the formation of radioactive dihydrouracil, carbamoyl-β-alanine, carbon dioxide and, in addition, α-[1-^{14}C]alanine, some unidentified compounds and an organic acid which is chromatographically identical with succinic acid.

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Immunological Properties of Exopenicillinase Synthesized by *Bacillus cereus* 569/H in the Presence of Amino Acid Analogues

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Previous experiments with *Staphylococcus aureus* 524 have shown that the replacement in the growth medium of the essential amino acid, arginine, by the structural analogue canavanine led to continued protein synthesis, but no production of active forms of phosphatase, β-galactosidase, hyaluronidase nor of a lytic enzyme which digested cell-wall preparations from *Micrococcus lysodeikticus*. To explain this finding it was suggested that protein synthesized in the presence of canavanine contained that amino acid in place of arginine and that this resulted in inactive forms of the enzymes (Richmond, 1959*a*). This possibility was supported by the detection of canavanine in the protein fraction of organisms incubated under these conditions (Richmond, 1959*b*).

It would seem that the one feasible method of testing this hypothesis would be by detecting an immunological cross-reaction between the inactive enzyme protein and a specific antibody against the normal enzyme. Unfortunately specific antisera against the enzymes studied in *S. aureus* were not available nor did it seem practicable to purify these enzymes to the degree necessary for the preparation of such sera. Accordingly, the effect of canavanine was studied on penicillinase formation by *Bacillus cereus* 569 (inducible strain) and *B. cereus* 569/H (constitutive strain). This system was

chosen as the relevant enzyme has been crystallized (Kogut, Pollock & Tridgell, 1956), its biosynthesis has been investigated extensively (Pollock, 1956*b*; Pollock & Kramer, 1958) and highly specific antisera were available together with techniques for their use in the isolation of small quantities of pure exopenicillinase (Pollock, 1956*a*; Pollock & Kramer, 1958). Further, the rate of protein 'turnover' has been measured in the constitutive strain (Urbá, 1959).

In addition to canavanine, the phenylalanine analogue *p*-fluorophenylalanine has also been used in this work, since it has been studied in detail (Munier & Cohen, 1956, 1959; Cohen & Munier, 1959; Cowie, Cohen, Bolton & de Robichon-Szulmajster, 1959) and shown to replace phenylalanine but not tyrosine in the protein fraction of *Escherichia coli*. Further, a supply of *p*-fluorophenyl[β-^{14}C]alanine was available, whereas no radioactive isomers of canavanine could be readily obtained.

This paper describes preliminary experiments on the effect of canavanine and *p*-fluorophenylalanine on growth and enzyme formation by *B. cereus* 569 and 569/H, together with a detailed description of the immunological properties of the penicillinase formed in the presence of these unnatural amino acids. Unlike *S. aureus* 524, this strain of *B. cereus* is not exacting for either arginine or phenylalanine and, as a result, the effect of the externally added amino acid analogues may be altered by the presence of the endogenously synthesized normal amino acids. To assess the importance of these possibilities an analysis of the effect of *p*-fluorophenylalanine on cell metabolism and penicillinase synthesis in *B. cereus* 569/H was carried out and is the subject of the subsequent paper (Richmond, 1960). Attempts to interpret the immunological differences exhibited by the penicillinase formed in the presence of *p*-fluorophenylalanine in the light of the effect of this amino acid on growth and penicillinase synthesis will be deferred to the following paper.

MATERIALS AND METHODS

Organisms. The inducible strain (569) and the constitutive mutant strain (569/H) of *B. cereus* used in these studies have been described previously (Kogut *et al.* 1956; Pollock, 1956*a*, *b*). Induction of the inducible strain (569) was carried out where required with 1 unit of benzylpenicillin/ml. as described by Pollock (1952).

Media. The casein-hydrolysate medium has been described by Kogut *et al.* (1956). The fully defined amino acid medium (18AA medium) was the same as that used for growth of *S. aureus* 524SC (Richmond, 1959*a*), except that nicotinic acid and thiamine hydrochloride, being unnecessary for the growth of *B. cereus*, were omitted. For specific purposes 18AA medium was modified by omitting arginine [17AA(−Arg) medium] or phenylalanine [17AA(−Phe) medium].

Amino acids. I am indebted to Dr J. Mandelstam for a gift of L-canavanine, which had been prepared from jack beans. The β-(*p*-fluorophenyl)alanine and further samples of L-canavanine sulphate were obtained from L. Light and Co. Ltd, Colnbrook, Bucks, England. DL-β-(*p*-Fluorophenyl)[β-^{14}C]alanine was obtained from the Commissariat à l'Energie Atomique, Gif-sur-Yvette, (Seine et Oise), France. It was diluted before use with unlabelled DL-β-(*p*-fluorophenyl)alanine to a specific activity of 0·17 μc/μmole.

Anti-exopenicillinase. Two preparations of immune-rabbit γ-globulin with similar neutralization characteristics were provided by Dr M. R. Pollock for use in these studies. The first (prepared from rabbit 362; see Pollock, 1956*a*) was used in the majority of experiments and had a titre of about 10^4 neutralization units/ml. The second preparation was made by Pollock & Kramer (1958) and had a titre of $1{\cdot}2 \times 10^5$ neutralization units/ml. Both preparations were diluted in 0·9% sodium chloride solution containing 0·8 mM-oxine to a titre of about 450 neutralization units/ml. and stored at −20°.

