We wish to express our thanks to Dr Melvin Cohn for much valuable advice during the preliminary work on methods of purification, and one of us (M.R.P.) is greatly indebted to Dr Jacques Monod of the Institut Pasteur, Paris, for the hospitality of his laboratory in which much of the earlier work on isolation of penicillinase was carried out. We are also grateful to Dr T. S. Work for help in the chromatographic estimation of amino acids. The photographs were taken by Mr M. R. Young.

The Perkin–Elmer Tiselius apparatus used for electrophoresis analysis was purchased by means of a grant from Eli Lilly and Co.

REFERENCES

Abraham, E. P. (1951). In *The Enzymes*, vol. 1, part 2, p. 1170. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press.

Abraham, E. P. & Chain, E. (1940). *Nature, Lond.*, **146**, 837.

Baudet, P. & Hagemann, G. (1954). *Experientia*, **10**, 374.

Hall, J. R. & Ogston, A. G. (1956). *Biochem. J.* **62**, 401.

Henry, R. J. & Housewright, R. D. (1947). *J. biol. Chem.* **167**, 559.

Kogut, M., Pollock, M. R. & Tridgell, E. J. (1956). *Biochem. J.* **62**, 391.

Low, B. W. & Richards, F. M. (1954). *J. Amer. chem. Soc.* **76**, 2511.

Ma, T. S. & Zuazaga, G. (1942). *Industr. Engng Chem. (Anal.)*, **14**, 280.

McQuarrie, E. B. & Liebmann, A. J. (1944). *Arch. Biochem.* **5**, 307.

Morgan, J. F. & Campbell, M. E. (1947). *J. biol. Chem.* **169**, 337.

Murao, S. (1950). *Symp. Enz. Chem. (Japan)*, **5**, 81.

Pollock, M. R. (1950). *Brit. J. exp. Path.* **31**, 739.

Pollock, M. R. & Torriani, A.-M. (1953). *C.R. Acad. Sci., Paris*, **237**, 276.

Roland, J. F. & Gross, E. M. (1954). *Analyt. Chem.* **26**, 502.

Sneath, P. H. A. (1955). *J. gen. Microbiol.* **13**, 561.

Wainwright, S. D. (1950). Thesis submitted for Ph.D. degree, University of London.

Purification of Penicillin-Induced Penicillinase of *Bacillus cereus* NRRL 569: A Comparison of its Properties with those of a similarly Purified Penicillinase produced Spontaneously by a Constitutive Mutant Strain

By MARGOT KOGUT,* M. R. POLLOCK and E. J. TRIDGELL
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 8 *July* 1955)

Ever since the phenomenon of microbial enzyme adaptation began to be studied seriously it has been of obvious interest and importance to determine the nature of the substance or substances responsible for the increase in enzyme activity that occurs after treatment of cells with the specific inducer. Although it has often been assumed—in many cases not unreasonably—that the development of enzyme activity corresponded to the production of an equivalent amount of some distinct, enzymically active protein, direct proof of this assumption (for *Escherichia coli* β-galactosidase induction) was not provided until quite recently (Cohn & Monod, 1951). A further query, which could not be answered until the induced enzyme had been isolated and purified, was whether it showed any peculiarities or distinctive features that might place it in a class apart from 'ordinary' (constitutive) enzymes produced by cells spontaneously in the absence of inducer. More specifically this question may take the form of the problem as to whether a spontaneously produced (i.e. basal or constitutive) enzyme belongs to the same molecular species as the catalytically similar enzyme evoked after addition of a specific inducer. If the 'spontaneous' and the induced enzymes consisted of different types of protein molecule, the mechanisms for their production would probably be quite distinct; and the inducer would have to be considered as initiating the formation of a new kind of protein instead of merely increasing the rate of production of a molecular species already formed to some extent in its absence. The 'unitary hypothesis', discussed by Cohn & Monod (1953), which postulates basically similar mechanisms for the formation of constitutive and induced enzymes, would be thus greatly weakened.

This question, which we have attempted to answer in this paper, can be best approached by the comparative study of a purified induced enzyme and the catalytically similar enzyme formed by the same, or closely related, cells in the absence of inducer. Ideally, such a comparison should be made between purified induced enzyme and the constitutive moiety (usually referred to as 'basal enzyme') produced by the same cells without

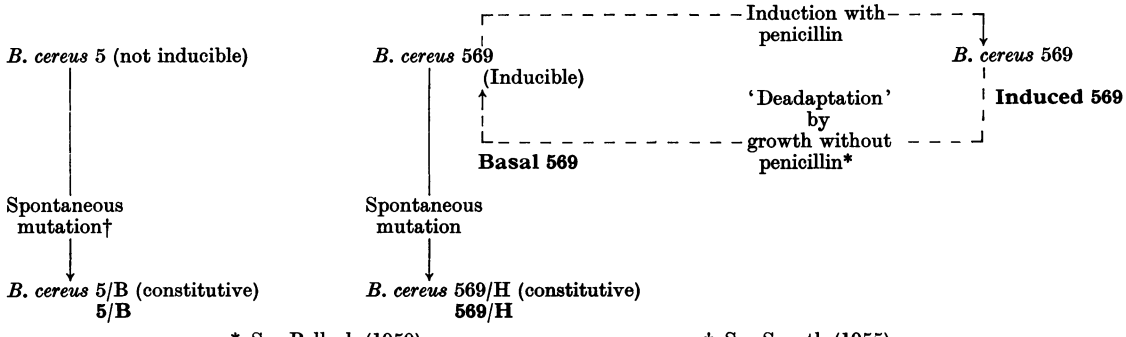
* Present address: Agricultural Research Council, Unit for Microbiology, University of Sheffield.

added inducer. So far, however, this has not been achieved because of the very small amounts of basal enzyme formed by most inducible cultures. Comparisons have indeed been made with impure enzyme preparations in the case of the β-galactosidase of *Esch. coli* (Monod & Cohn, 1952), the exocellular penicillinases of *Bacillus subtilis* and *B. cereus* (Manson, Pollock & Tridgell, 1954), and with partially purified β-galactosidase preparations from *Neurospora* (Landman, 1954). No significant differences were detected. As pointed out by Manson *et al.* (1954), however, analysis of impure preparations does not permit an accurate comparison of properties such as sedimentation and diffusion constants, specific activity, electrophoretic mobility, salt-solubility, amino acid analysis, etc., which might be expected to disclose small differences more readily than measurement of pH/activity curves, affinity constants, thermal stability, etc., carried out by these groups of workers. Indeed, this argument was reinforced by the finding that the penicillinases from *B. cereus* 5/B (see Sneath, 1955) and from penicillin-induced *B. cereus* 569 do not differ significantly in pH/activity relationships or in Michaelis constants (Pollock & Tridgell, unpublished), although they were later (see Table 3) found to be quite distinct in physicochemical properties.

The nearest approach to a comparison of purified constitutive and induced enzymes produced in the same organism would be provided by a comparative study of the enzyme produced by closely related strains, differing only in the extent to which addition of inducer was essential for maximal enzyme formation. *B. cereus* 5, which is the parent strain of *B. cereus* 5/B (Sneath, 1955), and which produces no penicillinase detectable by manometric assay, cannot be induced to form the enzyme by treatment with penicillin; nor has an inducible mutant ever been isolated from either 5 or 5/B. A constitutive mutant has, however, been successfully isolated from the inducible strain 569. Table 1 illustrates the relationship of the strains referred to and the enzymes they synthesize. This constitutive strain, 569/H, produces about the same enzyme activities as 5/B under similar conditions, but is distinguishable from 569 only by its independence of penicillin for the formation of large quantities of penicillinase. It seemed, therefore, to be a satisfactory source of enzyme for comparison with the induced enzyme of 569.

