The Composition of β -Lactamase I and β -Lactamase II from *Bacillus cereus* 569/H

By S. KUWABARA, E. P. ADAMS* AND E. P. ABRAHAM
Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, U.K.

(Received 19 January 1970)

1. Crystalline β -lactamase I from *Bacillus cereus* 569/H yielded only amino acids on acid hydrolysis, but crystalline β -lactamase II from the same organism yielded also substantial quantities of neutral sugars and amino sugars. 2. Analysis with an amino acid analyser indicated that the two enzymes were similar though not identical in overall amino acid composition. Analysis of neutral and amino sugars as their silyl derivatives by gas-liquid chromatography showed that the carbohydrate moiety of β -lactamase II contained residues of glucose, galactose, mannose, fucose, glucosamine and galactosamine. 3. After oxidation and hydrolysis both β -lactamases gave small amounts of cysteic acid. After treatment of inactive Zn^{2+} -free β -lactamase II with *N*-ethylmaleimide or iodoacetate enzymic activity was not restored by the addition of Zn^{2+} .

Although β -lactamase I and β -lactamase II are both produced by *Bacillus cereus* 569 and 569/H the latter enzyme differs from the former in requiring Zn^{2+} as a cofactor (Sabath & Abraham, 1966), in showing a wider range of activity, in being less readily inactivated by heat (Kuwabara & Abraham, 1967) and in having a higher molecular weight (Lloyd & Peacocke, 1970). The present paper gives the results of studies of the composition of crystalline preparations of the two enzymes.

MATERIALS AND METHODS

Materials

Enzyme preparations. These were the crystalline preparations of β -lactamase I (stage 8) and β -lactamase II (stage 7) described by Kuwabara (1970). Buffers B and BZ are also as described by Kuwabara (1970).

Thiol reagents. *N*-Ethylmaleimide and iodoacetic acid were from British Drug Houses Ltd., Poole, Dorset, U.K.

Determinations of protein and thiol groups

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Crystalline bovine serum albumin (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.) was used as a standard. Thiol groups were determined by the method of Ellman (1959).

* Present address: John Curtin School of Medical Research, Canberra, A.C.T., Australia.

Paper chromatography and electrophoresis

Paper chromatograms were run on Whatman no. 1 paper in butan-1-ol-acetic acid-water (4:1:4, by vol.). Electrophoresis on Whatman no. 1 paper was carried out at 75 V/cm in pyridine-acetate buffer, pH 4.5 (50 mm with respect to acetate), in an apparatus similar to that of Katz, Dreyer & Anfinsen (1959). Amino acids were detected on paper with ninhydrin. Hexosamines were detected by the method of Partridge (1948).

Quantitative amino acid analysis

Amino acids in acid hydrolysates of β -lactamases (200–700 μg) were determined (a) as described by Moore, Spackman & Stein (1958) and Spackman, Stein & Moore (1958) (with an amino acid analyser from Evans Electro-selenium Ltd.) and (b) in a manner similar to that of Hamilton & Anderson (1959) (with an amino acid analyser from the Locarte Co.). Methods (a) and (b) were used with the enzyme preparations (a) and (b) respectively described in Table 1. Hydrolysis was with 6M-HCl at 110°C for 17 h. Cyst(e)ine was determined as cysteic acid as described by Moore (1963). The losses of serine and threonine on hydrolysis were assumed to be 10% and 5% respectively. Tryptophan was determined after alkaline hydrolysis of the enzymes as described by Noltmann, Mahowald & Kuby (1962). The tyrosine/tryptophan ratio was measured spectroscopically by the methods of Holiday (1936) and of Bencze & Schmid (1957).

Carbohydrate analysis

Determinations of glucosamine and galactosamine in hydrolysates used for amino acid analysis were made in the amino acid analysers.

For the determination of the composition of carbohydrate, neutral sugar and amino sugar residues were

converted into silyl derivatives, which were resolved by g.l.c. (Hedgley & Overend, 1960). The method used was adapted from that of Sweeley & Walker (1961) and Sweeley, Bentley, Makita & Wells (1963).

Methyl glycoside formation. Samples (1–4 mg) of β -lactamase were incubated with 4.0 ml of anhydrous methanolic 0.5 M-HCl for 20 h at 74°C in a sealed Pyrex tube. The solvent and acid were removed on the rotary evaporator at reduced pressure.

