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Preparation of Gram Quantities of a Purified R-Factor-Mediated Penicillinase from *Escherichia coli* Strain W3310

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Purified penicillinase, in gram quantities, has been prepared from *Escherichia coli* strain W3310 by using methods developed to handle large amounts of material. The final product had a specific enzyme activity of 3.08 units/μg of protein, which was over twice as high as that reported previously (Datta & Richmond, 1966). The purified enzyme was similar to that from *E. coli* strain TEM, but different in molecular weight and some other respects. The differences observed may be a result of the greater purity obtained.

Production and purification processes for R-factor-mediated penicillinases from *Escherichia coli* have been described by Datta & Richmond (1966), Sawai *et al.* (1970) and Lindquist & Nordstrom (1970). The enzyme yields per ml of culture were 200 and 600 in the case of Datta & Richmond (1966) and Sawai *et al.* (1970) respectively. The units used by Lindquist & Nordstrom (1970) are not comparable. However, the quantities of purified enzymes produced were small: 1.8mg from 3 litres of culture (Datta & Richmond, 1966), 15.7mg from 10 litres of culture (Sawai *et al.*, 1970) and 0.64mg from 12 litres of culture (Lindquist & Nordstrom, 1970).

In the experiments reported in the present paper units of penicillinase activity are expressed as μmol of substrate transformed/min at 30°C and pH7.0. However, it should be noted that other authors with whose work comparisons are made (Datta & Richmond, 1966; Sawai *et al.*, 1970; Melling & Ford, 1971) used the unit defined by Pollock & Torriani (1953), which relates activity to μmol of substrate transformed/h at 30°C and pH7.0. Thus 60 of the units defined by Pollock & Torriani (1953) are equivalent to one of the present international enzyme units.

To allow a detailed appraisal of the characteristics of a penicillinase mediated by the TEM R-factor (Datta & Kontomichalou, 1965), including the amino acid sequence, much larger quantities of the enzyme were required than had been produced hitherto.

Yields of 5000 enzyme units/ml of culture were obtained (Melling & Ford, 1971) from *E. coli* strain W3310, which carried the TEM R-factor (M. H. Richmond, personal communication). This corresponds to 1000 units/mg dry wt. of bacteria and thus the preparation of gram amounts of purified enzyme required kilogram quantities of bacteria as a starting material. The procedures reported here were therefore developed for work on a large scale.

Experimental

Culture methods

E. coli strain W3310 containing the TEM R-factor was obtained from Professor M. H. Richmond, Dept. of Bacteriology, University of Bristol. The organism was maintained on 1% nutrient agar plates (Novick, 1962) containing 25μg of ampicillin/ml. Cultures for penicillinase production were grown in the medium described by Novick (1962) at 37°C in a 400-litre fermenter. The fermenter (76cm diam. × 137cm high) was fitted with four baffles (6.3cm × 72cm) spaced vertically at equal distances around the vessel wall. The stirrer was fitted with two 30.5cm impellers, each having four blades (4.4cm high × 8.3cm long), extending to the periphery of the impeller disc. The general construction of the culture vessel and associated equipment was similar to the 100-litre vessel described by Elsworth & Stockwell (1968). The pH was automatically maintained at 6.8 by using phosphoric acid and the cultures were aerated with 300 litres of air/min and stirred at 250rev./min. When the penicillinase titre reached a maximum, after about 15h of incubation, the bacteria were collected by centrifugation and washed with 0.1M-sodium phosphate buffer, pH7.0. The washed cell paste was stored at −20°C until required.

General methods

Enzyme assays. Penicillinase assays with sodium benzyl penicillin as substrate were done by the

micro-iodometric method of Novick (1962) or by a spectrophotometric variation of the Perret (1954) assay (Sherratt, 1969). Units of penicillinase activity are expressed as μmol of substrate transformed/min at 30°C and pH7.0.

Protein estimation. Protein was normally measured by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard. Elution of protein from columns was followed by measuring the E_{254}.

Ion-exchange celluloses. DEAE-celluloses DE11 and DE52 obtained from Whatman Products, H. Reeve Angel, London E.C.4, U.K., were prepared for use according to the maker's directions.

