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A Convenient Method of Preparation of High-Activity Urease from *Canavalia ensiformis* by Covalent Chromatography and an Investigation of its Thiol Groups with 2,2'-Dipyridyl Disulphide as a Thiol Titrant and Reactivity Probe

By ROGER NORRIS and KEITH BROCKLEHURST
Department of Biochemistry and Chemistry, St. Bartholomew's Hospital Medical College, University of London, Charterhouse Square, London EC1M 6BQ, U.K.

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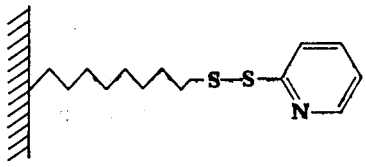
1. A convenient method of preparation of jack-bean urease (EC 3.5.1.5) involving covalent chromatography by thiol–disulphide interchange is described. 2. Urease thus prepared has specific activity comparable with the highest value yet reported (44.5±1.47kat/kg, $K_m = 3.32 \pm 0.05$mM; $k_{cat.} = 2.15 \times 10^4 \pm 0.05 \times 10^4 s^{-1}$ at pH7.0 and 38°C). 3. Titration of the urease thiol groups with 2,2'-dipyridyl disulphide (2-Py–S–S–2-Py) and application of the method of Tsou Chen-Lu [(1962) *Sci. Sin.* **11**, 1535–1558] suggests that the urease molecule (assumed to have mol.wt. 483000 and $\varepsilon_{280} = 2.84 \times 10^5$ litre·mol^{-1}·cm^{-1}) contains 24 inessential thiol groups of relatively high reactivity (class-I), six 'essential' thiol groups of low reactivity (class-II) and 54 buried thiol groups (class-III) which are exposed in 6M-guanidinium chloride. 4. The reaction of the class-I thiol groups with 2-Py–S–S–2-Py was studied in the pH range 6–11 at 25°C ($I = 0.1$ mol/l) by stopped-flow spectrophotometry, and the analogous reaction of the class-II thiol groups by conventional spectrophotometry. 5. The class-I thiol groups consist of at least two sub-classes whose reactions with 2-Py–S–S–2-Py are characterized by (a) $pK_a = 9.1$, $\tilde{k} = 1.56 \times 10^4 M^{-1} \cdot s^{-1}$ and (b) $pK_a = 8.1$, $\tilde{k} = 8.05 \times 10^2 M^{-1} \cdot s^{-1}$ respectively. The reaction of the class-II thiol groups is characterized by $pK_a = 9.15$ and $\tilde{k} = 1.60 \times 10^2 M^{-1} \cdot s^{-1}$. 6. At pH values 7–8 the class-I thiol groups consist of approx. 50% class-Ia groups and 50% class-Ib groups. The ratio class Ia/class Ib decreases as the pH is raised according to a pK_a value $\geq$ approx. 9.5, and at high pH the class-I thiol groups consist of at most 25% class-Ia groups and at least 75% class-Ib groups. 7. The reactivity of the class-II thiol groups towards 2-Py–S–S–2-Py is insensitive to the nature of the group used to block the class-I thiols. 8. All the 'essential' thiol groups in urease appear to be reactive only as uncomplicated thiolate ions. The implications of this for the active-centre chemistry of urease relative to that of the thiol proteinases are discussed.

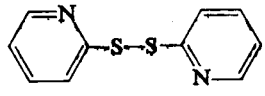
Urease (EC 3.5.1.5) can be extracted from jack-bean (*Canavalia ensiformis*) meal and reproducibly purified to high specific activity by the method of Blakeley *et al.* (1969*a*). A chloroform/acetone-dried powder of jack-bean meal is extracted with 30% (v/v) acetone containing 1% 2-mercaptoethanol at 39°C for 5min. The filtrate is left for 48h at 4°C to provide crystalline material that is then dissolved in buffer, dialysed and further purified by recycling upward-flow gel filtration on Sephadex G-200.

We have reported a simple general method (covalent chromatography by thiol–disulphide interchange) for the purification of thiol enzymes (Brocklehurst *et al.*, 1973, 1974) and its application in the immobilization of commercial samples of urease (Carlsson *et al.*, 1974), and it seemed possible that this technique might successfully be substituted for the recycling gel-filtration stage of the procedure of Blakeley *et al.* (1969*a*) to provide a more rapid and convenient method of preparation of high-activity urease.

The present paper reports that this modification using a Sepharose–glutathione–2-pyridyl disulphide gel (compound I) does provide urease with catalytic-activity characteristics closely similar to those of urease prepared by the method of Blakeley *et al.* (1969*a*). It reports also a study of the urease thiol groups by using 2,2'-dipyridyl disulphide (2-Py–S–S–2-Py, compound II) as a thiol-titrant and reactivity probe. The results of the present study suggest that if the essential thiol group of the urease subunit is part of the catalytic site, the active-centre chemistry of urease is very different from that of the thiol proteinases. Urease appears to undergo a conformational



(I) Sepharose–glutathione–2-pyridyl disulphide



(II) 2,2′-dipyridyl disulphide

change dependent on an ionization characterized by pK_a approx. 9.5 which affects the reactivity characteristics of some of the inessential thiol groups.

Materials and Methods

Urea (cyanate- and NH_3-free) was the 'ultra pure' product of Mann Research Laboratories, New York, NY, U.S.A. 2,2′-Dipyridyl disulphide was the product of Aldrich Chemical Co., Wembley, Middx. HA0 1PY, U.K. 5,5′-Dithiobis-(2-nitrobenzoic acid) (Nbs_2), *N*-ethylmaleimide and 2-mercaptoethanol were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Benzofuroxan (benzo-2-oxa-1,3-diazole *N*-oxide) was a gift from Dr. J. A. L. Herbert, who prepared it while working in this Department on another project. Bio-Gel A-50m was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A., and Sepharose 2B and Sephadex G-25 were from Pharmacia Fine Chemicals AB, Uppsala, Sweden. CNBr was purchased from Fluka A.G., Buchs SG, Switzerland. Guanidinium chloride (Aristar grade) and Nessler's reagent were the products of BDH Chemicals, Poole, Dorset, U.K. Nessler's reagent was stored in the dark and fresh batches of reagent were used if the solution became discoloured. Jack-bean meal was obtained from Sigma. For routine preparation of high-activity urease only batches with a creamy colour were used; those with a green colour tended to provide urease of low activity. Commercial preparations of urease were obtained from Sigma (types IV, VI, and VII) and from Boehringer Corp. (London), Uxbridge, London W5 2TZ, U.K.

