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L-Serine Dehydratase from *Arthrobacter globiformis*

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1. L-Serine dehydratase (EC 4.2.1.13) was purified 970-fold from glycine-grown *Arthrobacter globiformis* to a final specific activity of 660 μmol of pyruvate formed/min per mg of protein. 2. The enzyme is specific for L-serine; D-serine, L-threonine and L-cysteine are not attacked. 3. The time-course of pyruvate formation by the purified enzyme, in common with enzyme in crude extracts and throughout the purification, is non-linear. The reaction rate increases progressively for several minutes before becoming constant. The enzyme is activated by preincubation with L-serine and a linear time-course is then obtained. 4. The substrate-saturation curve for L-serine is sigmoid. The value of $[S]_{0.5}$ varies with protein concentration, from 6.5 mM at 23 μg/ml to 20 mM at 0.23 μg/ml. The Hill coefficient remains constant at 2.9. 5. The enzyme shows a non-specific requirement for a univalent or bivalent cation. Half-maximal activity is produced by 1.0 mM-$MgCl_2$ or by 22.5 mM-KCl. 6. L-Cysteine and D-serine act as competitive inhibitors of L-serine dehydratase, with K_i values of 1.2 and 4.9 mM respectively. L-Cysteine, at higher concentrations, also causes a slowly developing irreversible inhibition of the enzyme. 7. Inhibition by $HgCl_2$ (5 μM) can be partially reversed in its initial phase by 1 mM-L-cysteine, but after 10 min it becomes irreversible. 8. In contrast with the situation in all cell-free preparations, toluene-treated cells of *A. globiformis* form pyruvate from L-serine at a constant rate from the initiation of the reaction, show a hyperbolic substrate-saturation curve with an apparent K_m of 7 mM and do not require a cation for activity.

A variety of enzymes are known that are capable of deaminating L-serine to produce pyruvate and NH_3. They can be considered in three classes. (1) Dehydratases that are reported to be specific for L-serine, such as the enzyme from *Escherichia coli* described by Alföldi *et al.* (1968). (2) Dehydratases that act on other substrates, most frequently L-threonine, as well as on L-serine, usually at similar rates. Some enzymes of this type, e.g. the 'biosynthetic' and 'degradative' L-threonine dehydratases (EC 4.2.1.16) of *E. coli* and *Salmonella typhimurium*, have been extensively studied (Umbarger, 1973), though more often for their activity on threonine than for that on serine. (3) Enzymes primarily catalysing a different reaction but also able to catalyse the deamination of L-serine, e.g. the B protein of tryptophan synthetase (EC 4.2.1.20) of *E. coli* (Crawford & Ito, 1964) and cystathionine synthetase (EC 4.2.1.22) of rat liver (Nagabhushanam & Greenberg, 1965).

Of these three classes, the enzymes that are specific for L-serine have been least studied. Such enzymes have been reported in crude extracts, or have been partially purified, from *E. coli* (Alföldi *et al.*, 1968), *S. typhimurium* and *Bacillus cereus* (Raskó *et al.*, 1969), *Streptomyces rimosus* (Szentirmai & Horvath, 1962), *Bacillus alvei* (Griffiths & DeMoss, 1970), *Bacillus circulans* (Nabe, 1971) and *Corynebacterium* sp. (Morikawa *et al.*, 1974), but only the enzyme from *Clostridium acidi-urici* (Carter & Sagers, 1972) has hitherto been purified to homogeneity.

The present paper describes the purification of a specific L-serine dehydratase (EC 4.2.1.13) from the aerobic soil bacterium, *Arthrobacter globiformis*, and compares the properties of the purified enzyme with those observed with toluene-treated whole cells. A preliminary report from this laboratory (Bridgeland & Jones, 1965), based on studies with enzyme preparations only slightly purified from the crude extract, suggested that the properties of the enzyme changed significantly when it was released from its cellular environment. The present work confirms and extends these observations by using the purified enzyme.

Materials and Methods

Materials

L-Serine (chromatographically homogeneous) was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.; D-serine from Koch–Light Laboratories,

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Colnbrook, Bucks., U.K., and from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.; 1,2-diamino-4-nitrobenzene from Ralph N. Emmanuel Ltd., Wembley, Middx., U.K.; NADH from the Boehringer Corp. (London) Ltd., London, W.5., U.K.; lactate dehydrogenase (from rabbit muscle) from Sigma (London) Chemical Co. Ltd.; and DEAE-cellulose (type DE52, microgranular) from W. and R. Balston (Modified Cellulose) Ltd., Maidstone, Kent, U.K.

Organism and growth conditions

Arthrobacter globiformis N.C.I.B. strain 8602 was maintained by monthly subculture on slopes of the medium described below, solidified with 1.5% agar. Cultures were grown at 30°C for 36–48h and stored at room temperature.

The medium for growth had the following composition (per litre): KH_2PO_4, 7.65g; K_2HPO_4, 10.9g; $MgSO_4,7H_2O$, 0.2g; $FeCl_3,6H_2O$, 2.7mg; $CaCl_2$, 40mg; glycine, 7.5g; biotin, 100μg; final pH, 6.4. Glycine, biotin and $CaCl_2$ solutions were sterilized separately and added aseptically to the sterile basal medium. For growth on glucose, glycine was replaced by glucose (7.5g/l) plus NH_4Cl (3g/l).

Cultures were grown at 30°C in 800ml batches of medium in 2-litre baffled Erlenmeyer flasks and aerated by shaking on an orbital shaker at 120rev./min. Large-scale cultures were grown, also at 30°C, in 15-litre batches in 20-litre carboys. They were vigorously aerated by passing air, sterilized by passage through columns of sterile cotton-wool, into the culture through two spargers. Cultures were inoculated with a cell suspension prepared from a recently grown culture to give an initial cell density of 0.03–0.05mg dry wt./ml of culture. Growth was continued until a cell density of 1.0mg dry wt./ml had been reached, by which stage the growth rate was declining. The mean generation time on glycine was 2.6h and on glucose 2.3h.

Bacterial growth was assessed by measurement of A_{680} and the value was converted into mg dry wt./ml of culture by comparison with a previously constructed standard curve.

