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Physical Properties and Subunit Structure of L-Asparaginase Isolated from *Erwinia carotovora*

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1. L-Asparaginases from *Erwinia carotovora* and *Escherichia coli* (EC2 enzyme) are both capable of inhibiting and eliminating certain types of tumour cells. The *Er. carotovora* enzyme is a more basic protein, however, and in contrast with the EC2 enzyme it contains neither tryptophan nor cystine, and disulphide bonds are therefore absent. The molecule is very stable in solution from pH3.0 to about pH12.0, and is somewhat more stable at alkaline pH than is the *Esch. coli* enzyme. Calculations based on a $s^0_{20,w}$ 7.43S and a sedimentation-equilibrium molecular weight of 135000±10000 give a frictional ratio (f/f_0) of 1.08. The molecular conformation is therefore very compact in solution, and the electron microscope shows the negatively stained molecules as almost spherical particles with a diameter of 7.2±0.7nm. 2. Sedimentation-velocity and equilibrium ultracentrifugation, in 5–8M solutions of urea and guanidinium chloride, and also electrophoresis in sodium dodecyl sulphate–polyacrylamide gel, reveal a dissociation of the native protein molecule into four subunits of similar molecular weight in the range 32500–38000. The enzymically inactive subunits can be physically reassembled into an active tetramer when urea is removed by dialysis. Although the subunit structures of the *Er. carotovora* enzyme and the *Esch. coli* enzyme molecules are similar, the secondary bonding forces holding the subunits together in the tetramer are somewhat stronger in the *Er. carotovora* enzyme. 3. The optical-rotatory-dispersion (o.r.d.) parameters that characterize the Cotton effects arising from ordered structure in the molecule are $[m']_{233} = -3522 \pm 74°$ and $[m']_{200} = 9096 \pm 1700°$. These show very marked changes as the secondary structure is disrupted and the molecule dissociates into subunits. A correlation pathway was traced on the basis of o.r.d. parameters and enzyme activity as the polypeptide chains were denatured and renatured (and reconstituted) into active molecules after the dilution of solutions in urea. Subunits resulting from treatment with sodium dodecyl sulphate do not show the typically disordered o.r.d. profile, but nevertheless they are inactive.

L-Asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) catalyses the deamidation of L-asparagine to produce L-aspartic acid and ammonia. Although Clementi (1922) had reported its presence in guinea-pig serum, the anti-tumour properties of the enzyme were only recognized some time later when Broome (1961) discovered that the regression of lymphosarcoma transplants in mice treated with guinea-pig serum (Kidd, 1953) was due to the nutritional dependence of the malignant cells on exogenous L-asparagine. Cells resistant to the effect of the enzyme either contain an L-asparagine synthetase or are able to produce it when the deficiency arises. Yellin & Wriston (1966*a*,*b*) purified the protein in sufficient amount to characterize it in the ultracentrifuge and reported a molecular weight of 138000 and $s^0_{20,w}$ 6.55S.

Bacteria contain L-asparaginase with anti-tumour activity (Mashburn & Wriston, 1963; Campbell *et al.*, 1967; Rowley & Wriston, 1963), and until recently *Escherichia coli* has been the only source of enzyme used for cancer therapy. Of special note are the reports of remissions in cases of acute lymphoblastic leukaemia (Adamson & Fabro, 1968; Old *et al.*, 1968; Grundmann & Oettgen, 1970).

This has stimulated the large-scale production of highly purified bacterial L-asparaginase and a major part of the research to date is based on several commercially available preparations isolated from mutants of *Esch. coli*. Crystalline material has been produced by the Biochemisches Laboratorium der Farbenfabriken Bayer A.-G. (Wuppertal-Elberfeld, West Germany) from *Esch. coli* A.T.C.C. 9637 and A.T.C.C. 11303 (Arens *et al.*, 1970) and the Lilly Research Laboratories of Eli Lilly and Co. (Indianapolis, Ind., U.S.A.) from *Esch. coli* A.T.C.C. 13706 (Ho *et al.*, 1970; Frank *et al.*, 1970). Amorphous L-asparaginase produced by the Squibb Institute for Medical Research (New Brunswick, N.J., U.S.A.) has been studied by Whelan & Wriston (1969) and Kirschbaum *et al.* (1969). The physical, chemical and physiological properties of these enzyme

preparations have been reviewed by Grundmann & Oettgen (1970), Cooney & Handschumacher (1970) and Wriston (1971).

The only other bacterium that has been used for the large-scale production and purification of L-asparaginase is *Erwinia carotovora* (Wade *et al.*, 1968; Wade & Rutter, 1970). Clinical trials carried out in the U.K. have shown that the Bayer, Squibb and the M.R.E. *Er. carotovora* bacterial asparaginases are roughly comparable in their clinical efficacy with minor differences in toxicity and various side effects (Beard *et al.*, 1970; Wade & Rutter, 1970; Crowther, 1971). As the L-asparaginases from *Esch. coli* and *Er. carotovora* possess different immunological specificities (MacLennan *et al.*, 1971) they offer an important alternative therapy if a patient happens to develop hypersensitivity to one or the other of the enzymes (Crowther, 1971).

The *Er. carotovora* enzyme can be crystallized (North *et al.*, 1969), and its molecular weight is about 135000, being composed of four subunits each with a molecular weight of about 35000 (Miller *et al.*, 1971*a*,*b*). We now present the full evidence for the subunit structure and how the subunits may be reassembled to produce an active tetramer. O.r.d. (optical-rotatory-dispersion) studies are described that help in understanding the conformational changes that take place before and after dissociation into subunits. Comparisons are drawn between the *Er. carotovora* and *Esch. coli* enzymes, which differ in amino acid composition, isoelectric point and immunological specificity.

Materials and Methods

Batch production of L-asparaginase from Er. carotovora

Buck *et al.* (1971) outlined a procedure for the growth of a mutant of *Er. carotovora* N.C.P.P.B. 1066 at 37°C in 400-litre stirred deep-culture vessels in a medium containing 4% light-grade Yeatex (English Grains Co. Ltd., Burton on Trent, Staffs., U.K.) and 1.7% sodium glutamate. The crude enzyme was extracted at pH12.5 and then adsorbed on CM-cellulose. After elution the enzyme was precipitated with 60%-saturated $(NH_4)_2SO_4$ and adsorbed again on DEAE-cellulose. Crystals from 40–50% ethanol had a specific activity of 650–700 units/mg of protein.

Solutions of amorphous protein (7–14mg/ml) were sterilized by filtration (Millipore GS filter, pore size 0.22μm) and then adjusted to pH7.4 with phosphoric acid before being sealed in ampoules and stored at −20°C.

Determination of protein and enzyme activity

Technicon AutoAnalyzer equipment was used for the parallel determination of protein and asparaginase activity. A full description of the technique is given by Wade & Phillips (1971). One unit of L-asparaginase activity is defined as that which catalyses the liberation of 1.0μmol of NH_3/min at 37°C under the standard conditions of the assay.

Determination of protein by absorption in the ultraviolet

Measurements of the extinction at 280nm in a Unicam SP.500 or Beckman DB spectrophotometer were used to determine protein concentrations for physical studies. A slight variation in the extinction coefficient was found with different batches of L-asparaginase at pH7.4. The value adopted was $E^{1.0\%}_{1.0cm}$ 6.1±0.3 at 280nm. A sample of Bayer *Esch. coli* L-asparaginase contained approx. 30% of diffusible material (presumably a preservative), which was removed by thorough dialysis. The extinction coefficient of this material was higher, 7.7, and the E_{280}/E_{260} ratio was 2.1 compared with 2.76 for the *Er. carotovora* enzyme.

Determination of protein by the refractive increment

This method of determining protein concentration gave the most consistent results, although its accuracy depends on a thorough dialysis beforehand. The refractive increment between the dialysed solution and diffusate was measured at 546nm in the Brice–Phoenix differential refractometer. A specific refractive increment of 0.188ml·g^{-1} was used to convert $(\Delta n)_{dr}$ into protein concentration.

Sedimentation velocity

Sedimentation analysis and the measurement of sedimentation coefficients has been described in previous work (Cammack & Wade, 1965; Cammack *et al.*, 1970). Rayleigh interference fringe photographs were recorded on OAG plates (Kodak Ltd.). Estimates of $(\Delta n)_{uc}$ were made from the area of schlieren peaks but more accurately from interference patterns. All the experiments were carried out at 25°C.

Determinations of molecular weights by electrophoresis in sodium dodecyl sulphate–polyacrylamide gels

Standard procedures published by Shapiro *et al.* (1967) and Weber & Osborn (1969) were followed. The gels contained 0.1% (w/v) sodium dodecyl sulphate and 5% acrylamide (Kodak Ltd., Kirkby, Liverpool, U.K.). The extent to which the native molecule could be dissociated in 0.1% sodium dodecyl sulphate was found to depend on several factors. To guarantee a complete dissociation into subunits it was essential to incubate at 37°C for 24h. The

source of the enzyme and freeze-drying were found to affect the results.

Moving-boundary electrophoresis

A Spinco model H apparatus was used as described by Tozer *et al.* (1962) and Cammack & Wade (1965). The buffer solutions covering the pH range 6.0–9.5 were prepared by the method of Miller & Golder (1950) but modified to include 0.1 *I* of the buffer salt and 0.1 *I*-NaCl, making a total ionic strength of 0.2 *I*.

Sedimentation equilibrium

Molecular weights were determined by the 'short-column' sedimentation-equilibrium method with Rayleigh interference optics (Van Holde & Baldwin, 1958; Richards & Schachman, 1959; Yphantis, 1964; Chervenka, 1968). A critical review of the equations used in the calculation of molecular weights has been produced by Creeth & Pain (1967). The runs were performed in a 'double-sector' cell fitted with sapphire windows to minimize the distortion and loss of fringes at high rotor speeds. A micro-syringe was used to fill the cell with 0.1 ml of dialysed protein solution (2.5 mm column) and 0.12 ml of diffusate, which were supported on 0.02 and 0.01 ml respectively of FC43 immiscible fluid (Beckman Instruments Inc., Palo Alto, Calif., U.S.A.). Equilibrium for 'low-speed' conditions with solutions of 1–4 mg of protein/ml (i.e. 4–16 fringes) was established within 24 h at 6800 rev./min in the J rotor. The 'high-speed' meniscus-depletion method of Yphantis (1964) required solutions of 0.1–1 mg of protein/ml centrifuged at 17000 rev./min for 24 h.

The equations for the calculation of molecular weights are:

$$M = \frac{2RT}{(1-\bar{v}\rho)\omega^2} \cdot \frac{\Delta c}{c_0} \cdot \frac{1}{r_b^2 - r_m^2} \quad \text{('low-speed')}$$

$$M = \frac{2RT}{(1-\bar{v}\rho)\omega^2} \cdot \frac{\mathrm{d}(\ln c)}{\mathrm{d}(r^2)} \quad \text{('high-speed')}$$

in which R is the gas constant, T the absolute temperature (298°K), $\bar{v}$ the partial specific volume (0.739), ρ the solvent density, ω the angular velocity of the rotor, r the distances from the axis of rotation, Δc the difference in the concentration from the meniscus to the base of the column at equilibrium, and c_0 the initial concentration.

