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.

REFERENCES

- Adams, M. H. (1942). *J. exp. Med.* **76**, 175.
 Boyd, W. C. (1956). *Fundamentals of Immunology*, 2nd ed. New York: Interscience Publishers Inc.
 Campbell, D. H. & Fourt, L. J. (1939). *J. biol. Chem.* **129**, 385.
 Cohen, G. W. & Munier, R. (1959). *Biochim. biophys. Acta*, **31**, 347.
 Cowie, D. B., Cohen, G. N., Bolton, E. & de Robichon-Szulmajster, H. (1959). *Biochim. biophys. Acta*, **34**, 39.
 Henion, W. F., Mansour, T. E. & Bueding, E. (1955). *Exp. Parasit.* **4**, 40.
 Kantorowicz, O. (1951). *J. gen. Microbiol.* **5**, 276.
 Kogut, M., Pollock, M. R. & Tridgell, E. J. (1956). *Biochem. J.* **62**, 391.
 Krebs, E. G. & Najjar, V. A. (1948). *J. exp. Med.* **88**, 599.
 Macfarlane, M. G. & Knight, B. C. J. G. (1941). *Biochem. J.* **35**, 884.
 Mandelstam, J. & Rogers, H. J. (1959). *Biochem. J.* **72**, 654.
 Munier, R. & Cohen, G. N. (1956). *Biochim. biophys. Acta*, **21**, 592.
 Munier, R. & Cohen, G. N. (1959). *Biochim. biophys. Acta*, **31**, 378.
 Pollock, M. R. (1952). *Brit. J. exp. Path.* **33**, 587.
 Pollock, M. R. (1956*a*). *J. gen. Microbiol.* **14**, 90.
 Pollock, M. R. (1956*b*). *J. gen. Microbiol.* **15**, 156.
 Pollock, M. R. & Kramer, M. (1958). *Biochem. J.* **70**, 665.
 Pollock, M. R. & Perret, C. J. (1951). *Brit. J. exp. Path.* **32**, 387.
 Richmond, M. H. (1959*a*). *Biochem. J.* **73**, 155.
 Richmond, M. H. (1959*b*). *Biochem. J.* **73**, 261.
 Richmond, M. H. (1960). *Biochem. J.* **77**, 121.
 Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T. & Britten, R. J. (1955). In *Studies of biosynthesis in Escherichia coli*. Washington: Carnegie Institution of Washington (Publication No. 607).
 Urbá, R. C. (1959). *Biochem. J.* **71**, 513.

Biochem. J. (1960) **77**, 121

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

By M. H. RICHMOND

National Institute for Medical Research, The Ridgeway, Mill Hill, London, N.W. 7

(Received 20 January 1960)

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 immuno-

logical 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 (μ g. of *p*-fluorophenylalanine/ μ g. of exopenicillinase-type protein). Further, as the specific enzyme activity (enzyme units/ μ g. of exopenicillinase-type protein) may be impaired under these conditions (Richmond, 1960), enzyme activity cannot be used as an

accurate measure of the quantity of enzyme protein present in the preparations. It has, however, proved possible to measure the quantity of protein indirectly by following the incorporation of L-[¹⁴C]valine. The differential rate of incorporation of this amino acid into exopenicillinase-type protein preparations was unaffected by the presence of *p*-fluorophenylalanine and the total amount of radioactivity incorporated was a measure of the L-valine content of the exopenicillinase-type protein. This, in turn, was related to total quantity of protein in the exopenicillinase-type protein preparations and allowed the calculation of specific enzyme activities. In parallel experiments it was possible to measure the incorporation of *p*-fluorophenyl[β -¹⁴C]alanine into exopenicillinase-type protein and associate this with the effect of the analogue on the specific enzyme activity (enzyme units/ μ g. of exopenicillinase-type protein). Further experiments, in turn, associated these changes with the abnormal immunological properties of exopenicillinase-type proteins.