Harvesting of organisms

Cells were harvested by centrifuging at 23000*g* for 15min at 0–4°C, and washed by resuspending in 10mM-Tris/HCl buffer, pH8.0, and again centrifuging. The washed cells were suspended in 10mM-Tris/HCl buffer, pH8.0. With large batches of cells the initial centrifugation was performed in a Sharples continuous-flow centrifuge (Sharples Centrifuges Ltd., Camberley, Surrey, U.K.) at 20000rev./min and a flow rate of approx. 200ml/min.

Toluene-treatment of cells

Toluene (0.025ml/ml of cell suspension) was added to the cell suspension (adjusted to a cell density of 1.0mg dry wt./ml) and vigorously mixed on a Vortex mixer. The mixture was incubated at 30°C for 15min. During the incubation period the mixing was repeated from time to time. At the completion of the incubation period, the suspension was diluted 10–20-fold with 10mM-Tris/HCl buffer, pH8.0, and used immediately.

Preparation of cell-free extracts

Dynomill extraction. Extracts were usually prepared by using a Dynomill laboratory mill, type KDL (Willy A. Bachofen, Basel, Switzerland; obtained through Glen Creston, Stanmore, Middx., U.K.), which agitates a mixture of glass beads and cell suspension by means of rapidly rotating vanes.

Glass beads (0.1mm diam.; 510ml packed volume) were placed in the 600ml continuous-flow grinding container of the Dynomill, and the vessel was filled with cell suspension (approx. 200ml; 40mg dry wt./ml). The agitator vanes were set to rotate at 3000rev./min and the cell suspension was pumped through the container at a flow rate of about 2.5l/h. The temperature of the emerging broken-cell suspension was kept below 15°C by circulating brine cooled to −15°C through the jacket of the grinding vessel. When all the cell suspension had been pumped into the grinding vessel, that remaining in the vessel was washed out with about 250ml of 10mM-Tris/HCl buffer, pH8.0. The treated cell suspension was centrifuged at 40000*g* for 40min at 4°C. All the L-serine dehydratase activity was in the supernatant.

Other methods of cell breakage were used occasionally, especially when small batches of cells were being processed.

Ultrasonication. The cell suspension, cooled to 4°C and placed in a beaker surrounded by an ice/water mixture, was treated with ultrasound for a total of 2.5min, with intervals for cooling, with an MSE 60W ultrasonic oscillator (M.S.E., Crawley, Sussex, U.K.) operating at 1.2–1.5A.

Braun shaker. Bacterial suspension (35ml) was mixed with 25ml of glass beads (0.1mm diam.) and shaken at 4000 oscillations/min in a Braun shaker (B. Braun, Melsungen, W. Germany) for two 1min periods separated by a cooling period. The temperature of the suspension was maintained at 0–10°C by passing liquid CO_2 over the shaking vessel.

French pressure cell. Cell suspension, cooled to 4°C, was passed twice through a French pressure cell (American Instrument Co., Silver Springs, MD, U.S.A.) at a pressure of 82.8MPa (12000lb/in^2).

Lysozyme treatment. Cells, harvested early in the exponential phase of growth, were treated by the method of Sistrom (1958), using 40mM-Tris/HCl

buffer, pH 8.0, containing 2.3 mM-EDTA (sodium salt).

Cell debris was removed by centrifuging as described above.

Enzyme assays

Three methods were used to assay L-serine dehydratase activity, all of which measured the formation of pyruvate. The basal assay mixture contained, in a final volume of 1.0 ml: 50 mM-Tris/HCl buffer, pH 8.2, 10 mM-$MgCl_2$ and 50 mM-L-serine (adjusted to pH 8.2 with KOH). The enzyme sample was added last to initiate the reaction. Assays were performed at 30°C.

Method 1: continuous measurement of pyruvate formation by coupling to lactate dehydrogenase. The basal assay mixture was supplemented with approx. 10 units of lactate dehydrogenase and 0.225 μmol of NADH, and NADH oxidation followed at 340 nm. For quantities of L-serine dehydratase greater than 1 unit, the quantity of lactate dehydrogenase was correspondingly increased and 0.9 μmol of NADH added. In this case, the oxidation of NADH was followed at 366 nm, at which wavelength the molar extinction coefficient of NADH is 3300 litre·mol^{-1}·cm^{-1} (Bergmeyer, 1962). This method was used in all kinetic experiments with the purified enzyme, but could not be used with crude preparations, which contained NADH oxidase in high activity.

Method 2: continuous measurement of pyruvate formation by means of its absorbance at 315 nm. In the assay mixture given above, pyruvate shows a broad absorption peak in the region 300–330 nm, with a maximum at 315 nm ($\varepsilon = 22.0$ litre·mol^{-1}·cm^{-1}). This low molar extinction coefficient necessitates the addition of at least 2 units of L-serine dehydratase activity to the assay mixture, and suits this method to the assay of highly active enzyme preparations. It was used, especially, in monitoring enzyme activity at the completion of each stage of the purification procedure.

Method 3: colorimetric estimation of pyruvate as its 2,4-dinitrophenylhydrazone (Friedemann & Haugen, 1943). Assay mixtures, as above, were incubated for 10 min, at the end of which time 0.33 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 M-HCl was added to each mixture. The mixtures were left for 5 min at room temperature, 2 ml of 2 M-NaOH was then added to each, and after a further 10 min the A_{445} was measured. This method was used for experiments with toluene-treated cells (in which case $MgCl_2$ was omitted from the assay mixture), with crude preparations containing high NADH oxidase activity, in experiments where large numbers of enzyme samples were to be assayed, e.g. column fractions, and when substances that would inhibit lactate dehydrogenase, e.g. mercurial compounds, were present in the assay mixture.

Definition of unit of L-serine dehydratase activity. One unit of L-serine dehydratase activity is defined as the activity forming 1 μmol of pyruvate/min at 30°C, when the reaction is proceeding at a linear rate.