Measurements of Δc in the 'low-speed' runs are based on the differential number of fringes (ΔJ) that resolve between the meniscus and the base of the column, and c_0 on the total number of fringes (J_0) produced by a sedimentation-velocity boundary that is usually formed in the synthetic-boundary cell. The 'low-speed' method is particularly subject to error caused by molecular heterogeneity (Creeth & Pain, 1967), and as a routine it is essential to increase the rotor speed to confirm that J_0 does not decrease with time, correction being made for radial dilution.

Optical rotatory dispersion

The o.r.d. spectra were measured on a FICA Spectropol 1b spectropolarimeter with quartz cells (Hellma-Suprasil) of 0.2–10 mm path length. The specific optical rotation was calculated from the relationship:

$$[\alpha]_\lambda = \frac{\alpha_\lambda}{lc}$$

where α_λ is the observed rotation at wavelength λ, l the path length (in dm) and c the concentration (in g/ml).

The reduced residue rotation was calculated by assuming a mean residue weight of 113 and a mean refractive index, n, for the solutions of 1.3915, in the relationship below:

$$[m']_\lambda = \frac{\text{Mean residue wt.} \times [\alpha]_\lambda}{100} \cdot \frac{3}{(n^2+2)}$$

Stock solutions were prepared from frozen and freeze-dried samples of L-asparaginase in phosphate buffer, pH 7.4, containing 0.1 *I*-sodium phosphates and 0.1 *I*-NaCl. Solutions for measurement of optical rotation at various pH values and urea concentrations were made by dilution of 0.05–0.25 ml of stock solution to 4.5 ml with the appropriate solvent, producing a final protein concentration of 0.24–1.5 mg/ml. Unless stated otherwise the results refer to 25°C.

Amino acid analysis

Samples (1.6 mg) were hydrolysed in 6M-HCl in sealed tubes under N_2 at 105°C for 24, 48 and 72 h and analysed in a Technicon TSM1 AutoAnalyzer.

Tests were made on larger samples for tryptophan (5 mg) and cystine (10 mg). For tryptophan, hydrolysis was carried out in 5M-NaOH at 105°C for 18 h in polypropylene tubes sealed inside a glass tube. This sample was analysed in the Technicon Auto-Analyzer by using a modified programme to resolve tryptophan; another sample was tested directly with *p*-dimethylaminobenzaldehyde (Graham *et al.*, 1947.) Approx. 1.7 mg of the sample in 0.5 ml of the hydrolysate was treated with 2.5 ml of 0.5% *p*-dimethylaminobenzaldehyde in conc. HCl and then allowed to stand for 30 min in the dark. This was followed by addition of 2–3 drops of 0.2M-$NaNO_2$ and 2.5 ml of ethanol. No blue colour developed

after 30min whereas a 0.02μM-tryptophan solution produced a distinct blue colour.

For cystine the sample was oxidized with performic acid and then hydrolysed for examination in the Technicon AutoAnalyzer. A sample was also tested by the Saville diazotization technique (Saville, 1958; Todd & Gronow, 1969). A 10mg sample of asparaginase in 1ml of 6M-HCl was reduced with 30mg of Zn dust in a boiling-water bath, and then cooled in ice before addition of 0.25ml of nitrous acid (1vol. of 0.12%-$NaNO_2$ and 4vol. of 1.0M-H_2SO_4). After 5min at 0°C 0.1ml of a 1% solution of ammonium sulphamate was added, and after mixing well, 2ml of a solution consisting of 1vol. of a 1% solution of $HgCl_2$ in 0.4M-HCl and 2vol. of a 0.5% solution of sulphanilic acid and a 0.005% solution of naphthylethylenediamine hydrochloride, in 5% acetic acid, was also added. After 30min the presence of cysteine is indicated by a pink colour measured by extinction at 550nm. Asparaginase samples were negative. Samples of bovine plasma albumin (1mg) and bovine γ-globulin (2.5mg) (Armour Laboratories, Hampden Park, Eastbourne, Sussex) treated in the manner described both produced deep-pink colours, also a sample that contained 0.05μM-cysteine gave a very definite pink colour.

Electron microscopy

Enzyme solutions containing 1mg and 0.1mg of protein/ml in sodium phosphate buffer, pH7.4, were mixed with an equal volume of negative stain, either 1% (w/v) phosphotungstic acid, pH7.2, or 2% (w/v) ammonium molybdate, pH5.5. One drop of the resulting mixture was placed on a very thin [about 1.5nm (15Å)] carbon film, which was itself supported by a thicker carbon film containing holes and mounted on a copper specimen grid. The specimens were examined in a Philips EM300 electron microscope, with an accelerating voltage of 60kV and a 30μm objective aperture.

Results

Physical homogeneity in the ultracentrifuge

Solutions of the purified enzyme of specific activity 500–750 units/mg of protein were examined at regular intervals in the ultracentrifuge for physical homogeneity. After dialysis in 0.1*I*-sodium phosphate buffer, pH7.4, containing 0.1*I*-NaCl, the solutions were equilibrated for 1h at room temperature before being used to fill the cell. Fig. 1 is typical of the many sedimentation diagrams recorded for L-asparaginase. The optical recoveries expressed as $(\Delta n)_{uc}/(\Delta n)_{dr}$ were invariably high, confirming that the 7.4S peak represented 95–98% of the non-diffusible material under test. Traces of 3S and 9S components were sometimes detected. Batch AF1 was a special preparation in which the alkali extraction method was not used. This had a specific activity of 400 units/mg of protein and produced a normal sedimentation diagram with s value 7.32S.

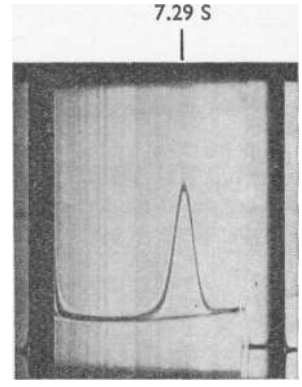


Fig. 1. *Physical homogeneity of Er. carotovora* L-*asparaginase stored at* −20°*C*

Batch CB4 (initially 750 units/mg of protein) examined at 12mg/ml after being stored as a frozen solution in buffer for 9 months (670 units/mg of protein). Sedimentation diagram of a solution in buffer, pH7.4, containing 0.1*I*-sodium phosphate and 0.1*I*-NaCl, exposure after 65min at 44000rev./min, with the phase-plate set at 60°.

Routine storage of frozen solutions or freeze-dried material at −20°C did not result in any obvious sign of physical deterioration, although the specific activity was diminished by about 10% after 18 months. Dialysed solutions were found to be stable for at least 30 days at 2–4°C, for up to 5 days at room temperatures of 18–20°C and for 2 days at 37°C.

Dependence of sedimentation on protein concentration

The dependence of the sedimentation coefficient on protein concentration was almost negligible from 0.5 to 12mg/ml at pH7.4 in 0.2*I* buffer (Fig. 2). In this solvent the values for the expression $s = s^0_{20,w}(1-kc)$ are $s = 7.43(1-0.021c)$ and the standard error of the $s^0_{20,w}$ is ±0.19S. Absence of any spontaneous dissociation into subunits was confirmed down to 0.25mg/ml, and there was no formation of polymeric species at concentrations as high as 15mg/ml. Superficially, at least, we could find no evidence of rapidly reversible monomer–polymer equilibria that might have been disturbed by sedimentation. Usually this type of system is recognized by an abnormally diffuse velocity boundary or poorly resolved bimodal peaks, as outlined originally by Gilbert & Jenkins (1956). Good examples of these

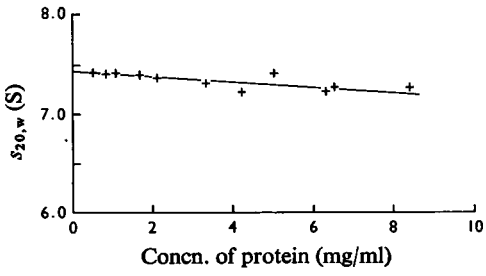


Fig. 2. *Sedimentation coefficient ($s_{20,w}$) of Er. carotovora* L-*asparaginase as a function of protein concentration in buffer, pH*7.4, *containing* 0.1 *I-sodium phosphate and* 0.1 *I-NaCl.*

The experimental details are given in the text. The $s^0_{20,w}$ value was 7.43±0.19S.

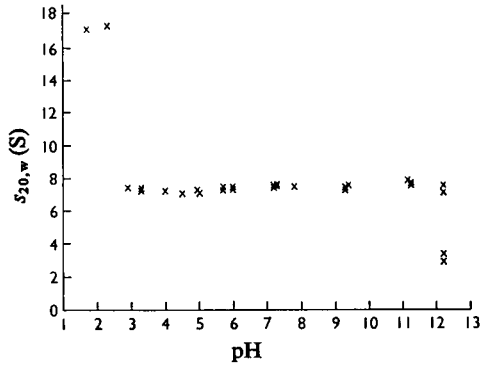


Fig. 3. *Sedimentation coefficients ($s_{20,w}$) of Er. carotovora* L-*asparaginase as a function of pH for solutions containing* 2.5 *mg of protein/ml in* 0.2 *I buffers prepared according to data in Miller & Golder* (1950)

The solutions were analysed after 1 and 7–10 days at 4°C. Refer also to Fig. 5 for information on instability at pH12.2.

anomalous sedimentation diagrams have been described for Botulinus toxin by Cammack (1960).

Concentration-dependence was investigated also in 0.1 *I*-sodium acetate–acetic acid buffer containing 0.1 *I*-NaCl at pH4.9 [$s = 7.09(1-0.026c)$] and in 0.1 *I*-glycine–NaOH buffer containing 0.1 *I*-NaCl at pH9.4 [$s = 7.54(1-0.054c)$] with results similar to those obtained in pH7.4 buffer.

pH stability

Solutions containing about 2.5mg of protein/ml were dialysed for 24h at 2–4°C against 0.2 *I* buffer solutions in the pH range 2.3–12.2. Both the sedimentation coefficients and enzyme activities remained constant within experimental error from pH3.0 to 11.3 after 7–10 days' storage at 4°C (Fig. 3). There was obvious instability below pH3.0 and in the alkaline pH region above pH11.9.

A more detailed study of physical instability at pH11.5, 11.7 and 11.9 is shown in Fig. 4, which confirmed that a detectable falling off in enzyme activity occurred at pH11.9 after 48h at 20°C. A 40% loss in activity was combined with approx. a 10% breakdown of the 7.4S molecule into 3.7S material. Similar physical changes were observed in the ultracentrifuge when the solution was adjusted initially to pH12.2 (Figs. 5*a*, 5*b* and 5*c*), but they were more rapid and within 24h the loss in activity was approx. 80%.