Preparation of bacteria for experimental purposes. About 0·2 ml. of a spore suspension (approx. 3×10^8 spores/ml.) was used to inoculate 20 ml. of casein-hydrolysate medium and incubated in stationary culture at 35° overnight. After 16 hr. the culture was diluted tenfold with fresh casein-hydrolysate medium and shaken at the maximum rate (86 cyc./min.; 1 ft. throw) of a graded shaker (Kantorowicz, 1951) until growth was exponential. When the culture had reached a density of 0·2–0·4 mg. dry wt. of bacteria/ml., the organisms were removed by centrifuging, washed in 0·9% sodium chloride solution and resuspended in the experimental media. If the subsequent experiments were to be carried out in amino acid media, organisms grown up in casein-hydrolysate medium were given a period of growth in 18AA, 17AA(−Arg) or 17AA(−Phe) medium, as required, to accustom them to the change in growth conditions.

Crude exopenicillinase. Crude preparations of both normal and abnormal penicillinase consisted of the supernatant growth medium remaining after removal of the organisms from appropriate cultures by centrifuging. These preparations were diluted for enzyme estimation or neutralization experiments in 1% of gelatin in water containing 0·8 mM-oxine to give a final concentration of about 20–30 enzyme units/ml. For certain purposes crude enzyme preparations were freed of low-molecular-weight contaminants by dialysis at 2° against four changes of 50 vol. of mM-Na_2HPO_4–KH_2PO_4 buffer, pH 7·0.

Preparation of cell fractions. The 'protein + cell wall' fraction of organisms was prepared as described for *S. aureus* 524 SC by Richmond (1959*a*, *b*). Cell-wall preparations were made by the method of Mandelstam & Rogers (1959). Free amino acids were liberated from these fractions by hydrolysis in 6N-HCl for 16 hr. at 105° in a sealed tube. The amino acids were separated by descending chromatography in butanol–acetic acid–water (60:10:27, by vol.). The locations of radioactive spots on the chromatograms were detected by radioautography as described by Roberts, Abelson, Cowie, Bolton & Britten (1955).

Assay systems. The technique of penicillinase assay and the titration of anti-exopenicillinase antiserum have been described elsewhere (Kogut *et al.* 1956; Pollock, 1956*a*).

RESULTS

Effect of canavanine and p-*fluorophenylalanine on growth and enzyme formation*

Cultures of *B. cereus* 569 and 569/H grew exponentially up to a density of about 0·8 mg. dry wt. of bacteria/ml. in the basal 18AA, 17AA(−Phe) and 17AA(−Arg) media used in these experiments. In 18AA and 17AA(−Phe) media the mean generation time of the organisms was about 50 min. whereas in 17AA(−Arg) medium it was 60–65 min.

Fig. 1 shows the growth curves obtained when organisms growing exponentially in 18AA medium were centrifuged, washed in 0·9% sodium chloride solution and immediately resuspended in 17AA(−Arg) medium containing 50 μg. of canavanine/ml. or in 17AA(−Phe) medium containing 50 μg. of *p*-fluorophenylalanine/ml. The culture incubated in the presence of canavanine grew only for about 60 min., but that in *p*-fluorophenylalanine increased at a lower exponential rate than the control for about 60 min., before becoming approximately linear with respect to time. The 'linear' growth phase may continue in the presence of *p*-fluorophenylalanine until the culture reaches a density of greater than 0·8 mg. dry wt. of organisms/ml.

Cultures of *B. cereus* 569/H growing exponentially in fully synthetic amino acid medium synthesized penicillinase at a constant differential rate and the total penicillinase activity of cultures under these conditions may be ascribed to three main penicillinase fractions. An exocellular fraction (α- or exo-

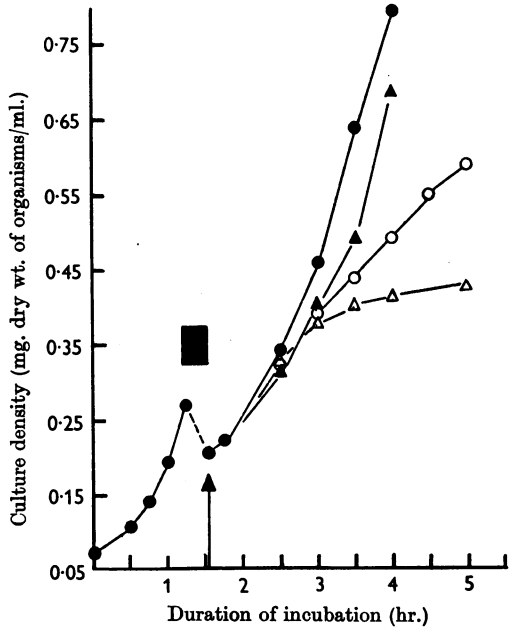

Fig. 1. Effect of canavanine or *p*-fluorophenylalanine on the growth of cultures of *Bacillus cereus* 569/H. ●, 18AA or 17AA(−Phe) medium; ▲, 17AA(−Arg) medium; ○, 17AA(−Phe + *p*-fluorophenylalanine) medium; △, 17AA(−Arg + canavanine) medium. *p*-Fluorophenylalanine or canavanine was added at arrow. Solid rectangle indicates time taken to centrifuge organisms and to resuspend them in the experimental media.