Low-cation assay mixture. For experiments on the effect of added cations on enzymic activity, it was necessary to lower the cation content of the assay mixture by omitting Tris/HCl buffer and adjusting the pH of L-serine to 7.0, instead of 8.0. The low-cation assay mixture, which was used with assay method 1, the lactate dehydrogenase method, contained: 50 mM-L-serine (adjusted to pH 7.0 with KOH), 10 units of lactate dehydrogenase [exhaustively dialysed against water to remove $(NH_4)_2SO_4$], 0.225 mM-NADH and the enzyme sample. The final pH was 6.7. The mixtures were estimated to contain 0.5 mM-Na^+ (introduced with the NADH) and approx. 0.5 mM-K^+ (introduced in the L-serine solution).

Protein, serine and ammonia determinations

Protein concentration was determined either by the method of Warburg & Christian (1942) or by that of Lowry *et al.* (1951) with bovine serum albumin as standard. The protein content of cell suspensions was determined by heating the suspension with an equal volume of 2 M-NaOH at 100°C for 10 min, neutralizing with HCl and determining protein in samples of the resulting clear solution by the method of Lowry *et al.* (1951).

Serine was measured by the chromotropic acid method (Frisell *et al.*, 1954) and ammonia by using glutamate dehydrogenase as described by Holzer *et al.* (1964).

Polyacrylamide-gel electrophoresis

Gel electrophoresis was performed with a Shandon Electrophoresis Unit (Shandon Scientific Co., London N.W.10, U.K.) at 4°C and a current of 5 mA per tube. Two gel systems were used in the analysis of protein samples: (i) the system of Davis (1964), with a Tris/glycine buffer, pH 8.9; (ii) a system at pH 6.8, with Tes [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid]/triethanolamine buffer (Orr *et al.*, 1972). In both cases gels 7.0 cm long and containing 7.5% (w/v) acrylamide were used. The enzyme sample (up to 150 μl), solid sucrose (7.5 mg/ml of sample) and Bromophenol Blue (10 μl of a solution containing 1 mg/ml) were mixed before layering on to the top of the gel.

Protein was detected by staining the gel with 1% Naphthalene Black in 7% (v/v) acetic acid. Gels were destained electrophoretically.

L-Serine dehydratase activity was detected by incubating the gels in a mixture containing 500 mM-L-serine, 10 mM-$MgCl_2$ and 50 mM-Tris/HCl buffer, pH 8.2, for between 0.5 and 2 h and then heating them at 90°C in 0.6% 1,2-diamino-4-nitrobenzene in 2 M-HCl for 30 min. The position of L-serine dehydrat-

ase was indicated by a red band on a yellow background (Gannon & Jones, 1977). Gels stained for activity in this manner could be subsequently stained for protein as described above.

Concentration of enzyme samples

Dilute enzyme samples were concentrated by ultrafiltration with either an Amicon Ultrafiltration cell, model 202 (Amicon Corp., Lexington, MA, U.S.A.), or a Chemostat C50 Ultrafiltration cell (Chemlab Instruments Ltd., Ilford, Essex, U.K.). In both cases an Amicon UM-10 membrane, which retains substances having a mol.wt. greater than about 10000, was used and a pressure of 345kPa (50lb/in^2) applied. The procedure resulted in a small loss (up to about 10%) of enzymic activity.

Enzyme purification

The procedure is summarized in Table 1. Except where noted otherwise below, all steps were carried out at 4°C.

Step 1: extraction and treatment with deoxyribonuclease and ribonuclease. Cells derived from three 15-litre cultures of *A. globiformis* were extracted with glass beads by using the Dynomill as described above, yielding about 1.25 litres of cell-free extract. (Extracts prepared by ultrasonication or by treatment in the Braun shaker have been successfully taken through the purification procedure without the need for any modifications.) The extract was treated with 4mg each of deoxyribonuclease and ribonuclease per litre of extract at 15°C for 30min. This treatment caused no change in activity, but improved the fractionations obtained in later steps.

Step 2: heat treatment in the presence of $CaCl_2$. Solid $CaCl_2$ was added, with stirring, to the extract to give a final concentration of 0.1M. During this addition the pH of the solution was maintained at 8.0 by the addition of 1M-Tris. The mixture was placed in a water bath at 70°C, stirred until its temperature had risen to 40°C and then transferred to a water bath at 40°C. After 10min at 40°C, the mixture was cooled to less than 20°C in an ice/water mixture. The resulting precipitate was inactive and was removed by centrifugation at 40000*g* for 40min at 4°C.

Step 3: $(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ (209g/l) was added slowly with stirring to the supernatant from step 2 to give a saturation of 35%. The pH was maintained at 8.0 by the addition of 1M-Tris. The mixture was kept at room temperature for 30min before being centrifuged at 40000*g* for 30min at 4°C. The inactive precipitate was discarded. The $(NH_4)_2SO_4$ saturation of the supernatant was then increased to 53% by adding solid $(NH_4)_2SO_4$ (115g/l) slowly with stirring. The pH was maintained at 8.0 with 1M-Tris and, after standing at room temperature for 30min, the mixture was centrifuged at 40000*g* for 30min. The supernatant contained less than 5% of the L-serine dehydratase activity and was discarded. The precipitate was dissolved in a minimum volume (3–6ml) of 10mM-potassium phosphate buffer (pH6.7)/0.5mM-EDTA (buffer A).

Step 4: chromatography on DEAE-cellulose. A column (15cm long×6.5cm diam.; bed volume 500ml) of DEAE-cellulose was equilibrated with buffer A. The redissolved precipitate from step 3 was diluted with 500ml of this buffer in order to lower the concentration of $(NH_4)_2SO_4$ carried over from step 3 to a point at which it would not interfere with the binding of the enzyme to the DEAE-cellulose. This solution was applied to the column at a flow rate of 500ml/h and the column was eluted first with buffer A and then with a 2-litre gradient (0.15–0.6M) of KCl in buffer A. Fractions (10ml) were collected. The L-serine dehydratase activity was eluted in a broad peak between 1.1 and 1.4 litres, after the main peak of protein. Fractions showing a specific activity greater than 20μmol/min per mg of protein were combined for further purification.