Sephadex. Sephadex G-75 purchased from Pharmacia, Uppsala, Sweden was used according to the maker's directions.

Starch-gel electrophoresis. This was done by the method of Smithies (1955) by using Connaught hydrolysed starch obtained from Connaught Medical Research Laboratories, Toronto, Ont., Canada.

Molecular weights. Determinations of molecular weights were performed by gel filtration on Sephadex G-75 (superfine grade) (Andrews, 1964) and by electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate (Weber & Osborn, 1969).

Amino acid analysis. The method of Spackman *et al.* (1958), with a temperature of 105°C for hydrolysis, was employed with a model 120C amino acid analyser (Beckman Instruments Inc., Palo Alto, Calif., U.S.A.). Tryptophan was determined by the method of Spies & Chambers (1948). Cysteine was determined by treating the protein with iodoacetic acid (BDH Ltd., Poole, Dorset, U.K.) and measuring carboxymethylcysteine in an acid hydrolysate of the protein (Gurd, 1967).

Carbohydrate. The carbohydrate content of protein samples was determined by the method of Devor (1950).

Results

Release of the penicillinase

The penicillinase in *E. coli* strain W3310 is cell-associated, with little in the culture supernatant; therefore a cell-breaking procedure was required. Datta & Richmond (1966) used ultrasonic treatment, but this was not suitable in the present case where multi-kilogram quantities of bacteria were involved. A Manton–Gaulin homogenizer (APV Ltd., Crawley, Sussex, U.K.) operated at 50 litres/h was used for bacterial disruption. Maximum release of the penicillinase occurred after one pass of an aqueous suspension of bacteria (10% dry wt.) through the machine at a pressure of 55.12MN/m² (8000lb/in²), as shown in Table 1.

Purification of the penicillinase

The purification was done in two parts; first, the processing of the broken-cell suspension to give about 5×10^6 units of penicillinase with a specific enzyme activity of 283 units/mg of protein, then purification in batches of 1.6×10^6 enzyme units to give material with a specific enzyme activity of 3083 units/mg of protein. The two parts of the process are summarized in Tables 2 and 3.

Part I. Centrifugation. The broken-cell suspension had a high viscosity and was treated with 0.4μg of deoxyribonuclease/ml before centrifugation to improve the recovery of supernatant (Melling & Atkinson, 1972). The suspension was centrifuged at 13000*g* at 8°C and a flow rate of 20 litres/h in a Sharples no. 6 open clarifier.

Ammonium sulphate fractionation. The supernatant was fractionated with ammonium sulphate and Fig. 1 shows the results of a small-scale process. In the routine preparation solid ammonium sulphate (reagent grade; May and Baker, Dagenham, Essex, U.K.) was added to the supernatant to give a 20% (w/v) solution. This was centrifuged as above, the deposit discarded and the ammonium sulphate content of the supernatant increased to 56% (w/v). The resulting deposit, collected by centrifugation, contained the penicillinase, which was stable in this condition for at least 6 months when stored at 4°C.

Dialysis. The ammonium sulphate precipitate was dissolved in water and dialysed against demineralized water in a continuous dialyser (Heppell Engineering, Harlow, Essex, U.K.) to decrease the conductivity to 1000μsiemens. The enzyme solution was then dialysed to equilibrium with the appropriate buffer system. For small-scale work dialysis tubing was used.

Table 1. *Release of penicillinase from Escherichia coli strain W3310*

Samples of an aqueous suspension of *E. coli* (10%, w/v) were passed through a Manton–Gaulin homogenizer operated at various pressures. Penicillinase and protein determinations were made on the supernatant after centrifuging at 10000*g* for 15min.

Operating pressure		Penicillinase	Protein
(MN/m²)	(lbs/in²)	(units/ml)	(mg/ml)
0	0	45	2.9
13.78	2000	300	19.0
27.56	4000	510	52.3
41.34	6000	591	63.3
55.12	8000	600	62.6
68.90	10000	585	61.3

Table 2. *Summary of part I of the purification process for penicillinase from Escherichia coli strain W3310*

Experimental details are given in the text.