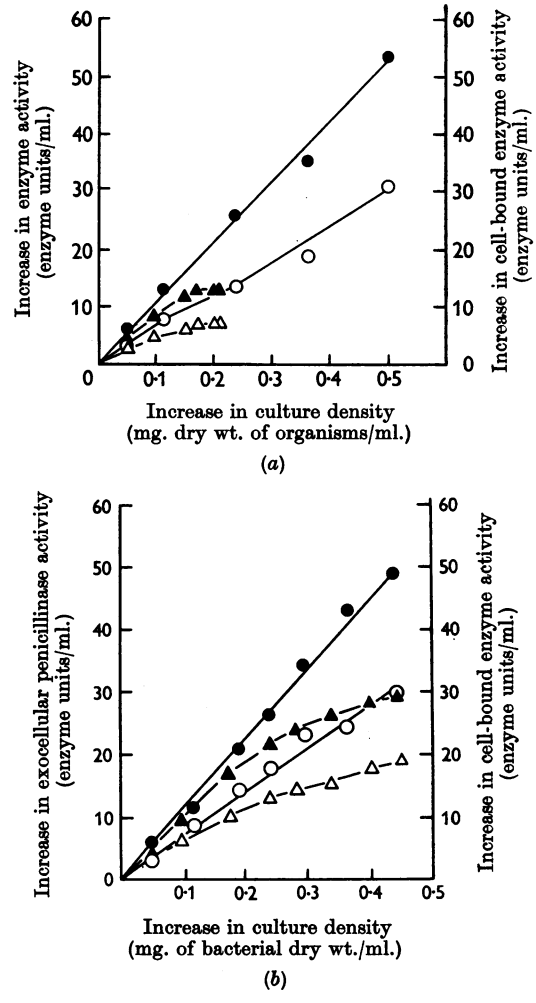

Fig. 2. Effect of (*a*) canavanine and (*b*) *p*-fluorophenylalanine on the differential rate of appearance of penicillinase fractions in cultures of *B. cereus* 569/H. (*a*) Exopenicillinase: ●, 17AA(−Arg) medium; ▲, 17AA(−Arg + canavanine) medium. Total cell-bound penicillinase: ○, 17AA(−Arg) medium; △, 17AA(−Arg + canavanine) medium. (*b*) Exopenicillinase: ●, 17AA(−Phe) medium; ▲, 17AA(−Phe + *p*-fluorophenylalanine) medium. Total cell-bound penicillinase: ○, 17AA(−Phe) medium; △, 17AA(−Phe + *p*-fluorophenylalanine) medium.

penicillinase) and a cell-bound fraction, about 40% of which is α-penicillinase that has been adsorbed on to the cell surface [the β-penicillinase of Pollock (1956*b*)] and the remaining 60% an immunologically and physicochemically distinct penicillinase (γ-penicillinase). The relative proportions of these fractions do not vary significantly during normal exponential growth (see Pollock, 1952, 1956*b*). As both canavanine and *p*-fluorophenylalanine have a marked effect on growth, they would be expected to have an effect on the absolute amount of penicillinase appearing in the culture medium. To determine whether either amino acid analogue inhibited penicillinase formation to a greater extent than it inhibited growth or whether there was any disproportionate effect on any of the penicillinase fractions, the differential rate of synthesis of total cell-bound and exocellular enzyme was followed in cultures incubated in 17AA(−Phe) medium containing 50 μg. of *p*-fluorophenylalanine/ml. and in 17AA(−Arg) medium containing 50 μg. of canavanine/ml. The differential rate of formation of both penicillinase fractions is markedly impaired in the presence of canavanine (Fig. 2*a*). The effect may be observed within 15 min. of addition of the analogue and affects the two enzyme fractions to the same extent. On the other hand, the rate of synthesis in the presence of *p*-fluorophenylalanine (Fig. 2*b*) is only slightly affected within 45 min. of addition of the analogue and thereafter falls to about 40% of the control rate. This lower rate of penicillinase synthesis in the presence of *p*-fluorophenylalanine may continue for 2–3 hr. under these conditions. Further, if organisms incubated in this way for 90 min. are centrifuged and immediately resuspended in fresh 17AA(−Phe) medium containing *p*-fluorophenylalanine, the turbidity of the culture continues to increase linearly with time and exopenicillinase is then being synthesized at about 40% of the differential rate attained in the control.

Protection from canavanine and p-*fluorophenylalanine inhibition with arginine and phenylalanine*

The addition of 50 μg. of canavanine or *p*-fluorophenylalanine/ml. to cultures growing exponentially in 'S' peptone broth (Pollock & Perret, 1951) or 18AA medium had no effect on the growth rate or the differential rate of enzyme synthesis. The presence of arginine in cultures containing canavanine, or of phenylalanine in those containing *p*-fluorophenylalanine, protected the organisms from inhibition. Fig. 3*a* shows the effect of adding arginine (5 μg./ml.) and Fig. 3*b* that of adding phenylalanine (5 μg./ml.) to cultures incubated for varying periods of time in 17AA(−Arg) medium