Step 5: chromatography on Sephadex G-100. To avoid cracking of the Sephadex G-100 column it was necessary to decrease the salt concentration in the enzyme-containing fractions from step 4. This was achieved by concentrating the combined fractions (approx. 250ml) to a volume of 15ml by ultrafiltration, diluting the concentrated enzyme solution to 50ml with buffer A, and again concentrating the solution

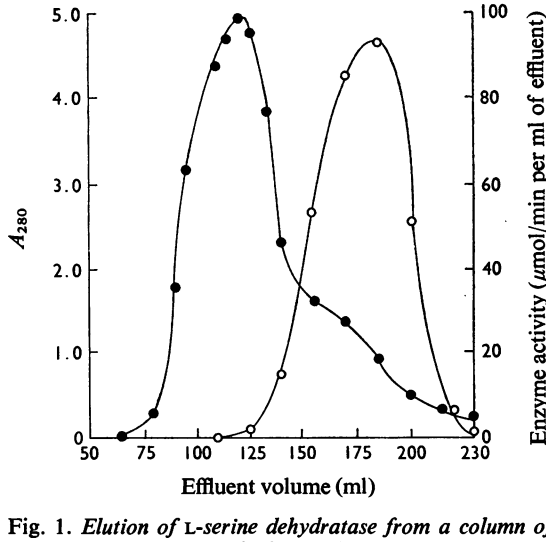

Fig. 1. *Elution of L-serine dehydratase from a column of Sephadex G-100*

Protein solution from step 4 (6.5ml containing 500mg of protein and 15100 units of enzyme activity) was eluted as described in the text. ○, L-Serine dehydratase activity assayed by method 3; ●, A_{280}.

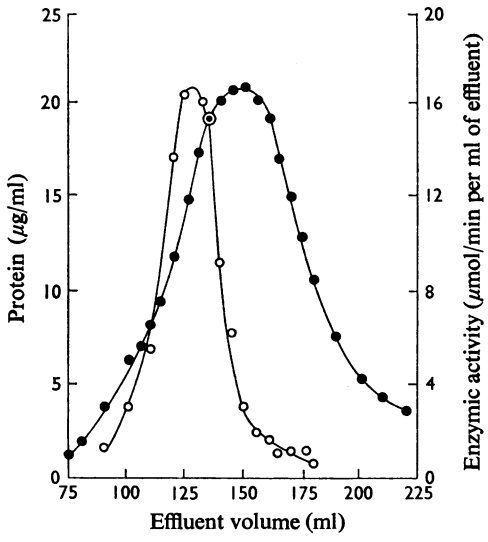

Fig. 2. *Elution of* L-*serine dehydratase from Sephadex G-100 in the presence of* 150 mM-D-*serine*
Protein solution from step 5 (1.5ml containing 9mg of protein and 1500 units of enzyme activity) was pre-incubated with D-serine and $MgCl_2$ and then eluted from a column of Sephadex G-100 with buffer containing 150mM-D-serine, as described in the text. ○, L-Serine dehydratase activity assayed by method 3; ●, protein concentration.

to 6ml or less by ultrafiltration. This sample was placed on a column (56cm×2.7cm) of Sephadex G-100 that had been equilibrated with buffer A containing 0.15M-KCl. The column was eluted with this buffer at a flow rate of 30ml/h (Fig. 1). Fractions (5ml) with a specific activity greater than 80μmol/min per mg of protein were combined and concentrated to a volume of 3–6ml by ultrafiltration.

Step 6: chromatography on Sephadex G-100 in the presence of D-*serine.* $MgCl_2$ and D-serine were added to the concentrated enzyme solution from step 5 to give final concentrations of 10mM and 150mM respectively, and the mixture was incubated at 15°C for 15min. This treatment causes dimerization of the enzyme (F. Gannon & K. M. Jones, unpublished observations). The treated enzyme sample was placed on a column (55cm×2.7cm) of Sephadex G-100 that had been equilibrated with buffer A containing 150mM-D-serine and 75mM-KCl. The column was eluted with the same buffer at a flow rate of 30ml/h (Fig. 2). Fractions (5ml) having a specific activity greater than 400μmol/min per mg of protein were pooled, concentrated by ultrafiltration, and stored at −70°C.

Results

Enzyme activity in toluene-treated cells

When cells of *A. globiformis*, grown on glycine as source of carbon and nitrogen, were toluene-treated and incubated in a mixture containing L-serine and buffer, pyruvate was formed at a rate that was constant for at least 30min and was directly proportional to the quantity of cells added to the incubation mixture. The specific activity of L-serine dehydratase in toluene-treated glycine-grown cells was 0.5–2.0μmol of pyruvate formed/min per mg of cell protein at 30°C.

Effect of L-*serine concentration.* The curve relating L-serine concentration to the rate of pyruvate production was hyperbolic, giving a linear double-reciprocal plot. The apparent K_m value was 7mM-L-serine and was unaffected by the concentration of toluene-treated cells used in the determination.

Effect of cations. Addition of $MgCl_2$ or KCl to the incubation mixture did not increase the dehydratase activity of toluene-treated cells. Indeed, 10mM-$MgCl_2$ caused 50% inhibition of activity.

Glucose-grown cells. When cells were grown with glucose plus NH_4Cl in the medium, in place of glycine, the activity was much lower, 0.015–0.03μmol of pyruvate formed/min per mg of cell protein, but displayed the same properties as those described for glycine-grown cells.

Enzyme activity in cell-free preparations

In contrast with the situation with toluene-treated cells, pyruvate formation by cell-free extracts of *A. globiformis* in a mixture containing L-serine, $MgCl_2$ and buffer (see the Materials and Methods section) did not proceed in a linear fashion. Instead, the rate of pyruvate formation increased progressively over a period of several minutes before a constant rate was achieved (Fig. 3). The same non-linear time-course for the enzyme-catalysed reaction was found with enzyme samples taken at each stage of the purification of L-serine dehydratase and with the purified enzyme itself.