Er. carotovora L-asparaginase is evidently more stable than the *Esch. coli* enzyme in the alkaline pH region. According to Frank *et al.* (1970) the Lilly enzyme preparation is completely dissociated into 1.8S subunits within 30min of adjusting the pH to 11.8. A direct comparison in Figs. 6(*a*) and 6(*b*) of the Bayer *Esch. coli* enzyme and the *Er. carotovora* enzyme dialysed for 24h at 4°C in 0.1 *I*-glycine–NaOH buffer, pH11.9, with 0.1 *I*-NaCl followed by 1h at 20°C confirms there is a significant difference in their stability.

Solutions prepared from freeze-dried material

No difference was observed in the ultracentrifuge between solutions examined shortly after thawing and those dialysed for at least 48h. Kirschbaum *et al.* (1969) have reported varying proportions of 4S (mol.wt. 64500), 5.6S (mol.wt. 125000) and 8.6S (mol.wt. 250000) components during the first 3h of preparing a solution from a dried enzyme preparation. Changes in the molecular dispersion of the enzyme after its reconstitution in buffered saline could be important clinically.

A sample of *Er. carotovora* L-asparaginase was freeze-dried from a solution containing 0.01M-salt and then redissolved in 0.2 *I*-phosphate buffer, pH7.4. Only a trace of insoluble residue was visible, which represented less than 1% of the total solid resuspended. The earliest photograph that could possibly be taken in the first stage of the ultracentrifuge run was recorded 10min after reaching a speed of 59780rev./min and 30min after redissolving the protein. At this time, and for runs performed 24 and 48h later, the 7.4S peak remained the dominant feature of the sedimentation diagram, and this applied also to other solvent systems of ionic strength ranging from 0.02 *I* to 0.5 *I*. A very similar result was obtained with a sample of the Bayer *Esch. coli* L-asparaginase in 0.2 *I*-sodium phosphate buffer, pH7.4.

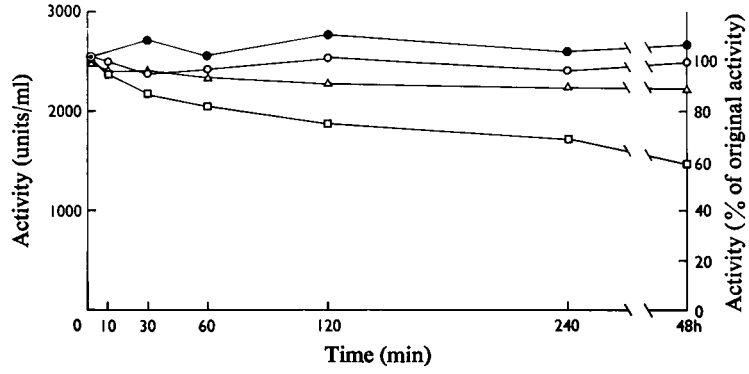


Fig. 4. *Stability of Er. carotovora* L-*asparaginase activity at* 20°C *for solutions in various buffers*

The enzyme (4.7mg/ml) was dissolved in the following buffers: ●, pH7.4, 0.05*I*-sodium phosphate+0.1*I*-NaCl; ○, pH11.5, △, pH11.7, and □, pH11.9, all 0.05*I*-glycine–NaOH+0.1*I*-NaCl.

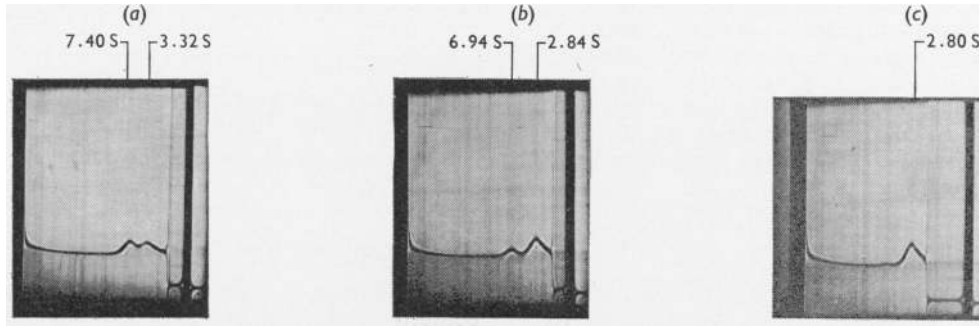


Fig. 5. *Breakdown of Er. carotovora* L-*asparaginase at alkaline pH*

The solution contained 2.5mg of protein/ml with an initial activity of 1500 units/ml, and was stored at 4°C in 0.1*I*-NaCl+0.1*I*-glycine–NaOH buffer initially at pH12.2±0.1, but this decreased by at least 1pH unit during storage. Sedimentation diagrams were recorded after centrifugation for 26–31min at 59780rev./min with the phase-plate set at 60°. (*a*) Stored for 24h, activity 275 units/ml; (*b*) stored for 48h, activity 290 units/ml; (*c*) stored for 10 days, activity 23 units/ml.

Amino acid composition

The amino acid compositions of the *Er. carotovora* enzyme and the Bayer, Squibb and Lilly *Esch. coli* enzymes are compared in Table 1. There is little distinction on this basis among the *Esch. coli* enzyme preparations, but the *Er. carotovora* enzyme is clearly more basic. It contains less aspartic acid (or asparagine) and rather more arginine, and there are also differences in the isoleucine, leucine and histidine contents. Most notable is the absence of cystine, which excludes the possibility of inter- or intramolecular disulphide bonds in the molecule, whereas *Esch. coli* asparaginase is reported to have a single intramolecular disulphide bond per subunit (Frank *et al.*, 1970; Glossmann & Bode, 1971). Tryptophan is absent, but the tyrosine contents of the *Er. carotovora* and *Esch. coli* enzymes are similar.

Isoelectric point

Electrophoretic mobilities were measured in 0.2*I* buffer systems as a function of pH. In Fig. 7 the isoelectric pH may be interpolated at zero mobility as p*I* $7.6_5 \pm 0.1$. The mobility of the Bayer *Esch. coli* enzyme at pH5.0 in 0.1*I*-sodium acetate–acetic acid buffer with 0.1*I*-NaCl was $-0.35 \times 10^{-5}\,cm^2 \cdot s^{-1} \cdot V^{-1}$. By extrapolation to zero mobility the p*I* of this more acidic enzyme is estimated to be 4.6–4.8. Isoelectric

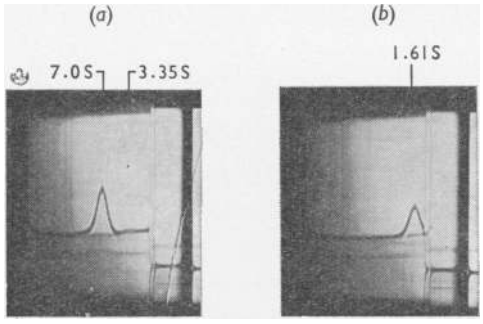


Fig. 6. *Comparison of the stability of Er. carotovora and Esch. coli* L-*asparaginases at pH* 11.9

The solutions were prepared initially in 0.1 *I*-sodium phosphate buffer, pH 7.4, +0.1 *I*-NaCl and then dialysed together in the same flask, which contained 0.1 *I*-glycine–NaOH buffer, pH 11.9, +0.1 *I*-NaCl at 4°C for 24h. Solutions were aged for 1h at 25°C before examination in the untracentrifuge at 59780rev./min. Exposures were taken after 34min with the phase-plate set at 60°. (*a*) *Er. carotovora* L-asparaginase batch CB7, concentration 5.8mg/ml, specific activity 600units/mg; (*b*) *Esch. coli* L-asparaginase (Bayer), concentration 2.9mg/ml, specific activity 15 units/mg.

focusing methods in Ampholine materials gave evidence of isoenzymes in the pH range 4.8–5.2 (Arens *et al.*, 1970). Applying the same method to the *Er. carotovora* enzyme, H. K. Robinson & H. E. Wade (unpublished work) resolved only two isoelectric bands, one being very faint; the isoelectric point estimated for the major band was p*I* 8.6±0.1.

Molecular weight

Preliminary estimates of the molecular weight by the sedimentation-equilibrium method were 128000–145000 and by X-ray crystallography were 116000–140000 (North *et al.*, 1969).

In Table 2 there is a summary of molecular weights obtained for two different enzyme preparations, and included are the experimental conditions that were used to achieve sedimentation equilibrium. The molecular weights obtained for a crystalline sample of bovine plasma albumin agree satisfactorily with published values of 66500–68500.

Consistently lower values for the molecular weight were produced by the 'low-speed' method. Because of the sensitivity of this method to aggregated material that may be excluded in the determination of c_0 (J_0) (see, e.g., Creeth & Pain, 1967), we are more inclined to give credence to the results obtained by the 'high-speed' method, which were 141000–144000. The results as a whole cover the range 125000–144000.

Subunits of L-*asparaginase*

In contrast with *Esch. coli* L-asparaginase the *Er. carotovora* enzyme failed to undergo a complete dissociation into 1.4S subunits after 48h dialysis at 4°C against 0.2 *I*-phosphate buffer, pH 7.4, containing 8.0M-urea. Under these conditions only a 50% dissociation was achieved. To increase the amount of dissociation in 6.5–8.0M-urea the pH has to be adjusted well away from the isoelectric region to pH 3.6 or 10.0. At pH 10.0, as the urea concentration was increased the degree of dissociation observed in the ultracentrifuge (Figs. 8*a*, 8*b*, 8*c*, 8*d* and 8*e*) was related to a proportionate decrease in activity (Table 3), which eventually became zero in 6.5M-urea coincident with a 100% dissociation into subunits (Fig. 8*e*). Fortunately urea does not interfere with the chemistry of the assay procedure. This was confirmed by assays in 0.5 *I*-glycine–NaOH buffer, pH 10, containing 6.0M-urea. In this solvent the enzyme molecule remains undissociated and shows normal activity. The results in Table 3 are satisfactory evidence of the 1.4S subunit being inactive.

Dialysis against 0.2 *I*-sodium phosphate buffer, pH 7.4, containing 4.0M-guanidinium chloride produced a 100% dissociation into subunits and was clearly more effective than urea at the same molarity. This treatment is not exactly comparable with urea, since it combines hydrogen-bond- and hydrophobic-bond-breaking properties with a significant increase in the ionic strength of the solvent. The activity of the subunits in this experiment could not be determined, as the guanidinium chloride produced a heavy precipitate on application of the normal assay procedure.

Molecular weight of the subunit

The reliability of molecular weights determined by the sedimentation-equilibrium method in non-ideal solvent systems such as those above has been discussed by Casassa & Eisenberg (1964) and Creeth & Pain (1967). A thorough dialysis to equilibrium is essential, and whenever it is practical the measurements should be made close to the isoelectric point of the protein.