Munier & Cohen (1956, 1959) have reported that *p*-fluorophenylalanine completely replaces phenylalanine in proteins synthesized in the presence of the analogue. As normal exopenicillinase from *B. cereus* 569 (inducible strain) and 569/H (constitutive mutant strain) contains about seven phenylalanine residues, it would be interesting to determine the degree of replacement of phenylalanine in exopenicillinase-type proteins by the *p*-fluoro derivative. For technical reasons (see below) it is not possible to measure this by the effect of *p*-fluorophenylalanine on [¹⁴C]phenylalanine incorporation into exopenicillinase-type proteins and therefore the degree of replacement must be estimated indirectly. There is no increase in the phenylalanine content of the total cell protein or the total exocellular protein after incubation for about 60 min. in the presence of the analogue. Subsequently the *p*-fluoro derivative replaced an increasing number of the phenylalanine residues. As the relative proportion of *p*-fluorophenylalanine appearing in a number of protein fractions from the organisms (including exopenicillinase-type proteins) is constant under these conditions it is likely that most of the phenylalanine in the exopenicillinase-type proteins has been replaced by the *p*-fluoro derivative. The implications of these findings are discussed in the light of the experiments outlined above and the results reported by Richmond (1960).

MATERIALS AND METHODS

Organisms and media. The organisms used in these studies, together with their preparation for experimental purposes and the composition of casein-hydrolysate medium, 18AA medium and 18AA(-Phe) medium have

been described by Richmond (1960). For specific purposes, 18AA medium was modified by omitting valine [17AA(-Val) medium] or phenylalanine and valine [16AA(-Phe-Val) medium].

Amino acids. The β -*p*-(fluorophenyl)[β -¹⁴C]alanine was obtained from the Commissariat à l'Energie Atomique, Gif-sur-Yvette (Seine et Oise), France, at a specific activity of 2.35 μ C/ μ mole. The L-[¹⁴C]valine was generally labelled and was obtained from The Radiochemical Centre, Amersham, Bucks, at a specific activity of 4.0 μ C/ μ mole.

Anti-exopenicillinase. Neutralization experiments were carried out with rabbit anti-exopenicillinase γ -globulin no. 362; it had a titre of about 10⁴ neutralization units/ml. For experiments involving precipitation of exopenicillinase preparations (see Results section) a preparation made by Pollock & Kramer (1958) was used; it had a titre of 1.2 $\times$ 10⁶ neutralization units/ml. Both anti-penicillinase 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°.

Chemical fractionation. The following fractionation procedure was used to isolate the internal 'pool', lipid, ethanol-soluble protein, total nucleic acid and protein + cell-wall fractions (see McQuillen & Roberts, 1954). Samples containing about 1.0-2.0 mg. dry wt. of organisms were centrifuged at 3000 g, for 15 min. and the supernatant growth medium was discarded.

(1) Internal 'pool'. The organisms were resuspended in 5% (w/v) trichloroacetic acid and stored at 2° for 2-3 hr. The supernatant obtained after centrifuging was pooled with a similar supernatant obtained by a second extraction and constituted the internal 'pool' fraction.

(2) Lipid and ethanol-soluble protein. The insoluble residue obtained from (1) was extracted twice with aqueous ethanol (75%, v/v) at 75°. The extracts obtained on centrifuging were pooled and constituted the mixed lipid + ethanol-soluble protein fraction.

(2a) The ethanol-soluble protein was separated from the lipid by mixing the extract obtained in (2) with 3 vol. of ether + 3 vol. of water. After thorough mixing the ether layer was removed and the water phase, which contains the ethanol-soluble protein fraction, was dried *in vacuo*.

(3) Total nucleic acids. The cell pad obtained in (2) was extracted three times for 20 min. with 5% trichloroacetic acid at 95° and the extracts were combined. More than 90% of the total nucleic acid in the organisms may be extracted in this way.

(4) The residue from (3) constituted the protein + cell-wall fraction. It was washed successively with aqueous ethanol (75%, v/v) and ether and dried in a stream of air. (Yield, 45-60% of the original dry wt. of the organisms.)

Hydrolysis. Hydrolysis to liberate peptides from the protein + cell-wall fraction was carried out in 12N-HCl at 35°. Samples removed at intervals and analysed by chromatography and ionophoresis showed that hydrolysis for 36 hr. gave the most satisfactory yield of peptide material. To liberate free amino acids, the protein + cell-wall fraction was hydrolysed for 16 hr. in 6N-HCl at 105° in a sealed tube. The HCl was removed *in vacuo*.