Many variations of the standard assay conditions were tested to exclude the possibility that this non-linear time-course might be an artifact caused by the particular conditions used. None of the following variations significantly affected the shape of the time-course of the reaction. (i) Following the time-course by each of the assay methods described in the Materials and Methods section and by measuring the disappearance of serine or the appearance of NH_3; (ii) using extracts, or enzyme preparations purified from them, made by any of the methods given in the Materials and Methods section; (iii) purifying the L-serine either by recrystallizing it twice from 50% (v/v) ethanol or by chromatography on Dowex-50

(H^+ form) followed by recrystallization; (iv) varying the pH of the assay mixture in the range from 6.0 to 9.0; (v) substituting a variety of cationic and anionic buffers for Tris/HCl; (vi) substituting other salts, containing univalent or bivalent cations, for $MgCl_2$; (vii) performing the assays at temperatures between 4° and 40°C; (viii) making extracts from glucose-grown cells.

Effect of toluene. The possibility that the linear time-course, observed in toluene-treated cells, might result from the presence of toluene was examined in two ways. First, toluene was added to assay mixtures. Both with crude extracts and with purified enzyme preparations, a non-linear time-course was obtained, similar to that found in such preparations in the absence of toluene. Secondly, extracts were prepared from toluene-treated cells by ultrasonication and by lysozyme treatment. As with extracts of untreated cells, these extracts showed a non-linear time-course of pyruvate formation.

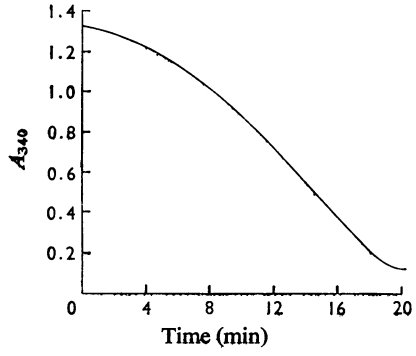

Fig. 3. *Time-course of* L-*serine dehydratase reaction with purified enzyme*

Pyruvate formation was followed by monitoring the A_{340} of an assay mixture (method 1) containing 0.04 unit of L-serine dehydratase purified to step 6.

Effect of products. The results described above suggest that the non-linear time-course is not an artifact, but a genuine property of the enzyme-catalysed reaction, once the enzyme has been extracted from the bacterial cell. It would seem to result from an interaction between the enzyme and one or more of the components of the reaction mixture. The time required to reach maximum activity would be readily explained if the enzyme was activated by one of the products of the reaction. However, when pyruvate (1 mM) and NH_4Cl (1 mM) were included in the assay mixture or when the enzyme was preincubated with these compounds, the progress curve of the reaction was identical with that of a control from which pyruvate and NH_4Cl had been omitted.

Preincubation experiments. When enzyme was preincubated in a mixture of 50mM-Tris/HCl buffer, pH8.2, and 10mM-$MgCl_2$ (an assay mixture lacking serine) and, after 15min at 30°C, a sample was transferred to a complete assay mixture, a non-linear time-course, very similar to that obtained in a comparable standard assay, was obtained. If, however, the preincubation mixture contained 50mM-L-serine, in addition to buffer and $MgCl_2$, the time-course of pyruvate formation was linear from the moment that the sample was transferred into the fresh assay mixture.

These experiments demonstrate that the activation of the enzyme is caused by incubating it with L-serine, and not by any other component of the reaction mixture. It also suggests that the low rate of this interaction is responsible for the progressively increasing rate of pyruvate formation observed in the absence of preincubation.

In the remainder of this paper the reaction rates being considered are the linear rates of pyruvate formation attained by the enzyme, unless specifically stated otherwise.

Table 1. *Purification of* L-*serine dehydratase*

Enzyme activity was assayed by method 2. Only a part, usually about one-quarter, of the material from step 5 was purified through step 6 at any one time. The values quoted for step 6 have been scaled up to correspond to the treatment of the whole of the material derived from step 5.

Step	Treatment	Total protein	Total activity (μmol/min)	Recovery of activity (%)	Specific activity (μmol/min per mg of protein)	Increase in specific activity
1	Extract, treated with ribonuclease and deoxyribonuclease	49.6g	34000	100	0.68	—
2	Heat/$CaCl_2$ precipitation	23.5g	28400	84	1.21	1.8
3	$(NH_4)_2SO_4$ fractionation	8.4g	24100	71	2.85	4.2
4	DEAE-cellulose chromatography	500mg	15100	44	30.2	44
5	Sephadex G-100 gel filtration	40mg	6800	20	170	250
6	Sephadex G-100 gel filtration in the presence of D-serine	5mg	3300	10	660	970

Properties of the purified enzyme

The purification procedure, given in detail in the Materials and Methods section and summarized in Table 1, resulted in a 970-fold purification of the L-serine dehydratase from glycine-grown *A. globiformis* and yielded an enzyme preparation with a specific activity of 660 μmol of pyruvate formed/min per mg of protein. On polyacrylamide-gel electrophoresis at pH 6.8, this preparation showed a single band of protein, which coincided in position with the L-serine dehydratase activity. When the same preparation was analysed on pH 8.9 gels, two closely juxtaposed major bands and one minor band of protein were observed. The two major bands both fell within the confines of the band given by the activity stain, so that it was not possible to say whether both had enzymic activity.

Stability. In crude extracts, enzymic activity was stable over many months when stored at −20°C and at a pH between 7.0 and 8.0. After step 3 of the purification procedure, the pH of maximum stability shifted to about 6.7. Enzyme from step 5, at protein concentrations above 1 mg/ml, could be stored at −70°C without significant loss over long periods, but after step 6 (protein concentration 0.6 mg/ml) about 30% of activity was lost in 1 month even at −70°C.

Substrate specificity. The enzyme was highly specific for L-serine. When L-threonine, DL-homoserine, L-cysteine, L-alanine or D-serine (each at 50 mM) was used as substrate, the reaction rate was less than 1% of that observed with L-serine.

Effect of substrate concentration. The graph of linear rate against substrate concentration gave a sigmoid curve (Fig. 4). Values of the substrate concentration giving half-maximal velocity, $[S]_{0.5}$, and of the Hill coefficient, h, were calculated from plots of $\log[v/(V_{max.}-v)]$ against $\log[S]$. Values of $[S]_{0.5}$ varied with the protein concentration (Fig. 4), from 6.5 mM-L-serine at a protein concentration of 23 μg/ml to 20 mM at 0.23 μg of protein/ml. The Hill coefficient, however, remained unchanged at 2.9 ± 0.3 (ten determinations). A sigmoid relationship, with values of $[S]_{0.5}$ and h in the same ranges, was also observed in crude extracts and at each stage of the purification procedure.