Stage	Procedure	Amount: Volume (l)	Amount: Weight (g)	$10^{-6}\times$Total enzyme activity recovered (units)	Specific enzyme activity (units/mg of protein)	Recovery (%): Per stage	Recovery (%): Overall
1	Homogenization	12		4.46	7.13	100	100
2	Centrifugation	60		6.41	12.25	142	143
3	First ammonium sulphate fractionation (20%, w/v)	60		9.37	51.31	146	209
4	Second ammonium sulphate fractionation (56%, w/v)		2424	9.23	66.83	98	206
5	Dialysis	8		9.15	68.00	99	205
6	Absorption and elution from DE11 cellulose						
	(*a*) First three pooled eluates	4.5		5.10 } 6.87	283.33	56 } 75	114 } 153
	(*b*) Second three pooled eluates	4.5		1.76	91.72	19	39
7	Freeze-drying						
	(*a*) First three pooled eluates		145	5.00 } 6.75	283.33	98 } 98	111 } 150
	(*b*) Second three pooled eluates		148	1.75	91.67	99	39

Table 3. *Summary of part II of the purification process for penicillinase from Escherichia coli strain W3310*

The starting material was a portion of the freeze-dried product from stage 7 (Table 1). Experimental details are given in the text.

Stage	Procedure	Amount: Volume (ml)	Amount: Weight (mg)	$10^{-6}\times$Total enzyme activity recovered	$10^{-2}\times$Specific enzyme activity (units/mg of protein)	Recovery (%): Per stage	Recovery (%): Overall
8	Dialysis	1100		1.98	1.56	100	100
9	Chromatography on DE52 cellulose	400		1.87	4.33	94	94
10	Gel filtration on Sephadex G-75	200		1.77	26.50	94	89
11	Chromatography on DE52 cellulose	80		1.70	30.83	96	85
12	Dialysis and freeze-drying		550	1.70	30.83	100	85

Absorption and elution from DE11 cellulose. For ease and speed of operation it was intended to use a batch absorption and elution process.

Initial observations indicated that the conditions used by Datta & Richmond (1966), i.e. 0.01M-sodium phosphate buffer at pH7.0, gave relatively poor absorption of the enzyme. Only 1.83×10^3 units of penicillinase were absorbed/g of dry DE11 cellulose. Therefore to have absorbed the normal batch of about 6.6×10^6 units would have required

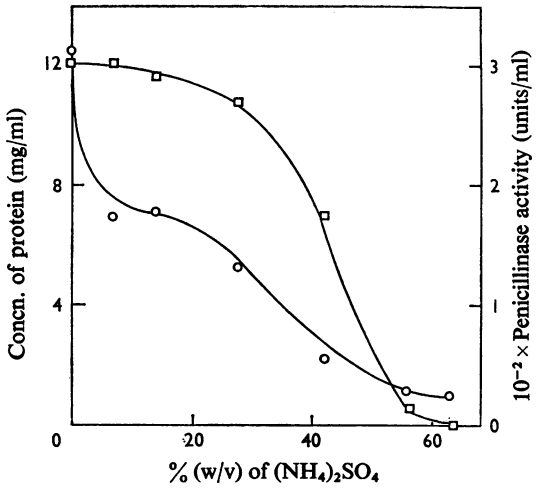

Fig. 1. *Ammonium sulphate fractionation of a crude penicillinase solution*

The penicillinase solution, obtained by bacterial disruption and centrifugation, was added to suitable quantities of ammonium sulphate to produce 100ml volumes of the required ammonium sulphate concentration. The protein (o) and penicillinase (□) contents of the supernatants were measured after centrifugation at 15000*g* for 15min.

