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Behaviour of Tritium-Labelled Isopenicillin N and 6-Aminopenicillanic Acid as Potential Penicillin Precursors in an Extract of *Penicillium chrysogenum*

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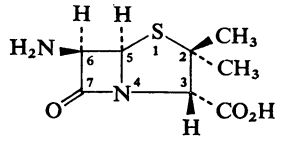
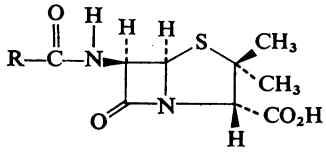
1. ^3H was incorporated into solvent-soluble penicillin from isopenicillin N and 6-aminopenicillanic acid ^3H-labelled in the 2β-methyl group when the labelled compounds were incubated with a crude extract of *Penicillium chrysogenum*. 2. With a soluble protein fraction of the extract incorporation from isopenicillin N occurred on addition of phenylacetyl-CoA. 3. Labelled benzylpenicillin was isolated after incubation of the crude extract with phenylacetyl-CoA and isopenicillin and the addition of unlabelled benzylpenicillin as a carrier. 4. No incorporation of ^3H into solvent-soluble penicillin was detected on incubation of these extracts with penicillin N.

The finding of 6-aminopenicillanic acid (compound I) in fermentations of *Penicillium chrysogenum* to which no side-chain precursor of a penicillin (compound II) had been added raised the question as to



(I)



(II)

whether 6-aminopenicillanic acid is an intermediate in penicillin biosynthesis (Batchelor *et al.*, 1959). Several enzymes have been described which catalyse the formation of a penicillin from 6-aminopenicillanic acid. Bacterial penicillin acylases bring about the reversible hydrolysis of benzylpenicillin to 6-aminopenicillanic acid and the transfer of a phenylacetyl group from phenylacetylglycine to 6-aminopenicillanic acid (Kaufmann & Bauer, 1964; Cole, 1969). A penicillin acyltransferase from *P. chrysogenum* catalyses the isotopic exchange of ^{35}S between certain penicillins and 6-aminopenicillanic acid (Pruess & Johnson, 1967). A 6-aminopenicillanic acid acyltransferase produces penicillins from an acyl-CoA and 6-aminopenicillanic acid (Spencer, 1968; Gatenbeck & Brunsberg, 1968; Brunner *et al.*, 1968). It has been suggested that these activities in extracts of *P. chrysogenum* are associated with a single thiol-dependent enzyme (Spencer & Maung, 1970).

The discovery of penicillin N, with a δ-(D-α-aminoadipyl) side chain (RCO in compound II), in the culture fluid of a strain of *Cephalosporium acremonium* (Newton & Abraham, 1954) and of the tripeptide, δ-(α-aminoadipyl)cysteinylvaline, in the mycelium of *P. chrysogenum* (Arnstein & Morris, 1960) led to the suggestion that penicillin N was a precursor of benzylpenicillin (and other penicillins with non-polar side chains) produced by the latter organism. Evidence for the view that α-aminoadipic acid has a role in the biosynthesis of benzylpenicillin was obtained by Somerson *et al.* (1961), who showed that production of the latter was stimulated by DL-α-aminoadipic acid but inhibited by L-lysine. The subsequent finding of isopenicillin N [with a δ-(L-α-aminoadipyl) side chain] in *P. chrysogenum* (Flynn *et al.*, 1962; Cole & Batchelor, 1963) and of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine in *C. acremonium* (Loder & Abraham, 1971) made isopenicillin N a more likely precursor than penicillin N itself (Demain, 1966). Vanderhaeghe *et al.* (1968) reported that neither isopenicillin N nor penicillin N was hydrolysed to 6-aminopenicillanic acid by the penicillin acylase from a strain of *P. chrysogenum*. However, Goulden & Chattaway (1968) showed that mutants of *P. chrysogenum* which were unable to synthesize α-aminoadipic acid were unable to synthesize benzylpenicillin unless α-aminoadipic acid was added to the culture medium.

Loder (1972) and Abraham (1974) reported briefly that the addition of either 6-aminopenicillanic acid or synthetic isopenicillin N to a crude extract of *P. chrysogenum* increased the ability of the latter to incorporate ^{14}C from [^{14}C]phenylacetyl-CoA into

benzylpenicillin. The present paper describes experiments with isopenicillin N, penicillin N and 6-aminopenicillanic acid labelled with ^3H in the ring system (Usher *et al.*, 1975). These experiments were designed to show whether one or more of the labelled compounds yield radioactive solvent-extractable penicillins, such as benzylpenicillin, in an extract of *P. chrysogenum*.

