. (1928). *J. biol. Chem.* **76**, 149.
- Tanis, R. J. & Naylor, A. W. (1968). *Biochem. J.* **108**, 771.
- Varner, J. E. (1960). In *The Enzymes*, vol. 4, p. 247. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York: Academic Press Inc.
- Waley, S. G. & Watson, J. (1953). *Biochem. J.* **55**, 328.