Table 4. *Effect of pH, concentration and buffer composition on the amount of penicillinase absorbed per gram of DE11 cellulose*

Known amounts of DE11 cellulose were equilibrated to various buffer systems and then added to solutions of penicillinase which had been dialysed against the appropriate buffer. The penicillinase solutions were assayed before and after 30min of equilibration with the DE11 cellulose.

pH	10^{-3} × Penicillinase units absorbed/g dry wt. of DE-11 cellulose: Sodium phosphate buffer (0.01M)	Tris buffer (0.02M)
6.5	1.16	—
7.0	1.83	—
7.5	2.50	—
8.0	3.33	—
8.2	4.17	15.65
9.0	—	26.66

Concentration of phosphate buffer, pH8.2 (M)	10^{-3} × Penicillinase units absorbed/g dry wt. of DE11 cellulose
0.001	10.33
0.01	4.17
0.1	Nil

4–5kg of DE11 cellulose and involved very large elution volumes.

The results in Table 4 show that increasing the pH of the phosphate buffer to 8.2 improved absorption; a decrease in buffer concentration further increased the amount of enzyme absorbed. However, the use of tris buffer (Table 4) produced a marked improvement in absorption capacity. For routine use, 0.02M-tris buffer, pH8.2, was the system selected since, although the absorption capacity was higher at pH9.0, the enzyme could not be eluted by increasing the buffer concentration at this pH.

In the large-scale purification process 500g of DE11 cellulose was equilibrated to 0.02M-tris buffer, pH8.2, and added to the enzyme solution. The suspension was stirred for 30min and the DE11 cellulose collected by filtration and washed three times with 5-litre volumes of 0.02M-tris buffer, pH8.2. This removed unabsorbed protein and pigment. The cellulose was then treated with successive 1.5-litre volumes of 0.3M-tris buffer, pH8.2, and the eluates were collected by filtration after 30min of stirring.

Dialysis and freeze-drying. The eluates from the DE11 cellulose were pooled (see Table 2), dialysed continuously against 0.02M-tris buffer, pH8.2, and freeze-dried in 7.5-litre batches. The freeze-dried material was stored at −20°C until required.

Part II. Dialysis. A typical batch containing about 1.6×10^6 units of enzyme activity was dissolved in water and dialysed against 6×8-litre volumes of water at 4°C; when the pH of this solution increased to about pH8.5 it was adjusted to pH7 with 1M-HCl. Ultimately the solution was adjusted to pH7.0 with 1M-HCl and to a conductivity of 80μsiemens with 0.05M-tris–HCl buffer, pH7.0.

Chromatography on DE52 cellulose. The solution of penicillinase was applied at 150ml/h to a column (20cm×2.5cm) of DEAE-cellulose previously equilibrated to 0.02M-tris–HCl buffer at pH7.0; no enzyme was detected in the eluate at this stage. The column was washed with 250ml of 0.02M-tris–HCl buffer, pH7.0, and a linear gradient consisting of 350ml of 0.02M-buffer and 350ml of 0.05M-buffer was applied. Fractions (10ml) were collected. The pooled fractions from this column were dialysed exhaustively against distilled water at 4°C and freeze-dried; no detectable loss of enzyme activity occurred on freeze-drying.

Separation through Sephadex G-75. The freeze-dried material was dissolved in 10.0ml of 0.04M-

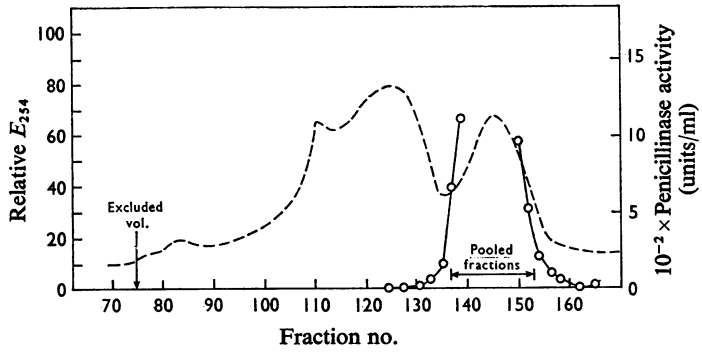

Fig. 2. *Elution profile of penicillinase from Sephadex G-75*

About 10^8 units of penicillinase in 10ml of 0.04M-sodium phosphate buffer, pH7.0, were applied to a column (90cm×6cm) of Sephadex G-75. The flow rate was 100ml/h and 10.0ml fractions were collected. The E_{254} of the effluent was monitored directly and was plotted relative to a buffer blank (---), and the fractions were assayed for penicillinase activity (o). Fractions 137–153 inclusive were pooled and concentrated.