Table 1. *Origin and relationship of bacterial strains and penicillinases referred to in the text*

The names of the strains are printed in italics and those of the enzymes in heavy type.



* See Pollock (1950). † See Sneath (1955).

Pollock & Torriani (1953) isolated a highly active preparation of induced 569 penicillinase from 50 l. of culture in a peptone medium. But although this preparation showed a single peak on electrophoresis, ultracentrifugal analysis (Dr A. G. Ogston, private communication) indicated heterogeneity, and significantly different sedimentation constants and boundary-gradient shapes were obtained with two separate batches prepared by similar techniques. It was thought possible that some contaminating protein ingredient of the peptone might have formed complexes with the penicillinase and thus have been responsible for these inconsistent results. The successful purification of *B. cereus* 5/B exocellular penicillinase (Pollock, Torriani & Tridgell, 1956) produced in a casein-hydrolysate medium encouraged the hope that the same medium and methods could be employed for the production and purification of 569 (induced) and 569/H penicillinases.

METHODS OF PRODUCTION AND PURIFICATION

General

Penicillinase assay, nitrogen estimations and specific activity measurements on enzyme samples. These were made as described in the preceding paper (Pollock *et al.* 1956).

Organisms. (*a*) *B. cereus*, NRRL 569 (referred to as 569): the inducible strain used for previous work on penicillinase adaptation (see Pollock, 1953). Treatment of cells with penicillin will cause up to a 500-fold increase in the rate of penicillinase formation.

(*b*) *B. cereus* 569/H (referred to as 569/H). This constitutive mutant, derived from 569 was isolated as follows. As previously pointed out (Manson *et al.* 1954), inability to isolate a constitutive mutant by subculture of the inducible strain in a penicillin-containing medium could be attributed to the negligible selective advantage that such a mutant might have in the presence of rapidly inducible parent cells. For this reason no velvet-pad isolation (Lederberg & Lederberg, 1952) was ever attempted. Instead, five large square dishes (normally used for antibiotic assay; Kantorowicz, 1951) were prepared as follows: bottom layer: 150 ml. of 'S' peptone broth, pH 7·6 (Pollock & Perret, 1951) containing 1·5% agar; middle layer: 100 ml. of 'S' broth with 3% agar inoculated with 10^6 spores of 569; top layer: 100 ml. of 'S' broth with 3% agar. All layers contained 4% of Andrade indicator (colourless at pH 7·6). The plates were incubated at 30° for 16 hr. Growth appeared as a semi-confluent 'haze' of micro-colonies. A solution of penicillin (10 ml.; 2×10^5 units/ml.) was added to each plate, which was left on a levelled table at room temperature and carefully inspected every 10 min. After 45 min. one small pink area (indicating hydrolysis of penicillin to penicilloic acid) was noted in one of the plates. The agar including this pink spot was cut out, fragmented, and incubated for 4 hr. in 5 ml. of broth, a drop of which was then streaked on plates of nutrient agar containing Andrade indicator. Incubation of these plates overnight at 35°, followed by addition of concentrated penicillin solution, resulted in rapid development of pink colour around 30% of the colonies, showing them to be constitutive penicillinase producers; the 569/H strain was thus purified. In order to be certain that this single micro-colony was derived directly from 569, and was not an aerial contaminant, the whole procedure was repeated with spores of a doubly marked strain (isolated by Dr P. H. A. Sneath) derived in two successive steps from 569. This double mutant (569/f/1) could be identified by its resistance to streptomycin and by the formation of a characteristic yellow pigment which was liberated into the surrounding medium. A constitutive penicillinase mutant was isolated in a similar manner from a total 5×10^6 spores of 569/f/1, and, on testing, was found to be streptomycin-resistant and capable of producing a yellow pigment. It thus seemed reasonable to conclude (*a*) that 569/H was likewise derived by mutation from 569, and (*b*) that the transformation was due to a single spontaneous mutation, occurring *before* addition of the penicillin, which can have acted (in the 45 min. elapsing between its addition and evidence of penicillinase action) only as an indicator of enzyme already formed by the micro-colony under test.

Medium (CH/C). This comprised: Difco casamino acids (technical), 10 g.; KH_2PO_4, 2·72 g.; sodium citrate, 5·88 g. dissolved in 200 ml. of water; conc. NaOH solution added to give pH 7·2, and water to 1 l. It was sterilized by autoclave. A portion (10 ml.) of a solution containing 0·51 g. of $MgSO_4,7H_2O$ and 3 ml. of a 0·16% solution of $(NH_4)_2Fe(SO_4)_2,6H_2O$ was autoclaved and added to the solution, separately.

Enzyme production

The first attempt at large-scale production and isolation of *B. cereus* 569 induced penicillinase from a casein-hydrolysate culture in a 320 l. deep fermenter ended in failure. The reasons for this were: (1) Low enzyme titre following induction in the casein-hydrolysate medium; (2) inactivation of the enzyme by excessive foaming, which could not be completely controlled in the deep fermenter, even by continual addition of Silicone antifoam; (3) large losses of enzyme which occurred during the preliminary concentration of 320 l. of supernatant fluid by distillation under reduced pressure (lasting 2·5 days) and final concentration by pressure dialysis.

It was clear that, before proceeding further, it would be necessary to solve the problems (*a*) of enzyme stabilization in the casein-hydrolysate medium during and immediately after culture, and (*b*) of rapid and efficient concentration of enzyme from large volumes of culture supernatant fluid. The first problem was solved by Mlle Torriani of the Institut Pasteur, Paris, who found that the incorporation of 0·02 M potassium citrate in the medium prevented losses of enzyme during and after growth, and who generously passed on this information to us. A proteinase liberated by the cells into the culture medium had been previously suspected of causing inactivation of penicillinase. Thus it seems possible that the citrate prevents destruction of the penicillinase by reducing the concentration of Ca^{2+} and Mg^{2+} ions, thereby inhibiting the production or action, or both, of the proteinase (Gorini & Fromageot, 1950).

The second problem was solved by the chance observation that the enzyme was completely removed from solution after passage through an ordinary, porosity-grade 5, bacteriological sintered-glass filter. It could be largely recovered by treatment of the glass filter with a high concentration of phosphate buffer. Small-scale trials with the fine-mesh glass powder used for making these filters (Baird and Tatlock) showed that 95% of the enzyme could be absorbed on the glass from the casein-hydrolysate–citrate medium and up to 90% rapidly eluted by 0·5-saturated $(NH_4)_2SO_4$ solution at pH 8·5. This elution technique offered the additional advantage of allowing the removal, at the same time as the glass, of any proteins which, unlike penicillinase, were insoluble in 0·5-saturated $(NH_4)_2SO_4$. The penicillinase eluted from glass was later found to be practically free from other protein. Thus in one simple manoeuvre it was possible both to concentrate and purify the enzyme from large quantities of culture, with relatively slight loss.

Absorption on glass surfaces has also been tried by Rasmussen (1954), who used pulverized quartz sand for the partial purification of testicular hyaluronidase. In general, however, it seems that this technique might be better suited for the concentration and purification of proteins from large volumes of very dilute solutions.

A large batch of *B. cereus* 569 uninduced culture was also grown, primarily in order to obtain enough 'basal' enzyme at comparatively high concentration to permit an immunological study of its properties, including precipitation and absorption of antibodies prepared against the induced enzyme (Pollock, 1956).

The methods adopted for the various enzyme preparations differed only as follows: (*a*) Basal enzyme. Inoculum: strain 569; no additions to medium during growth.

(*b*) Induced enzyme. Inoculum: strain 569; penicillin (final concn. 1 unit/ml.) was added to the culture when the concentration of cells reached 0·1 mg. dry bacterial wt./ml. and further penicillin (final concn. 100 units/ml.) when a concentration of 0·25 mg./ml. was attained. (*c*) Constitutive enzyme. Inoculum: strain 569/H; no additions.