Urease (*EC* 3.5.1.5)

(*a*) *From jack-bean meal.* Fully active urease containing 24 inessential thiol groups/molecule with relatively high reactivity towards 2-Py–S–S–2-Py at pH7 was prepared from jack-bean meal. The procedure consisted of extraction, crystallization and recrystallization by the method of Blakeley *et al.* (1969*a*) followed by covalent chromatography by using Sepharose–glutathione–2-pyridyl disulphide gel (Brocklehurst *et al.*, 1973, 1974). Recrystallized urease (prepared from 850g of jack-bean meal) was homogenized in 15ml of 0.05M-Tris/HCl buffer, pH7.2, containing 1mM-2-mercaptoethanol and 1mM-EDTA, and this was subjected to centrifugation at 35000*g* and 4°C for 30min. The precipitate was discarded and the supernatant was stored at 4°C until required. Provided that 2-mercaptoethanol is present, such urease solutions can be stored without deterioration for up to 9 months. Before covalent chromatography, 2-mercaptoethanol was removed from the urease solution by gel filtration on a column (19cm×3cm) of Sephadex G-25 with 0.1M-Tris/HCl, pH7.2, as running buffer. The protein-containing eluate (20ml, containing approx. 40mg of protein) was applied to a column (100cm×4cm) of Sepharose–glutathione–2-pyridyl disulphide gel equilibrated with 0.1M-Tris/HCl buffer, pH7.2. The column was washed with the same buffer until values of E_{280} and E_{343} of the eluate had fallen to 0.03. Urease was released from the column where it was bound as a mixed disulphide by washing with 30mM-L-cysteine in the Tris/HCl buffer. The gel-bound urease makes a convenient storage form, and urease can be released when required, and freed from L-cysteine by gel filtration on Sephadex G-25.

When it was necessary, urease was concentrated at 4°C by using solid polyethylene glycol.

In early preparations further purification was attempted by application of 20mg of urease in 0.1M-Tris/HCl buffer, pH7.2, containing 0.1M-KCl, 1mM-EDTA and 1mM-2-mercaptoethanol to a column (42cm×2cm) of Bio-Gel A-50m. The eluate was assayed for ureolytic activity by Nesslerization and the peak fractions were desalted by gel filtration on Sephadex G-25. Since no significant increase in specific activity was achieved by the Bio-Gel step this was omitted in the routine preparation of urease for kinetic studies. The covalent-chromatography step achieves separation from non-thiol-containing material including nucleic acids, as evidenced by an increase in E_{280}/E_{260} from 1.13 to 1.9, and polysaccharides, as measured by acid hydrolysis of samples of dried protein and determination of reducing sugars (Hassid & Abraham, 1957).

(*b*) *Commercial urease preparations.* These were treated with 30mM-dithiothreitol in, e.g., 0.1M-Tris/HCl buffer, pH7.2, containing 1mM-EDTA for 30min and low-molecular-weight material was then removed by gel filtration on Sephadex G-25. These preparations were then subjected to covalent chromatography, as described above.

Ureolytic-assay procedures

To determine the kinetic parameters characteristic of the ureolytic activity of urease prepared by covalent chromatography, the titrimetric assay described by Blakeley *et al.* (1969*a*) was used. The reactions were followed at pH7.0 and 38°C with Radiometer equipment.

For routine assays and experiments in which rapid determination of urease activity was required, the release of NH_3 was determined by Nesslerization. To stoppered test tubes each containing 2ml of 0.2M-Tris/HCl buffer, pH7.2, containing 1mM-EDTA and 1mM-2-mercaptoethanol were added portions (20–50μl) of a urease solution such that the rate of liberation of NH_3 was approx. 20μg/min. In experiments in which some of the enzyme was present in a blocked state, e.g. by reaction with 2-Py–S–S–2-Py, 2-mercaptoethanol was omitted. The reaction was allowed to proceed for 1min at 23°C with constant shaking. Over this time-interval the reaction rate was found to be constant, and the pH increased by less than 0.1 unit. Portions (100μl) of the reaction mixture were transferred to tubes containing 1ml of 1M-NaOH and the resulting solutions were diluted with 4ml of deionized water and then treated with 100μl of Nessler's reagent. E_{436} was measured by using a Cary 16K spectrophotometer, and the activity was calculated by using a standard curve relating to NH_3 solutions of known concentration. Control experiments demonstrated that the NH_3 content of the atmosphere was negligible.

Thiol determinations

These were made as a routine with 2-Py–S–S–2-Py as titrant and $\Delta\varepsilon_{343} = 8.08\times10^3/\{1+(1.6\times10^{-10}/[H^+])\}M^{-1}\cdot cm^{-1}$ (Little, 1971; Brocklehurst & Little, 1973; Stuchbury *et al.*, 1975). In reactions with Nbs_2 and *N*-ethylmaleimide, concentrations were calculated by using $\Delta\varepsilon_{412} = 1.36\times10^4M^{-1}\cdot cm^{-1}$ (Ellman, 1959) and $\Delta\varepsilon_{310} = -620M^{-1}\cdot cm^{-1}$ (Andrews & Reithel, 1970) respectively.

Determination of the relationship between loss of enzymic activity and titration of the urease thiol groups

This relationship was investigated by two methods: (i) comparison of the complete thiol-titration progress curves with activity-loss profiles both determined by using an excess of thiol reagent over the urease thiol groups, and (ii) measurement of activity loss during titration of separate samples of urease with different sub-stoicheiometric amounts of titrant.

(i) Two identical solutions of urease (2ml of 0.3μM-protein) at 25.0°C were allowed to react with 2-Py–S–S–2-Py (200μM in the reaction mixture) at various pH values between 7 and 8.5 obtained by using potassium phosphate buffers prepared from stock solutions of 0.1M-KH_2PO_4 and 0.1M-K_2HPO_4. In one of the solutions at a given pH the release of 2-thiopyridone (Py-2-SH) was followed by recording the increase in E_{343} by using a Cary 16K spectrophotometer. The loss of enzymic activity in the other reaction mixture was measured by withdrawing 50μl portions immediately after the addition of the 2-Py–S–S–2-Py and at approx. 3min intervals thereafter and assaying them immediately by Nesslerization. Control experiments showed that 2-Py–S–S–2-Py and Py-2-SH in the concentrations used in these experiments do not interfere with the Nesslerization, although at concentrations higher than 250μM, addition of Py-2-SH to Nessler's reagent produced a white precipitate. Although in general it was found to be more convenient to carry out the spectral analysis of the thiol titration and the activity-loss experiment with separate reaction mixtures, in some cases both experiments were performed by using the same sample of reaction mixture. Similar results were obtained with both procedures.

(ii) A given solution of urease (2ml of 1–2μM-protein) was titrated in a stepwise procedure with 10μl portions of 1.5mM-2-Py–S–S–2-Py. Several such experiments were carried out in the phosphate buffers, pH7–8.5, and release of Py-2-SH was recorded at 343nm. When the reaction with a given portion of 2-Py–S–S–2-Py was complete, a 10μl sample of the reaction mixture was removed for measurement of ureolytic activity by Nesslerization. Another 10μl portion of 1.5mM-2-Py–S–S–2-Py was then added and the process was repeated until the ureolytic activity had fallen to zero.

Kinetics of the reactions of the urease thiol groups with 2-Py–S–S–2-Py at 25°C

(i) *Class-I thiol groups.* The reactions of these groups with 2-Py–S–S–2-Py were studied at 343nm by using a Durrum stopped-flow spectrophotometer connected to a Tektronix oscilloscope. One syringe contained urease (0.2–1μM-protein) in buffer and the other contained 2-Py–S–S–2-Py (600μM–1.5mM) in buffer.

(ii) *Class-II thiol groups.* The reactions of these thiol groups with 2-Py–S–S–2-Py were studied with a Cary 16K spectrophotometer by using (*a*) urease containing its full complement of thiol groups and (*b*) urease preparations in which the class-I thiol groups had been modified in various ways (see below). The reactions were carried out in a reaction volume of 2.2ml containing 150–750μM-2-Py–S–S–2-Py and 0.2–0.5μM-native urease or 3–7μM-modified urease.