Re-N-acetylation. The mixture of methyl glycosides was dissolved in 1.0 ml of anhydrous methanol-acetic anhydride (3:1, v/v). Acetylation of the free amino groups was catalysed by the presence of 10–20 mg of silver acetate. The reaction was carried out at room temperature in the dark, with an occasional shaking, for 24 h. Insoluble catalyst was removed by centrifugation and washed with 2 × 2 ml of anhydrous methanol. Supernatant and washings were combined and evaporated to dryness on the rotary evaporator at reduced pressure; the final traces of acetic anhydride were removed *in vacuo* over NaOH pellets.

Silylation. The above residue was dissolved, under anhydrous conditions, in pyridine-hexamethyldisilazane-trimethylsilyl chloride (10:2:1, by vol.), and kept at room temperature for 30 min during which time NH_4Cl separated out. The solvent and excess of reagent were removed by reduced-pressure distillation at a temperature below 35°C, and the trimethylsilyl ethers were extracted into redistilled *n*-hexane.

The yield of carbohydrate derivatives was determined by weighing and the method was monitored by using hog gastric mucin, which contained the following sugars: fucose, galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine.

G.l.c. of the silyl derivatives. The apparatus used was the Pye 104 model 64 gas chromatograph fitted with dual columns and hydrogen flame ionization detectors. The 150 cm coiled glass columns used were packed with 5% Apiezon-L grease on Gas-Chrom P (Applied Science Laboratories, State College, Pa., U.S.A.), operated at 170°C with oxygen-free nitrogen as carrier gas at a flow rate of 80 ml/min. The detectors were maintained at 220°C. The trimethylsilyl ethers (100 μg amounts), in hexane, prepared from β -lactamase, were chromatographed separately and together with the trimethylsilyl ethers prepared from hog gastric mucin used as a standard.

Relative retention values for some of the major components for the trimethylsilyl derivatives from β -lactamase II [α -methyl (tetra-*O*-trimethylsilyl)galactopyranoside = 1.00] coincided with those of the corresponding components from hog gastric mucin and standards. The relative composition was determined by calculating the area under the peak from the relationship: peak height × width at half the height.

Reaction of Zn^{2+} -free β -lactamase II with thiol reagents

Zn^{2+} -free enzyme. A solution of twice-crystallized β -lactamase II (3.03 mg) in buffer BZ (1 ml) was dialysed for 6 h against sodium citrate buffer, pH 4.5 (5 mM with respect to Na^+), containing 1 M-NaCl and then for 18 h against 0.1 M-tris-acetate buffer, pH 7.0. The resulting solution showed no β -lactamase activity, but at least 80%

of the original activity (with either cephalosporin C or benzylpenicillin as substrate) could be restored by addition of ZnSO_4 to give a final concentration of 0.33 mM.

Reaction with *N*-ethylmaleimide and iodoacetate. To samples of a solution of Zn^{2+} -free β -lactamase II (about 3 mg/ml in 1 mM-buffer BZ) were added different amounts of a solution of *N*-ethylmaleimide in a mixture of ethanol and 5 mM-tris-acetate buffer, pH 7.0 (1:10, v/v). The final concentration of ethanol varied from 0.01 to 0.05% (v/v). The resulting solutions were kept for 15 min at 30°C. A solution of ZnSO_4 (final concn. 0.33 mM) was then added to each sample and the β -lactamase II activity determined.

To further samples of the Zn^{2+} -free enzyme were added aqueous solutions of iodoacetate. After 15 min at 30°C a solution of ZnSO_4 (final concn. 0.33 mM) was added and the β -lactamase activity was determined.

RESULTS

Composition of β -lactamase I and β -lactamase II

Amino acids. Only amino acids were found in hydrolysates of β -lactamase I. However, hydrolysates of β -lactamase II also contained neutral sugars and amino sugars. The results of amino acid analysis of β -lactamase I and β -lactamase II are given in Table 1. In view of the presence of amino sugars in the preparation of β -lactamase II determinations of the protein content of this preparation by the method of Lowry *et al.* (1951) could not be regarded as reliable. The amount of destruction of amino sugars on hydrolysis was not determined. Because of these uncertainties the values given for the amino acids and amino sugars in β -lactamase II are relative and are based on an arbitrary value of 4 residues for histidine so that comparison of the relative composition of hydrolysates of β -lactamase II with those of β -lactamase I can easily be made.