Spores were inoculated into a small volume (100 ml.) of S broth and the resulting culture added to 5 l. of the CH/C medium and shaken at 35° until an opacity corresponding to about 0·5 mg. dry bacterial wt./ml. was attained. This was added to 320 l. of CH/C medium in a 100 gal. deep fermenter at 35°, and the mixture stirred rapidly and aerated by a sparger from below. Growth was allowed to continue up to a cell concentration of about 1·0–1·5 mg./ml. Foaming was troublesome towards the end of growth and was controlled by addition of minimal quantities (usually 20–50 g.) of silicone A antifoam dissolved in light petroleum. The pH increased slightly up to about 7·8 and was prevented from rising higher by addition of conc. HCl. The whole culture was then cooled to 0° and the cells separated in a Sharples centrifuge. Only about 15% of the total enzyme activity remained associated with the cells.

For 569/H, enzyme production was combined, on the ground of economy, with an experiment on the incorporation of 8-azaguanine into bacterial ribonucleic acid undertaken by Matthews & Smith (unpublished). This involved the addition of 40 μg. of 8-azaguanine/ml. to the culture at a bacterial density of 0·8 mg./ml. and subsequent incubation for a further 2·5 hr., during which period further growth and enzyme production were almost completely inhibited. In all other respects (apart from the absence of penicillin) the culture was grown in the same manner as 569, and since 90% of the enzyme had been formed before addition of 8-azaguanine it was not thought likely at the time that the preparation, referred to as 569/H(1), would be significantly affected.

Isolation of enzymes

Here again the methods used for the various types of enzyme differed only in minor details, except where specifically indicated. The basic procedure was as follows:

Absorption on, and elution from, glass. Glass powder (1 kg.) was added to the supernatant fluid and left therein overnight (90–95% maximal absorption is, in fact, attained within 3 hr.), with occasional stirring. The glass was then removed in the Sharples centrifuge and treated with 500 ml. of acetone, previously cooled to −20°, in order to remove lipids which otherwise proved troublesome during subsequent ammonium sulphate precipitation. It was later found preferable to wash the remaining acetone off the glass with two lots (each 1 l.) of 0·02 M potassium citrate (pH 7·0) before eluting the enzyme. But in the preparations referred to here the glass was treated, immediately after centrifuging from acetone, with three successive lots of 400 ml. of 0·5-saturated $(NH_4)_2SO_4$ solution containing 0·1 M potassium phosphate brought to pH 8·5 by addition of aqueous NH_3 solution.

Elution is very rapid and the glass can be spun off as soon as it has been evenly suspended in the $(NH_4)_2SO_4$ solution. It was found that 1 g. of glass would absorb approximately 1 mg. of penicillinase, so that adjustment of the quantity added may be necessary for very high enzyme titres. Large quantities of other protein may, however, inhibit absorption. It was found impossible, for example, to absorb even small amounts of penicillinase in the presence of 1% of gelatin. The glass powder can be used again (preferably after 'titration' of a sample with culture supernatant fluid containing penicillinase to determine its enzyme-absorbing power) after washing with distilled water, warming with 10% (w/v) NaOH solution until no further NH_3 is released and subsequent washing with water to remove the NaOH, followed by drying at 105°.

Ammonium sulphate fractionation. The enzyme dissolved in the combined eluates was precipitated at +2° by addition of more $(NH_4)_2SO_4$ to 1·0 saturation (707 g. of the salt dissolved in 1 l. of water) at pH 7·0 and the mixture set aside overnight at −2°. The precipitate (later found to be almost pure penicillinase in the batches of induced and constitutive enzymes) was filtered off on a Büchner funnel, dissolved in a minimal volume (usually about 50 ml.) of 0·1 M potassium phosphate buffer, pH 7·0, and dialysed free of $(NH_4)_2SO_4$ against three changes of 0·01 M potassium phosphate buffer, pH 7·0. After this dialysis, the sac containing the enzyme solution (now increased in volume to about 100 ml.) was dialysed against $(NH_4)_2SO_4$ solution (pH 7·0), which was adjusted so that the concentration, after equilibration, would be 0·60 saturated. The slight precipitate that appeared was spun off and discarded, and the supernatant dialysed successively against 0·67-, 0·75- and 0·83-saturated $(NH_4)_2SO_4$ at pH 7·0. The fractions precipitated at 0·67- and 0·75-saturated $(NH_4)_2SO_4$ (containing about 80% of the total enzyme) were combined and dissolved in a small volume (about 10 ml.) of 0·1 M potassium phosphate, pH 7·0, and dialysed against 0·1 M potassium phosphate, pH 7·0, to remove all $(NH_4)_2SO_4$.

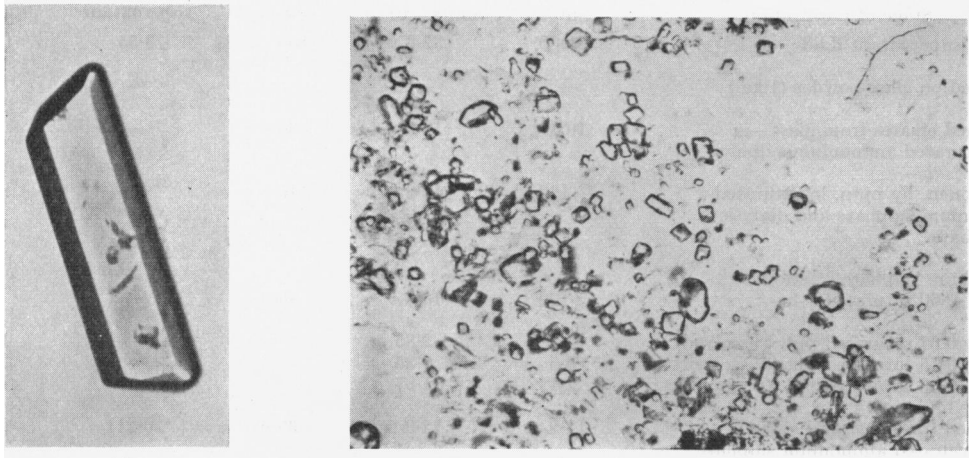
Ethanol fractionation. The 569/H penicillinase was further fractionally precipitated with ethanol (previously cooled to −20° and added slowly, with constant stirring, to the enzyme in 0·1 M phosphate at −2°), at concentrations rising from 36 to 50% (v/v). No increase in specific activity was obtained by this ethanol fractionation, and it was not applied to the 569 induced enzyme. The ethanol-precipitated 569/H enzyme fractions were dissolved in 0·1 M potassium phosphate, pH 7·0, and concentrated by precipitation in saturated $(NH_4)_2SO_4$, redissolved in 4·0 ml. of the phosphate buffer and dialysed free from $(NH_4)_2SO_4$ against 0·1 M potassium phosphate, pH 7·0.

With very small samples it was possible to elute up to 75% of the enzyme absorbed on the glass, but with the larger batches it was not possible to elute more than 60%. Some loss may have occurred during acetone treatment, owing to denaturation of the enzyme. Subsequent losses—during $(NH_4)_2SO_4$ and ethanol fractionations—were relatively small. With care, an overall yield of about 50% could be obtained after the $(NH_4)_2SO_4$ fractionation, when purification of the enzyme is completed. With the basal 569 enzyme, so little precipitate was obtained on addition of saturated $(NH_4)_2SO_4$ after elution from glass that it was all dissolved in only 10 ml. and, after dialysis against 0·1 M potassium phosphate, pH 7·0, was precipitated in one step with 0·83-saturated ammonium sulphate and the precipitate finally dissolved in 1·4 ml. of 0·1 M potassium phosphate, pH 7·0, and not further treated.

Table 2 gives a full summary of the isolation procedures, yields and specific activities of the three preparations.