Blocking of the class-I thiol groups

(i) *With 2-Py–S–S–2-Py, Nbs_2 or N-ethylmaleimide.* The class-I thiol groups of urease were blocked by

allowing 2ml samples of the enzyme (5–7 μM) to react with a succession of 0.2ml portions of 1.5mM-2-Py–S–S–2-Py or -*N*-ethylmaleimide or 0.4mM-Nbs_2 in 0.05M-Tris/HCl buffer, pH7.0, until a 2–3% decrease in ureolytic activity was observed in one of the samples thus analysed; this corresponded to reaction of 22–23 thiol groups per urease molecule. Reaction was then essentially stopped by lowering the pH to 6.3 (by addition of a few drops of 0.3M-KH_2PO_4). In later experiments it was found to be more convenient to add a small excess of thiol reagent over the total thiol content of the enzyme and rapidly lower the pH after 22–23 thiol groups per molecule had reacted (as adjudged by E_{343} analysis). The modified urease was then separated from low-molecular-weight material by gel filtration on Sephadex G-25 equilibrated with pH6.3 buffer.

(ii) *With benzofuroxan.* This compound has been developed as a chromogenic oxidizing agent for protein thiol groups (M. Shipton, T. Stuchbury, K. Brocklehurst, J. A. L. Herbert & H. Suschitzky, unpublished work). When used to oxidize low-molecular-weight thiols, the products are the disulphide and *o*-benzoquinone dioxime. When steric constraints prevent disulphide formation, as, e.g., in the papain active centre, the oxidation products are sulphur oxy acids.

Urease, with its class-I thiol groups oxidized by benzofuroxan and its class-II thiol groups intact, was prepared by reaction of urease (3 μM-protein) with benzofuroxan (1.5mM) at pH8.5 until a decrease in ureolytic activity of 2–3% was observed. The pH was then adjusted to 7.2 by addition of 0.3M-KH_2PO_4 and the modified urease isolated by gel filtration on Sephadex G-25 at the same pH. The nature of this oxidized urease preparation was not investigated. In earlier experiments in which the class-I thiol groups were allowed to undergo air-oxidation for approx. 2–3 weeks, Bio-Gel analysis (see below) suggested that the product was a mixture of sulphur oxy acids and aggregates formed by intersubunit disulphide formation.

Chromatography of urease and air-oxidized urease on Bio-Gel A-50m

The elution profiles on Bio-Gel A-50m of freshly prepared urease and urease stored for 2–3 weeks in 5mM-potassium phosphate buffer, pH8.2, in the absence and presence of 1mM-2-mercaptoethanol, were compared. Storage in the absence of 2-mercaptoethanol resulted in the loss of at least 80% of the class-I thiol groups. Each urease preparation (1ml) was fractionated on a column of Bio-Gel A-50m (42cm×2cm) by using 0.1M-potassium phosphate buffer, pH7.1, containing 0.1M-KCl. Fractions (3ml) were analysed by measurement of E_{280}. With freshly prepared urease or urease stored in 2-mercaptoethanol, the protein was eluted as a single peak in fractions 30–35, whereas with oxidized urease the protein was eluted as a much broader band in fractions 20–35, indicating the presence of aggregates of mol.wt. > 483000 [see also Fishbein *et al.* (1970) and Fishbein & Nagarajan (1971)].

Protein determinations

Protein determinations with purified urease were made by measurement of E_{280}. Blakeley *et al.* (1969*a*) give $E_{280} = 0.589$ for a concentration of urease of 1mg/ml in 1cm cells and, assuming a mol.wt. for urease of 483000, this corresponds to $\varepsilon_{280} = 2.84 \times 10^5 M^{-1} \cdot cm^{-1}$. The value for E_{280} given by Blakeley *et al.* (1969*a*) is in good agreement with that reported by Reithel & Robbins (1967), but is considerably smaller than that reported by Gorin & Chang-Chen Chin (1966). Spectroscopic analysis of urease concentrations is further complicated by the recent discovery that nickel is bound to the enzyme (see Dixon *et al.*, 1975*a*,*b*, 1976). For crude urease preparations and for urease derivatives, protein was determined by the biuret method and the Folin method respectively (see Layne, 1957).

Buffers and pH measurement

The following buffers were used as a routine: pH6–8, KH_2PO_4/K_2HPO_4; pH7–8, Tris/HCl; pH9–10.9, $Na_2CO_3/NaHCO_3$; pH11–12, KH_2PO_4/NaOH. These were made up at three times the concentrations given for $I = 0.1$ mol/l and 25°C by Long (1971), and diluted to various extents for use in kinetic and chromatographic work.

The pH values of reaction mixtures used for kinetic experiments were recorded immediately after each run with Radiometer equipment.

Results and Discussion

Preparation of urease by covalent chromatography and a convenient storage form of urease

Covalent chromatography (see Brocklehurst *et al.*, 1973, 1974) separates urease from nucleic acids, carbohydrates and traces of proteins containing thiol groups with only low reactivity towards 2-Py–S–S–2-Py that are present in the crystalline urease preparation (see the Materials and Methods section). This technique uses a much quicker and less elaborate process than recycling chromatography on Sephadex G-200.

The Sepharose gel/urease mixed disulphide provides a convenient storage form of urease; when required, e.g. for kinetic studies, urease may be released by treatment of the conjugate in stirred suspension with a low-molecular-weight thiol and separated from the gel by vacuum filtration.

Table 1. *Comparison of (A) urease prepared by covalent chromatography, (B) urease prepared by recycling upward-flow gel chromatography on Sephadex G-200 and (C) crystalline urease prepared by solvent extraction without either of the further treatments specified in (A) and (B)*

The reaction conditions and values of the various parameters used in calculations are given in the Materials and Methods section; values marked * were calculated from the values of specific activity in (units/ml)/E_{280} given in Blakeley *et al.* (1969*a*). For preparation A results are means±S.E.M. of five determinations.

Urease preparation	E_{280}/E_{260}	Specific activity (units/mg)	Specific activity (kat/kg)	K_m (mM)	$10^{-4} \times k_{cat.}$ (s^{-1})	Reference
A	1.9	2674±88	44.57±1.47	3.32±0.05	2.15±0.05	The present work
B	1.8–1.9	2532–2709	(42.1–45.1)*	3.28	2.34	Blakeley *et al.* (1969*a*)
					(2.04–2.18)*	The present work
C	1.13	approx. 1767	approx. 29	—	—	The present work; Blakeley *et al.* (1969*a*)

J. Carlsson, I. Olson & R. Axén (personal communication) have prepared urease of specific activity similar to that of urease prepared by the present method (see Table 1) by using covalent chromatography without prior crystallization of the urease.

They applied an ethanolic extract of jack-bean meal to an agarose–hydroxypropyl ether–2-pyridyl disulphide gel synthesized by the method of Axén *et al.* (1975). This gel has a mixed disulphide content that is approximately ten times that of the gel used in the present work. This more highly substituted gel was used in an attempt to minimize competition by other thiols present in the jack-bean-meal extract for the disulphide sites on the gel used in the covalent-chromatography step. Whether the omission of the crystallization step has consequences for the urease preparation that are not reflected in its ureolytic activity, e.g. its state of aggregation, remains to be determined.

Catalytic activity

Table 1 shows that urease prepared by covalent chromatography is indistinguishable in its catalytic characteristics from urease prepared by the method of Blakeley *et al.* (1969*a*). The attempted further purification of urease prepared by covalent chromatography by gel filtration on a column of Bio-Gel A-50m provided an increase in specific activity of only approx. 1%. Bio-Gel A-50m chromatography has been used to separate proteins in the mol.wt. range 100000–several millions (Vogel *et al.*, 1971).