Variation of the L-serine concentration did not affect the non-linear progress curve of the reaction.

Effect of pH. The enzyme showed a broad pH optimum with maximum activity between pH 7.5 and 8.0.

Effect of cations. When the purified enzyme was tested in the low-cation assay mixture, without any added salt, its activity was less than 2% of that observed in the standard mixture. The addition to the assay mixtures of a variety of salts containing univalent or bivalent cations, including KCl, NaCl, $(NH_4)_2SO_4$, Tris hydrochloride, $MgCl_2$, $MnCl_2$ and $CaCl_2$, stimulated enzymic activity. A hyperbolic relationship was found between salt concentration and the linear rate of pyruvate formation. The same value for maximum rate was obtained with all the salts tested, but the concentrations giving a half-maximal rate were markedly different (Table 2). The bivalent cations were much more effective, giving a half-maximal rate at concentrations of 0.6–1.0 mM, whereas the univalent cations were required at concentrations close to 20 mM to produce the same effect. When combinations of cations were tested at concentrations of each which, individually, would give less than half-maximal activity, their stimulatory effects on enzymic activity were additive. On the other hand, when a saturating concentration of one cation was present the addition of another had no effect.

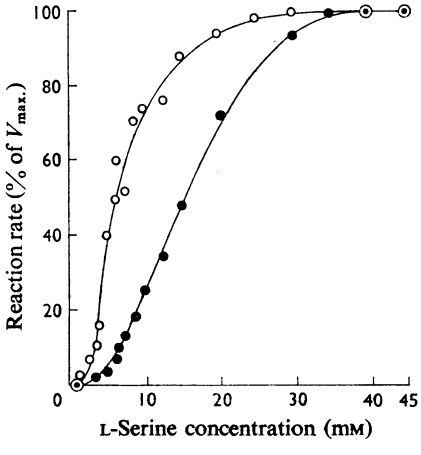

Fig. 4. *Effect of L-serine concentration on enzymic activity* Linear rates were determined by assay method 1 and are expressed as a percentage of $V_{max.}$. Enzyme from step 5 of the purification procedure was used at protein concentrations of 23 μg/ml (○) and 0.23 μg/ml (●).

Table 2. *Activation of L-serine dehydratase by cations* Linear rates of pyruvate formation were determined by method 1, with 0.3 unit of enzyme purified to step 5, in low-cation assay mixtures with the addition of appropriate concentrations of one of the salts listed below. The concentration of salt giving half-maximal activity was calculated from a double-reciprocal plot of rate against salt concentration for each compound.

Salt	Concentration giving half-maximal activity (mM)
$MgCl_2$	1.0
$CaCl_2$	0.8
$MnCl_2$	0.6
$(NH_4)_2SO_4$	8.0 (≡16.0 mM-NH_4^+)
NH_4Cl	20.0
KCl	22.5
NaCl	22.0

Concentrations of $MgCl_2$ above 40mM were inhibitory, the activity falling to 50% of its maximum value at 80mM-$MgCl_2$. On the other hand, KCl caused no inhibition at concentrations up to 250mM.

Pyridoxal phosphate. The addition of 1mM-pyridoxal phosphate to the assay mixture did not increase enzymic activity nor did it alter the non-linear time-course of the reaction. The same results were obtained when the pyridoxal phosphate was preincubated with the enzyme for 30min.

The enzyme lost 70–97% of its activity after dialysis against 10mM-Tris/HCl buffer (pH7.8)/1mM-EDTA, either alone or containing 100mM-L-cysteine, 50mM-hydroxylamine or 100mM-D-serine. However, inclusion of 1mM-pyridoxal phosphate in the assay mixture or preincubation of the inactivated enzyme with it did not restore activity.

Other activators. Many compounds have been reported to stimulate the activity of L-serine dehydratase from different sources. None of the following compounds either stimulated the activity of the enzyme from *A. globiformis* or affected its time-course: AMP, ADP, ATP (all at 1mM), cyclic AMP (0.1mM), biotin (0.01mM), $FeSO_4$ (10mM), reduced glutathione (10mM) and dithiothreitol (20mM).

Inhibitors of L-*serine dehydratase*

L-*Cysteine and* D-*serine.* When L-cysteine or D-serine was added to the assay mixture, L-serine dehydratase activity was inhibited. The nature of these inhibitions was examined by the procedures developed by Cleland (1963, 1967). The linear rates of pyruvate formation were determined in a series of assays at different concentrations of substrate and inhibitor. At each inhibitor concentration, the slope and the intercept on the $1/v$ axis of the plots of $1/v$ against $1/[S]$ were calculated. Although the enzyme gave a sigmoid relationship between v and [S], the double-reciprocal plots were linear if substrate concentrations above the K_m value were used. Cleland (1967) has stated that a range above K_m, although not ideal, is acceptable if it is not possible to use a wider range. The calculated values of slope and intercept are plotted against inhibitor concentration, for L-cysteine, in Fig. 5 and, for D-serine, in Fig. 6. It can be seen that the slope varied markedly with inhibitor concentration, whereas the intercept remained nearly constant. This behaviour suggested that the compounds were acting as competitive inhibitors. To confirm this conclusion, the original rate data were fitted to the equations for competitive and non-competitive inhibition. The calculated values of the kinetic constants and their standard errors are shown in Table 3. With both inhibitors, the errors, especially in K_{is} and K_{ii}, were greater when the data were fitted to the equation for non-competitive inhibition than when they were fitted to the competitive case. It is

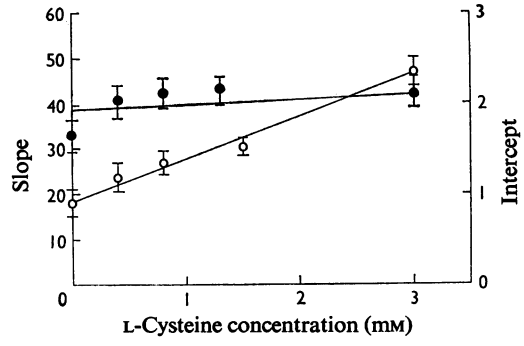

Fig. 5. *Inhibition of* L-*serine dehydratase by* L-*cysteine* Linear rates were measured by assay method 1, with 0.4 unit of enzyme purified to step 5, at L-serine concentrations of 6, 10, 12.5, 20 and 30mM and each of the L-cysteine concentrations noted on the graph. For each L-cysteine concentration, the values of the slope and of the intercept on the $1/v$ axis for plots of $1/v$ against $1/[S]$ were calculated by linear regression. Slopes (○) and intercepts (●), and their standard errors, are in arbitrary units.