Crystallization. After several unsuccessful attempts to crystallize the induced 569 enzyme from $(NH_4)_2SO_4$

solution, it was twice precipitated from solution in 0·1 M potassium phosphate pH 7·0 by 50% (v/v) ethanol, then dissolved in the minimum amount (4 ml.) of 10^{-2} M potassium phosphate, pH 7·0, at +2° and suspended in a sealed bath of 40% (v/v) ethanol at −2°, the technique described by Pollock *et al.* (1956) for the recrystallization of *B. cereus* 5/B penicillinase being used. After 28 days the enzyme solution was removed from the bath, further ethanol added until precipitation just began and left at −2°. Thirteen days later, long columnar crystals began to appear and collected slowly during the following 5 days (Fig. 1*a*). These crystals were examined by Mrs O. Kennard in a polarizing microscope and compared with crystals of the 5/B penicillinase (Pollock *et al.* 1956). Their extreme



(*a*) (*b*)

Fig. 1. Crystals of penicillin-induced penicillinase from *B. cereus* NRRL 569. *a*, ×90; *b*, ×400.

Table 2. *Purification of penicillinase produced by* (*a*) B. cereus 569/*H* (*constitutive strain*) *without penicillin treatment* ('*constitutive enzyme*')

	(*a*) 569/H (constitutive enzyme)			
Stage of purification	Vol. (ml.)	Total activity (units × 10^{-7})	Yield (% orig. activity)	Specific activity (u. × 10^{-6}/mg. protein N)
Culture supernatant fluid	320 000	44·2	100	—
↓				
Absorbed on glass powder (1 kg.)	—	—	—	—
↓				
Combined eluates from glass—in 0·5 saturated ammonium sulphate	1 310	14·8	33·5	—
↓				
After concn. by pptn. in saturated ammonium sulphate and dialysis v. phosphate	106	13·2	30·0	2·16
↓				
Ammonium sulphate fractions:				
0·60 saturated soln.	49·5	12·5	27·3	—
↓				
0·67 saturated ppt. (dissolved)	8·5	4·55	—	2·13
0·75 saturated ppt. (dissolved)	6·0	4·32	—	1·89
0·83 saturated ppt. (dissolved)	3·2	0·83	—	1·27
↓				
Combined fractions pptd. at 0·67 and 0·75 saturated ammonium sulphate dissolved in phosphate	14·5	8·9	20·2	2·11
Ethanol fractions:				
36% ppt. (dissolved)	3·0	4·23	—	2·22
40% ppt. (dissolved)	2·0	1·34	—	2·27
43% ppt. (dissolved)	1·5	0·54	—	2·18
50% ppt. (dissolved)	1·5	1·38	—	1·93

Table 2 (*cont.*). (*b*) B. cereus 569 (*inducible strain*) (*i*) *with penicillin treatment* ('*induced enzyme*'); (*ii*) *without penicillin* ('*basal enzyme*')

(*b*) 569. (i) Induced (induced enzyme)

Stage of purification	Vol. (ml.)	Total activity (units × 10^{-7})	Yield: % original activity	Yield: mg. protein N/l. supernatant	Specific activity (u. × 10^{-6}/mg. protein N)
Culture supernatant fluid	320 000	32·6	100	3·55	0·287
↓ Absorbed on glass powder (1 kg.)	—	—	—	—	—
↓ Combined eluates from glass—in 0·5 saturated ammonium sulphate	1010	19·5	60·0	—	—
↓ After concn. by pptn. in saturated ammonium sulphate and dialysis v. phosphate	199	16·2	49·8	—	2·25
↓ Ammonium sulphate fractions: 0·60 saturated soln.	100	15·0	46·0	—	—
↓ 0·67 saturated ppt. (dissolved)	4·9	2·85	—	—	1·95
0·75 saturated ppt. (dissolved)	6·5	9·35	—	—	2·30
0·83 saturated ppt. (dissolved)	2·4	1·87	—	—	1·68
↓ Combined fractions pptd. at 0·67 and 0·75 saturated ammonium sulphate dissolved in phosphate	11·9	12·9	39·6	0·211	2·18
Crystals	—	—	—	—	2·02

(*b*) 569. (ii) Not induced (basal enzyme)

Stage of purification	Vol. (ml.)	Total activity (units × 10^{-7})	Yield: % original activity	Yield: mg. protein N/l. supernatant	Specific activity (u. × 10^{-6}/mg. protein N)
Culture supernatant fluid	260 000	0·071	100	—	—
↓ Absorbed on glass powder (1 kg.)	—	—	—	—	—
↓ Combined eluates from glass—in 0·5 saturated ammonium sulphate	2 200	0·0213	30·0	—	—
↓ After concn. by pptn. in saturated ammonium sulphate and dialysis v. phosphate	14·0	0·0062	8·8	—	—
↓ Ammonium sulphate fractions: 0·60 saturated soln.	11·5	0·0066	9·3	—	—
↓ 0·67 saturated ppt. (dissolved)	—	—	—	—	—
0·75 saturated ppt. (dissolved)	—	—	—	—	—
0·83 saturated ppt. (dissolved)	—	—	—	—	—
↓ Combined fractions pptd. at 0·67 and 0·75 saturated ammonium sulphate dissolved in phosphate	1·4	0·0058	8·2	0·0055	0·040

fragility made detailed crystallographic studies difficult. Both types of crystals were birefringent. Although very similar in form they were easily distinguishable by their angles of extinction, which differed by about 16°.

The yield of large crystals was poor, and there was a tendency towards gel formation at −2°. Repetition of the ethanol treatment applied to the mother liquor produced more crystals which, however, were firmly fixed to the sides of the glass tube and were exceedingly fragile, so that after freeing them into a suspension they appeared to be fragmented and irregular (Fig. 1*b*). A solution of these crystals in dilute potassium phosphate (pH 7·0) showed a typical protein absorption curve between 240 and 300 mμ. and had a specific penicillinase activity of $2{\cdot}02 \times 10^6$ units/mg. N, which is not significantly different from that of the mother liquor.

COMPARISON OF INDUCED AND CONSTITUTIVE PENICILLINASE

Crystallization was not achieved before the work reported here had been completed. All physicochemical analyses were done on the purified enzyme preparation before crystallization.

569 (*induced*) *and* 569/*H enzymes*

Electrophoresis. Both preparations were studied at pH 8·4 and 5·5 in the Perkin–Elmer electrophoresis apparatus. Fig. 2 shows tracings of the boundary patterns at pH 8·4, and the mobilities are recorded in Table 3. Both preparations show single peaks, even after 4 hr.; but whereas those of the 569 induced enzyme are almost symmetrical, the 569/H(1) penicillinase gives indications of some heterogeneity. The isoelectric point, for both enzymes, must be slightly below pH 5·5.

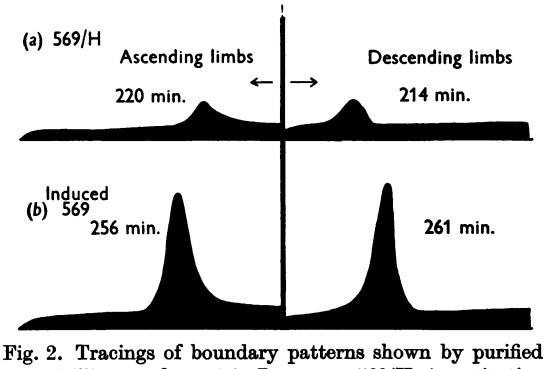


Fig. 2. Tracings of boundary patterns shown by purified penicillinases from (*a*) *B. cereus* 569/H (constitutive enzyme: concn., approx. 0·3%) and (*b*) *B. cereus* 569 (induced enzyme: concn., approx. 1·0%) on electrophoresis in glycine–NaCl buffer, pH 8·4, $\mu = 0{\cdot}2$.