Table 4 lists molecular weights of 34700–38900 for the subunit. They depend on the assumption that:

$$(1-\bar{v}\rho) = (1-\phi\rho) = \left(\frac{\partial\rho}{\partial c}\right)^{0}_{\mu}$$

Table 1. *Amino acid composition of* L-*asparaginases*

Amino acid	Amino acid composition of *Er. carotovora* enzyme (μmol/mg of protein)	Amino acid composition of *Er. carotovora* enzyme (g of amino acid residue/100g of protein)	Amino acid composition of *Esch. coli* enzyme (g of amino acid residue/100g of protein) Bayer prep. (Arens *et al.*, 1970)	Squibb prep. (Whelan & Wriston 1969)*	Lilly prep. (Ho *et al.*, 1970)*
Aspartic acid	1.04†	11.9_5	16.9_4	15.5_6	17.6_3
Threonine	0.78†	7.9_0	9.6_0	9.1_9	10.0_1
Serine	0.52†	4.5_7	4.1_4	3.9_6	3.9_2
Glutamic acid	0.62	8.0_1	7.9_7	8.1_6	7.3_7
Proline	0.37	3.5_7	3.6_9	3.5_3	3.2_0
Glycine	0.99	5.6_9	4.6_1	4.6_7	4.9_7
Alanine	0.86	6.1_2	6.7_5	6.4_6	7.0_4
Valine	0.88‡	8.7_4	9.4_1	9.0_1	10.4_0
Methionine	0.19	2.5_1	1.8_7	2.3_9	1.5_8
Isoleucine	0.50	5.6_5	4.3_0	4.1_1	4.0_5
Leucine	0.84	5.5_8	7.5_2	7.2_0	7.4_7
Tyrosine	0.37†	6.0_9	5.4_2	5.9_3	5.3_9
Phenylalanine	0.20†	2.9_7	3.4_9	4.0_1	3.5_3
Histidine	0.16	2.1_5	1.3_0	1.2_4	1.2_4
Lysine	0.54	6.9_8	7.9_1	8.1_6	8.0_9
Arginine	0.48	7.4_7	3.7_1	4.2_6	3.2_9
Cystine/2	0.00§	0.0	0.4_8	0.4_6	0.6_2
Tryptophan	0.00§	0.0	0.8_8	1.6_9	0.5_6
Ammonia	0.74‖				

* Calculated from published results.
† Extrapolated to 0h hydrolysis from 24, 48 and 72h results.
‡ Increased with hydrolysis time, 72h results used.
§ Analyses for cystine and tryptophan, each by two distinct methods, detected significantly less than 1 residue/molecule.
‖ Not included in second column.

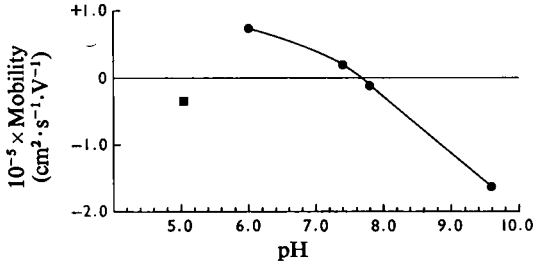


Fig. 7. *Electrophoretic mobility of Er. carotovora* L-*asparaginase as a function of pH in* 0.2*I buffers prepared according to the data in Miller & Golder* (1950)

●, *Er. carotovora* L-asparaginase (2mg/ml), the interpolated value for the isoelectric point of which is p*I* 7.65; ■, *Esch. coli* L-asparaginase (0.8mg/ml), a sample of the Bayer material for which the extrapolated value of the isoelectric point is about p*I* 4.8. The electrophoresis was carried out in a Spinco model H moving-boundary apparatus.

where $(\partial\rho(\partial c)^0_\mu$ is the density increment of the macromolecule at constant chemical potential μ of diffusible solutes, and ϕ is the apparent specific volume, as defined originally by Casassa & Eisenberg (1964). In effect the sedimentation-equilibrium plot gives a slope equal to the product $M\ (\partial\rho/\partial c)$ and the value of $(\partial\rho/\partial c)$ should be determined independently as described by Reisler & Eisenberg (1969).

However, for dilute protein solutions in 0.1–0.2*I* buffers the error in molecular weight as a result of using $(1-\bar{v}\rho)$ in place of $(\partial\rho/\partial c)$ is apparently neglible (Creeth & Pain, 1967, p. 245). In high concentrations of urea or guanidinium chloride the error can be much greater. Quite arbitrarily we have applied a correction of −0.01 to $\bar{v}$ (Tanford *et al.*, 1967) to illustrate the comparatively large effect (−7%) on the value of the molecular weight. It appears that any forthcoming correction to these experimental values for the molecular weight of the subunit, on the basis of Casassa–Eisenberg (1964) theory, would still support a four-subunit structure for native asparaginase.

Table 2. *Molecular-weight determinations by sedimentation equilibrium*

The solvents used were: 1, 0.1 *I*-NaCl+0.1 *I*-sodium phosphates (6.8mM-NaH_2PO_4+31.1mM-Na_2HPO_4), pH 7.4; 2, 0.1 *I*-KCl+20mM-tris, pH7.3; 3, 0.1 *I*-sodium acetate+67mM-acetic acid, pH4.8.

Sample	Concentration (mg/ml)	Solvent	Speed (rev./min)	Time* (h)	Molecular weight 'Low speed'	Molecular weight 'High speed'
L-Asparaginase batch CB4	2.5	1	6800	17	125000	
	4.0	1	6800	17	133000	
L-Asparaginase batch CB4	0.1	1	17000	17		144000
	0.3	1	17000	17		143000
L-Asparaginase batch CB5	2.0	1	6800	17	132000	
	4.0	1	6800	17	130000	
L-Asparaginase batch CB5	0.1	1	17000	25		141000
Bovine plasma albumin†	2.1	2	10590	24	66100	
	4.4	2	10590	24	72940	
Bovine plasma albumin†	0.6	3	20410	24		73120

* Time at which no significant change had occurred during at least the preceding 3–4h.

† Contained not more than 5% dimer. Published results for the molecular weight are 66500–68500, and the presence of 5% dimer would increase these values by about 3400.

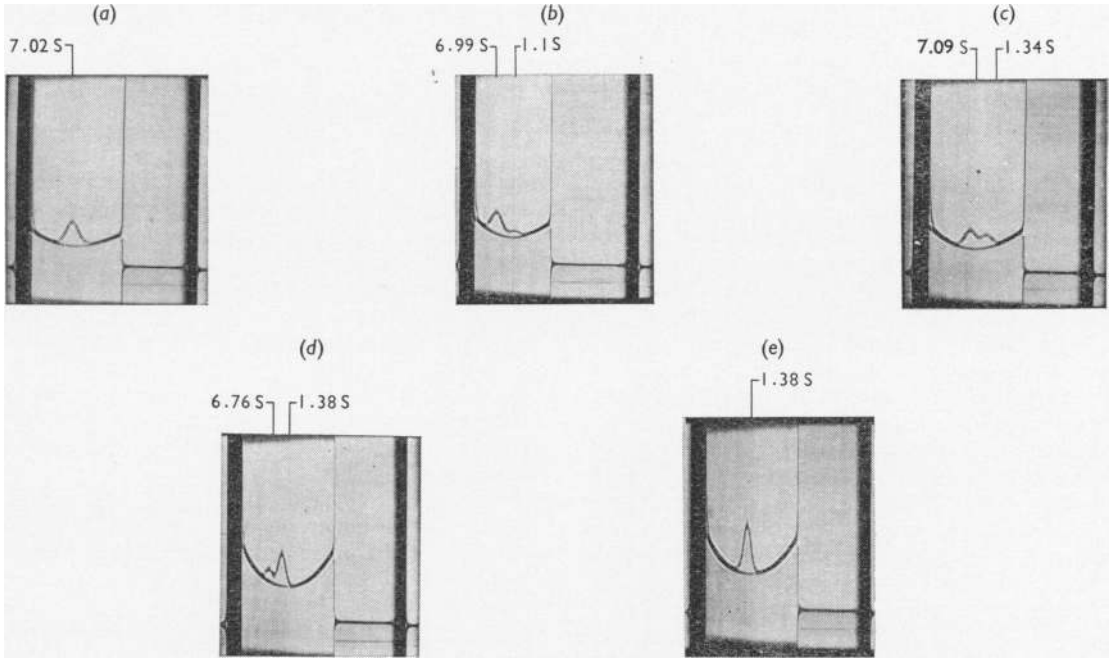


Fig. 8. *Dissociation of Er. carotovora* L-*asparaginase into subunits by dialysis*

Dialysis was carried out into solutions of urea in 0.1 *I*-glycine–NaOH buffer, pH10, +0.1 *I*-NaCl: (*a*) 4M-urea; (*b*) 5M-urea; (*c*) 5.5M-urea; (*d*) 6M-urea; (*e*) 6.5M-urea. Sedimentation diagrams were recorded in a capillary-type synthetic-boundary cell after centrifugation for: (*a*) 40min at 42040rev./min; (*b*) and (*c*) 44min at 47660rev./min; (*d*) and (*e*) 24 min at 59780rev./min., with the phase-plate set at 60°.

Molecular weights by the sodium dodecyl sulphate–polyacrylamide-gel electrophoresis method

A typical calibration plot is shown in Fig. 9, in which there is very nearly a linear relationship between the log (molecular weight) and the relative mobility of a series of standard proteins after electrophoresis in 0.1% sodium dodecyl sulphate–5% polyacrylamide gel. Usually the proteins are given a

Table 3. *Dissociation of* L-*asparaginase into subunits and loss of specific activity in the presence of urea in* 0.1 *I-NaCl*+0.1 *I-glycine–NaOH buffer, pH* 10.0±0.1

The percentage dissociation was determined from the ratio of 1.4S to 7.4S schlieren peak areas of the ultracentrifuge runs shown in Fig. 8.

Concn. of urea (M)	Approx. % dissociation into 1.4S subunits	Specific activity (units/mg of protein)
4.0	0	470
5.0	15	393
5.5	40	270
6.0	80	38
6.5	100	0.5

Table 4. L-*Asparaginase subunit molecular weights determined by sedimentation equilibrium*

Experimental details and discussion of these results are given in the text.

Partial specific volume ($\bar{v}$) (ml/g)	Dissociated subunit mol.wt.	
	8M-Urea	4M-Guanidinium chloride
0.739*	37200	38900
0.729†	34700	36800

* Calculated from the amino acid analysis (Cohn & Edsall, 1943).
† Correction applied (Tanford *et al.*, 1967).

preliminary treatment with 0.1% sodium dodecyl sulphate for 2h at room temperature.

Plate 1 shows the results obtained with freeze-dried *Er. carotovora* L-asparaginase, Lyovac *Esch. coli* L-asparaginase from Merck, Sharp and Dohme Research Laboratories (West Point, Pa., U.S.A.) and the Bayer, Squibb and Lilly *Esch. coli* enzyme preparations. It is noteworthy that the *Er. carotovora* enzyme was incompletely dissociated and gave two bands of molecular weights 125000 and 37500, whereas the *Esch. coli* enzyme samples were completely dissociated into subunits producing a single band of molecular weight 35000–40000. It was found with the *Er. carotovora* enzyme that frozen solutions stored at −20°C were even more resistant to dissociation in sodium dodecyl sulphate and only by applying more vigorous treatment, namely incubation at 37°C in 0.1% sodium dodecyl sulphate for at least 5h, was it possible to obtain results comparable with those for the *Esch. coli* enzyme.