The amino acid composition found for β -lactamase I showed an overall similarity to that given by Citri & Pollock (1966) for highly purified extracellular penicillinase from *B. cereus* 569/H, although the values for methionine, threonine, serine and tyrosine were significantly higher. However, cyst(e)ine, which was reported to be absent from the penicillinase preparation by Citri & Pollock (1966), was apparently present as cysteic acid in a hydrolysate of β -lactamase I that had been oxidized by performic acid under the conditions described by Moore (1963). β -Lactamase II closely resembled β -lactamase I in much of its relative amino acid composition, but it appeared to contain relatively more valine and relatively less alanine and isoleucine and to yield more cysteic acid after oxidation.

The u.v.-absorption spectra of β -lactamase I and β -lactamase II showed a change in E_{max} from 280 nm to 293 nm with an increase in pH from 7.6 to 13 that is characteristic of the change resulting from a mixture of tyrosine and tryptophan residues.

Table 1. *Amino acid and amino sugar composition of β -lactamase I and β -lactamase II*

The crystalline enzyme preparations analysed were (a) those used for the determination of molecular weights (Kuwabara, 1970; Lloyd & Peacocke, 1970) and (b) similar preparations from different culture supernatants. Hydrolysis and analysis in amino acid analysers were carried out as described in the Materials and Methods section. The two sets of values under (b) were obtained from two successive determinations with the same preparation. Values in parentheses (c) are those for residues/mol given by Citri & Pollock (1966). Values for tyrosine and tryptophan determined spectrophotometrically by the methods of Holiday (1936) (*) and of Bencze & Schmid (1957) (†) are included. The values for β -lactamase II are relative to an arbitrary value of 4 residues for histidine. No correction has been made for destruction of amino sugars on hydrolysis.

Amino acid or amino sugar	Number of residues					
	β -Lactamase I (27 800 g)			β -Lactamase II (relative)		
	(a)	(b)	(c)	(a)	(b)	(c)
Lys	21	21	(21)	27	20	18
His	4	3	(5)	4	4	4
Arg	15	12	(10)	11	10	9
Cys†	—	0.3	0.4	—	1	0.9
Asp	31	31	(31)	32	34	30
Thr	20	21	(13)	18	18	16
Ser	14	16	(5)	14	19	17
Glu	23	23	(22)	22	28	25
Pro	9	13	(10)	8	13	12
Gly	20	20	(19)	19	25	22
Ala	31	28	(33)	25	22	20
Val	11	14	(17)	14	23	20
Met	4	4	(1)	3	3	3
Ile	19	17	(22)	16	13	12
Leu	19	18	(18)	20	21	18
Tyr	9	9	(4)	8	13	11
	11†	8*		7†	5*	
Phe	9	8	(6)	7	—	—
Trp	4			2		
	2†	3*		2†	3*	
Glucosamine	—	—		26	29	
Galactosamine	—	—		20	22	

† Determined as cysteic acid.

A plot of $E_{1\text{cm}}^{0.1\%}$ at 293nm against pH is shown in Fig. 1 and the results of a calculation of the tyrosine and tryptophan content of the enzymes by two different methods from the absorption curves at pH 13 are given in Table 1.

Carbohydrate content of β -lactamase II. When acid hydrolysates of β -lactamase II were run in the amino acid analysers two ninhydrin-positive compounds emerged from the column after phenylalanine in positions corresponding to those of glucosamine and galactosamine respectively (Table 1). After two-dimensional paper electrophoresis at pH 4.5 for 20 min and chromatography in butan-1-ol-acetic acid-water, a cherry-red spot was revealed by the acetylacetone-*p*-dimethylaminobenzaldehyde reagent and was indistinguishable in position (10.5 cm from the origin towards

the cathode and R_F 0.2) from that given by glucosamine or galactosamine.

The results of carbohydrate analyses of preparations of β -lactamase II by g.l.c. of silyl derivatives of neutral sugar and amino sugar residues are given in Table 2. The analyses showed close agreement and revealed the presence of glucose, galactose, mannose and fucose, as well as glucosamine and galactosamine. The amount of galactosamine, relative to the amount of glucosamine, was smaller than that obtained with the amino acid analyser, but the reason for this discrepancy has not been ascertained. There was no evidence of the presence of uronic acids or of muramic acid. The yield of trimethylsilyl glycopyranosides indicated that the amount of carbohydrate in the enzyme was not less than 20%.