Solubility in ammonium sulphate. Small samples of both enzymes were dialysed against increasing concentrations of $(NH_4)_2SO_4$ at pH 7·0; and at each concentration, after equilibration had been reached, the precipitate was spun off and the enzyme activity in the supernatant fluid measured in triplicate. No further direct determinations of protein N were made because (as shown in Table 2) the specific activities of fractions precipitated at concentrations of $(NH_4)_2SO_4$ between 0·60 and 0·75 saturation did not, in either case, show any marked differences. The work of Itano (1953) on detection of differences between the electrophoretically indistinguishable human haemoglobins b (sickle-cell) and d by comparing their solubilities in high concentration of sodium sulphate suggested that analogous differences might be detected in these two penicillinase preparations. Fig. 3, however, shows that no differences in solubility were apparent.

Ultracentrifuge. A determination of sedimentation and diffusion constants of 569 induced and 569/H(1) enzyme preparations was carried out by Hall & Ogston (see Addendum). In view of the striking similarity between the two enzyme preparations when compared by other criteria, the slight, but significant differences in sedimentation and diffusion constants (giving, however, on calculation, similar molecular weights) were thought possibly to be due to artifacts arising from effects of the ethanol precipitation on the 569/H preparation. In order to test this possibility 30 mg. of the 569 induced enzyme, dissolved in 2·0 ml. of 0·1 M potassium phosphate pH 7·0, were precipitated with 75% (v/v) ethanol and redissolved in 3·0 ml. of the buffer and dialysed against a saturated

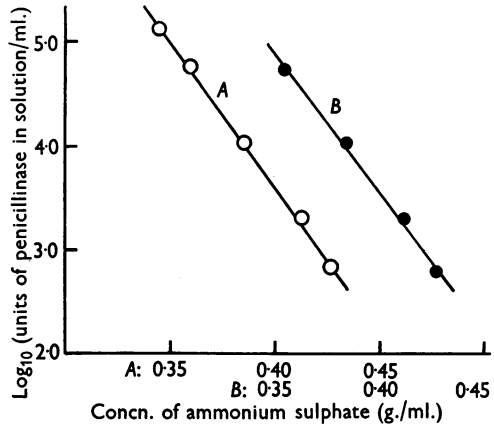


Fig. 3. Solubilities in ammonium sulphate of penicillinases from: *A*, *B. cereus* 569/H (constitutive enzyme); *B*, *B. cereus* 569 (induced enzyme); expressed by plotting $\log_{10}$ (units of enzyme activity remaining in solution/ml.) (ordinate) against the concentration of ammonium sulphate at +2°, pH 6·44–6·70 (abscissa).

solution of $(NH_4)_2SO_4$. The precipitate was dissolved in 1·5 ml. of 0·1 M phosphate and dialysed against a buffer solution containing 0·1 M-NaCl, 0·067 M-KH_2PO_4 and 0·05 M-Na_2HPO_4, pH 7·2. This solution of the enzyme (concn. approx. 0·9 %) was run in the Spinco analytical centrifuge (18 mm. cells) at 50950 r.p.m. and the sedimentation boundary compared with that of a sample of the same enzyme, untreated with ethanol, run under identical conditions. No significant differences in boundary shape or sedimentation rate were observed, and it was therefore concluded that the differences found in the first place between 569 induced and 569/H(1) could not be attributed to the ethanol precipitation of the latter. Thus the possibility had to be seriously considered that the addition of 8-azaguanine to the culture (see above) might have affected the properties of the 569/H(1) enzyme preparation in some way. A small fresh batch of 569/H penicillinase was therefore prepared from 10 l. of culture distributed in ten 5 l. conical flasks shaken at 35°. The methods of production and extraction were, proportional to the quantity of enzyme formed, exactly those described under 'Methods of production and purification', except that the culture was incubated for 40 hr. and a higher enzyme titre (4700 units/ml. of culture supernatant fluid) so obtained. The overall yield at the stage of concentration by precipitation with saturated ammonium sulphate was 52 %. Thereafter, the enzyme was precipitated three times with 0·75-saturated $(NH_4)_2SO_4$ at +2° (the precipitate being dissolved in, and dialysed against, 0·1 M potassium phosphate pH 7·0 between successive precipitations); it was finally dissolved in 2·5 ml. of 0·1 M potassium phosphate pH 7·0 at a concn. of about 1 %. The enzyme was found to have a specific activity of $1·95 \times 10^6$ units/mg. of N. The results of an ultracentrifugal analysis of this control 569/H(2) preparation (see Addendum and Table 3) showed that it differed significantly from 569/H(1), suggesting that addition of 8-azaguanine may possibly have affected the properties of the penicillinase subsequently isolated. Moreover, the sedimentation and diffusion constants of 569/H(2) do not differ significantly from those of the 569 induced penicillinase.

A comparison of the biological and physicochemical properties of the enzyme preparations is summarized in Table 3.

569 *Basal enzyme*

The total amount of protein obtained after fractionation of the uninduced 569 culture supernatant was only about 2·5 %, and the total enzyme activity only 0·02 %, of that obtained from the induced culture grown to the same bacterial density (Table 2). The percentage recovery was much less than with the other preparations, probably because of the losses due to the inevitably

Table 3. *Summary of physicochemical properties of purified penicillinase from* B. cereus 569, *induced with penicillin* ('*induced enzyme*') *and from* B. cereus 569/*H*, *untreated with pencillin* ('*constitutive enzyme*')

Comparative figures for *B. cereus* 5/B constitutive penicillinase (see Pollock *et al.* 1956) are given in the last column in order to illustrate some of the properties in which it differs from the 569 and 569/H enzymes.

	569/H Constitutive penicillinase		569 Induced penicillinase	5/B Constitutive penicillinase
	Prep. (1)	Prep. (2)		
Electrophoretic mobility (cm.²/v/sec.)				
Glycine–NaCl, $\mu = 0·2$, pH 8·4				
Ascending boundary	$-1·73 \times 10^{-5}$	—	$-1·68 \times 10^{-5}$	—
Descending boundary	$-1·65 \times 10^{-5}$	—	$-1·70 \times 10^{-5}$	$-3·48 \times 10^{-5}$
Acetate, $\mu = 0·2$, pH 5·5				
Ascending boundary	$-0·55 \times 10^{-5}$	—	$-0·63 \times 10^{-5}$	—
Descending boundary	$-0·45 \times 10^{-5}$	—	$-0·49 \times 10^{-5}$	—
Specific enzyme activity (u./mg. of protein N)	$2·11 \times 10^6$	$1·95 \times 10^6$	$2·02 \times 10^6$	$1·53 \times 10^6$
Sedimentation constants $S_{20,w}$ (Hall & Ogston, 1956)				
Approx. protein concn. 0·9 %	$2·83 \times 10^{-13}$	$2·65 \times 10^{-13}$	$2·68 \times 10^{-13}$	—
0·3 %	$2·80 \times 10^{-13}$	—	$2·66 \times 10^{-13}$	$2·82 \times 10^{-13}$
Diffusion coefficient (Hall & Ogston, 1956)	$9·01 \times 10^{-7}$	$8·38 \times 10^{-7}$	$8·28 \times 10^{-7}$	$7·80 \times 10^{-7}$
Molecular weight (Hall & Ogston, 1956)	30 600	30 800	31 500	35 200
Molecular activity (molecules substrate hydrolysed/mol. of enzyme at 30°, pH 7·0)	$1·62 \times 10^5$	$1·53 \times 10^5$	$1·60 \times 10^5$	$1·48 \times 10^5$
Salting-out constant expressed as mean decrease in $\log_{10}$ units of enzyme activity remaining in solution/ml./mg./ml. increase in concn. of ammonium sulphate (+2°) (see Fig. 3)	0·0754	—	0·0748	—
$E^{280\,m\mu.}_{1\,cm.}$ at concn. of 1·0 mg. N/ml.	6·35	—	6·0	7·35

greater dilution of the enzyme that occurred during the first stages of fractionation after elution from the glass. No accurate quantitative study of the basal enzyme, from a physicochemical point of view, was of course possible; but there was enough material for some quantitative, immunological precipitation tests.