sodium phosphate buffer, pH7.0, and applied to a column (90cm×6cm) of Sephadex G-75, which had previously been equilibrated and packed in 0.04M-sodium phosphate buffer, pH7.0. This buffer was pumped through the column at 100ml/h and 8.0ml fractions were collected. The elution profile is shown in Fig. 2. The pooled fractions were dialysed and adjusted to pH7.0 and a conductivity of 220μsiemens with 0.1M-sodium phosphate buffer, pH7.0.

Rechromatography on DE 52 cellulose. The above solution was applied to a column (10cm×2.5cm) of DEAE-cellulose equilibrated in 0.002M-sodium phosphate buffer, pH7.0. The column was washed with 8mM-buffer and then with a linear gradient consisting of 250ml of 8mM-buffer and 250ml of 16mM-buffer; these molarities were critical for good results. The flow rate was 50ml/h and 6.0ml fractions were collected. The elution profile is shown in Fig. 3. The pooled fractions were dialysed against water and freeze-dried. Fig. 4 shows the results of starch-gel electrophoresis of samples of this preparation before and after this final stage.

Properties of the penicillinase

Homogeneity. The purified protein was homogeneous as judged by starch-gel electrophoresis at pH8.5 and 4.0 and by its elution profile in gel-filtration and ion-exchange chromatography. A very faint band of contaminating protein of low molecular weight was detected by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. The total carbohydrate content corresponded to 0.8mol of reducing sugar/mol of protein.

Enzymic properties. The following characteristics were determined for the purified protein by using sodium benzylpenicillin as substrate: specific activity, 3083 units/mg of protein; K_m, 12μM; turnover number, 6×10^4mol/min per mol of enzyme. The relative activities for benzylpenicillin, ampicillin and ceporin were 100, 112 and 125% respectively (J. Fleming, personal communication).

Molecular weight. Determinations of molecular weight by gel filtration, sodium dodecyl sulphate–polyacrylamide-gel electrophoresis and calculation from the amino acid composition gave values of 2.1×10^4, 2.7×10^4 and 2.2×10^4 respectively. The total number of tryptic peptides is in approximate accord with the calculated value. There are 9 lysine residues and 11 arginine residues per molecule, from which one would expect 21 tryptic peptides. Between 20 and 25 tryptic peptides have been isolated in the course of amino acid sequence analysis (G. K. Scott, unpublished work).

Amino acid composition. Table 5 gives the amino acid composition of the purified protein. The cysteine content was 0.9mol of carboxymethylcysteine/mol of iodoacetic acid-treated protein; the tryptophan content was 3mol/mol of protein.

Molar extinction coefficient. The purified protein had a molar extinction coefficient of 2.14×10^4. The calculated value on the basis of 3mol of tryptophan and 3mol of tyrosine/mol of protein was 2.08×10^4 (Beaven & Holiday, 1952).

Discussion

The final recovery of purified enzyme from the procedure described above exceeded 100% when compared with the penicillinase content of the