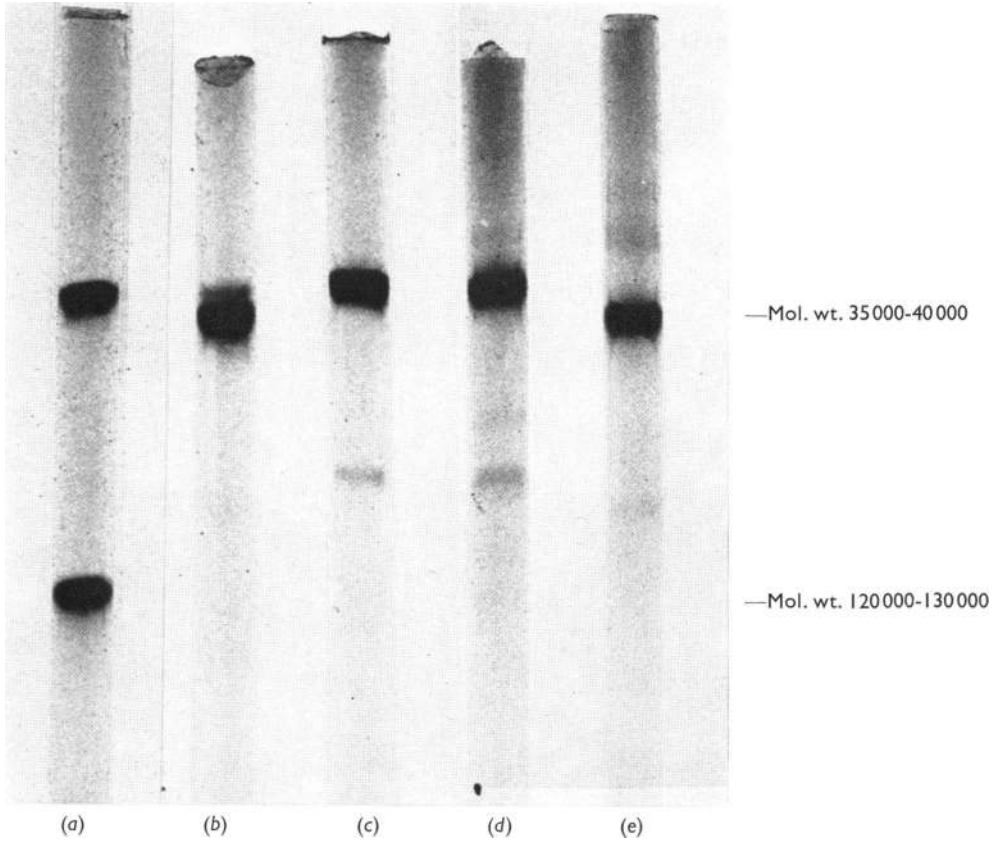
Reassembly of subunits into an active polymer

Removal of the urea by thoroughly dialysing the treated protein solutions against 0.2 *I*-sodium phosphate buffer, pH 7.4, at 4°C produced greater than 85% recovery of the enzyme activity if the initial protein concentration was not above 3mg/ml (Table 5). Poor recovery was obtained at much higher concentrations of 6–18mg/ml, and as the urea was being dialysed out of the sac the solution became opalescent and usually formed a precipitate at room temperature. Centrifugation of the suspension followed by an assay of the enzyme activity of the clear supernatant (Table 5) confirmed that the bulk of the activity was in the supernatant fraction and that the residue was mainly denatured protein. A physical reassembly of 7.4S molecules from subunits was demonstrated in the ultracentrifuge (Fig. 10), but traces of incompletely reassembled material, e.g. a 4.3S component in Fig. 10, were regularly seen in the sedimentation diagrams.

Urea was removed by dialysis against 0.1 *I*-glycine–NaOH buffer, pH 10.0, containing 0.1 *I*-NaCl in place of the phosphate buffer, pH 7.4, and this produced a 3.7S component that might be a refolded inactive subunit. Further dialysis from pH 10.0 back to pH 7.4 led to reassembly, although some precipitation occurred.

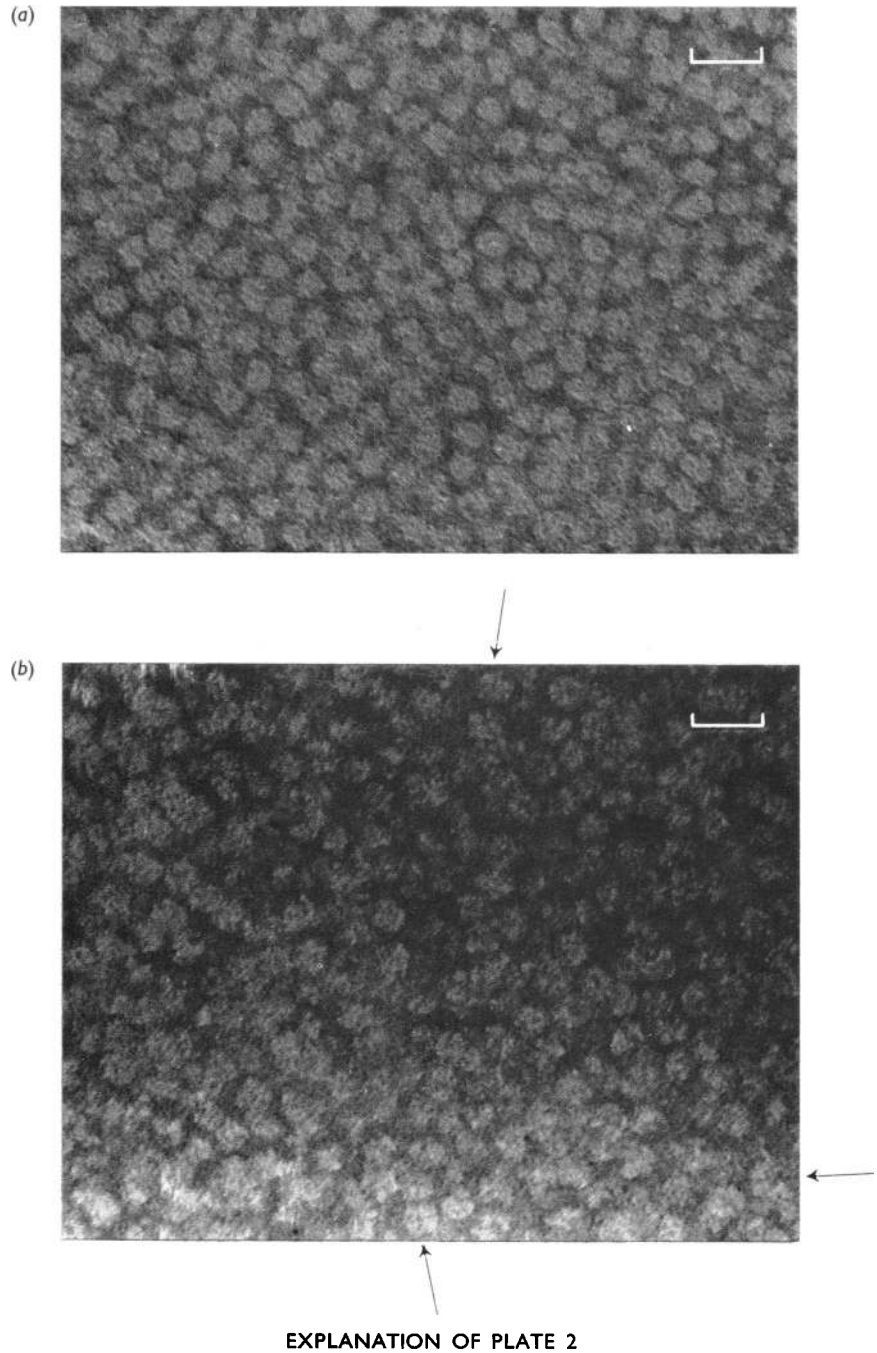
Electron microscopy

Electron micrographs obtained from samples of the *Er. carotovora* and *Esch. coli* enzymes stained with ammonium molybdate are reproduced at a final magnification of 450000 in Plate 2(*a*) and 2(*b*). A comparison of the photographs reveals an important difference in the penetration of the quaternary structure of individual molecules by the negative stain. In Plate 2(*a*) the *Er. carotovora* enzyme



EXPLANATION OF PLATE 1

Comparison of *Er. carotovora* L-asparaginase and *Esch. coli* L-asparaginase preparations by the method of sodium dodecyl sulphate–polyacrylamide-gel electrophoresis under the same conditions as those outlined in Fig. 9. Samples were: (*a*) *Er. carotovora* L-asparaginase batch CB5 (freeze-dried material); (*b*) Merck *Esch. coli* L-asparaginase; (*c*) Bayer *Esch. coli* L-asparaginase; (*d*) Squibb *Esch. coli* L-asparaginase; (*e*) Lilly *Esch. coli* L-asparaginase.



EXPLANATION OF PLATE 2

Electron micrographs of *Er. carotovora* L-asparaginase and *Esch. coli* L-asparaginase (Bayer) preparations negatively stained with ammonium molybdate. (*a*) *Er. carotovora* L-asparaginase batch CB5. In this experiment the penetration of the quarternary structure is insufficient to obtain a resolution of the subunits. The diameter of the particles is 7.2 ± 0.7 nm; a scale superimposed on the photograph indicates a length of 20 nm. (*b*) *Esch. coli* L-asparaginase (Bayer). The particles in this case have a characteristic mottled appearance as a result of better penetration by the negative stain, and in several particles located by the arrows the cleft separating the four subunits may be clearly seen.

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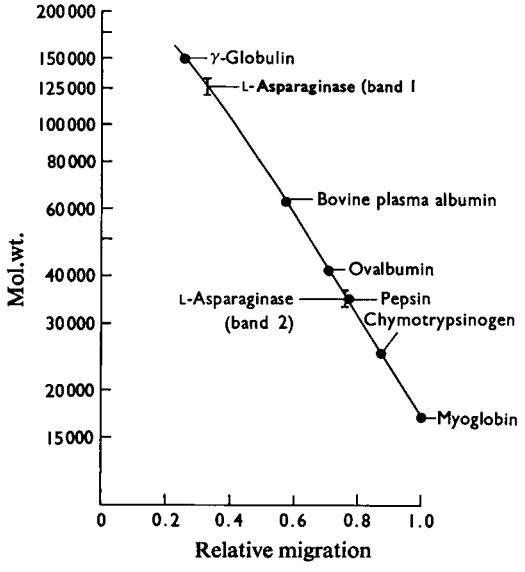

Fig. 9. *Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of Er. carotovora* L-*asparaginase and six reference proteins*

The reference proteins were: rabbit γ-globulin (mol.wt. 150000), bovine plasma albumin (mol.wt. 68000), ovalbumin (mol.wt. 43000), pepsin (mol.wt. 35000), chymotrypsinogen (mol.wt. 25000) and myoglobin (mol.wt. 17200). Protein solutions (1mg/ml) were treated for 2h at 20°C in 0.1% sodium dodecyl sulphate in 0.1*I*-sodium phosphate buffer, pH7.4, and 10μl was applied to individual 0.1% sodium dodecyl sulphate–5% polyacrylamide gels and run in parallel for 2h at a potential of 40V (7cm gel) and a current of 8mA/gel. In this experiment the L-asparaginase resolved as band 1 (mol.wt. 125000) and band 2 (mol.wt. 35200).

molecules resolve as uniform, compact particles that contrast exceedingly well against the electron-dense background. Their diameter is 7.2±0.7nm. In Plate 2(*b*) the *Esch. coli* enzyme produces a more diffuse particle with a characteristic mottling where the ammonium molybdate has penetrated the structure. In contrast with the *Er. carotovora* enzyme, several of the particles in Plate 2(*b*) can be identified as a tetramer, showing a typical 'hot-cross-bun' striation across the surface. This result is in complete agreement with the findings of Irion & Voigt (1970).