Ionophoresis. Peptides were separated in collidine-acetic acid-water (0.7:0.3:100, by vol.), pH 7.0 (Newton & Abraham, 1954). The neutral and acidic bands obtained at this pH were eluted in water and run again in 2.0M-acetic acid (pH 2.4). Ionophoresis was carried out in an apparatus

of the type described by Kunkel (1954), with a potential gradient of about 15 v/cm.

Chromatography. Free amino acids obtained by hydrolysis of the protein + cell-wall fraction were separated by descending chromatography in butanol-acetic acid-water (60:10:26, by vol.). For quantitative determination of the relative quantities of tyrosine, phenylalanine and leucine + isoleucine (+*p*-fluorophenylalanine, if present) hydrolysates of the protein + cell-wall fraction were transferred quantitatively to Whatman no. 3 paper as 5 cm. bands at the origin. The chromatograms were developed for 19–20 hr. and the location and quantity of the amino acids present determined as described by Mandelstam & Rogers (1959). The $P_{\text{phenylalanine}}$ values of the tyrosine, valine + methionine and leucine + isoleucine + *p*-fluorophenylalanine spots were about 0.68, 0.87 and 1.16 respectively. The quantity of *p*-fluorophenyl[β - ^{14}C]alanine present was determined by estimating the radioactivity present in a representative sample removed from the eluate containing leucine + isoleucine + *p*-fluorophenylalanine after development with ninhydrin. Separate experiments showed that known quantities of *p*-fluorophenyl[β - ^{14}C]alanine treated on paper with ninhydrin were not appreciably volatile unless the papers were moist and heated to temperatures greater than 50°.

Isolation and estimation of total exocellular protein. Samples of about 10–20 ml. were removed from cultures and centrifuged at 3000 *g*, for 30 min. The supernatant growth medium was decanted into tubes and centrifuged again under the same conditions for a further 30 min. The supernatant obtained in this way was passed through an Oxoid membrane filter and mixed with sufficient 50% (w/v) trichloroacetic acid to give a final concentration of 5%. After standing overnight at 2° the protein precipitates were centrifuged at 3000 *g* for 30 min.; the centrifuge tubes were then turned in the centrifuge cups through 180° and the precipitates resedimented at 3000 *g* for a further 30 min. The supernatants were decanted and the protein precipitates estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). Suitable dilutions of crystalline bovine-serum albumin (Armour Laboratories Ltd.) were used as standards. When it was not required to estimate the total amount of exocellular protein (e.g. when radioactive determinations only were required), 100 μg . of serum albumin/ml. was added to the filtrate before precipitation. The 'carrier' greatly facilitated handling of the precipitates and the centrifuging time could be reduced to one period of 20 min. at 3000 *g*.

Radioactivity determinations. Samples were prepared for counting as described by Richmond (1959). Counting was continued until more than 10^3 counts above background had been registered, and the samples were assumed to be infinitely thin unless they contained more than 0.5 mg. dry wt. of material/cm.² A sample of [^{14}C]valine containing 1.0 μmc registered 193 counts/min. in the equipment used.

Determination of differential rates. Unless otherwise stated, all differential rates have been measured as described by Richmond (1959). The growth of cultures was followed turbidimetrically and the culture density determined by reference to a standard curve. When incubation in the presence of *p*-fluorophenylalanine was continued for long periods, it was found experimentally that the standard curve no longer gave a true estimate of dry weight of organisms present in these cultures. This was due to the

lower scattering caused by the increased size of organisms obtained under these conditions (see Mitchell, 1949). In practice, however, all differential rates have been measured on the basis of the standard curve; the resulting apparent stimulation in differential rate of the inhibited culture, due to this procedure, is only about 10% above normal after incubation for 2 hr. in the presence of *p*-fluorophenylalanine and increases of this order have been discounted.

Purification of radioactive exopenicillinase and exopenicillinase-type protein. Both exopenicillinase and exopenicillinase-type protein were purified as described by Pollock & Kramer (1958) except that non-radioactive 'carrier' exopenicillinase was not added because it has been shown that exopenicillinase synthesized in the presence of *p*-fluorophenylalanine has abnormal neutralization characteristics (Richmond, 1960) and may not therefore be a true carrier.