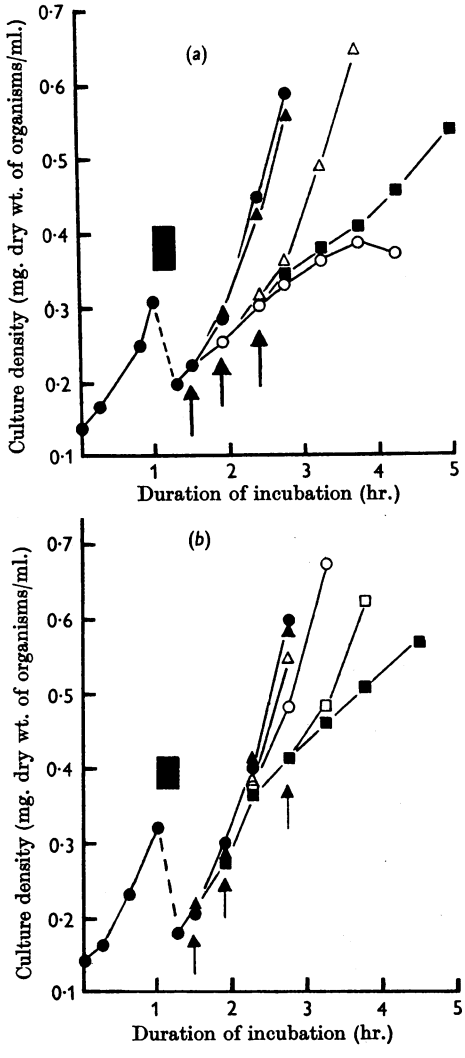

Fig. 3. Reversal of inhibitory effects on growth of (*a*) canavanine by arginine and (*b*) *p*-fluorophenylalanine by phenylalanine. (*a*) Organisms were grown in 17AA(−Arg) medium (●) for 1 hr. and then centrifuged and resuspended in experimental media (for duration of centrifuging see solid rectangle). Experimental media: ●, 17AA(−Arg) medium; ○, 17AA(−Arg+canavanine) medium (canavanine added at 1 hr. 20 min.); 17AA(−Arg+canavanine) medium with reversing concentrations of arginine added at intervals: ▲, at 1 hr. 30 min.; △, at 1 hr. 55 min.; ■, at 2 hr. 25 min. (see arrows). (*b*) Organisms were grown in 17AA(−Phe) medium for 1 hr. (●) and then centrifuged and resuspended in the experimental media (for duration of centrifuging see solid rectangle). Experimental media: ●, 17AA(−Phe) medium; ■, 17AA(−Phe+*p*-fluorophenylalanine) medium (*p*-fluorophenylalanine added at 1 hr. 30 min.); 17AA(−Phe+*p*-fluorophenylalanine) medium with reversing concentrations of phenylalanine added at intervals: ▲, at 1 hr. 30 min.; ○, at 1 hr. 55 min.; □, at 2 hr. 45 min. (see arrows).

containing 50 μg. of canavanine/ml. and 17AA(−Phe) medium containing 50 μg. of *p*-fluorophenylalanine/ml. respectively. Arginine added at the same time as canavanine completely protects the culture from inhibition; delay in addition of arginine leads to an increased lag period before the resumption of exponential growth. On the other hand, addition of phenylalanine leads to resumption of the full exponential growth rate within 40 min. even when incubation in the presence of *p*-fluorophenylalanine has continued for 90 min. In all cases, restitution of exponential growth was accompanied by a normal differential rate of synthesis of all the penicillinase fractions.

Incorporation of canavanine and p-*fluorophenylalanine into the protein fraction of organisms*

In view of the reports of incorporation of canavanine into protein of *S. aureus* (Richmond, 1959*b*) and *p*-fluorophenylalanine into *E. coli* (Munier & Cohen, 1956, 1959; Cohen & Munier, 1959), and the inhibitory action of these two analogues on growth and enzyme formation reported above, it was of interest to see whether these compounds were incorporated into the protein fraction of *B. cereus*. Cultures grown for 90 min. in 17AA(−Arg) medium containing 50 μg. of canavanine/ml. were precipitated with an equal volume of 10% trichloroacetic acid, containing 10 mg. of L-arginine hydrochloride/ml., and then fractionated as described by Richmond (1959*a*, *b*). In this way it was possible to show the presence of canavanine in the protein+cell-wall fraction of *B. cereus*. As canavanine cannot be detected in cell-wall preparations isolated from this strain after growth in canavanine, it seems likely that the analogue is incorporated in the protein fraction of the organisms.

The incorporation of *p*-fluorophenylalanine into the protein fraction of *B. cereus* was followed by growing cultures in 17AA(−Phe) medium containing *p*-fluorophenyl[β-^{14}C]alanine (final concentration 50 μg./ml. at 0·17 μc/μmole). The rate of incorporation of radioactivity increased during the first 45 min., thereafter reaching a constant rate with respect to time. The protein+cell-wall fraction was prepared from organisms grown under these conditions for 90 min., hydrolysed in 6N-HCl to liberate free amino acids and the presence of *p*-fluorophenylalanine was shown by radioautography after separating the hydrolysate by chromatography in butanol–acetic acid–water. The radioactive material obtained in this way was further identified as *p*-fluorophenylalanine by the 'finger-print' technique of Roberts *et al.* (1955). No other radioactive spot was detected in the hydrolysate prepared in this way.

Immunological properties of exopenicillinase synthesized in the presence of amino acid analogues

The results reported above imply therefore that the incorporation of the analogues into the protein fraction of the organisms interferes with the appearance of penicillinase activity to a greater extent than growth. An analysis of this problem is the subject of the succeeding paper; this paper is concerned with the immunological properties of the exopenicillinases formed in the presence of canavanine and *p*-fluorophenylalanine.