Titration of the urease thiol groups by using Nbs_2 and 2-Py–S–S–2-Py

Many attempts have been made to titrate the thiol groups of urease with a variety of titrants [see Reithel (1971) and references therein]. Comparison of the value of the cysteic acid content of urease obtained after performic acid oxidation and hydrolysis (Reithel & Robbins, 1967) with thiol-titration data obtained by using *N*-ethylmaleimide and Nbs_2 as titrants in denaturing media and in peptic digests (Andrews & Reithel, 1970) suggests that the urease molecule [mol.wt. approx. 483000 (Sumner *et al.*, 1938; see also Blakeley *et al.*, 1969*b*; Dixon *et al.*, 1975*a*)] contains approx. 83–85 mol of thiol/mol of protein and no disulphide bonds. In the present work we have titrated the urease thiol groups with 2-Py–S–S–2-Py in the presence of 6M-guanidinium chloride (see the Materials and Methods section) and obtained a value of 84±1 mol of thiol/mol of protein, in good agreement with the earlier reports.

Attempts to titrate accurately the thiol groups with appreciable reactivity in native urease have not hitherto been as successful as the corresponding experiments carried out in denaturing media. This appears to have been due in part to the use of urease preparations of variable quality and in part to the use of titrants not entirely suited to the reactivity characteristics of the thiol groups of native urease. Most studies have indicated that urease contains 22–28 mol of thiol/mol of protein of mol.wt. 483000 that react with thiol reagents without marked activity loss.

Studies with *N*-ethylmaleimide and Nbs_2 as titrants (Ambrose *et al.*, 1951; Gorin & Chang-Chen Chin, 1965; Andrews & Reithel, 1970) indicated that, in addition to these inessential thiol groups, urease has four to nine thiol groups/molecule that are essential for activity. The pK_a values (approx. 9; see below) of the urease essential thiol groups and the low reactivity of these thiol groups combine to make accurate titrations that use the poorly electrophilic *N*-ethylmaleimide in approximately neutral media rather difficult. The much more reactive thiol reagent Nbs_2 would be expected to be a much better titrant for the essential urease thiols. Even with their urease preparation of reproducibly high activity, Blakeley *et al.* (1969*b*) reported, without further explanation, that titration with Nbs_2 did not permit them to define unequivocally the number of essential thiol groups in the 483000-mol.wt. species.

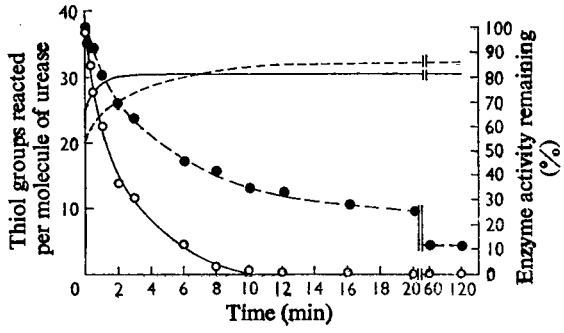


Fig. 1. *Comparison of 2-Py–S–S–2-Py and Nbs_2 as thiol titrants for and inhibitors of urease*

Reactions were carried out at pH8.35, 25.0°C and $I = 0.1$ mol/l with $[\text{urease}]_0 = 2\,\mu$M and $[\text{2-Py-S-S-2-Py}]_0 = 400\,\mu$M or $[Nbs_2]_0 = 400\,\mu$M; for further details, see the text. 2-Py–S–S–2-Py reaction: ——, thiol titration; ○, activity loss. Nbs_2 reaction: – – – –, thiol titration; ●, activity loss.

One of the problems encountered in using Nbs_2 as a thiol titrant for urease is that although reaction of urease with excess of Nbs_2 at pH8.3 appears to be complete after approx. 31 thiol groups/molecule have reacted, approx. 10% of the original ureolytic activity remains. A similar finding was reported by Andrews & Reithel (1970) for the reaction at pH7.1 (33 thiol groups/molecule reacted, leaving 18% of the original activity). Fig. 1 demonstrates that this problem does not exist when 2-Py–S–S–2-Py instead of Nbs_2 is used as titrant; ureolytic activity is abolished during reaction of 30 ± 1 thiol groups/molecule.

In an attempt to determine the number of 'essential' thiol groups in urease from results similar to those of Fig. 1 for the 2-Py–S–S–2-Py reaction, it seemed appropriate to apply a simple method suggested in a little-quoted paper by Tsou Chen-Lu (1962) [for an application of this method to the study of the essential carboxyl groups of pepsin, see Paterson & Knowles (1972)]. The experimental part of the method of Tsou Chen-Lu (1962) consists of allowing an enzyme to react with a chemical reagent that causes activity loss and is specific for a particular type of functional group. Subsequent discussion in the present paper is given in terms of specific modification of thiol groups. The number of essential groups is determined by measuring (*a*) the residual catalytic activity and (*b*) the number of thiol groups modified, in samples of partially modified enzyme. Consider an enzyme, such as urease, with a total of n thiol groups per molecule, which can be subdivided on the basis of their reactivity towards the modifying reagent into three types. Type-I groups, of which there are s per molecule, react most rapidly, and this type contains no essential groups. Type-II groups, of which there are p per molecule, react appreciably only after reaction of the type-I groups has almost reached completion. Among the p groups of type II is a number, (i), of essential groups. Type-III groups, of which there are $(n-s-p)$ per molecule, react only very slowly, or not at all, with the modifying reagent.

When the reaction of the type-I groups is essentially complete, and the reaction of the type-II groups has occurred to a discernible extent, the relationship of the fraction, a, of the initial activity remaining is related to the total number of groups modified per molecule by eqn. (1).

$$a^{1/i} = 1 + \frac{s}{p} - \frac{m}{p} \qquad (1)$$

Application of eqn. (1) relies on the assumption that catalytic activity is completely abolished by the modification of one essential group per molecular unit of enzyme to which fractional activity, a, relates. The method of Tsou Chen-Lu (1962) is readily extended to include oligomeric enzymes if it is assumed that they are composed of identical subunits and if subunit interaction is neglected. For such enzymes eqn. (1) may be written as eqn. (2) in which q is the number of subunits per molecule and the other parameters are as defined above, except that i is now the number of essential groups per subunit instead of per molecule.

$$\left(\frac{a}{q}\right)^{1/i} \equiv a^{1/i} = 1 + \frac{s/q}{p/q} - \frac{m/q}{p/q} = 1 + \frac{s}{p} - \frac{m}{p} \qquad (2)$$

Thus the linear plot of $a^{1/i}$ against m provides, according to eqn. (2), the number of essential groups per subunit (i) and the numbers of groups of type I (s) and of type II (p) per molecule.

Fig. 2 shows a typical set of results for the urease/2-Py–S–S–2-Py reaction at pH values between 7 and 8. In this pH range, five sets of data were analysed, and in each case the best straight line was obtained with $i = 1$. These data gave as values of the other characterizing parameters, $p = 5.9 \pm 0.2$ and $s = 24.3 \pm 0.2$.