Immunology

A detailed immunological comparison, by neutralization and precipitation tests reported elsewhere (Pollock, 1956), showed no significant differences between the three preparations 569/H, 569 (induced) and 569 (basal), although they were easily distinguishable from the 5/B penicillinase.

(*b*) A sample (250 ml.) of supernatant fluid was concentrated to about 50 ml. by evaporation at 37° under reduced pressure in the apparatus of Craig (1950). The concentrate was dialysed under pressure against 10^{-3} M potassium phosphate (pH 7·0) until further reduced to about 2·0 ml., when the sac was tied off, and further dialysed against five changes of 1 l. of 10^{-3} M potassium phosphate pH 7·0. A certain amount of insoluble matter remained, but the whole was made up to a volume of 7·5 ml., and 1·5 ml. samples were analysed for N content in duplicate before and after removal of insoluble matter by centrifuging.

Table 4 summarizes the results found with cultures grown to about the same bacterial density as in the large preparation batches. The figures for total non-dialysable N show fair agreement (within 20 %) with those for protein N estimated against an albumin standard. The amount of exocellular protein formed by these strains is relatively low, corresponding to only about 3 % of the dry weight of the cells. However, penicillinase forms a high proportion of this exocellular protein, amounting to nearly one-third (26 %) in the constitutive *B. cereus* 5/B and nearly one-tenth in the induced 569 culture.

Table 4. *Exocellular protein concentrations in cultures of* B. cereus *grown in casein hydrolysate–casein (CH/C) medium*

Culture	Final growth yield (mg./ml.)	Penicillinase activity in culture supernatant (u./ml.)	Protein estimations (μg./ml. culture supernatant)		Kjeldahl N (μg./ml.)		Specific activity (u./mg. of total N)	Penicillinase N as % of total non-dialysable N
			Total protein (albumin standard)	Protein N (albumin standard)	Total non-diffusible	Soluble non-diffusible		
Uninoculated medium	~	~	<0·2	<0·03	<0·5	<0·5	~	~
569 (not induced)	1·4	5·5	41·5	6·6	5·08	3·99	$1·07 \times 10^3$	0·05
569 (induced)	1·35	$7·6 \times 10^2$	32·8	5·2	3·98	3·27	$1·91 \times 10^5$	8·9
569/H	1·3	$2·47 \times 10^3$	52·0	8·3	9·82	6·98	$2·52 \times 10^5$	11·7
5/B	1·52	$2·71 \times 10^3$	48·3	7·7	6·55	5·58	$4·13 \times 10^5$	26·5

Exocellular protein production by Bacillus cereus

Total protein estimations on the untreated culture medium after centrifuging off the cells was not done on the large batches. In order to determine the specific activity of the enzyme in the culture medium before its isolation, four 1 l. batches of 5/B, 569/H and 569, induced and uninduced, were incubated in 5 l. conical flasks, shaken at 35°, until a cell concentration of about 1·4 mg./ml. had been attained. The cells were removed by centrifuging and, after enzyme activity had been assayed, the protein content of the supernatant fluid was estimated by two different methods, as follows:

(*a*) The proteins in 2·5 ml. samples were first precipitated with 5 % trichloroacetic acid (TCA) and the precipitate was washed twice with 5 % TCA, dissolved in alkali and treated with the copper reagent and Folin reagent as described by Lowry, Rosebrough, Farr & Randall (1951), the blue colour being compared against standards of 25, 50, 75 and 100 μg. of crystalline bovine serum albumin.

DISCUSSION

The 569 induced and the 569/H penicillinases do not differ significantly in any of the properties investigated. The slight discrepancy, of less than 7 %, found between the two preparations of 569/H, in sedimentation and diffusion constants, must be provisionally ascribed to some unknown effect of the 8-azaguanine added to the culture medium of the 569/H(1) batch, although only 10 % of the total enzyme was formed after addition of this compound. The possibility that this guanine

analogue, which is known to be incorporated into the ribonucleic acid of *B. cereus* 569 and 569/H (Smith & Matthews, unpublished), may have influenced protein synthesis in some manner, is being further investigated.

The mean specific activity found for the 569 induced penicillinase was a little over twice that obtained on isolation of the enzyme from a peptone medium by another method (Pollock & Torriani, 1953). It seems likely, therefore, that earlier results were to some extent vitiated by contaminating proteins from the peptone forming relatively stable complexes with the enzyme. This may also partly explain the curious results of Baudet & Hagemann (1954), who claim to have obtained a 'highly purified' preparation of induced penicillinase, from the same strain (*B. cereus* 569) grown in a peptone medium, which had a specific activity less than 1/30 of the preparation described here.

The conclusion of Pollock & Torriani (1953) about the catalytic action of penicillin as inducer for penicillinase production in *B. cereus* remains, however, unaffected. Assuming a mol.wt. of 31000 and a specific activity of 2×10^6 units/mg. protein N, it can be calculated that in CH/C or broth at 35° the quantity of penicillinase formed by *B. cereus* 569 after induction by penicillin corresponds to the production of between 30 and 40 molecules of enzyme per hour for every atom of penicillin sulphur specifically fixed on the cells. It has previously been shown (Pollock & Perret, 1951; Pollock, 1952) that the rate of penicillinase formation is proportional to the amount of penicillin sulphur specifically fixed on the cells after treatment with ^{35}S-labelled penicillin and removal of all free penicillin by repeated washing or by destruction with penicillinase. In whatever manner penicillin functions as inducer of penicillinase, it must therefore act, directly or indirectly, in the role of catalyst. These results are consistent with the 'organizer' theory of enzyme induction proposed by Pollock (1953) and by Cohn & Monod (1953).

It is significant that, with the uninduced 569 culture, the amount of protein obtained by the procedure designed for specific isolation of induced or constitutive penicillinase was only 2·5% of that obtained from a parallel, induced culture of the same organism. This demonstrates that induction of penicillinase corresponds to the formation of a specific protein with distinct physicochemical properties. The fact that the specific activity of the basal enzyme preparation was only 2% of that of the induced preparation is not of any significance when the total material available for purification was so minute. If, indeed, the basal and induced enzymes are identical the total weight of basal enzyme available during the final stages of fractionation was only about 0·2 mg., obviously far too small an amount to isolate and purify by these methods. It seems much more likely that the low specific activity of the basal enzyme preparation is due to the presence of an impurity, small in absolute quantity but large in proportion to the amount of basal enzyme, which is possibly also present in the induced preparation. The presence of only 2·5% of impurity in an uncrystallized preparation of a protein might be considered hardly surprising, so that the finding of such small amounts of protein after fractionation of the uninduced preparation may, not unreasonably, be taken as indicating the approximate degree of purity of the induced enzyme preparation.

The similarity between the induced and constitutive enzymes in electrophoretic mobility at two pH values, specific activity, sedimentation and diffusion constants and solubility in ammonium sulphate, is all the more striking when the values are compared with those found for the penicillinase of a related strain of *B. cereus* (5/B) reported in the preceding paper (Pollock *et al.* 1956). Apart from the solubilities in ammonium sulphate, which were not studied in detail in the case of 5/B (though even here the results of fractionation at different concentrations strongly suggested a significant difference), the 569 and 569/H enzymes differ quite markedly from the 5/B enzyme in every character investigated, including immunological properties. This similarity between spontaneously produced and penicillin-induced penicillinase is consistent with the hypothesis that they are formed by fundamentally the same mechanism.