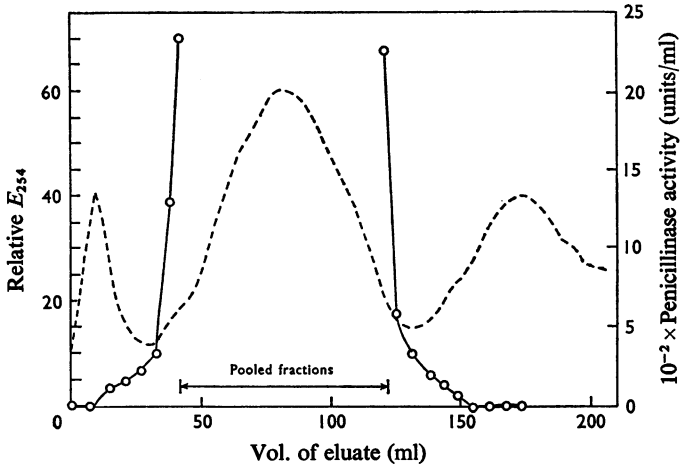

Fig. 3. *Elution profile of penicillinase from DEAE-cellulose*

This purification step was performed with the pooled material from the Sephadex G-75 eluate, after dialysis and adjustment of pH and concentration to match the 2mM-sodium phosphate buffer, pH7.0, in which the column (10cm×2.5cm) of DE52 cellulose was equilibrated. The flow rate was 50ml/h and 6.0ml fractions were collected. Protein was measured by monitoring the E_{254} of the effluent and plotting it relative to a buffer blank (---), and the fractions were assayed for penicillinase activity (o). Fractions 7–21 inclusive were pooled.

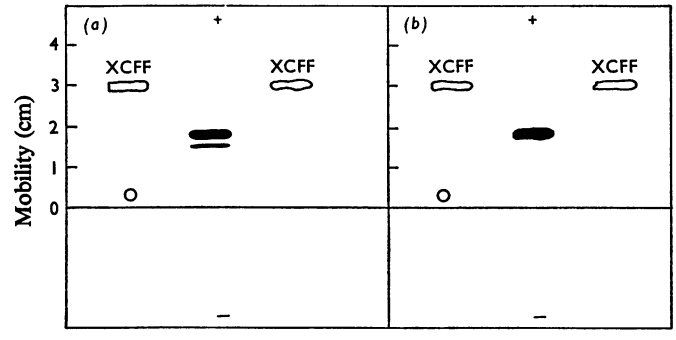

Fig. 4. *Starch-gel electrophoresis of samples of the preparation before (a) and after (b) the final DE52 cellulose chromatography stage, showing that this is essential for the removal of a contaminating protein*

The method of Smithies (1955) was used. The gels were stained for protein. Xylene Cyanol FF (XCFF) was used as a marker; it migrated towards the positive electrode. Staining for penicillinase revealed no activity associated with the protein, which was removed by the final DE52 cellulose chromatography stage. O represents the origin.

broken-cell suspension (Tables 1 and 2). However, in some preliminary experiments dilution of the homogenized cell suspension before ammonium sulphate fractionation was omitted and there was no increase in enzyme titre such as occurred in the large-scale preparation. These results are consistent with other findings (J. Melling & D. Westmacott, unpublished work) which indicate that this enzyme aggregates, probably with other proteins, and there may thus be shielding of the active site of some enzyme molecules in concentrated protein solutions.

In a small-scale preparation the binding capacity of the DEAE-cellulose may be of minor importance, but in the present case an improvement obtained by

Table 5. *Amino acid composition of penicillinase from Escherichia coli strain W3310*

Columns 1 and 2 show the numbers of residues calculated by analysing hydrolysed samples from two different preparations of the enzyme. Results are expressed in number of residues per molecule, assuming a molecular weight of 2.2×10^4. Column 3 gives the mean values to the nearest whole number. The values for threonine and serine were increased by 5 and 10% respectively to correct for losses due to hydrolysis. Methods for analysis are given in the text.

Amino acid	1	2	3
Lys	9.0	9.0	9
His	3.8	4.0	4
Arg	10.4	10.7	11
Asp	17.5	16.8	17
Thr	13.0	13.3	14
Ser	9.6	10.0	11
Glu	19.8	19.5	20
Pro	6.9	7.8	7
Gly	14.6	14.6	15
Ala	17.5	17.2	17
Val	9.2	8.8	9
Met	5.4	5.6	6
Ile	8.9	8.9	9
Leu	19.8	20.7	20
Tyr	3.1	3.1	3
Phe	4.0	4.0	4
Trp	—	—	3
Cys	—	—	1

replacing phosphate buffer with tris buffer (Table 4) greatly facilitated the large-scale process by a tenfold decrease in the amounts of DEAE-cellulose, and hence of eluate, required. The lower binding capacity in the presence of phosphate may have resulted from competition for sites between the enzyme and phosphate ions.