Optical rotatory dispersion

L-Asparaginase displays an o.r.d. profile typical for that of a globular protein with a small amount of ordered secondary structure in the form of α-helical segments (Figs. 11 and 15). The characteristic features of these spectra are a trough at 233nm, a shoulder in the region of 210–215nm and a peak at 200nm. The magnitude of the 'reduced residue rotation' at 233nm (−3522±74°) is consistent with a helical content of about 10% (Jirgensons, 1969), whereas the b_0 parameter (−147) calculated from the Moffitt–Yang equation (Moffitt & Yang, 1956) applied to dispersion data in the region 300–600nm gave a helical content nearer 20%.

As the pH of L-asparaginase solutions is raised to 11.8 and higher there is a change in the o.r.d. profile indicative of a transition to a less ordered conformation. The change is time-dependent and becomes more marked the higher the pH of the solution. The changes in the spectrum, especially in the region below 220nm, over the pH range 11.8–12.3 are shown in Fig. 12. Table 6 lists values of the 'reduced residue rotations' at 215 and 233nm in the pH range 7.3–12.3 at 3h and more than 48h after the solutions were made up.

Table 5. *Recovery of enzyme activity of* L-*asparaginase dissociated with* 8M-*urea at pH*10 *after dialysis into* 0.1*I-NaCl*+0.1*I-sodium phosphate buffer, pH*7.4

The initial specific activity of the L-asparaginase solution was 550 units/mg before the addition of urea, 0.1 unit/mg in urea. A solution of L-asparaginase at 18mg/ml was dissociated into subunits by dialysis into 8M-urea. This solution was diluted with the diffusate and then dialysed at 4°C into the buffer, pH7.4. Some precipitation occurred in these solutions, especially as they warmed to 20°C. Specific activities are given of the suspension and the supernatant after centrifugation for 15min at 10000*g* ($r_{av.}$ 10cm).

Total protein before centrifugation		Soluble protein in supernatant after centrifugation		Recovery (% of initial activity)
(mg/ml)	(units/mg)	(mg/ml)	(units/mg)	
18.0	140	5.1	510	26
6.0	320	4.6	430	60
3.1	470	3.0	430	89
1.3	450	1.2	480	90

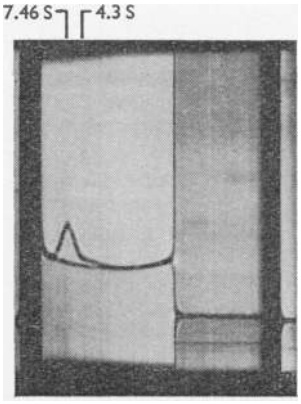

Fig. 10. *Physical reassembly of Er. carotovora* L-*asparaginase from its subunits after the removal of* 6M-*urea at pH*10 (*cf. Fig.* 8) *by dialysis at* 4°C *against* 0.1 *I-sodium phosphate buffer, pH*7.4, +0.1 *I-NaCl*

Initially the solution contained 3.8mg of protein/ml with a specific activity of 670 units/mg; after dissociation and reassembly the solution contained 3.3mg/ml with a specific activity of 490 units/mg. The sedimentation diagram was recorded 17min after centrifugation at 59780rev./min in a capillary-type synthetic-boundary cell. The phase-plate was set at 60°.

Similar but somewhat larger changes in the o.r.d. profile are observed on treatment of the L-asparaginase solution at pH10.5 with urea (Fig. 11) which are also time-dependent. These results may be correlated with those obtained in the ultracentrifuge (Table 3 and Figs. 8*a*, 8*b*, 8*c*, 8*d* and 8*e*) when the molecule was observed to dissociate into subunits. The o.r.d. spectra in Fig. 11 refer to equilibration after 24h at 25°C in the various concentrations of urea indicated. If the results are presented as shown in Fig. 13, in which the values of $[m']_{215}$ and $[m']_{233}$ are given as a function of the urea concentration, it becomes evident that the breakdown of the secondary structure occurs between 4M-urea and 6M-urea and therefore approximately parallel with the dissociation observed in the ultracentrifuge.

When the experiments in urea were repeated at pH7.4 instead of pH10.5 the changes in the o.r.d. profiles were very similar, but, as might be expected from the ultracentrifuge study, which showed only partial dissociation in 8.0M-urea at pH7.4, the equivalent profiles in Fig. 14 were shifted to higher concentrations of urea. A 'back-titration' was performed on the solutions in urea by dilution with sodium phosphate buffer, pH7.4, as shown in Figs. 14(*a*) and 14(*b*). The pathway of the 'forward' and 'back' titrations could be followed by measuring the parameter $[m']_{215}$ and the specific activity as a function of urea concentration. It is important to note that dissociation experiments in urea at pH10 and 7.4 were performed by dialysis at 4°C and aging for 1h at 20°C before the ultracentrifuge run, whereas in the o.r.d. experiments (Figs. 14*a* and 14*b*) the solutions were aged for 24h at 25°C.

After treatment of L-asparaginase with 0.1% sodium dodecyl sulphate for several hours at 37°C, up to a point where gel electrophoresis indicated a complete dissociation into subunits, the o.r.d. spectrum in Fig. 15 gave evidence of an increase in order rather than the expected decrease, and this was made even clearer by the circular-dichroism spectra in Fig. 16. Although the subunits produced o.r.d. and circular-dichroism spectra typical of a native protein molecule, they were inactive.

The effect of the sodium dodecyl sulphate on the o.r.d. profile of the *Er. carotovora* enzyme was time-dependent, whereas experiments with the Merck *Esch. coli* enzyme gave the spectrum of the sodium dodecyl sulphate–enzyme complex within the time normally required to measure the spectrum. These results were therefore entirely consistent with the differences observed between the *Er. carotovora* and *Esch. coli* enzymes in the sodium dodecyl sulphate–polyacrylamide-gel electrophoresis experiments.

Discussion

A study of the properties of the *Er. carotovora* L-asparaginase has confirmed that it is a different protein with a more basic amino acid composition and higher isoelectric point than any of the *Esch. coli* preparations studied. On the other hand, with the exception of the Squibb *Esch. coli* enzyme, the molecular parameters and subunit structure are remarkably similar. The *Er. carotovora* enzyme has molecular weight 135000±10000, $s^0_{20,w}$ 7.43S and subunit molecular weight 32500–38000. The Squibb, Bayer and Lilly *Esch. coli* enzymes have been studied by sedimentation-equilibrium (Whelan & Wriston, 1969; Kirschbaum *et al.*, 1969; Frank *et al.*, 1970), Sephadex G-200 chromatography (Arens *et al.*, 1970), electron microscopy (Irion & Voigt, 1970) and X-ray crystallography (Born & Bauer, 1970; Epp *et al.*, 1971), and the range of molecular weights published is 118000–142000.

Until recently there was some doubt as to whether the *Esch. coli* enzyme was composed of four or six subunits. Some early sedimentation-equilibrium measurements in urea and guanidinium chloride produced rather lower values for the molecular weight of the subunit, i.e. 19000–24000 (Whelan & Wriston, 1969; Kirschbaum *et al.*, 1969), and these were supported by estimates of the minimum molecular weight calculated from amino acid analyses (Whelan

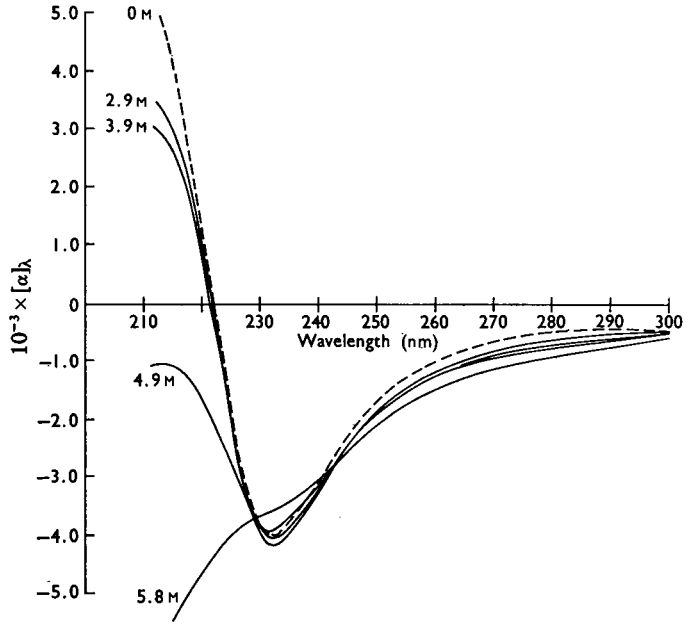

Fig. 11. *O.r.d. spectra of Er. carotovora* L-*asparaginase*

The enzyme (0.24mg/ml) was dissolved in solutions of 0.1*I*-glycine–NaOH buffer, pH10.5, containing 0.1*I*-NaCl and 0–5.8M-urea (concentrations indicated by individual curves in the figure). Recorded after 24h equilibration at 25°C.

& Wriston, 1969; Arens *et al.*, 1970), although *N*-terminal amino acid analyses indicated a subunit molecular weight of 35000 (Arens *et al.*, 1970), much closer to the accepted value.

There seems to be very good evidence from sodium dodecyl sulphate–polyacrylamide-gel electrophoresis that all the *Esch. coli* preparations examined (i.e. Bayer, Squibb, Merck and Lilly) and the *Er. carotovora* enzyme are composed of four subunits of similar size (Plate 1), and this has been confirmed for the Bayer *Esch. coli* enzyme by Glassmann & Bode (1971), who obtained a subunit molecular weight 35500 by the same gel-electrophoresis method.

The question of whether the *Esch. coli* enzyme molecule contains four or six subunits would appear to have been settled by the sedimentation-equilibrium results of Frank *et al.* (1970) which gave a subunit molecular weight 33000, and the resolution of the quaternary structure of the tetramer by electron microscopy (Irion & Voigt, 1970) and X-ray crystallography (Epp *et al.*, 1971).