Materials and Methods

Materials

Coenzyme A (lithium salt) was from Sigma Chemical Co., London S.W.6, U.K. A solution of β-lactamase I from *Bacillus cereus* (penicillinase) was provided by Dr. M. Brightwell (see Davies *et al.*, 1974). Sephadex G-25 (coarse) was from Pharmacia Fine Chemicals, Uppsala, Sweden. Benzylpenicillin (sodium salt) was from Glaxo Laboratories Ltd., Greenford, Middx., U.K. Hi-Soy flour was from the British Arkady Co. Ltd., Old Trafford, Manchester, U.K., and corn-steep liquor from Garton Sons and Co. Ltd., London S.W.11, U.K. Sand (fine sifted) and other reagents (AnalaR grade where available) were from BDH Chemicals Ltd., Poole, Dorset, U.K. The sand was washed successively with 3M-HCl, 2M-NaOH, 1% (w/v) EDTA and water and then dried before use.

Phenylacetyl-CoA was prepared by the method of Stadtman (1957). Isopenicillin N, penicillin N and 6-aminopenicillanic acid labelled with ^3H in the 2-β-methyl group were synthesized as described by Usher *et al.* (1975).

Methods

Culture and growth conditions. The strain of *P. chrysogenum* (SC 6140) used in these experiments was kindly provided by The Squibb Institute for Medical Research, Princeton, N.J., U.S.A. Freeze-dried cultures were stored in ampoules. Distilled water was added to the ampoules and the cultures were transferred to the surface of glycerol–agar slopes (Gailey *et al.*, 1946) and incubated at 27°C for 7–14 days. The surface growth from a 6oz medical flat was transferred to a 500ml baffled Erlenmeyer flask (Smith *et al.*, 1967) containing 100ml of germination medium. The latter had the following composition: Hi-Soy flour, 30g; glucose, 1.5g; sucrose, 15g; lard oil, 5ml; water, 1litre. After growth at 27°C and at 140rev./min for 48h, portions (10ml) of the resulting culture were used to inoculate the fermentation medium (100ml) in 500ml wide-mouthed Erlenmeyer flasks. The composition of the fermentation medium was as follows: corn-steep liquor, 60g; lactose, 90g; $(NH_4)_2SO_4$, 4g; KH_2PO_4, 4g; Na_2SO_4, 1.5g; $CaCO_3$, 10g; soya-bean oil, 10ml; water, 1litre; side-chain precursor (1.0ml of a solution containing 20g of *N*-methylphenylacetamide/litre and 20g of potassium phenylacetate/litre) was added to each flask on transfer to the fermentation medium and at 24h intervals. The fermentation was carried out at 27°C and at 300rev./min for 96h.

Preparation of mycelial extract. Cultures were harvested after 96h, the beginning of the rapid phase of antibiotic production, and filtered through hardened paper in a Buchner funnel. The mycelium was washed on the filter with three 100ml portions of water. The yield of damp-dry mycelium was about 8g/flask.

Portions (usually 10.0g) of the damp-dry mycelium were ground by hand in a mortar (cooled in crushed ice) with an equal weight of acid-washed sand for 10min. Buffer (1ml/10g of mycelium) was added and the grinding continued for 5min. The buffer used (pH7.8) was based on that of Gatenbeck & Brunsberg (1968) and contained 0.2M-NaCl, 0.2M-Tris, 0.05M-KH_2PO_4, 0.001M-EDTA. The paste was centrifuged at 2500*g* for 5min and the turbid supernatant (approx. 4.0ml), which was free from sand and whole cells, was removed and kept cold. This is referred to subsequently as 'crude extract'. Its protein content, determined by the microbiuret procedure of Goa (1953), was 23.7mg/ml. A particulate fraction of the disrupted mycelium was separated from the sand by three resuspensions of the 2500*g* pellet in buffer (each approx. 8ml) followed by decantation and centrifugation at 2500*g* for 5min. The particulate material from 2g of mycelium was resuspended in 2ml of buffer.

For some experiments the crude extract was centrifuged at 20000*g* and at 4°C for 30min. The clear supernatant (approx. 5ml) was removed and applied to a column (25cm×1.5cm diam.) of Sephadex G-25 (coarse) in the pH7.8 buffer described above at 4°C. Elution was carried out with the same buffer and 2ml fractions were collected. The protein-containing fractions (approx. 10ml) were pooled.