Thiol content of β -lactamase I and β -lactamase II. For the determination of thiol groups by the method of Ellman (1959) the final solutions (3.2ml, pH 8.0) contained 1.22mg of β -lactamase I and 1.25mg of β -lactamase II protein respectively and were 6M with respect to guanidine. The extinctions at 412nm (0.065 and 0.084 respectively relative to the control solutions) corresponded with the

presence of 0.45mol of SH group/27800g of β -lactamase I protein and 0.35mol of SH group/27800g of β -lactamase II protein. In the former case the maximum extinction was obtained at the first reading and in the latter after 15 min.

Reaction with thiol reagents

Reactions with N-ethylmaleimide and iodoacetate. After treatment of a solution of Zn^{2+} -free β -lactamase II at pH 7.0 with 0.1mM-N-ethylmaleimide, only 30% of the original activity (with either benzylpenicillin or cephalosporin C as substrate) was restored by the addition of Zn^{2+} . With 1mM-N-ethylmaleimide this irreversible inactivation was virtually complete.

Only 30% of the original enzymic activity was restored by Zn^{2+} after reaction of Zn^{2+} -free β -lactamase II with 0.01mM-iodoacetate at pH 7.0 and no activity was restored when the concentration of the latter was raised to 1mM.

No inactivation of native (Zn^{2+} -containing) β -lactamase II in 1mM-buffer BZ was detected after treatment with 0.1mM-N-ethylmaleimide or 0.1mM-iodoacetate. However, in higher concentrations of iodoacetate (1mM) nearly complete inactivation was observed.

Treatment of β -lactamase I with 1mM-N-ethylmaleimide or 1mM-iodoacetate under the conditions used with β -lactamase II resulted in no significant loss of activity.

Fig. 1. Change of E_{293} with pH. ○, β -Lactamase I; ●, β -lactamase II. The pH was raised by addition of 1M-NaOH to solutions of recrystallized β -lactamase I (998 μ g/ml) in 0.1mM-buffer B and recrystallized β -lactamase II (1016 μ g/ml) in 0.1mM-buffer BZ (for details see the text).

DISCUSSION

The molecular weight calculated by Lloyd & Peacocke (1970) from sedimentation-equilibrium studies for β -lactamase II (35600) is higher than that for β -lactamase I (27800) and that calculated

Table 2. *Composition of the carbohydrate moiety of β -lactamase II*

Sample A was a preparation of β -lactamase that had been precipitated by $(NH_4)_2SO_4$ immediately before crystallization (Kuwabara, 1970). Sample B was material from the same batch after two crystallizations. Sample C was from a different batch at the same stage of purification as sample B. Amino sugars were determined as their *N*-acetyl derivatives. For details see the Materials and Methods section. The values are expressed as percentages by weight and as μ mol/100 μ mol of the total carbohydrate.

Sugar	Amount in sample					
	A		B		C	
	(% by wt.)	(μ mol/ 100 μ mol)	(% by wt.)	(μ mol/ 100 μ mol)	(% by wt.)	(μ mol/ 100 μ mol)
Fucose	6.8	8.1	7.5	8.8	7.0	8.3
Mannose	12.8	13.8	14.2	15.1	13.5	15.4
Galactose	23.7	25.6	25.2	26.9	24.4	26.1
Glucose	13.2	14.1	14.5	15.5	14.1	15.1
<i>N</i> -Acetylgalactosamine	15.4	13.6	13.4	11.7	14.2	12.4
<i>N</i> -Acetylglucosamine	28.2	24.8	25.2	21.9	26.8	23.6

earlier by Hall & Ogston (1956) for penicillinase from *B. cereus* 569/H (30 600). Hence the preparation of β -lactamase II would be expected to contain residues that are absent from β -lactamase I.

A striking difference in the composition of the crystalline preparations of β -lactamase I and β -lactamase II is the presence in the latter of a substantial carbohydrate fragment. The failure to find muramic acid in this fragment suggests that the latter is not derived from the murein of the cell wall.