The greater difficulty found in dissociating the subunits of the *Er. carotovora* enzyme molecule in urea and sodium dodecyl sulphate is a hint that the interaction of these subunits in the tetramer involves stronger secondary bonding forces than those that maintain the integrity of the *Esch. coli* enzyme. This statement applies only to solutions at neutral pH. Presumably the strength of the secondary bonds is a function of the isoelectric points. With p*I* 4.8–5.2 (Arens *et al.*, 1970) the *Esch. coli* enzyme molecule has a moderately high negative charge at pH7.4, whereas the *Er. carotovora* enzyme is isoelectric at this pH, and clearly the electrostatic attractive and repulsive forces generated (i.e. inter- and intramolecular) will differ. This was emphasized by the results in Figs. 8(*a*), 8(*b*), 8(*c*), 8(*d*) and 8(*e*), when it was necessary to adjust the pH several units away from the isoelectric point to obtain a complete dissociation into subunits in urea.

Some idea of the molecular conformation in solution can be deduced from the frictional coefficient, *f*, which was calculated from the Svedberg equation after resubstitution of RT/Nf for D the diffusion coefficient:

$$M = Nfs/(1-\bar{v}\rho)$$

The equation:

$$f = (f/f_0)_{\text{shape}}\,(1 + w/\bar{v}\rho)^{1/3}\,6\pi\,(3M\bar{v}/4\pi N)^{1/3}$$

provides the parameter $(f/f_0)_{\text{shape}}$, the frictional ratio, which is defined by Schachman (1959). This

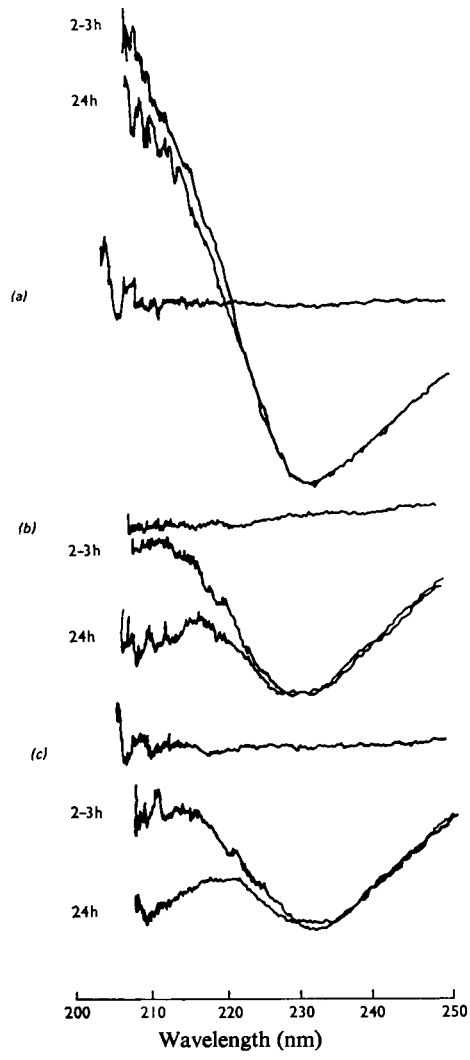

Fig. 12. *O.r.d. spectra of Er. carotovora* L-*asparaginase*

The enzyme (0.24mg/ml) was dissolved in 0.1 *I*-glycine–NaOH buffers containing 0.1 *I*-NaCl at: (*a*) pH11.8; (*b*) pH12.1; (*c*) pH12.3.

---

was used to give an axial ratio of an equivalent ellipsoidal model with hydrodynamic properties similar to those of the hydrated protein molecule. The value assumed for hydration in the equation above was $w = 0.3$ g/g of protein. The value of $(f/f_0)_{shape} = 1.08$ fits a prolate or oblate ellipsoid with an axial ratio of 2.5, and the molecular parameters published for the Bayer and Lilly *Esch. coli* enzyme preparations produce almost identical results, so that therefore all these molecules are very compact in solution.

In contrast, the value of 5.6S for the Squibb *Esch. coli* L-asparaginase (Kirschbaum *et al.*, 1969) is abnormally low when combined with the molecular weight 125000–139000, and as a consequence the axial ratio of a prolate ellipsoid deduced from these values is nearer 10. Conclusions drawn from this result are: (i) the molecule for some reason has a more highly expanded molecular conformation and/or may be more heavily hydrated, or (ii) the sedimentation coefficient may be in error. The decrease in sedimentation coefficient may possibly be due to the heat treatment at 55°C, which destroys the heat-labile EC-1 enzyme (clinically inactive) (see Campbell *et al.*, 1967; Whelan & Wriston, 1969): on the other hand the Bayer *Esch. coli* enzyme material is also prepared in this way and yet has a normal sedimentation coefficient.

The size and shape of the *Er. carotovora* enzyme molecule as deduced from the hydrodynamic data above is confirmed by the dimensions of the negatively stained particles resolved by the electron microscope (Plate 2*a*). The difficulty in resolving the subunit structure fits in very well with the idea of stronger interaction between subunits compared with the *Esch. coli* enzyme. Thus the *Esch. coli* enzyme molecule is easier to dissociate in urea and sodium dodecyl sulphate, and may even dissociate spontaneously on dilution.

Some caution is essential when making an estimate of the monomer/tetramer ratio from the schlieren diagrams reproduced in Fig. 8 (see also Table 3), especially if the results are used to determine the degree of dissociation applicable to any of the o.r.d. measurements in urea solutions. First, there is a very strong possibility of the considerable hydrostatic-pressure gradient in the ultracentrifuge disturbing mobile monomer–polymer equilibria. This pressure can be as high as 300 atm at the base of the solution column. Myosin (Josephs & Harrington, 1966, 1968; Kegeles & Johnson, 1970*a*,*b*) and α-chymotrypsin (Kegeles & Johnson, 1970*a*) are excellent examples of this phenomenon. Secondly, the o.r.d. measurements were, for technical reasons, restricted to lower concentrations than those commonly used in sedimentation, i.e. 0.24–1.5mg/ml compared with 5–8mg/ml, and there was the possibility of the denaturing agent being more effective under these conditions.

Breakdown of the L-asparaginase molecule in the pH region 11.8–12.3 is evident from the loss in enzyme activity with time in Fig. 4, the dissociation of the 7.4S molecule into inactive 2.8S subunits in Fig. 5 and the progressive changes in the o.r.d. spectra shown in Fig. 12 and Table 6. For the reasons already stated, the correlation of the degree of dissociation into subunits in urea solution (Fig. 8 and

Table 6. *Variation of principal o.r.d. parameters with pH of* L-*asparaginase solutions*

The concentration of L-asparaginase was 0.24–0.27mg/ml. Experimental details of these results are given in the text.

pH of protein solution	2–3h after solution		>48h after solution	
	$[m']_{233}$	$[m']_{215}$	$[m']_{233}$	$[m']_{215}$
7.3	−3450°	+3270°	−3450°	+3270°
8.8	—	—	−3520	+3300
10.5	—	—	−3430	+3270
11.3	−3380	+3300	−3380	+3300
11.9	−3430	+2950	−3430	+2300
12.1	−3400	−500	−3400	−2010
12.3	−3450	−1370	−3270	−2380

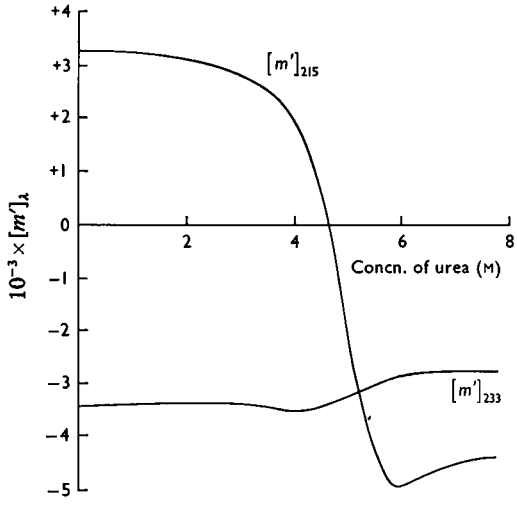

Fig. 13. *Reduced residue rotations* $[m']_\lambda$ *at* 233 *and* 215*nm of Er. carotovora* L-*asparaginase as a function of the urea concentration in* 0.1 *I-glycine–NaOH buffer, pH* 10.5, *containing* 0.1 *I-NaCl*

Table 3) with the magnitude of $[m']_{215}$ and $[m']_{233}$, plotted as a function of urea concentration in Fig. 13, must remain suspect until an alternative method is used to measure dissociation under the same experimental conditions as those used in the o.r.d. measurements. The application of the light-scattering method is an obvious answer, since, from a determination of weight-average molecular weights in urea solutions, one can determine the position of the monomer–tetramer equilibrium. But, on taking the results in Table 3 and Fig. 13 simply at their face value, the sharp change in $[m']_{215}$ above 5.0M-urea indicates a co-operative type of breakdown that results from a combination of denaturation and dissociation of the individual polypeptide chains. In the other set of experiments in urea at pH7.4 (Fig. 14*a*) a better correlation was achieved between the change in $[m']_{215}$ and dissociation in the ultracentrifuge, because the same solution was used in both experiments. This was made possible by making the o.r.d. measurements in a 0.2mm cell on solutions containing 1.5–2.0mg/ml. These results suggest that the sharp transition in $[m']_{215}$ occurs in parallel with dissociation. This does not resolve whether a certain amount of unfolding may take place before dissociation (or the converse), since the probable small amounts of dissociation involved are undetectable under the conditions used in the measurements.

Renaturation and reassembly of the individual polypeptide chains into an active tetramer can be carried out with a remarkably high degree of fidelity and excellent recovery of activity. This is perhaps surprising in view of the absence of intramolecular disulphide bonds, which normally would aid the preservation of ordered structure in solutions of the denaturing agent. However, the deliberate scission of the covalent disulphide bonds of the *Esch. coli* enzyme by Frank *et al.* (1970) before reassembly did not appear to affect their recoveries very much.

Some doubt exists as to the validity of the correlation made between asparaginase activity and physical properties in Table 3 and Figs. 14(*a*) and 14(*b*), since we are unable to decide what further change may take place on dilution of the enzyme for assay and incubation of it with substrate at 37°C. The work of Stellwagen & Schachman (1962) describes the reconstitution of aldolase from subunits produced in urea or by acid pH, and shows that recovery of activity is dependent on the pH and composition of the solvent. Dialysis as opposed to dilution was found to be important only in adjusting the pH after acid dissociation.