Incubation of isopenicillin N and 6-aminopenicillanic acid with extracts. Dithiothreitol was added to the whole extract, the Sephadex G-25 filtrate and to the particulate fraction to give a final concentration of 10mM. To 2ml portions of the extract or the protein fraction was added 8.0μCi of a solution (0.4μCi/μl) of ^3H-labelled isopenicillin N (371mCi/mmol) or 8.3μCi of a solution (0.55μCi/μl) of ^3H-labelled 6-aminopenicillanic acid (373mCi/mmol), both compounds being labelled in the 2-β-methyl group. Phenylacetyl-CoA (1μmol) was added in some experiments. The mixtures were incubated in stoppered tubes at 28°C for 2h and then kept frozen for 2h before use.

Analysis of the products. The incubation mixtures were thawed and 250μg of benzylpenicillin (sodium salt) was added to each. They were then centrifuged at 25000*g* and at 0°C for 20min and the supernatants

were removed and kept cold. Penicillin was extracted into butyl acetate (2×3ml) after adjustment of the pH of the aqueous phase to 2.0 with 1M-HCl and then re-extracted into 0.5mM-K_2HPO_4 (2ml) at pH6.5. The aqueous solution was divided into two parts and freeze-dried. The residue from half of the solution was dissolved in water and the remainder was dissolved in a solution of β-lactamase I from *B. cereus* (4.6×10^5 units/ml), and kept at room temperature (20°C) for 30min. Control samples of isopenicillin or 6-aminopenicillanic acid in buffer, but without enzyme, were treated in the same manner. Samples, usually one-sixth or one-eighth of the total, were applied to paper for (a) chromatography at 4°C in butan-1-ol–ethanol–water (4:1:5, by vol., top phase) on paper which had been dipped into 0.1M-sodium phosphate, pH6.2, blotted and dried in air and (b) electrophoresis at 13V/cm in collidine–acetate buffer (0.05M with respect to acetate), pH 7.0, for 3h. Benzylpenicillin was treated in the same manner and used as a marker.

The positions of benzylpenicillin and its penicilloate on paper were located from the markers by spraying the latter with 0.5M-NaOH followed by starch–iodine (Thomas, 1961). The radioactivity in these positions was determined by elution of the compounds from the paper with water and freeze-drying. The residue was then dissolved in a known volume of water and counted for radioactivity, after the addition of 3.0ml of the water-miscible scintillation fluid of Bruno & Christian (1960), in a Unilux 11A scintillation counter. The disappearance of radioactivity from the benzylpenicillin position and its appearance in the penicilloate position on treatment with penicillinase was also monitored by cutting the paper into 3cm × 1cm segments and immersing each in the scintillation fluid without prior elution. The counting efficiency of this method, however, was low (11–17%).

Isolation of ^{3}H-labelled benzylpenicillin. ^{3}H-labelled isopenicillin (8μCi) was incubated for 2h at 28°C with (1) phenylacetyl-CoA in the presence of 2ml of the extract of *P. chrysogenum* under the conditions described above and with (2) phenylacetyl-CoA under the same conditions except that the extract was replaced by water. The mixtures were centrifuged at 25000*g* for 20min and to each solution was added 120mg of solid benzylpenicillin (potassium salt). Each solution was then covered with methyl isopropyl ketone (20ml) and cooled to 0°C, after which the pH of the vigorously stirred mixture was decreased to 2.0 by addition of 0.1M-H_2SO_4. The layers were separated by centrifugation. The organic phase was washed with ice-cold water (3×5ml) and dried over anhydrous $MgSO_4$. A solution (0.12ml, 0.3mmol) of potassium 2-ethylhexanoate in butan-1-ol was added (Gourevitch *et al.*, 1962). White crystalline needles of benzylpenicillin (potassium salt; about 65mg) appeared on storage of the mixtures overnight. A portion of these products (30mg) was recrystallized by solution in water (0.2ml) and addition of acetone (6ml). The crystals were washed with acetone (2×5ml) and dried (18mg).

For determination of radioactivity, samples (10mg) of these products were weighed into a scintillation counting vial; water (0.1ml) was added, then 3.0ml of water-miscible scintillation fluid, and the sample counted for radioactivity for 1min.