In terms of the above analysis, the value of $i = 1$ implies that each catalytically active subunit of urease possesses one essential thiol group. It is important to emphasize that the values of $p = 6$ and $s = 24$ are dependent on the value of the optical factor used to calculate the molar concentration of urease from E_{280} measurement. The value used in the present work is that reported by Blakeley *et al.* (1969*a*), E_{280} for 1 mg/ml = 0.589, and a mol.wt. of 483000 was assumed. Although the present thiol-titration data might be taken to support the hexameric structure favoured by Blakeley *et al.* (1969*b*), they do not of course preclude other urease structures. We shall subsequently refer to the 24 inessential and exposed thiol groups of urease as the class-I thiol groups, the six essential thiol groups as the class-II thiol groups, and the 54 buried thiol groups as the class-III thiol

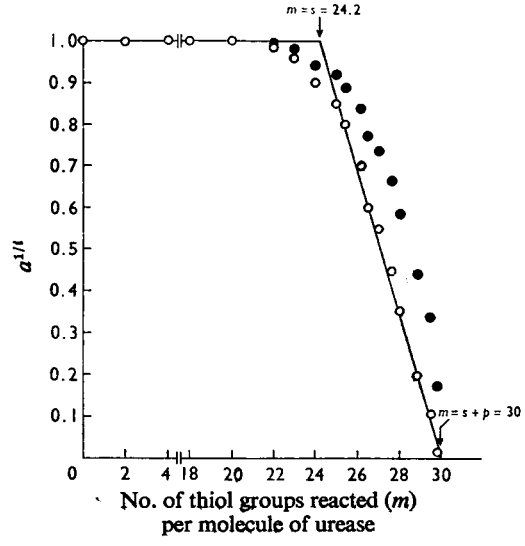


Fig. 2. *Tsou Chen-Lu* (1962) *plot of the urease/2-Py–S–S–2-Py thiol titration/activity-loss data*

Titration of the class-II thiol groups gives a linear plot (○) when $i = 1$ (i.e. one essential thiol group per subunit) but not (●), when $i = 2$. The intercepts of the linear part of the plot provide, when $a^{1/i} = 1$, the number of class-I thiol groups/molecule (s) and when $a^{1/i} = 0$, the number of class-I+class-II thiol groups/molecule ($s+p$).

groups. For simplicity, subsequent discussion is given in terms of a urease molecule composed of six subunits, each containing one essential thiol group, although this may well be an oversimplified picture, particularly if the essential thiol groups are not actually involved in catalysis. This possibility is suggested by the pH-dependence of the rate of their reaction with 2-Py–S–S–2-Py (see below). The question of the subunit structure of urease is further complicated by the work of Dixon *et al.* (1975*a*,*b*) (see also Dixon *et al.*, 1976), which suggests that (*a*) the mol.wt. of urease (483000; Sumner *et al.*, 1938) may need to be refined to either 420000 or 525000, and (*b*) urease contains 2 atoms of nickel/105000-mol.wt. species.

Characterization of the class-I and class-II thiol groups of urease by using 2-Py–S–S–2-Py as a reactivity probe

The value of reactivity probes, as against spectroscopic probes, for the study of conformational states of proteins has been emphasized by Byers & Koshland (1975), and we have demonstrated the particular value of 2-Py–S–S–2-Py in probing the environments of the active centres of the thiol proteinases (Brocklehurst & Little, 1970; Brocklehurst, 1974; Shipton *et al.*, 1975).

Reactivity probes can be used to obtain information about (i) the environment of the group undergoing modification, (ii) the possible plurality of conformational states containing the group and (iii) changes in environment consequent on, e.g. ligand binding, or dependent on syncatalytic effects (Christen & Riordan, 1970; Birchmeier *et al.*, 1972).

In the study of (ii) and (iii) it should be possible to use any reagent that reacts specifically with the functional group in question. Even for these types of study, 2-Py–S–S–2-Py possesses advantages over many other thiol reagents; it reacts specifically with thiol groups in a reaction that is essentially irreversible particularly at pH values of 9 or below (in contrast with Nbs_2), and is readily monitored by changes in u.v. absorption. In addition, the high reactivity of 2-Py–S–S–2-Py towards thiol groups in low-molecular-weight molecules means that even when thiol groups are located in proteins in relatively inaccessible sites it may be possible to quantify their reactions with this reagent. With less highly electrophilic reagents this is sometimes a problem. In the study of (i), disulphides containing the 2-pyridyl moiety (e.g. 2-Py–S–S–2-Py) are particularly valuable for the detection of thiol groups that interact with neighbouring acid/base groups to provide additional nucleophilic states in approximately neutral media (see Brocklehurst, 1974). This type of interaction characterizes the active centres of the thiol proteinases papain, ficin and bromelain (see, e.g., Shipton *et al.*, 1975) and may permit these enzymes to function effectively in neutral media.

In the present work, 2-Py–S–S–2-Py has been used to obtain information about the urease molecule of the three types outlined above.

The class-I thiol groups. Reactions of the (inessential) class-I thiol groups of urease with 2-Py–S–S–2-Py were studied in the pH range approx. 6–11 under pseudo-first-order conditions by using stopped-flow spectrophotometry. One syringe contained urease and the other contained 2-Py–S–S–2-Py, both in buffer (see the Materials and Methods section). In many reaction mixtures $[urease]_0$ was approx. 0.5 μM in protein, and $[2\text{-Py–S–S–2-Py}]_0$ was 375 μM. At all pH values studied, reaction of the class-I thiols produced Py-2-SH corresponding to reaction of 24 thiol groups per urease molecule. All of these progress curves were clearly not monophasic (see, e.g., Fig. 3). Each progress curve was resolved into two logarithmic plots (e.g. Fig. 4) apparently characteristic of two parallel first-order reactions, by the method given in Frost & Pearson (1961). In some cases, two stopped-flow traces were obtained by using different sweep times to facilitate analysis of the different parts of the progress curve. Plots similar to those shown in Fig. 4 provided the stoicheiometries and pseudo-first-order rate constants of each of the phases. The latter, together with relevant 2-Py–S–S–2-Py concentrations, were used to provide the corresponding second-order rate constants. At pH 7.5 the pseudo-first-order rate

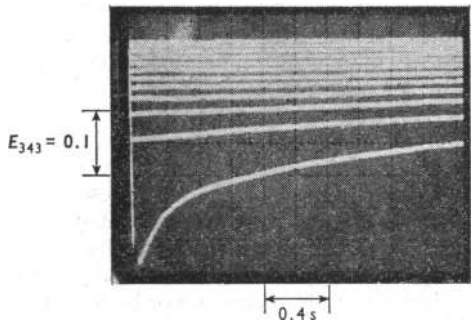


Fig. 3. *Stopped-flow record showing the inhomogeneity of the reaction of the class-I (inessential) thiol groups of urease with 2-Py–S–S–2-Py*

The reaction was carried out at pH9.56, 25.0°C and $I = 0.1$ mol/l with [urease class-I thiol groups]$_0 = 15\,\mu$M, and [2-Py–S–S–2-Py]$_0 = 375\,\mu$M.