For convenience, the immunological character chosen for study was the behaviour of the enzyme in the neutralization reaction between enzyme and specific antiserum. This test corresponds to a constant antigen titration and its characteristics for normal exopenicillinase from *B. cereus* 569 and 569/H have been fully investigated by Pollock (1956*a*). As antiserum is added to normal enzyme (see Fig. 4), the enzymic activity of the enzyme–antibody mixture falls until about 5% of the original activity remains. The disappearance of activity is proportional to the amount of antiserum added except at low antibody–enzyme ratios where the neutralizing ability of the antiserum is slightly impaired. To obtain a quantitative description of this process, the following neutralization characteristics (see Fig. 4) have been measured.

(*a*) Plateau zone (A, Fig. 4). This is the range of antibody–enzyme ratios below which the antiserum does not show its maximum neutralizing titre. The width of the plateau zone is measured as the quantity of antiserum (in neutralization units) equivalent to the distance A in Fig. 4. To deter-

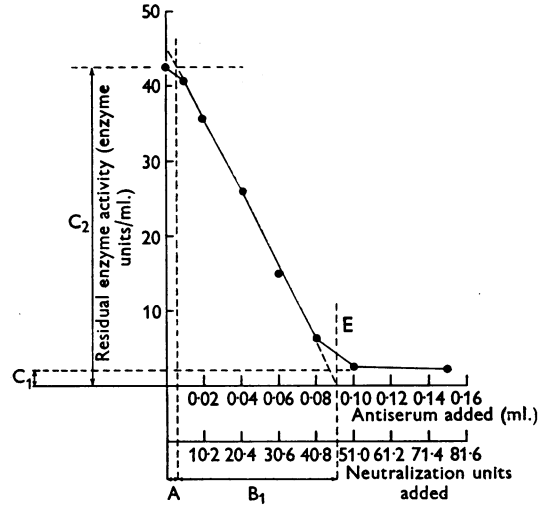

Fig. 4. Neutralization characteristics of normal exopenicillinase from *B. cereus* 569/H. For details see text.

mine this value exactly would require a large number of estimations and, consequently, the values quoted below are subject to large errors and are only approximate. In normal enzyme–antibody neutralization, with an initial enzyme titre of about 40 units/ml. the plateau zone corresponds to about 3·0 neutralization units.

(*b*) Neutralization phase (B_1, Fig. 4). This is the range of enzyme–antibody ratios lying between the plateau zone and the Equivalence Point (E, Fig. 4), over which the antiserum shows full neutralizing titre (measured in neutralization units/0·1 ml.). The maximum neutralization slope appears independent of initial enzyme concentration and is measured as C_2/B_1 (Fig. 4).

(*c*) Residual activity (C_1, Fig. 4). This is the amount of enzyme activity remaining at antibody–enzyme ratios greater than those required for maximum neutralization of enzyme. The percentage residual activity is 100 (C_1/C_2) (Fig. 4). Normal enzyme shows a residual activity of about 5–8 % of the initial enzyme titres.

Neutralization of enzyme synthesized in the presence of p-*fluorophenylalanine.* To test the neutralization characteristics of enzyme synthesized in the presence of *p*-fluorophenylalanine, organisms growing exponentially in 17AA(−Phe) medium were separated by centrifuging, washed and resuspended to a density of 0·2 mg. of bacteria/ml. in 17AA(−Phe) medium containing 50 μg. of *p*-fluorophenylalanine/ml. After incubation for 2 hr. (when the culture density is approx. 0·45 mg. dry wt. of bacteria/ml.), the organisms were removed by centrifuging and the supernatant liquid containing the exopenicillinase was mixed with one-third of its volume of 20 % (w/v) gelatin containing 1·6 mM-oxine for enzyme estimations. Normal exopenicillinase, prepared from a culture growing exponentially in 18AA medium, was diluted with fresh 18AA medium and oxine–gelatin to give approximately the same final titre as the material prepared in the presence of *p*-fluorophenylalanine. Fig. 5 compares the neutralization curves obtained when these enzyme preparations were titrated with anti-exopenicillinase serum. The enzyme synthesized in the presence of *p*-fluorophenylalanine differs from the normal in three respects. (1) The plateau zone is more marked with enzyme synthesized in the presence of *p*-fluorophenylalanine. In this experiment, the first 0·02 ml. of antiserum had little effect on the enzyme titre and the plateau zone, in this case, is equivalent to about 12–13 neutralization units/40 enzyme units, or about five times the normal. (2) The maximum neutralization slope of the antiserum (i.e. the slope of the neutralization curve during the neutralization phase) was about 55 % of that achieved with normal enzyme. (3) The residual activity after addition of 1·5 equivalents of antiserum (taking account of the lower neutralization titre) is about 1·2–1·4 times that found with normal enzyme.

The neutralization characteristics of varying concentrations of normal and analogue enzyme (made by diluting stock enzyme solutions in oxine–gelatin) are shown in Table 1. With increasing concentrations of enzyme but constant antibody–enzyme ratios, the plateau zone and neutralization slope were unaffected. The residual activity, however, is proportional to the initial enzyme titre.