Results

Incorporation of ^{3}H from ^{3}H-labelled isopenicillin N and 6-aminopenicillanic acid into solvent-extractable penicillin

Table 1 shows the amounts of radioactivity incorporated into solvent-extractable penicillin on incubation of isopenicillin N labelled with ^{3}H in the 2-β-methyl group with preparations of a crude extract, a soluble protein fraction and a particulate fraction respectively of *P. chrysogenum*. It also shows the incorporation of radioactivity in corresponding experiments in which labelled 6-aminopenicillanic acid was incubated with the crude extract and the particulate fraction. In some cases phenylacetyl-CoA was added to the mixture before incubation, although the crude extract itself, which was obtained from mycelium grown in the presence of potassium phenylacetate and *N*-methylphenylacetamide, presumably contained available side-chain precursor.

In a crude extract to which no phenylacetyl-CoA was added, ^{3}H was incorporated from isopenicillin N into solvent-extractable penicillinase-sensitive material which behaved like benzylpenicillin on paper chromatography in butan-1-ol–ethanol–water (4:1:5, by vol.) (Table 1, Expt. 1). No increase in incorporation was observed on addition of phenylacetyl-CoA (Table 1, Expt. 3). The radioactivity profiles obtained after analysis of the material extracted into butyl acetate by electrophoresis at pH7.0 and chromatography in butan-1-ol–ethanol–water before and after treatment with penicillinase are shown in Figs. 1 and 2 respectively. The addition of penicillinase to the extract caused a decrease in the radioactivity in the position of benzylpenicillin and an increase in the position of the corresponding penicilloate. However, since other solvent-extractable penicillins run to similar positions in both systems, the nature of the side chain was not revealed by these experiments. If a trace amount of ^{3}H-labelled isopenicillin N had been a contaminant of the extracted material this would not have been resolved from the solvent-soluble penicillins on electrophoresis. But in the chromatographic system used, isopenicillin N remains in a position close to the origin and is well

Table 1. *Incorporation of ^3H from labelled isopenicillin N and 6-aminopenicillanic acid into solvent-extractable penicillin by P. chrysogenum*

The crude extract of *P. chrysogenum* and the particulate and protein fractions derived from it were prepared as described under 'Methods'. In each experiment, 2 ml of enzyme preparation was used, the crude extract being derived from 5 g of damp-dry mycelium, the particulate fraction from 2.09 g and the protein fraction from 2.5 g. Phenylacetyl-CoA (1 μmol), ^3H-labelled isopenicillin N (IPN) (8.0 μCi) and 6-aminopenicillanic acid (6-APA) (8.3 μCi) were added where indicated. In control experiments buffer but no enzyme preparation was used. The mixtures were incubated for 2 h at 28°C. (For details see under 'Methods'.)

Expt.	Enzyme preparation	Additions: ^3H-labelled compound	Additions: Phenylacetyl-CoA	Total radioactivity (nCi) in: Butyl acetate extract	Total radioactivity (nCi) in: Residual aqueous phase	Change in radioactivity (nCi) with penicillinase treatment after paper chromatography in spots corresponding to: Benzylpenicillin	Benzylpenicilloate
1	Crude extract	IPN	−	315	3957	−111	+104
	Control		−	30	5561	−5.9	+5.5
2	Protein fraction	IPN	−	57	3450	−4.7	+5.0
	Protein fraction		+	109	3990	−25	+23
3	Crude extract	IPN	−	221	4190	−73	+34
	Crude extract		+	169	4600	−49	+20
	Particulate fraction		−	24	2100	−2.6	+1.1
	Particulate fraction		+	29	1200	−3.5	+1.7
4	Crude extract	6-APA	−	1350	2040	−249	+100
	Control		−	312	4900	−34	+5.7
5	Crude extract	6-APA	−	1080	2840	−119	+113
	Crude extract		+	1850	2930	−142	+128
	Particulate fraction		−	303	2380	−16	+13
	Particulate fraction		+	412	2570	−27	+42
6	Crude extract	IPN	−	338	3970	−68	+47
	Crude extract	6-APA	−	957	3130	−115	+109

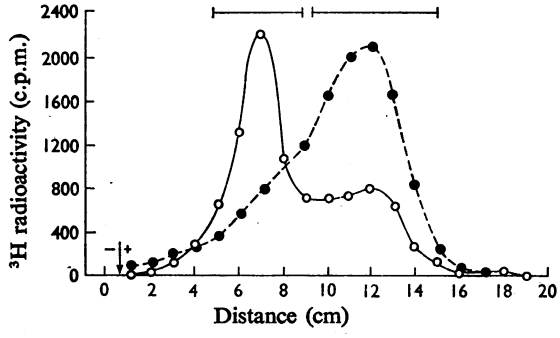


Fig. 1. *Radioactivity profiles after paper electrophoresis at pH 7.0*

○, Solvent-extractable material; ●, this material after treatment with β-lactamase. The bars indicate the locations of marker spots of benzylpenicillin (left) and benzylpenicilloate (right). The arrow marks the origin.