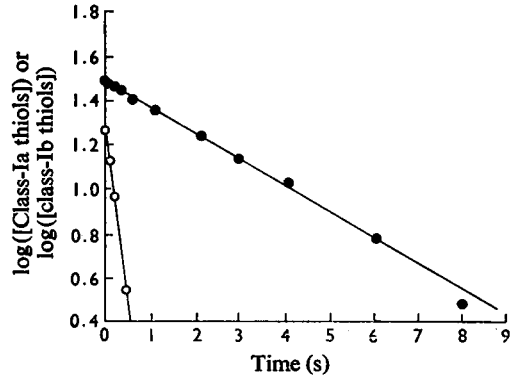


Fig. 4. *Resolution of the data of Fig. 3 into linear plots characteristic of two parallel (pseudo)-first-order reactions*

The data were treated as described by Frost & Pearson (1961); ○, reaction of the class-Ia thiol groups; ●, reaction of the class-Ib thiol groups.

constant for each of the phases was shown to increase linearly with the [2-Py–S–S–2-Py] up to 750 μM. Thus if these reactions proceed through the intermediacy of an adsorptive complex, they were studied at reagent concentrations much less than the dissociation constant of the enzyme–reagent complex. The pH-dependence of these apparent second-order rate constants, therefore, could provide information about native urease, although, if such complexes exist in non-equilibrium steady states, complications can exist (see Brocklehurst & Dixon, 1976).

In the pH range 6–8, the fast and slow phases of the reaction of the class-I thiol groups each correspond

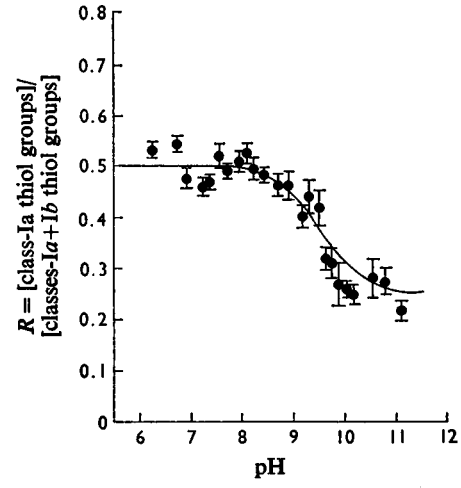


Fig. 5. *pH-dependence of R = [class-Ia thiol groups]/[classes Ia+Ib thiol groups]*

The standard errors relate to five experiments at each pH value. The line was calculated from $R = 0.25+0.25/(1+K/[H^+])$ in which p$K = 9.5$. A plateau value at high pH of 0.25 was assumed for R, and the data were analysed by unweighted regression of $1/(R-0.25)$ on $1/[H^+]$.

to 50% reaction. At higher pH values the stoicheiometry of the fast phase decreases and that of the slow phase increases correspondingly. The change in the stoicheiometries appears to follow a titration curve characterized by pK_a approx. 9.5, and, where the change appears to be complete at high pH, the stoicheiometry of the fast phase is 25% and that of the slow phase is 75% of the total reaction (see Fig. 5). It is difficult to obtain accurate data at high pH with 2-Py–S–S–2-Py as a thiol titrant, because the value of $\Delta\varepsilon_{343}$ for its reaction with thiols falls to essentially zero at pH values at which the product Py–2-SH (pK_a 9.8) exists as the anion (see the Materials and Methods section).

The pH-dependences of the second-order rate constants that characterize each of the phases of the reactions of the class-I thiol groups are shown as logarithmic plots in Fig. 6. The reaction of the fast phase [class-Ia thiol groups] is characterized by pH-independent rate constant $k = 1.56\times10^4\,M^{-1}\cdot s^{-1}$ and pK_a 9.1 and that of the slow phase (class-Ib thiol groups) by $k = 805\,M^{-1}\cdot s^{-1}$ and pK_a 8.1.

If it is assumed that the class-I thiol groups are distributed uniformly among six subunits, each subunit would contain four such thiol groups. Subsequent discussion is given in terms of a urease subunit containing four class-I thiol groups and one class-II (essential) thiol group (see below). In terms of this type of subunit, there are two class-Ia thiol groups

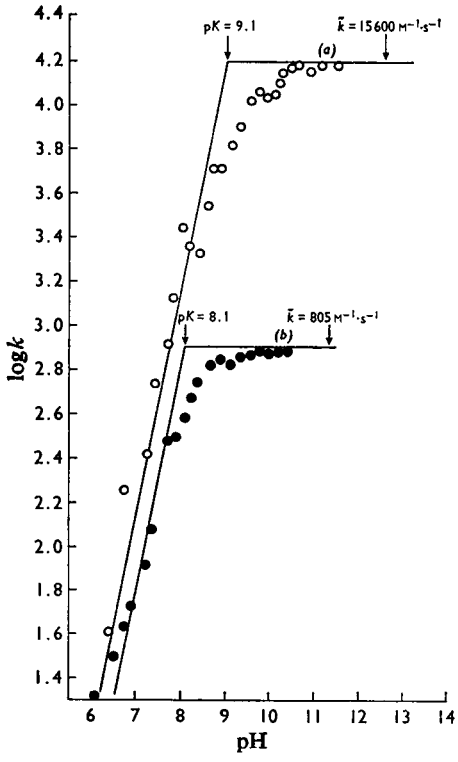


Fig. 6. *pH-dependence of the second-order rate constants for the reactions of 2-Py–S–S–2-Py with the class-Ia thiol groups of urease* (○) *and the class-Ib thiol groups of urease* (●) *at 25.0°C and I = 0.1 mol/l*

and two class-Ib thiol groups in approximately neutral media, whereas in more alkaline media the subunit contains one class-Ia thiol group and three class-Ib thiol groups. This finding suggests that urease undergoes a structural change dependent on an ionization (pK_a approx. 9.5) that results in a change in the ratio of class-Ia/class-Ib thiol groups. Urease that has been kept at pH11 for several hours retains its catalytic activity when assayed at pH7, so this change does not represent irreversible denaturation. The suggested conformational change that is permitted by the ionization of the 'group of pK_a9.5' could be triggered by reaction of some of the class-Ia thiol groups. If this is the case, then the stoicheiometry of the class-Ia reaction at high pH, i.e. six thiol groups per molecule, which could represent one thiol group per subunit, suggests that there may be no information transfer between the urease subunits, at least as monitored by this particular structural change, since this might be expected to result in less than six class-Ia thiol groups at high pH. The fact that urease preparations with mol.wts. much lower than 480000 retain substantial enzymic activity (see, e.g., Contaxis & Reithel, 1972) suggests independence of at least some of the subunits.

The simple analysis exemplified by Fig. 4 may of course be complicated by sequential changes in thiol reactivity imposed by modification of the most reactive thiol groups. Thus class-Ib thiol groups may possibly owe some of their reactivity characteristics to environmental changes (either direct steric shielding or induced conformational changes) provided by reaction of the class-Ia thiol groups.

The change in thiol reactivity controlled by the pK_a of 9.5 may help to characterize the pH-dependent structural change that permits the reassociation of the urease half-unit (mol.wt. 240000). Contaxis & Reithel (1971) reported that the urease half-unit prepared by dissociation of the urease molecule in 90% (v/v) propane-1,2-diol is stable for several weeks at pH9.2, but rapidly re-associates at pH7. The u.v.-absorption, optical-rotatory-dispersion and catalytic characteristics of the urease half-unit are similar to those of the urease molecule, mol.wt. 480000, which indicates that gross conformational changes do not occur in the dissociation or re-association processes.

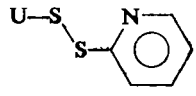
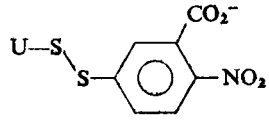
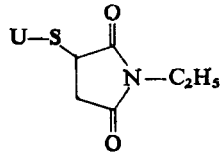
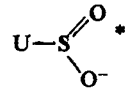
The parameters that characterize the pH-dependence of the reactivities of the class-I thiol groups are compared below with those that characterize the pH-dependence of the class-II (essential) thiol groups.