Mixtures of normal and abnormal enzyme containing equal amounts of enzyme activity show a neutralization curve intermediate between the normal and the abnormal type. The results shown in Table 2 were obtained by neutralizing a mixture of 20 units of normal + 20 units of abnormal enzyme. In this case the neutralization slope of the mixture was about 245 neutralization units/ml. compared

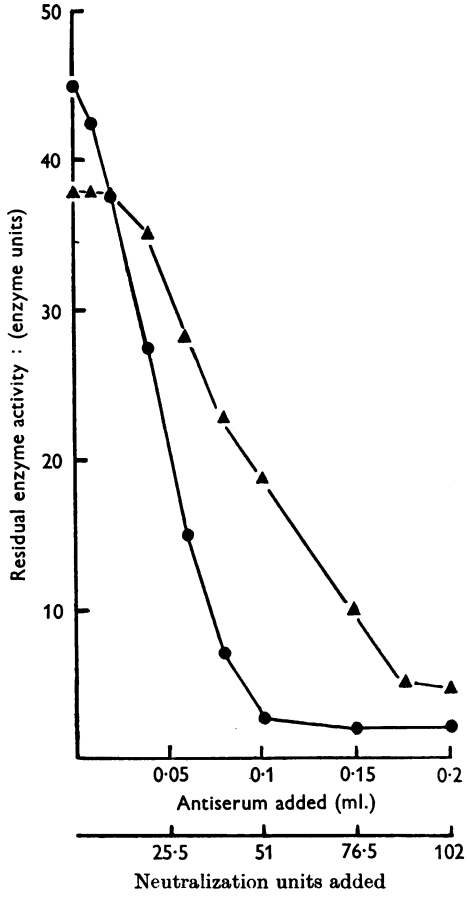

Fig. 5. Comparison of the neutralization characteristics of normal exopenicillinase (●) and that synthesized in the presence of *p*-fluorophenylalanine (▲).

with 420 neutralization units/ml. for normal and 115 neutralization units/ml. for abnormal enzyme. Although difficult to measure accurately, the plateau zone of the mixture was about three-quarters of that of unmixed abnormal enzyme of the same titre. The residual activity of mixtures of normal and abnormal enzyme was generally higher than that obtained with normal enzyme alone.

It follows from these experiments therefore that there is a *prima facie* case for believing that the enzyme synthesized in the presence of *p*-fluorophenylalanine reacts abnormally with antiserum prepared against pure normal exopenicillinase. There are, however, a number of alternative explanations which must first be excluded.

It is possible that some low-molecular-weight contaminant of the abnormal enzyme preparation not present, or present to a lesser extent, may interfere with the reaction between abnormal enzyme and antiserum. Salt is known to affect normal immunological precipitation reactions (see Boyd, 1956) but enzyme synthesized in the presence of *p*-fluorophenylalanine shows abnormal neutralization properties even after exhaustive dialysis against mM-Na_2HPO_4–KH_2PO_4 buffer, pH 7·0.

Interference by some large-molecular-weight compound, unrelated to penicillinase, is excluded by studying the effect on the neutralization of normal penicillinase of exocellular-protein preparations obtained by growing uninduced *B. cereus* 569 in an identical medium to that used for production of the abnormal enzyme. Addition of undiluted culture filtrates, obtained in this way, to normal enzyme preparations had no effect on the subsequent neutralization properties of the enzyme. On the other hand, culture filtrates of *B. cereus* 569, which had been induced with 1 unit of benzylpenicillin/ml. in the presence of 50 μg. of *p*-fluorophenylalanine/ml., yielded enzyme indistinguishable, immunologically, from abnormal enzyme prepared from the constitutive strain (see also Pollock, 1956*a*). Further, mixtures of equal activities of normal and abnormal enzyme from induced cultures behaved on neutralization with antiserum essentially as similar preparations made from the constitutive strain. As the only exocellular material known to be elaborated by *B. cereus* in response to addition of penicillin is penicillinase, it seems inescapable that the material reacting atypically with normal anti-exopenicillinase serum is a penicillinase or penicillinases with immunological properties modified by growth in the presence of *p*-fluorophenylalanine. The involvement of the analogue is confirmed by the fact that phenylalanine added to a growth medium containing *p*-fluorophenylalanine completely protects the culture from inhibition (even if the phenylalanine concentration is only about one-fifth of that of the *p*-fluoro derivative) and that the penicillinase synthesized under these conditions is indistinguishable from normal.

Up to this point, experiments on the immunological properties of penicillinase synthesized in the presence of *p*-fluorophenylalanine were all carried out on enzyme obtained from the growth medium after incubation of cells for 2 hr. in the presence of

Table 1. *Neutralization characteristics of exopenicillinase preparations*

The plateau zone was estimated as described in the text (see also Fig. 4). 0 represents a value too small for satisfactory estimation; e.u., enzyme units; n.u., neutralization units.

Normal exopenicillinase				*p*-Fluorophenylalanine exopenicillinase			
Enzyme concn. (e.u./ml.)	Plateau zone (n.u.)	Neutralization slope (n.u./0·1 ml.)	Residual activity (% of initial activity)	Enzyme concn. (e.u./ml.)	Plateau zone (n.u.)	Neutralization slope (n.u./0·1 ml.)	Residual activity (% of initial activity)
86	11±0·5	43	7·3	81	25±3·0	16·7	9·8
43	5±0·5	43	5·3	40·5	17±2·0	16·4	11·3
30·5	(1·5±0·5)	45	6·8	30·3	9±0·5	17·3	12·2
21·5	0	45	48	20·2	4±0·5	15·4	11·0

Table 2. *Neutralization characteristics of mixed exopenicillinase preparations*

For details see text; n.u., neutralization units.