If the end-products of penicillinase synthesis in the two cases are indistinguishable, renewed emphasis is laid on the importance of discovering in what ways the metabolic reactions underlying induced and constitutive enzyme synthesis do in fact differ. More specifically, it would be of interest, on the one hand, to examine experimentally the possibility (expressed in general terms as the 'unitary hypothesis' and discussed in detail by Cohn & Monod, 1953) that a constitutive penicillinase strain may synthesize the enzyme under the continuous stimulus of an endogenous inducer not formed in the inducible strain. On the other hand, it is possible that penicillin may evoke the formation of penicillinase in the inducible strain by antagonizing a specific inhibitor not present in the constitutive strain. Such ideas are amenable to an experimental approach and are worth testing if there is good reason for believing that the mechanisms of induced and constitutive enzyme synthesis are very closely similar. The absence of detectable differences in the properties of induced and constitutive penicillinase described here suggest that this is a reasonable assumption.

SUMMARY

1. Penicillin-induced penicillinase from *B. cereus* 569 grown in casein hydrolysate has been purified by absorption on fine-mesh glass powder, elution with 0·5-saturated $(NH_4)_2SO_4$ and fractionation between 0·60- and 0·75-saturated $(NH_4)_2SO_4$. It was finally crystallized from 30% ethanol. It appears to be homogeneous on electrophoretic and ultracentrifugal analysis; and the mol.wt. was found to be 31 000.

2. A constitutive penicillinase, produced without penicillin treatment by a strain (*B. cereus* 569/H) derived from *B. cereus* 569 by a spontaneous mutation, has been purified by the same technique and found not to differ significantly from the induced enzyme of its parent strain by any of the characters examined (specific activity, sedimentation and diffusion constants, electrophoretic mobility and salt solubility). This similarity is consistent with the hypothesis that induced and constitutive enzymes are formed by basically the same mechanism.

3. The constitutive penicillinase of *B. cereus* 569/H is, however, quite distinct physicochemically from the constitutive penicillinase of *B. cereus* 5/B.

4. Fractionation of the supernatant fluid from an uninduced culture of *B. cereus* 569 by the specific technique used for isolation of penicillinase yielded only 2·5% of the amount of protein obtained under comparable conditions from an induced culture. It is concluded that induction of penicillinase activity by penicillin corresponds to the formation of a specific, physicochemically distinct protein.

We wish to record our thanks to B. K. Kelly, C. W. Hale and C. B. Miller and other members of the staff of the Medical Research Council Antibiotics Research Establishment at Clevedon, Somerset, where the large-scale cultivation of *B. cereus* was carried out. We are also much indebted to Miss Joan Fleming for expert technical assistance; to Mr M. R. Young for taking the photographs; and to Mr J. Orr for carrying out the Spinco ultracentrifuge runs. The Perkin–Elmer Tiselius apparatus used for electrophoretic analysis was purchased by means of a grant from Eli Lilly and Co.

Dr Margot Kogut was on leave from the A.R.C. Unit of Microbiology, Sheffield.

REFERENCES

Baudet, P. & Hagemann, G. (1954). *Experientia*, **10**, 374.
Cohn, M. & Monod, J. (1951). *Biochim. biophys. Acta*, **7**, 153.
Cohn, M. & Monod, J. (1953). In *Adaptation in Micro-organisms*, p. 132. *3rd Symp. Soc. gen. Microbiol.* Ed. by Gale, E. F. & Davies, R. Cambridge University Press.
Craig, L. C. (1950). *Analyt. Chem.* **22**, 1462.
Gorini, L. & Fromageot, C. (1950). *Biochim. biophys. Acta*, **5**, 524.
Hall, J. R. & Ogston, A. G. (1956). *Biochem. J.* **62**, 401.
Itano, H. A. (1953). *Science*, **117**, 89.
Kantorowicz, O. (1951). *J. gen. Microbiol.* **5**, 357.
Landman, O. E. (1954). *Arch. Biochem. Biophys.* **52**, 93.
Lederberg, J. & Lederberg, E. M. (1952). *J. Bact.* **63**, 399.
Lowry, O. H., Rosebrough, M. J., Farr, A. L. & Randall, R. S. (1951). *J. biol. Chem.* **193**, 265.
Manson, E. E. D., Pollock, M. R. & Tridgell, E. J. (1954). *J. gen. Microbiol.* **11**, 493.
Monod, J. & Cohn, M. (1952). *Advanc. Enzymol.* **13**, 67.
Pollock, M. R. (1950). *Brit. J. exp. Path.* **31**, 739.
Pollock, M. R. (1952). In *Symposium sur la Biogénèse des protéines*, p. 67. *2nd Int. Congr. Biochem. Paris.*
Pollock, M. R. (1953). In *Adaptation in Micro-organisms*, p. 150. *3rd Symp. Soc. gen. Microbiol.* Ed. by Gale, E. F. & Davies, R. Cambridge University Press.
Pollock, M. R. (1956). *J. gen. Microbiol.* **14**, 90.
Pollock, M. R. & Perret, C. J. (1951). *Brit. J. exp. Path.* **32**, 387.
Pollock, M. R. & Torriani, A.-M. (1953). *C.R. Acad. Sci., Paris*, **237**, 276.
Pollock, M. R., Torriani, A.-M. & Tridgell, E. J. (1956). *Biochem. J.* **62**, 387.
Rasmussen, P. S. (1954). *Acta chem. scand.* **8**, 633.
Sneath, P. H. A. (1955). *J. gen. Microbiol.* **13**, 561.

ADDENDUM

Sedimentation and Diffusion of Samples of Penicillinase

By J. R. HALL* and A. G. OGSTON
Department of Biochemistry, University of Oxford

(*Received* 8 *July* 1955)

Purified samples of penicillinase, prepared as described in the two preceding papers, were examined by sedimentation and diffusion, in order to assess their molecular weights and degree of homogeneity.

* Rhodes Scholar, Balliol College, Oxford.

EXPERIMENTAL

Solutions. Sample 5/B was dialysed against buffer 0·2 M-NaCl, 0·0077 M-Na_2HPO_4, 0·0023 M-KH_2PO_4; other samples were dialysed against buffer 0·1 M-NaCl, 0·05 M-Na_2HPO_4, 0·0167 M-KH_2PO_4. After dialysis, the concentrations were measured refractometrically (Cecil & Ogston, 1951), assum-

ing a specific refractive increment of 0·00180: dilutions were made with diffusate.

Sedimentation. This was measured in a Svedberg oil-turbine ultracentrifuge at 900 rev./sec. by the method of Cecil & Ogston (1948). The modified schlieren optical system of Baldwin (1953*a*) was used to obtain the photographic records. From these the sedimentation coefficients were measured by the method of Cecil & Ogston (1948), from the maxima of the boundary gradient curves. Sedimentation homogeneity was tested in two ways: (i) The forms of the boundary gradient curves were tested by plotting $\log dn/dx$ against $(x-x_p)^2$, where x is the position in the boundary, measured from the centre of rotation; dn/dx is the corresponding value of the gradient of refractive index, obtained from the ordinate of the boundary gradient curve; x_p is the position of the maximum. For a symmetrical Gaussian boundary this plot is linear and identical for the two limbs of the curve.

(ii) The second moments, σ, of the gradient curves were measured from their maximum heights H and areas A: $\sigma^2=(A/H\sqrt{2\pi})^2$. Values of σ^2 were corrected for the non-uniformity of the centrifugal field by multiplying by the relevant value of $(1-\omega^2St)$, where ω is the angular velocity of rotation, S the sedimentation coefficient and t the time measured from the estimated beginning of sedimentation (Baldwin, 1953*a*, *b*); corrections of σ^2 for the concentration dependence of S were negligible and were not applied. For homogeneous solute the plot of σ^2 against t is linear, heterogeneity causing an upward curvature.