The class-II thiol groups. The reactions of 2-Py–S–S–2-Py with the thiol groups that are essential for ureolytic activity were studied in a number of ways. In most cases the class-I thiol groups were titrated with 2-Py–S–S–2-Py at pH7 until either 24 thiol groups/molecule had been titrated or until addition of further quantities of 2-Py–S–S–2-Py would have resulted in significant activity loss (approx. 23 thiol groups/molecule titrated; see Fig. 2). After removal of low-molecular-weight material by chromatography on Sephadex G-25, these partially blocked urease preparations were allowed to react with excess of 2-Py–S–S–2-Py under pseudo-first-order conditions at pH values in the range 6–11. The progress curves of these reactions should represent mainly the reactions of the six class-II (essential) thiol groups of the urease molecule. Similar rate constants for the reaction of the class-II thiol groups were obtained by using the technique described above and the last part of the multiphasic progress curve that results from admixture of excess of 2-Py–S–S–2-Py with native urease. When this reaction is studied by using a Cary 16K spectrophotometer instead of a stopped-flow method, the reaction of the class-Ia thiol groups appears to be essentially instantaneous; this phase is followed by a slower phase (the end of the class-Ib reaction), which merges into an even slower phase, which is mainly reaction of the class-II thiol groups.

The reactivity of the class-II thiol groups towards 2-Py–S–S–2-Py is insensitive to the group that blocks

Table 2. *Reactivity of the class-II thiol groups of urease towards 2-Py–S–S–2-Py at pH 7.68, $I = 0.1$ mol/l and 25.0°C*

The urease preparations in which the class-I thiol groups were blocked were approx. 1 μM with respect to protein, and [2-Py–S–S–2-Py] was 150–200 μM. Results are means±S.E.M. of the numbers of observations given in parentheses.

State of the class-I thiol groups [one blocked class-I thiol group per molecule of urease (U) is shown]	Second-order rate constant for reaction of the class-II thiol groups with 2-Py–S–S–2-Py (k) ($M^{-1} \cdot s^{-1}$)
	7.35±1.5 (5)
	6.75±2.1 (5)
	7.65±1.5 (5)
	5.2±1.3 (4)

* The primary oxidation product should be the sulphenic acid and this probably subsequently provides the sulphinic acid (depicted) and possibly the sulphonic acid; some intersubunit disulphide bond formation may occur.

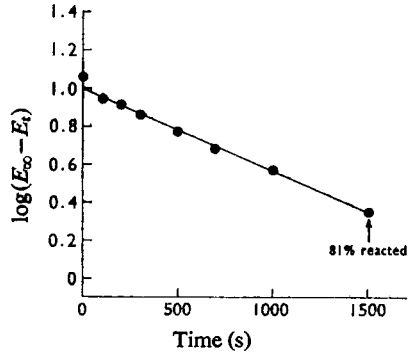


Fig. 7. *Demonstration of the probable homogeneity of the reaction of 2-Py–S–S–2-Py with the class-II (essential) thiol groups of urease at 25.0°C and $I = 0.1$ mol/l*

The class-I thiol groups had been blocked by reaction with 2-Py–S–S–2-Py as described in the text; [class II thiol groups]$_0$ = 7 μM and [2-Py–S–S–2-Py]$_0$ = 195 μM.

the class-I thiol groups. Thus the second-order rate constant for the reaction of 2-Py–S–S–2-Py with the class-II thiol groups at pH 7.68 is essentially the same whether the class-I thiol groups are blocked by reaction with 2-Py–S–S–2-Py, Nbs_2, *N*-ethylmaleimide or by oxidation to sulphur oxy acids (see Table 2). These preparations were subjected to Sephadex G-25 chromatography before being allowed to react with 2-Py–S–S–2-Py.

By contrast with the reactions of the class-I thiol groups, the reactions of the class-II thiol groups with 2-Py–S–S–2-Py appear to be monophasic at least up to approx. 80% of reaction (see Fig. 7 and compare with Fig. 4). In some runs, a small preliminary fast phase did appear to be revealed by logarithmic plots analogous to Fig. 7, but the rate constants of these phases had values similar to those of the reactions of the class-Ib thiol groups and probably arise from preparations in which these thiol groups were incompletely blocked.

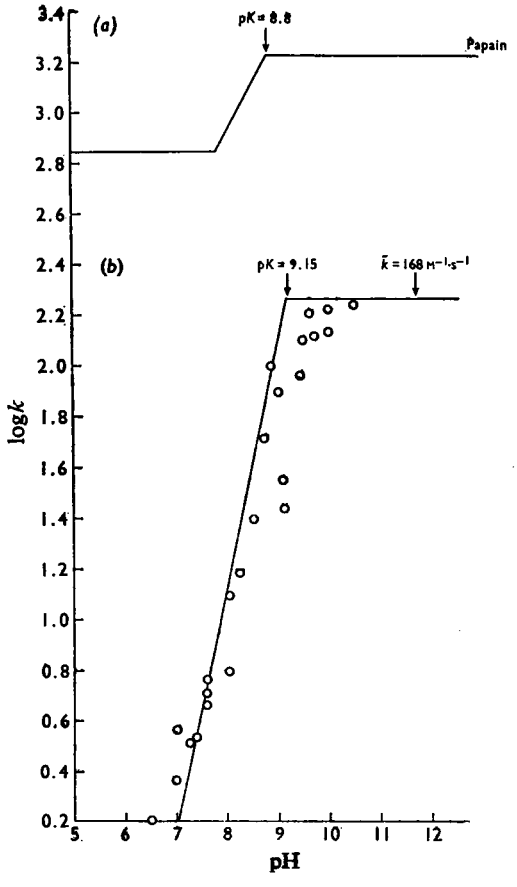


Fig. 8. *Comparison of the pH-dependences of the reactions of 2-Py–S–S–2-Py with the essential thiol groups of (a) papain and (b) urease (class-II thiol groups) at 25.0°C and I = 0.1 mol/l*

In the urease reactions, the class-I thiol groups were blocked by reaction with 2-Py–S–S–2-Py. The lines that characterize the papain reaction are theoretical for $k = 700+1700/(1+[H^+]/K)\,M^{-1}\cdot s^{-1}$ in which $pK = 8.8$ (Brocklehurst & Little, 1972).

pH-dependence of the reactions of 2-Py–S–S–2-Py with the class-Ia, class-Ib and class-II thiol groups of urease

As for the reactions of the class-I thiol groups, the observed first-order rate constant for the reactions of the class-II thiol groups with 2-Py–S–S–2-Py are linear in [2-Py–S–S–2-Py] up to 750 μM (at pH7.5).

The pH-dependences of the apparent second-order rate constants for the reactions of 2-Py–S–S–2-Py with the three accessible types of urease thiol group are shown as logarithmic plots in Figs. 6 and 8, and the characterizing parameters are compared in Table 3.