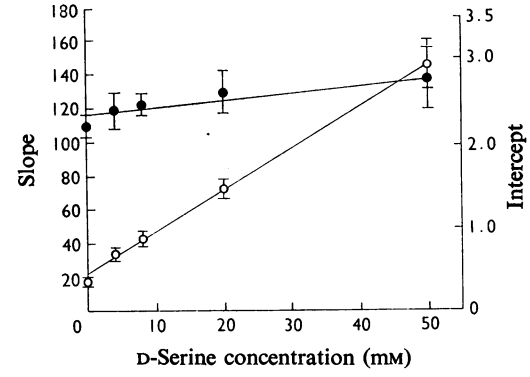

Fig. 6. *Inhibition of* L-*serine dehydratase by* D-*serine* Slopes (○) and intercepts (●) were calculated as in Fig. 5. L-Serine concentrations of 6, 10, 12, 30 and 50mM were used.

therefore concluded that L-cysteine and D-serine act as competitive inhibitors of L-serine dehydratase, and have K_i values of 1.2 and 4.9mM respectively.

The inhibition just described was reversible and was observed over time-intervals of a few minutes. When the enzyme was incubated for longer times with high concentrations of L-cysteine, irreversible inhibition occurred. In a mixture containing 150mM-L-cysteine, the activity of samples removed at timed intervals fell to 12% of that originally present over a period of 5h (Fig. 7). This inhibition was not caused

Table 3. *Inhibition of* L-*serine dehydratase by* L-*cysteine and* D-*serine*

Values of kinetic constants [K_{Is} and K_{Ii} are as defined by Cleland (1963)] and their standard errors were calculated by fitting the experimental data (see Figs. 5 and 6) to the equations for competitive and non-competitive inhibition by the methods of Cleland (1967).

Inhibitor	Type of inhibition	K_m (mM)	$V_{max.}$	K_{Is} (mM)	K_{Ii} (mM)
L-Cysteine	Competitive	8.4 ±1.4	0.50±0.02	1.16±0.18	—
	Non-competitive	10.08±1.89	0.54±0.04	1.87±0.68	6.9± 4.37
D-Serine	Competitive	8.16±1.05	0.46±0.02	4.87±0.60	—
	Non-competitive	8.41±1.15	0.47±0.02	5.38±1.13	142.7±237.9

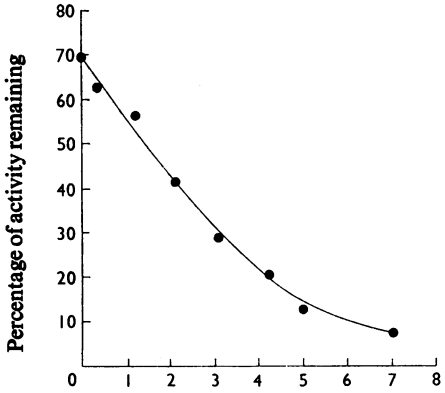

Fig. 7. *Irreversible inhibition of* L-*serine dehydratase by* 150 *m*M-L-*cysteine*

L-Serine dehydratase (25 units) was incubated at 15°C in a mixture (volume 1ml) containing: 150mM-L-cysteine (freshly neutralized to pH7); 10mM-Tris/HCl buffer, pH8.0; 10mM-$MgCl_2$. At the times shown, 20μl samples were transferred to assay mixtures (method 1) and the linear rate was determined. In a parallel experiment, L-serine dehydratase was incubated in a mixture lacking L-cysteine and the activity of 20μl samples determined by method 1 at the same times. The activity of the enzyme incubated with L-cysteine is recorded on the graph as a percentage of the activity of the enzyme incubated in its absence. The inhibition at zero time is a consequence of carrying over L-cysteine into the assay mixture to give a final concentration of 3mM.

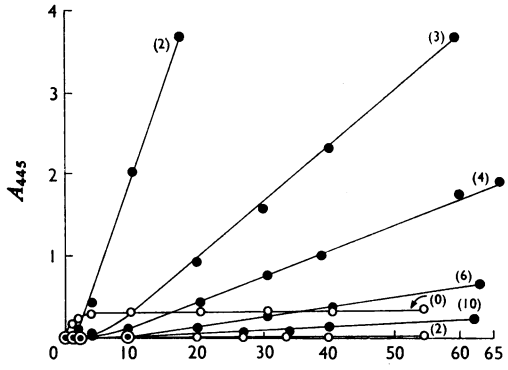

Fig. 8. *Effect of* L-*cysteine on the inhibition of* L-*serine dehydratase by* $HgCl_2$

Pairs of mixtures (vol. 8ml) each containing 50mM-Tris/HCl buffer, pH8.2, 10mM-$MgCl_2$, 5μM-$HgCl_2$ and L-serine dehydratase (1.5 units; step 3) were incubated at 30°C. At specified times between 0 and 10min, 1.0ml of 10mM-L-cysteine was added to one tube of a pair and 1.0ml of water to the other. After a further 30s, 1.0ml of 500mM-L-serine was added to both tubes. The time-course of pyruvate formation was followed by transferring, at timed intervals, a 1.0ml sample from each mixture into 0.33ml of 0.1% dinitrophenylhydrazine in 2M-HCl and determining pyruvate as described in assay method 3. On the graph, the value against each curve refers to the time (in min) of preincubation with $HgCl_2$ before the addition of L-cysteine or water. The times of incubation after the addition of L-serine are shown on the abscissa. ○, Assayed in the absence of L-cysteine; ●, assayed after the addition of L-cysteine.

by L-cystine, which might be formed from L-cysteine on standing.