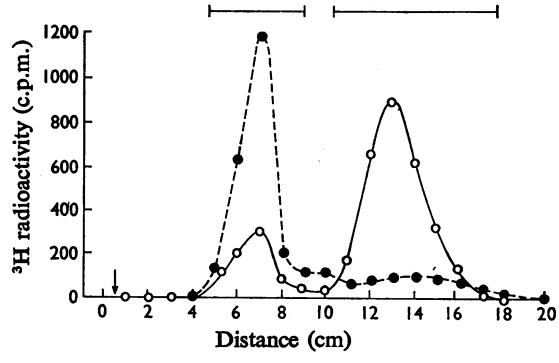


Fig. 2. *Radioactivity profiles after chromatography on paper in butan-1-ol–ethanol–water* (4:1:5, *by vol.*)

○, Solvent-extractable material; ●, this material after treatment with β-lactamase. The bars indicate the locations of marker spots of benzylpenicillin (right) and benzylpenicilloate (left). The arrow marks the origin.

separated from the penicillins with non-polar side chains.

When ^3H-labelled isopenicillin N was added to the protein fraction of the extract in the absence of phenylacetyl-CoA, the radioactivity in solvent-extractable penicillinase-sensitive material was similar to that found in a control containing no enzyme preparation. However, significant incorporation was obtained after addition of side-chain precursor to the protein fraction (Table 1, Expt. 2). With the particulate fraction of the extract incorporation of

radioactivity was low (Table 1, Expt. 3), even when allowance was made for the difference in the amount of mycelium from which the crude extract and particulate fraction were obtained (see under 'Methods').

In a further experiment, ^{3}H-labelled isopenicillin N and phenylacetyl-CoA were added to a crude extract and also to a control lacking the enzyme system. After incubation, unlabelled benzylpenicillin was added to the extract as a carrier. Radioactive benzylpenicillin was extracted into isopropyl methyl ketone and isolated as a crystalline potassium salt which had an antibacterial activity of 1820units/mg and a radioactivity of 0.97nCi/mg. The corresponding activities after recrystallization of this product from aq. acetone were 1540units/mg and 0.89nCi/mg. The corresponding values from the control incubation were 1645units/mg and 0.06nCi/mg, which became 1440units/mg and 0.06nCi/mg after recrystallization. When unlabelled benzylpenicillin was added to a solution of ^{3}H-labelled isopenicillin N alone and extracted in a similar way, the benzylpenicillin obtained after recrystallization gave activities of 0.059nCi/mg and 1720units/mg. The values obtained in these control experiments could have been due to carry-over of traces of radioactivity from the preparation of isopenicillin N and might have been decreased by more thorough washing of the isopropyl methyl ketone extract with water.

When ^{3}H-labelled 6-aminopenicillanic acid was incubated with the crude extract under conditions similar to those used with labelled isopenicillin N, the incorporation of ^{3}H into solvent-soluble penicillin was again observed, but with the particulate fraction of the extract the incorporation was considerably less (Table 1, Expts. 4 and 5). In a further experiment (Expt. 6) in which the labelled isopenicillin N and 6-aminopenicillanic acid were separately added to samples of the same crude extract, the incorporation from the latter appeared to be somewhat more efficient.

In a number of experiments in which a similar amount of ^{3}H-labelled penicillin N of the same specific radioactivity as the isopenicillin N was added to the crude extract, and also to the protein fraction in the presence and absence of phenylacetyl-CoA, no formation of a solvent-soluble penicillin was detected.

Discussion

The evidence obtained by Loder (1972) for the conversion of isopenicillin N, as well as 6-aminopenicillanic acid, into benzylpenicillin in an extract of *P. chrysogenum* was based on the apparent ability of the first two compounds to stimulate the incorporation of ^{14}C into benzylpenicillin from [^{14}C]phenylacetic acid and [^{14}C]phenylacetyl-CoA. It appeared that firmer conclusions could be drawn about this aspect of penicillin biosynthesis by the use of radioactive isopenicillin N and 6-aminopenicillanic acid labelled in the ring system.