It has been reported by Arcos (1968) that exo- β -lactamase produced by *B. cereus* 569/H can be separated by agar-gel electrophoresis into several active components and that some of the components are glycoproteins. The relative thermostability of β -lactamase II is a property that it shares with a number of glycoproteins and carbohydrate-protein complexes (Keller & Block, 1960; Putnam, 1965). However, the nature of the association of carbohydrate with protein and whether the carbohydrate is essential for the characteristic enzymic activity of β -lactamase II remain to be determined. The carbohydrate and protein were not dissociated by chromatography on DEAE-cellulose under the conditions used, but the loss of β -lactamase II activity when β -lactamase I is purified by adsorption on powdered glass or Celite (Sabath & Abraham, 1965; Kuwabara, 1970) raises the question whether a protein-carbohydrate complex is dissociated by this procedure.

The finding that β -lactamase II appears in the culture fluid before β -lactamase I is of interest in relation to Eylar's (1965) suggestion that the carbohydrate moiety of glycoproteins promotes the transport of these proteins from cells and needs play no functional role in their biological activity. Thus the glycoprotein ribonuclease B has been reported to have the same specific activity as ribonuclease A (Plummer & Hirs, 1963). But β -lactamase I and β -lactamase II differ clearly in their enzymic properties.

The changes in E_{293} shown in Fig. 1 indicate that most of the tyrosine residues in both enzymes ionize at a somewhat higher pK than normal unmasked tyrosine (pK 9.5–10). The presence of small amounts of an amino acid that behaved like cysteic acid in hydrolysates of β -lactamase I and β -lactamase II oxidized with performic acid is in apparent conflict with earlier reports that cyst(e)ine is absent from the penicillinase of *B. cereus* 569/H and from β -lactamases of other Gram-positive bacteria (Citri & Pollock, 1966; Pollock, 1968). Only a trace of cysteine was detected in hydrolysates of unoxidized β -lactamase I and none was found in those of β -lactamase II. The interpretation of these findings is complicated by the fact that only small amounts of the enzymes (<1 mg) were avail-

able for amino acid analysis, that cysteine has a low colour yield and is eluted with glutamic acid, and that carbohydrate is present in β -lactamase II (Schram, Moore & Bigwood, 1954). However, the yield of cysteic acid obtained after oxidation was less than 1 mol/mol of β -lactamase I, and it would also be less than 1 mol/mol of β -lactamase II if the molecular weight of the protein moiety of the latter enzyme were substantially lower than 27 800.

Determinations by the method of Ellman (1959) suggested that the preparations of both β -lactamase I and Zn^{2+} -free β -lactamase II contained free thiol groups, although the values obtained corresponded, with the former enzyme at least, to less than 1 mol of SH group/mol of protein. Sabath & Finland (1968) found that activation of crude β -lactamase II by Zn^{2+} was prevented by treatment with iodoacetate and with *N*-ethylmaleimide, and this has been confirmed in the present study with the purified enzyme. The simplest explanation of these findings is that a thiol group in β -lactamase II is involved in the binding of Zn^{2+} . But iodoacetate can react with histidine, methionine and lysine residues in protein as well as with the thiol group of cysteine (Gundlach, Stein & Moore, 1959). *N*-Ethylmaleimide can also react with residues other than a cysteine residue (Smyth, Blumenfeld & Konigsberg, 1964), and *N*-(*N'*-acetylsulphamoyl-phenyl)maleimide has been reported to react preferentially with the amino group of a lysine residue in glutamate dehydrogenase, even though the latter contains a free thiol group (Holbrook & Jeckel, 1969). Studies of the effect of thiol reagents on the apoenzyme of Zn^{2+} -requiring carboxypeptidase A led to the conclusion that a cysteine residue was one of the Zn^{2+} ligands (Vallee, Coombs & Hoch, 1960), but an X-ray-crystallographic analysis has shown that the two cysteine residues in this enzyme are linked by a disulphide bond and that the Zn^{2+} has three protein ligands provided by histidine, lysine and glutamic acid residues (Reeke *et al.* 1967; Lipscomb *et al.* 1968). In addition it appears that there may be other ligands that do not form part of the protein molecule. In view of these findings further studies will be required to establish whether a thiol group is a Zn^{2+} ligand in β -lactamase II.