With the *Er. carotovora* enzyme the optimum pH for obtaining a high recovery of activity during reconstitution of the tetramer is somewhere in the range pH5.0–7.0. Below pH4.0 and above pH8.0

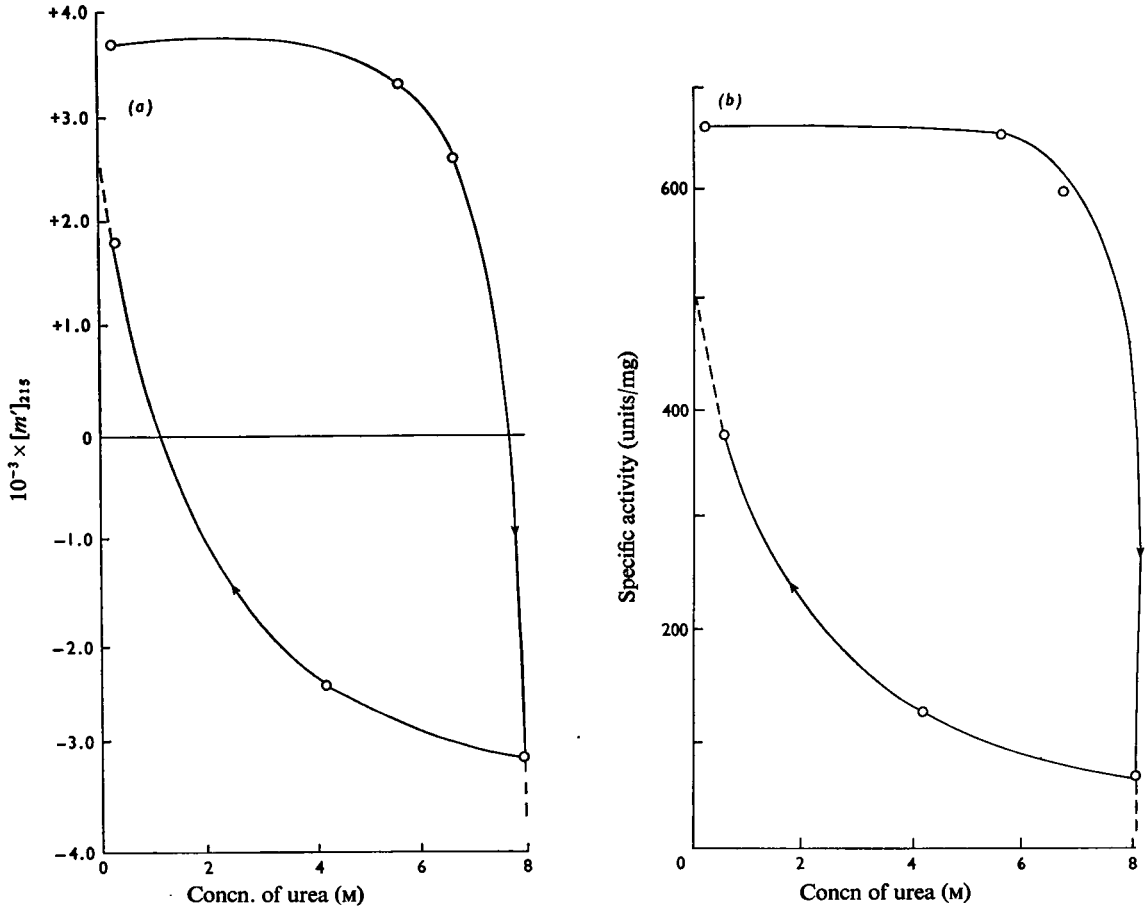

Fig. 14. *Comparison of the reduced residue rotations* $[m']_\lambda$ *at* 215 *nm* (*a*) *and enzyme activities* (*b*) *for solutions of Er. carotovora* L-*asparaginase*

The enzyme (1.5mg/ml) was dissolved in 0.1*I*-sodium phosphate buffer, pH7.4, containing 0.1*I*-NaCl and various concentrations of urea. The reduced residue rotations were obtained first by solution, aged for 24h at 25°C (forward direction), and subsequently by dilution with pH5 buffer (reverse direction).

there appears to be no recovery in activity. This explains the results in Table 3 and Figs. 14(*a*) and 14(*b*) which appear to conflict. In Table 3 the dilutions for assay were prepared in borate buffer, pH8.5 (see the Materials and Methods section), and therefore no recovery of activity can be expected and only the activity of the residual intact tetramers will be measured. The same applies to the 'forward pathway' in Figs. 14(*a*) and 14(*b*), but the 'backward pathway' has a different history in that dilutions from urea were prepared in sodium phosphate buffer, pH6.5, and not the borate buffer, pH8.5, therefore a partial or nearly complete recovery of activity can be expected.

The apparent increase in helix content of the subunits obtained by dissociation in sodium dodecyl sulphate (Figs. 15 and 16) is a similar phenomenon to that found with certain other proteins (Jirgensons, 1966, 1967). This increased order can arise in a number of ways: it may be that constraining forces operative in the tetramer when disrupted allow the subunits to adopt a more rigid conformation. The lack of unfolding in the backbone structure with dissociation by sodium dodecyl sulphate also suggests that the interactions holding the subunits together in the tetramer are predominantly hydrophobic in nature, whereas the forces responsible for the secondary structure in the subunits are primarily ionic and/or hydrogen-bonding in character.

The fact that the sodium dodecyl sulphate-dissociated subunit is inactive may arise because there

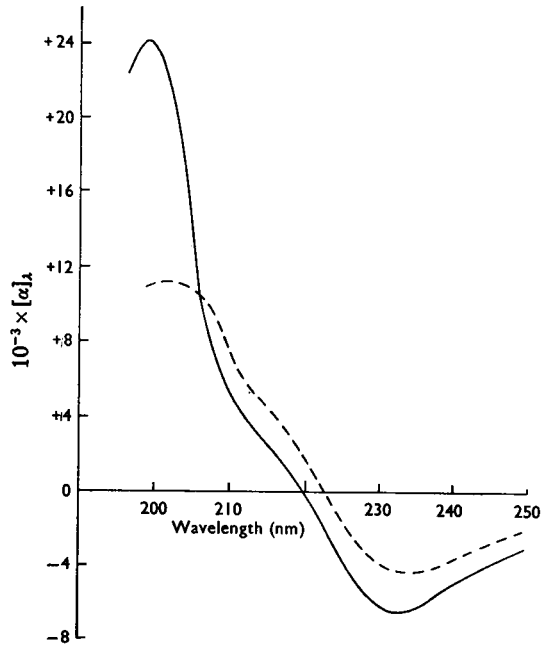

Fig. 15. *O.r.d. spectra of Er. carotovora* L-*asparaginase* (*concentration* 0.24 *mg/ml*) *in sodium phosphate buffer, pH* 7.4, *containing* 0.1 *I-NaCl*

Spectra were obtained before (----) and after (——) incubation with sodium dodecyl sulphate (0.1%, w/v) at 37°C.

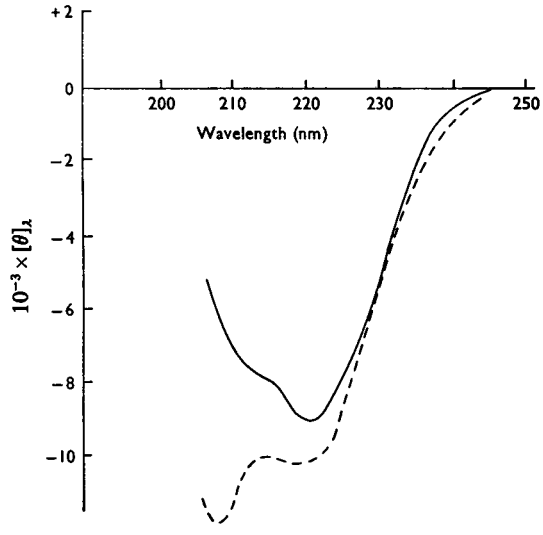

Fig. 16. *Circular-dichroism spectra of Er. carotovora* L-*asparaginase* (*concentration* 0.24 *mg/ml*) *in* 0.1 *I-sodium phosphate buffer, pH* 7.4, *containing* 0.1 *I-NaCl*

Spectra were obtained before (——) and after (---) incubation with sodium dodecyl sulphate (0.1%, w/v) at 37°C.

is a sufficient change in the conformation from the native structure (shown by the difference in the relative magnitudes of the Cotton effects in the circular dichroism spectra in Fig. 16) to perturb the active site. Alternatively the sodium dodecyl sulphate molecules bound to the protein may inhibit access to the active site. Partial recovery of the enzymic activity is observed in the presence of blood plasma but as yet there is no evidence to indicate whether the subunits are retaining their integrity or the tetramer is being reformed in the plasma.

There are important differences in molecular stability among various *Esch. coli* enzyme preparations as well as between *Esch. coli* and *Er. carotovora* L-asparaginase. The *Er. carotovora* enzyme does not show the same tendency to aggregate after being subjected to freeze-drying or dialysis (Ho & Milikin, 1970), and at no time did the sedimentation patterns give any hint of mass-action phenomena wherein the polymer/monomer ratio increases with protein concentration, as described for the Squibb *Esch. coli* enzyme by Kirschbaum *et al.* (1969). Significant amounts of aggregated material that contain two, three and even four of the normal tetramers were identified by Arens *et al.* (1970) by means of t.l.c. and Irion & Voigt (1970) by electron microscopy.

The general importance of aggregated forms of asparaginase is recognized in studies of the 'half-life' or 'clearance-rate' from the bloodstream after intravenous injection. Broome (1965) deduced that the relative ineffectiveness of a yeast L-asparaginase as an anti-tumour agent was the result of its very high molecular weight, 800000. Aggregates are more rapidly engulfed by macrophages and hence stimulate the formation of antibody and hasten the development of hypersensitivity.

It is evident that the *Er. carotovora* and Lilly *Esch. coli* asparaginases have a lot in common, as neither of these enzymes dissociates spontaneously on dilution (see Frank *et al.*, 1970), whereas both the Bayer and Squibb *Esch. coli* enzymes produce half-molecules (mol.wt. 64000) if diluted to less than 1 mg/ml (Kirschbaum *et al.*, 1969; Arens *et al.*, 1970). The clinical implications of these differences in molecular stability are noteworthy, since it seems reasonable to assume that the Bayer and Squibb *Esch. coli* asparaginases circulate in the bloodstream as smaller molecules, whereas the Lilly *Esch. coli* and *Er. carotovora* asparaginases are presumably intact as tetramers. In spite of these differences there is nothing

as yet to suggest that this affects their efficacy in cancer therapy. Nevertheless, if it is at all feasible to produce a stabilized ordered active subunit, this would be of great interest as it might provide better access to malignant tissue.

Note added in proof. Since this paper was submitted for publication we have had the opportunity to read a very recent report on the physical properties of Bayer *Esch. coli* L-asparaginase by Scholtan & Lie (1971). They have interpreted sedimentation-equilibrium data on the basis of Casassa & Eisenberg (1964) theory and determined the value of $(\partial\rho/\partial c)^0_\mu$ in two-component and three-component systems (Reisler & Eisenberg, 1969). The values of the partial specific volume, $\bar{v}$, and the apparent specific volume, ϕ, differ by about −0.004 to −0.007 in solutions of the protein dialysed against 6M-guanidinium chloride. The correction that we made on an empirical basis for *Er. carotovora* L-asparaginase was −0.01.

Scholtan & Lie (1971) report a molecular weight 129000±4000 and a subunit molecular weight 32700±2000 for the *Esch. coli* enzyme.

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