The final product was purified to a specific activity over twice as high as that achieved previously (Datta & Richmond, 1966). In pure form, the enzyme has a somewhat different Michaelis constant when compared with crude extracts of *E. coli* strain TEM, but the relative activities of the two preparations with respect to various substrates are almost identical.

Datta & Richmond (1966) concluded that the relatively small inhibitory effect of *p*-chloromercuribenzoate on penicillinase from *E. coli* strain TEM meant that there were probably no reactive thiol groups in the enzyme. The present study indicates that a cysteine residue is present.

The molecular weight data appear at first sight to be inconclusive, especially when the earlier value of 16700, obtained by ultracentrifugation, is considered (Datta & Richmond, 1966). There is evidence that some proteins give anomalous results in the gel-filtration method for molecular-weight determination (Andrews, 1964) and some doubt has recently been cast upon mobility in sodium dodecyl sulphate gel electrophoresis as a criterion of molecular weight (Tung & Knight, 1971). The value of 16700 was obtained with a preparation which may well have contained less than 50% penicillinase, and thus may be considerably in error.

If the analytical data and the gel-filtration estimate of molecular weight are considered as independent determinations which give approximately the same result, then a molecular weight of between 21 and 22×10^3 is arrived at. In addition the ultrafiltration characteristics of the enzyme also provide evidence for a molecular weight greater than 20×10^3 (J. Melling & D. Westmacott, unpublished work).

The penicillinases Ia and Ib purified by Sawai *et al.* (1970) and Yamagishi *et al.* (1969) and the penicillinase from *E. coli* R1 purified by Lindquist & Nordstrom (1970) are all from strains of *E. coli* and are all coded for by genes carried on resistance transfer factors. These enzymes, together with that from *E. coli* strain TEM, could be classified as type 1 penicillinases in the system of classification devised by Jack & Richmond (1970). These enzymes all have molecular weights in the region of 20×10^3–22×10^3 as estimated by gel filtration. The revised estimates of molecular weight for the penicillinase at present under consideration are in accord with the other penicillinases mentioned above.

References

Andrews, P. (1964) *Biochem. J.* **91**, 222

Beaven, G. H. & Holiday, E. R. (1952) *Advan. Protein Chem.* **7**, 319

Datta, N. & Kontomichalou, P. (1965) *Nature (London)* **208**, 239

Datta, N. & Richmond, M. H. (1966) *Biochem. J.* **98**, 204

Devor, J. W. (1950) *J. Amer. Chem. Soc.* **72**, 2004

Elsworth, R. & Stockwell, F. T. E. (1968) *Process Biochem.* **3**, 229

Gurd, F. N. (1967) *Methods Enzymol.* **11**, 532

Jack, G. W. & Richmond, M. H. (1970) *J. Gen. Microbiol.* **61**, 43

Lindquist, R. C. & Nordstrom, K. (1970) *J. Bacteriol.* **101**, 232

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265

Melling, J. & Atkinson, A. (1972) *J. Appl. Chem. Biotechnol.* **22**, 739

Melling, J. & Ford, J. W. S. (1971) *J. Gen. Microbiol.* **65**, iv

Novick, R. P. (1962) *Biochem. J.* **83**, 229

Perret, C. J. (1954) *Nature (London)* **174**, 1012
Pollock, M. R. & Torriani, A. M. (1953) *C.R. Acad. Sci.* **237**, 276
Sawai, T., Takahashi, K., Yamagishi, S. & Mitsuhashi, S. (1970) *J. Bacteriol.* **104**, 621
Sherratt, D. J. (1969) Ph.D. Thesis, University of Edinburgh
Smithies, O. (1955) *Biochem. J.* **61**, 629
Spackman, D. H., Stein, W. H. & Moore, S. (1958) *Anal. Chem.* **30**, 1190
Spies, J. R. & Chambers, D. C. (1948) *Anal. Chem.* **20**, 30
Tung, J. S. & Knight, A. (1971) *Biochem. Biophys. Res. Commun.* **42**, 1117
Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406
Yamagishi, S., O'Hara, K., Sawai, T. & Mitsuhashi, S. (1969) *J. Biochem. (Tokyo)* **66**, 11