We are grateful to the Medical Research Council for financial assistance, to Mrs Christina Walker and Mr Colin Castle for amino acid analyses and to Mrs P. B. Loder for thiol determinations.

REFERENCES

- Arcos, J. M. (1968). *Revta esp. Fisiol.* **24**, 137.
- Bencze, W. L. & Schmid, K. (1957). *Analyt. Chem.* **29**, 1193.
- Citri, N. & Pollock, M. R. (1966). *Adv. Enzymol.* **28**, 281.

- Ellman, G. L. (1959). *Archs Biochem. Biophys.* **82**, 70.
- Eylar, E. H. (1965). *J. theor. Biol.* **10**, 89.
- Gundlach, H. G., Stein, W. A. & Moore, S. (1959). *J. biol. Chem.* **234**, 1754.
- Hall, J. R. & Ogston, A. G. (1956). *Biochem. J.* **62**, 401.
- Hamilton, P. B. & Anderson, R. A. (1959). *Analyt. Chem.* **98**, 204.
- Hedgley, E. J. & Overend, W. G. (1960). *Chem. Ind.* p. 378.
- Holbrook, J. J. & Jeckel, R. (1969). *Biochem. J.* **111**, 689.
- Holiday, E. R. (1936). *Biochem. J.* **36**, 1795.
- Katz, A. N., Dreyer, W. J. & Anfinsen, C. B. (1959). *J. biol. Chem.* **234**, 2987.
- Keller, S. & Block, R. J. (1960). In *Analytical Methods of Protein Chemistry*, vol. 1, p. 9. Ed. by Alexander, P. & Block, R. J. Oxford: Pergamon Press Ltd.
- Kuwabara, S. (1970). *Biochem. J.* **118**, 457.
- Kuwabara, S. & Abraham, E. P. (1967). *Biochem. J.* **103**, 27 c.
- Lipscomb, W. N., Hartsuck, J. A., Reeke, G. N. jun., Quioco, F. A., Bethge, P. H., Ludwig, M. L., Steitz, T. A., Muirhead, H. & Coppola, J. G. (1968). *Brookhaven Symp. Biol.* **21**, 24.
- Lloyd, P. H. & Peacocke, A. R. (1970). *Biochem. J.* **118**, 463.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Moore, S. (1963). *J. biol. Chem.* **238**, 235.
- Moore, S., Spackman, D. H. Stein, W. H. (1958). *Analyt. Chem.* **30**, 1185.
- Noltmann, E. A., Mahowald, T. A. & Kuby, S. A. (1962). *J. biol. Chem.* **237**, 1146.
- Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
- Plummer, T. H., jun. & Hirs, C. H. W. (1963). *J. biol. Chem.* **238**, 1396.
- Pollock, M. R. (1968). *Ann. N.Y. Acad. Sci.* **151**, 502.
- Putnam, F. W. (1965). In *The Proteins*, vol. 3, p. 203. Ed. by Neurath, H. New York and London: Academic Press.
- Reeke, G. N., jun., Hartsuck, J. A., Ludwig, M. L., Quioco, F. A., Steitz, T. A. & Lipscomb, W. N. (1967). *Proc. natn. Acad. Sci. U.S.A.* **58**, 2220.
- Sabath, L. D. & Abraham, E. P. (1965). *Proc. 5th Inter-science Conf. Antimicrobial Agents and Chemotherapy*, p. 392.
- Sabath, L. D. & Abraham, E. P. (1966). *Biochem. J.* **98**, 11 c.
- Sabath, L. D. & Finland, M. (1968). *J. Bact.* **95**, 1513.
- Schram, E., Moore, S. & Bigwood, E. J. (1954). *Biochem. J.* **57**, 33.
- Smyth, D. G., Blumenfeld, O. O. & Konigsberg, W. (1964). *Biochem. J.* **91**, 589.
- Spackman, D. H., Stein, W. H. & Moore, S. (1958). *Analyt. Chem.* **30**, 1190.
- Sweeley, C. C., Bentley, R., Makita, M. & Wells, W. W. (1963). *J. Am. chem. Soc.* **85**, 2497.
- Sweeley, C. C. & Walker, B. (1961). *Analyt. Chem.* **36**, 1461.
- Vallee, B. L., Coombs, T. L. & Hoch, G. L. (1960). *J. biol. Chem.* **235**, 45.