	Normal exopenicillinase (42 units/ml.)	*p*-Fluorophenylalanine exopenicillinase (38 units/ml.)	Normal exopenicillinase (21 units/ml.) + *p*-fluorophenylalanine exopenicillinase (19 units/ml.)
Plateau zone (n.u.)	2·0±0·5	15±2·0	12±2
Neutralization slope (n.u./0·1 ml.)	42	17·5	24·5
Residual activity (% of initial activity)	7·2	9·8	11·5

the analogue. To study the immunological properties of enzyme found at various intervals after the addition of the analogue, a culture was incubated as described previously in 17AA(−Phe) medium containing 50 μg. of *p*-fluorophenylalanine/ml. and samples were removed at intervals for analysis. Table 3 shows the neutralization characteristics of the enzyme samples prepared in this way. The 'abnormality' of the enzyme (measured in terms of the width of the plateau zone and the neutralization slope) increases as incubation in the presence of *p*-fluorophenylalanine continues, reaching a maximum value after about 100–120 min. The relation of this finding to the increasing rate of *p*-fluorophenylalanine incorporation reported above has been investigated further in work described in the following paper (Richmond, 1960).

Neutralization of enzyme from canavanine-grown organisms. To test the neutralization characteristics of enzyme, formed in the presence of canavanine, organisms growing exponentially in 17AA(−Arg) medium were centrifuged down, washed in 0·9 % sodium chloride solution and resuspended to a density of about 0·2 mg. dry wt. of bacteria/ml. in 17AA(−Arg) medium containing 50 μg. of canavanine/ml. After incubation for 60 min. in this medium the organisms were removed by centrifuging and samples of exocellular enzyme prepared for titration with antiserum. Table 4 contains details of the neutralization characteristics of this enzyme preparation. As with exopenicillinase formed in the presence of *p*-fluorophenylalanine, the enzyme from canavanine-grown cells showed an increased width of plateau zone associated with a given amount of enzyme (measured in terms of activity). Under these conditions the plateau zone was equivalent to about 15 neutralization units/40 enzyme units. The residual enzyme activity was about 1·5 times the normal, but, unlike the enzyme from *p*-fluorophenylalanine-grown cells, the neutralization slope of the antiserum was about 85–90 % of that achieved with normal enzyme. Similar control experiments to those reported above showed that the abnormal neutralization characteristics of the enzyme formed in the presence of canavanine were due to a large-molecular-weight compound related to penicillinase. Neutralization of mixtures of equal amounts (on a basis of enzyme activity) of normal and abnormal enzyme showed similar characteristics to those reported previously; the width of the plateau zone was decreased by the presence of normal enzyme and the residual enzyme activity was related closely to the total initial enzyme titres. The changes observed in the neutralization slope during experiments of this kind were within the limits of experimental error, so it is not certain whether the addition of normal enzyme to enzyme formed in the presence of canavanine resulted in changes in the neutralization phase of the curve.

The neutralization characteristics of abnormal enzyme were examined at intervals after the addi-

Table 3. *Neutralization characteristics of exopenicillinase harvested at intervals after addition of* p-*fluorophenylalanine*

0 represents a value too low to measure accurately; e.u., enzyme units; n.u., neutralization units.

Time after addition of analogue (min.)	Enzyme titre (e.u.)	Plateau zone (n.u.)	Neutralization slope (n.u./0·1 ml.)	Residual activity (% of initial activity)
20	15	0	44	7·5
40	30	2±0·5	39	9·3
60	30	4±0·5	32	8·4
90	30	6±0·5	21	8·8
120	30	8±1·0	16·5	11·2
180	30	7±1·0	17·5	9·8
Normal enzyme	30	1·0±0·5	46·5	6·8

Table 4. *Neutralization characteristics of exopenicillinase synthesized in the presence of canavanine*

For details see text; e.u., enzyme units; n.u., neutralization units.

Exopenicillinase concn. (e.u./ml.)				
Normal enzyme	Canavanine-grown enzyme	Plateau zone (n.u.)	Neutralization slope (n.u./0·1 ml.)	Residual activity (% of initial activity)
—	40	16±2·0	33	10
—	30	9±1·0	33	13·2
—	20	5±0·5	34	11·3
30	—	2·0±0·5	37	6·8

tion of canavanine. The size of the plateau zone increased for only about 75 min. after the addition of the analogue, reaching a maximum value of about 18 neutralization units/40 enzyme units; the neutralization slope never fell below 85 % of the normal.

DISCUSSION

Growth of *B. cereus* in medium containing the amino acid analogues canavanine or *p*-fluorophenylalanine leads to the formation of material which reacts atypically with anti-exopenicillinase γ-globulin. As this material is formed by the inducible strain only after induction with penicillin, it is virtually certain that it must be a molecule related to penicillinase, rather than some non-specific factor resulting from the metabolic imbalance caused by addition of the analogues. As *p*-fluorophenylalanine is incorporated into the protein fraction of *B. cereus* 569/H grown in 17AA(−Phe) medium, and since Munier & Cohen (1956, 1959) and Cohen & Munier (1959) have shown that this analogue can replace phenylalanine in the protein fraction of cells grown in a glucose–ammonia–salts medium containing *p*-fluorophenylalanine, it seems possible that the material reacting atypically with anti-exopenicillinase γ-globulin consists of one or a number of abnormal penicillinases containing *p*-fluorophenylalanine in place of phenylalanine. Canavanine can be shown to be incorporated into the protein of *B. cereus* 569/H under suitable conditions and this could lead to the formation of abnormal penicillinase(s) containing canavanine in place of arginine.