The results obtained in the present work indicated that radioactivity from isopenicillin N labelled with ^{3}H in its 2-β-methyl group was incorporated into solvent-soluble penicillin and hence that the isopenicillin N was a substrate for an acyltransferase enzyme. Whether there is a transient formation of free 6-aminopenicillanic acid on the pathway from isopenicillin N to penicillins such as benzylpenicillin, or whether 6-aminopenicillanic acid is formed but not liberated from an enzyme complex, remains unknown. The extract was also shown to catalyse the conversion of 6-aminopenicillanic acid into a solvent-extractable penicillin, confirming earlier findings such as those of Gatenbeck & Brunsberg (1968). However, it has yet to be determined whether isopenicillin N and 6-aminopenicillanic acid are substrates for two different enzymes.

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References

Abraham, E. P. (1974) *Biosynthesis and Enzymic Hydrolysis of Penicillins and Cephalosporins*, University of Tokyo Press, Tokyo

Arnstein, H. R. V. & Morris, D. (1960) *Biochem. J.* **76**, 357–361

Batchelor, F. R., Doyle, F. P., Nayler, J. H. C. & Rolinson, G. N. (1959) *Nature (London)* **183**, 257–258

Brunner, R., Rohr, M. & Zinner, M. (1968) *Hoppe-Seyler's Z. Physiol. Chem.* **349**, 95–103

Bruno, G. A. & Christian, J. E. (1960) *J. Am. Pharm. Ass.* **49**, 560–561

Cole, M. (1969) *Biochem. J.* **115**, 733–739

Cole, M. & Batchelor, F. R. (1963) *Nature (London)* **198**, 383–384

Davies, R. B., Abraham, E. P. & Melling, J. (1974) *Biochem. J.* **143**, 115–127

Demain, A. L. (1966) *Biosynth. Antibiot.* **1**, 29–94

Flynn, E. H., McCormick, M. H., Stamper, M. C., De Valeria, H. & Godzeski, C. W. (1962) *J. Am. Chem. Soc.* **84**, 4594–4595

Gailey, F. B., Stefaniak, J. J., Olson, B. H. & Johnson, M. J. (1946) *J. Bacteriol.* **52**, 129–140

Gatenbeck, S. & Brunsberg, U. (1968) *Acta Chem. Scand.* **22**, 1059–1061

Goa, J. (1953) *Scand. J. Clin. Lab. Invest.* **5**, 218–222

Goulden, S. A. & Chattaway, F. W. (1968) *Biochem. J.* **110**, 55P–56P

Gourevitch, A., Holdrege, C. T., Hunt, G. A., Minor, W. F., Flanigan, C. C., Cheney, L. C. & Lein, J. (1962) *Antibiot. Chemother. (Washington, D.C.)* **12**, 318–324

Kaufmann, W. & Bauer, K. (1964) *Nature (London)* **203**, 520
Loder, P. B. (1972) *Postepy. Hig. Med. Dosw.* **26**, 493–500
Loder, P. B. & Abraham, E. P. (1971) *Biochem. J.* **123**, 477–482
Newton, G. G. F. & Abraham, E. P. (1954) *Biochem. J.* **58**, 103–111
Pruess, D. L. & Johnson, M. J. (1967) *J. Bacteriol.* **94**, 1502–1508
Smith, B., Warren, S. C., Newton, G. G. F. & Abraham, E. P. (1967) *Biochem. J.* **103**, 877–890
Somerson, N. L., Demain, A. L. & Nunheimer, T. D. (1961) *Arch. Biochem. Biophys.* **93**, 238
Spencer, B. (1968) *Biochem. Biophys. Res. Commun.* **31**, 170–175
Spencer, B. & Maung, C. (1970) *Biochem. J.* **118**, 29P–30P
Stadtman, E. R. (1957) *Methods Enzymol.* **3**, 931–941
Thomas, R. (1961) *Nature (London)* **191**, 1161–1163
Usher, J. J., Loder, P. B. & Abraham, E. P. (1975) *Biochem. J.* **151**, 729–739
Vanderhaeghe, H., Claesen, M., Vlietinck, A. & Parmentier, G. (1968) *Appl. Microbiol.* **16**, 1557–1563