The simplest interpretation (see Brocklehurst & Dixon, 1976) of the molecular pK_a values apparent in these profiles is that they characterize the three types of thiol group in urease that are accessible to electrophilic reagents. The pK_a values (8–9) are in the range frequently displayed by protein thiol groups. The values of the pH-independent rate constants, $\bar{k}$, in the simplest interpretation, characterize the reactions of the protein thiolate ions with unprotonated 2-Py–S–S–2-Py, and differences in the value of $\bar{k}$ reflect largely differences in the environment of the thiol groups. Table 3 also shows values of $\bar{k}$ for the reactions of a number of other thiolate ions with 2-Py–S–S–2-Py, for comparison. Differences in the pK_a values of the thiol groups should not contribute greatly to the differences in the values of $\bar{k}$, because the Brönsted coefficient for the reactions of low-molecular-weight thiols with 2-Py–S–S–2-Py is only approx. 0.25 (T. Stuchbury & K. Brocklehurst, unpublished work).

The reaction of the class-Ia thiolate ions is characterized by a value of $\bar{k}$ similar to that of the analogous thiolate ion of mercaptalbumin; that of the class-Ib thiolate ion is characterized by a value of $\bar{k}$ within a factor of 2–3 of those of the thiolate ions of the thiol proteinases, where, at least for papain, the thiol group resides in a groove in the protein molecule and that of the class-II (essential) thiolate ions is characterized by a value of $\bar{k}$ similar to the very low value of the reaction of pro-papain.

The very low reactivity of the class-II thiolate ions of urease may arise from restricted access of the probe molecule. These essential thiol groups may be located relatively deep inside the subunit structure in sites readily accessible only to molecules as small as the substrate urea. In this respect, the active-centre region of urease could differ from those of the thiol proteinases, which appear to be able to bind up to seven amino acid residues (Berger & Schechter, 1970). An intriguing aspect of the reactivity of these thiol groups is the possibility that it may be influenced by the presence of bound Ni^{2+} (Dixon *et al.*, 1975*b*).

The reactivities of the class-I thiol groups at pH7.8 are not affected by incorporating 0.2M-urea into the reaction mixtures. Increase in the reactivity of a group in an enzyme occasioned by the presence of the substrate as it is transformed into product has been called 'syncatalysis' (Christen & Riordan, 1970).

Perhaps the most striking feature of Fig. 6 and in particular of Fig. 8 is that, in contrast with the profile for the analogous reaction of the essential thiol group of the thiol proteinase papain (EC 3.4.22.2), at least the class-Ia and class-II thiol groups of urease appear to react with 2-Py–S–S–2-Py only as the uncomplicated thiolate ions. The absence of evidence for a second plateau in neutral media in the urease profiles suggests that there is no interaction of any of these thiol groups with another acid–base system, such

Table 3. *Parameters characterizing the reactions of 2-Py–S–S–2-Py with the thiolate ions of urease and with some other thiolate ions at 25.0°C and I = 0.1 mol/l*

Thiol producing the thiolate ion	Molecular pK_a value of the thiol producing the thiolate ion	Second-order rate constant (k) for the reaction of the thiolate ion with unprotonated 2-Py–S–S–2-Py ($M^{-1} \cdot s^{-1}$)	Reference
Urease (class-Ia)	9.1	15600	The present work
Urease (class-Ib)	8.1	805	The present work
Urease (class-II)	9.15	168	The present work
Pro-papain	7.6	233	Brocklehurst & Kierstan (1973)
Papain	8.8	1700	Brocklehurst & Little (1972)
Ficin	8.6	2230	J. P. G. Malthouse & K. Brocklehurst (unpublished work)
Mercaptalbumin	8.2	15000	J. P. G. Malthouse & K. Brocklehurst (unpublished work)
2-Mercaptoethano	9.43	65000	T. Stuchbury & K. Brocklehurst (unpublished work)
L-Cysteine ethyl ester zwitterion	7.45	31600	T. Stuchbury & K. Brocklehurst (unpublished work)

as a histidine imidazole group, that might be expected to produce a second nucleophilic state of the thiol group in neutral media. For the class-Ib thiol groups the deviation of the data points from the line of slope +1 suggests that at pH values below 6 the values of log$k$ could become pH-independent at a value greater than that calculated (see Brocklehurst & Little, 1972) for the reaction of the simple thiolate ion with 2-Py–S–S–2-Py$^+$H (approx. 0.04). The ability to magnify effects of interaction by making use of protonated or 'partially protonated' 2-Py–S–S–2-Py, which has been used successfully with the thiol proteinases, cannot be used with urease because it loses its activity in acidic media.

Whereas the class-I thiol groups of urease would not necessarily have been expected to exist in more than one nucleophilic state, the essential (class-II) thiol groups might have been expected to do so. The fact that no evidence could be obtained with the 2-Py–S–S–2-Py reactivity probe for a second nucleophilic state suggests the possibility either that the essential thiol groups are not part of the catalytic sites of urease or that if they are, the chemistry by which urease brings about the hydrolysis of urea may be very different from that used by the thiol proteinases to effect their catalytic functions. As pointed out by Blakeley *et al.* (1969*a*), the remarkable effectiveness of ureolytic action ($k_{cat.}$ is about 100 times higher than $k_{cat.}$ for the action of any peptidase in the hydrolysis of carboxamides) may point to an active-centre chemistry in urease which is different from that of the proteinases, and the presence of bound Ni^{2+} raises interesting mechanistic possibilities involving Lewis-acid catalysis (Dixon *et al.*, 1976).

Oxidation of the class-I thiol groups of urease on storage, and characteristics of commercial urease preparations

Storage of urease, prepared by covalent chromatography as described in the Materials and Methods section, as a 5 μM solution in the pH 8.2 buffer in the absence of reducing agents for 1–2 weeks results in the gradual loss of the class-I thiol groups. When more than 86% of these groups had disappeared, the preparations still had more than 90% of their original ureolytic activity. Andrews & Reithel (1970) had previously reported 'an aged sample of urease with no evident exterior SH groups remaining still retained 30% of its initial activity . . .'. These authors assumed that loss of the inessential thiol groups on storage is due to disulphide-bond formation, and some preparations of urease have been shown to contain aggregates containing intermolecular disulphide bonds (Creeth & Nichol, 1960). During this oxidation process, the reversal of the oxidation by treatment with 30 mM-dithiothreitol at pH 7.2 or pH 9.3 for 30 min became progressively less effective. This could be due to a slow conformational change occurring after disulphide-bond formation and rendering these bonds inaccessible to dithiothreitol or to oxidation to sulphinic acid or sulphonic acid through the intermediacy of relatively easily reducible sulphenic acids or to a combination of these processes. Chromatography of fresh and aged samples of urease on Bio-Gel A-50m showed that at least some aggregation had accompanied the loss of class-I thiol groups.

In view of the relative ease of oxidation of the class-I thiol groups of urease it seemed probable that commercial samples of urease might be irreversibly oxi-

dized to some extent. In contrast with freshly prepared urease (24 class-I thiol groups per molecule), commercial samples of urease were found to have the following numbers of class-I thiol groups/molecule: (a) Boehringer preparations, 5–20; (b) Sigma type IV preparations, usually three to six, sometimes none; (c) Sigma type VI preparations, 15–20; (d) Sigma type VII preparations, approx. 20. Attempts to reduce preparations (a)–(c) by the dithiothreitol treatment described above were unsuccessful. These changes probably account for the apparent specificity of the essential thiol groups of Sigma type IV urease for 2-pyridyl-containing disulphides observed in a study of the immobilization of this preparation by reaction with Sepharose–glutathione–2-pyridyl disulphide (Carlsson *et al.*, 1974).

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