Diffusion. This was measured in the Gouy diffusiometer at 25° (Coulson, Cox, Ogston & Philpot, 1948), using a modified cell (Hall, unpublished). The diffusion coefficients were obtained from the optical record by the method of Gosting & Kegeles (1947). For the one heterogeneous penicillinase 569/H(1) the extrapolation technique of Gosting & Akeley (1953) was used to determine the values of C_t (the theoretical maximum deflexion of light in the boundary).

RESULTS

Table 1 gives the values of the sedimentation and diffusion coefficients, corrected to their values in water at 20°. The molecular weights and frictional ratios have been calculated on the assumption that the partial specific volume is 0·75. Fig. 1 shows plots of $\log dn/dx$ against $(x-x_p)^2$. Fig. 2 shows plots of σ^2 (corrected) against t.

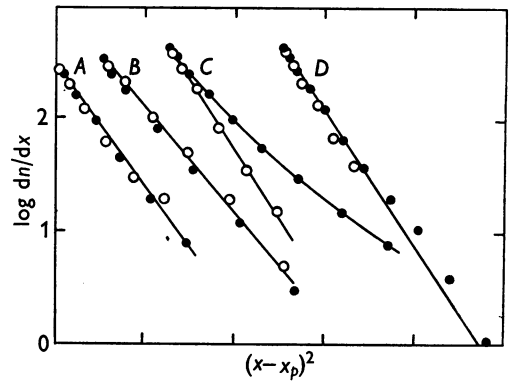


Fig. 1. Tests of the form of the sedimentation boundary-gradient curves by plotting (ordinate) $\log dn/dx$ against the square of distance from the centre of the boundary, $(x-x_p)^2$; $(x-x_p)^2$ and dn/dx are in arbitrary units. ●, Advancing limb; ○, trailing limb. *A*, Sample 5/B; *B*, sample 569 (induced); *C*, sample 569/H(1); *D*, sample 569/H(2).

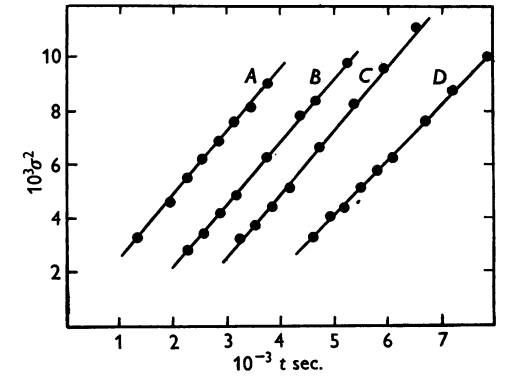


Fig. 2. Tests of homogeneity of sedimentation by plotting σ^2 (corrected) against time from the beginning of sedimentation. *A*, Sample 5/B; *B*, sample 569 (induced); *C*, sample 569/H(1); *D*, sample 569/H(2). The plots of *B*, *C* and *D* are displaced in the t axis.

Table 1. *Sedimentation and diffusion coefficients, molecular weights (M) and frictional ratios (f/f_0) of samples of penicillinase*

Sample	$10^{13}\times S_{20,w}$	$10^{7}\times D_{20,w}$	10^{-3} *M*	f/f_0
5/B	2·80 (0·475) 2·82 (0·237)	7·80 (0·24)	35·2	1·24
569 (induced)	2·68 (0·856) 2·66 (0·276)	8·28 (0·28)	31·5	1·22
569/H(1)	2·83 (0·873) 2·80 (0·300)	9·01 (0·31)	30·6	1·13
569/H(2)	2·65 (0·925)	8·38 (0·41)	30·8	1·21

The concentrations at which measurements were made (g./100 ml.) are given in brackets.

DISCUSSION

The plots in Figs. 1 and 2 show that samples 5/B, 569 (induced) and 569/H(2) appear to be homogeneous in sedimentation, judged both by the closely Gaussian form of the boundary-gradient curves and by the linearity of σ^2 against t. 569/H(1), however, has a markedly skew boundary, with an extended advancing limb; its plot of σ^2 against t shows a possibly significant curvature. Both these criteria indicate some degree of heterogeneity. Tests of homogeneity from the pattern of fringes in the Gouy diffusion record confirm these conclusions.

Samples 569 (induced) and 569/H(2) do not differ significantly from each other, but sample 5/B differs significantly from both. This is consistent with their being essentially different substances.

Sample 569/H(1) differs significantly from all others and in particular from sample 569/H(2). The differences of sedimentation and diffusion coefficients and the frictional ratio indicate some difference of molecular configuration, though the material is not entirely uniform. Sample 569/H(1) was precipitated by ethanol in the course of purification, whereas 569/H(2) was not. A test made with the Spinco ultracentrifuge at the National Institute of Medical research (Kogut, Pollock & Tridgell, 1956) showed that precipitation of 569 (induced) with ethanol produced no obvious change in its rate of sedimentation or in the form of its sedimentation boundary. It therefore appears possible that the differences in the conditions of culture may have caused the difference between 569/H(1) and 569/H(2).

SUMMARY

1. The diffusion and sedimentation of four samples of penicillinase have been observed.

2. Sample 5/B differs from all others; samples 569 (induced) and 569/H(2) appear to be identical; samples 569/H(1) and 569/H(2) are different.

3. All samples except 569/H(1) appear homogeneous, by the tests applied.

REFERENCES

Baldwin, R. L. (1953*a*). *Brit. J. exp. Path.* **35**, 255.

Baldwin, R. L. (1953*b*). D.Phil. Thesis: University of Oxford.

Cecil, R. & Ogston, A. G. (1948). *Biochem. J.* **43**, 592.

Cecil, R. & Ogston, A. G. (1951). *J. sci. Instrum.* **28**, 253.

Coulson, C. A., Cox, J. T., Ogston, A. G. & Philpot, J. St L. (1948). *Proc. Roy. Soc.* A, **192**, 382.

Gosting, L. J. & Akeley, D. F. (1953). *J. Amer. chem. Soc.* **75**, 5685.

Gosting, L. J. & Kegeles, G. (1947). *J. Amer. chem. Soc.* **69**, 2516.

Kogut, M., Pollock, M. R. & Tridgell, E. J. (1956). *Biochem. J.* **62**, 391.

The Biosynthesis of Kojic Acid

4. PRODUCTION FROM PENTOSES AND METHYL PENTOSES*

By H. R. V. Arnstein and R. Bentley

National Institute for Medical Research, Mill Hill, London, N.W. 7, and Graduate School of Public Health, University of Pittsburgh, Pittsburgh 13, Pennsylvania

(*Received* 11 *July* 1955)

The biosynthesis of kojic acid is of interest because it seems likely that the C_6 precursor of this mould metabolite is a compound synthesized by well-known reaction sequences of carbohydrate metabolism; however, because of features unique to the micro-organisms producing kojic acid, this precursor is no longer metabolized in the usual way, ultimately to carbon dioxide. Instead, special (shunt) reaction mechanisms convert the C_6 precursor into the relatively undegraded end product. It was hoped that a study of the biosynthesis of kojic acid in *Aspergillus flavus* would therefore provide information about both the general and special reaction sequences of carbohydrate metabolism in this organism. Some information has already been obtained from previous studies of kojic acid biosynthesis (Arnstein & Bentley, 1953*a–c*; Denison, Carson & Foster, 1954). In particular, it has been shown that the major pathway of kojic acid production from glucose is a direct conversion without splitting of the carbon chain. However, the experiments with labelled glucose, and also with a number of labelled small molecules (such as dihydroxyacetone, pyruvate, acetate, etc.) indicated a secondary pathway which was believed to involve triose phosphates (Arnstein & Bentley, 1953*a*, *c*).

It had at one time been supposed that, because the pentoses L-arabinose and D-xylose can also be converted into kojic acid by *Aspergillus* species

* Part 3: Arnstein & Bentley (1953*c*).