No similar irreversible inhibition was found when enzyme was incubated with 500mM-D-serine for periods up to 12h.

Mercury compounds. When $HgCl_2$ was added to the assay mixture, enzyme activity was inhibited, but the onset of inhibition was progressive rather than instantaneous. For example, with 5μM-$HgCl_2$, inhibition did not become complete until 4min after mixing the enzyme and $HgCl_2$. If the concentration of L-serine in the mixture was raised above the usual 50mM, the onset of complete inhibition was delayed still further, until 20min after mixing in the case of 350mM-L-serine.

In some circumstances, the inhibition caused by $HgCl_2$ could be reversed by L-cysteine. In these experiments, an L-cysteine concentration of 1mM was used, since this was insufficient to cause detectable irreversible inhibition and caused only a small degree (about 10%) of reversible inhibition in the presence of

50mM-L-serine. Two samples of L-serine dehydratase were incubated with 5 μM-$HgCl_2$, and, after a suitable period, L-cysteine was added to one mixture to give a final concentration of 1mM and a similar volume of water was added to the other. After a further 30s, L-serine (final concn. 50mM) was added to both mixtures and the time-course of pyruvate formation determined. The results of such experiments for periods of incubation with $HgCl_2$ up to 10min are shown in Fig. 8. When the activity was measured without adding L-cysteine, inactivation by 5 μM-$HgCl_2$ was complete within 2min. The addition of 1mM-L-cysteine, however, partially overcame the inhibition. As the period of incubation with $HgCl_2$ before the addition of cysteine was lengthened, an increasing proportion of the inhibition was not reversed by L-cysteine and, after 10min treatment with $HgCl_2$, L-cysteine had no effect.

The possibility that the biphasic nature of the inhibition by $HgCl_2$ might be the result of it acting as a bifunctional reagent, the inhibition being reversible after it had acted at one site but not after it had acted at two, was discounted by the finding that exactly similar results were obtained with the monofunctional reagent, *p*-hydroxymercuribenzoate.

Discussion

In a number of respects the L-serine dehydratase of *A. globiformis* shows characteristics that are also found in enzymes of this type derived from other sources. For example, the high degree of specificity for L-serine, the K_m value in the millimolar range, the broad pH optimum somewhat on the alkaline side of neutrality and the susceptibility to inhibition by L-cysteine, D-serine and mercury compounds have all been reported in other serine dehydratases.

It has frequently been assumed that serine dehydratases contain a pyridoxal phosphate prosthetic group. Although this is well proved for many enzymes of this type, e.g. D-serine dehydratase from *E. coli* (Dowhan & Snell, 1970*a*,*b*) and both the 'biosynthetic' and 'degradative' L-threonine/L-serine dehydratases (Umbarger, 1973), only one specific L-serine dehydratase, that from *Cl. acidi-urici*, has been shown by spectroscopic methods to contain pyridoxal phosphate (Carter & Sagers, 1972). With other L-serine-specific enzymes the situation is less clear. This is certainly true of the *A. globiformis* enzyme. No evidence was obtained of stimulation of activity by pyridoxal phosphate, and, although the enzyme is inactivated by procedures that have been used to dissociate pyridoxal phosphate enzymes, pyridoxal phosphate did not restore activity. The results of inhibitor studies and of spectroscopic investigations of the purified enzyme have been inconclusive (E. S. Bridgeland, F. Gannon & K. M. Jones, unpublished work). In summary, the question whether L-serine dehydratase from *A. globiformis* contains pyridoxal phosphate remains, for the moment, undecided.

In some of its other properties, the *Arthrobacter* enzyme is more remarkable. The first of these is the non-linear time-course of the reaction, which has been shown to result from a slow activation of the enzyme by its substrate, L-serine. When the studies from which the present work has developed were reported (Bridgeland & Jones, 1965), this phenomenon was considered most unusual. Since then, Frieden (1970) has drawn attention to a number of related examples and has coined the term 'hysteretic enzyme' to describe enzymes that respond slowly to rapid changes in the concentration of a ligand. He also discussed the possible importance of hysteretic enzymes in the regulation of metabolic pathways. For reasons explained below, however, there is no justification for attaching any regulatory significance to the hysteretic response of L-serine dehydratase from *A. globiformis*.

The second unusual feature is the non-specific requirement for a cation for activity. Although other serine dehydratases are stimulated to various degrees by cations and the enzyme from *Cl. acidi-urici* shows a specific requirement for Fe^{2+} (Carter & Sagers, 1972), the *Arthrobacter* enzyme is unusual in being virtually inactive in the absence of an added cation and in being non-specific in its requirement.

The third feature worthy of comment is the difference in properties between the enzyme *in situ* in toluene-treated cells and *in vitro* in cell-free preparations, both crude extracts and highly purified enzyme. In toluene-treated cells, the enzyme (i) shows a linear time-course of reaction, (ii) gives a hyperbolic saturation curve for L-serine, and (iii) shows no cation requirement. In contrast, in extracts and purified enzyme preparations, it (i) shows a non-linear time-course, the rate of pyruvate formation increasing for several minutes before becoming constant, (ii) gives a sigmoid relationship between serine concentration and linear rate, and (iii) requires cations for activity. The properties shown *in vitro* are all properties that have been suggested to contribute to the regulation of metabolic pathways in living cells. These properties could readily be fitted into a plausible scheme of control of glycine and serine metabolism in *A. globiformis*. And yet the absence of these properties when the enzyme is studied *in situ* makes it clear that they are accidental and do not play a regulatory role. When these features were first reported (Bridgeland & Jones, 1965), they constituted the first recorded instance where an enzyme was shown to display different properties in extracts and in toluene-treated cells. Further examples of this kind have since been reported in other organisms (Weitzman, 1973; Reeves & Sols, 1973), and greater caution is now generally exercised in extrapolating from enzyme properties observed *in vitro* to the situation *in vivo*.

The work reported here serves to emphasize again the need for such caution.

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