Superficially, the abnormal characteristics of the enzymes formed in the presence of either analogue are similar. In both cases the main abnormality is the appearance of the plateau zone. This change is difficult to interpret. The neutralization of a preparation of normal exopenicillinase probably involves a reaction between a single homogeneous antigen (the exopenicillinase) and a heterogeneous collection of γ-globulin molecules with varying efficacy in the neutralization of the enzyme. Neutralization of abnormal enzyme is more complicated, for there is good reason to believe (see Richmond, 1960) that a heterogeneous collection of penicillinase molecules is formed in the presence of *p*-fluorophenylalanine and that these may react to a varying extent with the heterogeneous collection of antibody molecules. A reaction of this kind could readily lead to a modified neutralization curve.

It is conceivable that the presence of the plateau zone could imply that the abnormal enzyme preparations are mixtures containing normal enzyme together with a proportion of abnormal molecules, which are devoid of enzyme activity but which have a higher affinity than normal enzyme for the antiserum. Normal enzyme, however, which is known to be a single protein (Kogut *et al.* 1956), shows a small plateau zone and this is probably just an expression of impaired neutralization at low antibody–enzyme (antigen) ratios. Although a plateau zone has not been specifically reported in connexion with the neutralization of other enzymes by antisera, it is frequently encountered in immunological precipitation reactions (see Boyd, 1956) and may be detected in the enzyme neutralization curves published by several authors (e.g. Campbell & Fourt, 1939; Macfarlane & Knight, 1941; Adams, 1942; Krebs & Najjar, 1948; Henion, Mansour & Bueding, 1955). Under these circumstances therefore it seems more likely that the increased width of the plateau zone is due to the presence of abnormal penicillinase(s) with impaired specific activity and ability to be neutralized by normal antiserum. The lowered differential rate of penicillinase synthesis occurring after the addition of the analogues (see Fig. 2), and the lowered neutralization slope exhibited by the antisera, could be due to the formation of penicillinase-like proteins with lowered specific enzyme activity.

The nature of the material reacting atypically with anti-exopenicillinase γ-globulin is discussed further at the end of the next paper (Richmond, 1960).

SUMMARY

1. Addition of canavanine to cultures of *Bacillus cereus* growing exponentially in fully synthetic amino acid media, lacking arginine, leads to complete inhibition of growth and exopenicillinase formation after incubation for about 90 min. in the presence of the analogue. The differential rate of exopenicillinase formation is, however, markedly inhibited before growth ceases completely.

2. Addition of *p*-fluorophenylalanine to cultures growing exponentially in a fully synthetic medium lacking phenylalanine leads to impaired growth which nevertheless continues in an approximately linear manner for 4–5 hr. During this period the differential rate of exopenicillinase synthesis is increasingly inhibited, falling to about half normal after 90 min. incubation in the presence of the analogue.

3. Comparison of the immunological neutralization of exopenicillinase formed in the presence of canavanine or *p*-fluorophenylalanine with normal exopenicillinase suggests that the abnormal enzyme preparations may have impaired specific enzyme activity and lowered efficiency of reaction with specific anti-exopenicillinase serum.

4. The appearance of the abnormal material may be induced in the inducible (569) strain of

B. cereus under the conditions necessary for induction of penicillinase in normal cultures.

5. The immunological 'abnormality' of exopenicillinase preparations obtained from cultures increases as incubation in the presence of the amino acid analogues is prolonged.

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Incorporation of DL-β-(*p*-Fluorophenyl)[β-^{14}C]alanine into Exopenicillinase by *Bacillus cereus* 569/H

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Experiments reported in the preceding paper (Richmond, 1960) have shown that addition of *p*-fluorophenylalanine to cultures of *Bacillus cereus* 569/H (constitutive strain) led to the formation of material which has the properties of immunologically abnormal penicillinase(s). Munier & Cohen (1956, 1959) and Cohen & Munier (1959) have reported that *p*-fluorophenylalanine replaces phenylalanine but not tyrosine in the total cell-protein fraction of *Escherichia coli* and Vaughan & Steinberg (1958) have detected [^{3}H]-*p*-fluorophenylalanine in purified ovalbumin and lysozyme isolated from hen-oviduct preparations that had been incubated with tritiated *p*-fluorophenylalanine. Kruh & Rosa (1959) incubated rabbit reticulocytes *in vitro* with [^{14}C]-*p*-fluorophenylalanine and detected the analogue in haemoglobin prepared from the cells. In view of these findings, it was decided to measure the incorporation of *p*-fluorophenylalanine into the exopenicillinase fraction of *B. cereus* 569/H to try to relate the degree of incorporation of the analogue with the abnormal immunological properties of the enzyme (see Richmond, 1960).

Unfortunately the one feasible way of isolating pure exopenicillinase in the quantity available from these experiments is by precipitation with specific antiserum (Pollock & Kramer, 1958). This immediately limits the types of exopenicillinase molecules studied in this work to those precipitable with antiserum, and it is proposed to call this group of proteins the 'exopenicillinase-type proteins'. The reasons for believing that the exopenicillinase-type proteins are, in fact, related to exopenicillinase are given by Richmond (1960).

A further complication of this method of isolation of the exopenicillinase-type protein is that it is impossible to obtain an accurate measure of the specific *p*-fluorophenylalanine incorporation (μg. of *p*-fluorophenylalanine/μg. of exopenicillinase-type protein). Further, as the specific enzyme activity (enzyme units/μg. of exopenicillinase-type protein) may be impaired under these conditions (Richmond, 1960), enzyme activity cannot be used as an