The validity of this method for purifying the exopenicillinase preparation depends upon the following considerations.

(a) All the enzyme proteins comprising the abnormal preparation react with glass powder. To test this point, crude preparations (see Richmond, 1960) of normal or abnormal exopenicillinase were adsorbed by step-wise addition of increasing quantities of glass powder. Fig. 1 shows that about three times the amount of glass powder required for the normal preparation had to be added for maximum adsorption of the abnormal penicillinase. The total amount of enzyme adsorbed was, however, approximately the same in both cases (90%). The elution patterns of the normal and abnormal enzyme preparations were similar in these experiments; the immunological characteristics of the eluted enzyme, the enzyme not adsorbed on glass and the enzyme initially added to the experiment were approximately the same (see Table 1). It appears therefore that the 'abnormal' enzyme behaves in a manner sufficiently homogeneous to allow use of adsorption on to glass powder as a step in the purification, and there is no

Fig. 1. Adsorption of normal exopenicillinase and exopenicillinase-type protein on to glass powder. ●, Normal enzyme; ○, exopenicillinase-type protein

Table 1. *Neutralization characteristics at various stages of purification of exopenicillinase-type protein preparations from Bacillus cereus 569/H grown in the presence of p-fluorophenylalanine*

Normal enzyme was purified as described by Pollock & Kramer (1958). The abnormal enzyme was purified in a similar way except that three times the quantity of glass powder was used per unit of enzyme activity (see text and Fig. 1). e.u., Enzyme units; n.u., neutralization units.

Enzyme preparation	Plateau zone (n.u.)	Neutralization slope (n.u./0.1 ml.)	Residual activity (% of initial enzyme activity)	Initial enzyme activity (e.u./ml.)
Crude normal	4 ± 1	43	5.7	35
Crude abnormal	17 ± 2	18.3	9.3	27
Residue after maximum glass adsorption	18 ± 2	16	9.8	17
Eluted enzyme	15 ± 2	19.5	9.5	43

evidence for preferential elution of any one fraction of the mixed exopenicillinase-type molecules. In addition, it was not possible to detect the non-adsorption of any cross-reacting protein devoid of enzyme activity.

(b) Addition of 1.5 equivalent quantities of antiserum completely precipitated (> 90%) normal exopenicillinase preparations (Pollock & Kramer, 1958). To check that the same was true of abnormal enzyme preparations, samples were titrated with antiserum to estimate the equivalence point and then treated with 1.5 equivalence doses of antiserum at concentrations of enzyme at which precipitation occurred. In all cases 85% or more of the enzyme activity was precipitated. The equivalence point of abnormal enzyme treated in this way was frequently two to three times as high as that of the normal enzyme. In no case was there any sign of solubility of enzyme-antiserum precipitates in excess of antibody (cf. Boyd, 1956). With this method of purification there is a high inherent risk of contamination of the enzyme-antiserum precipitates with non-specific radioactivity. The level of contamination was controlled in parallel experiments by incubating a culture of the uninduced inducible strain (569) under identical conditions to the experimental culture of the constitutive strain (569/H) and then adding non-radioactive 'carrier' exopenicillinase at the end of the experiment. Isolation of the exopenicillinase from such cultures, followed by radioactivity determinations, controlled the level of contamination both by high-molecular-weight materials precipitated with the anti-exopenicillinase-enzyme precipitates and by non-specific adsorption of low-molecular-weight radioactive material on to the precipitates [see Pollock & Kramer (1958) for detailed discussion of these points].

RESULTS

Preliminary experiments on the effect of *p*-fluorophenylalanine on exopenicillinase formation showed that an independent method of measuring the protein content of exopenicillinase-type protein was necessary before any deductions could be made about the effect of the analogue on specific enzyme activity. Because preliminary experiments were hopeful, but despite earlier evidence to the contrary (Halvorson & Spiegelman, 1952), it was decided to see whether the incorporation of amino acids other than phenylalanine into exopenicillinase could be used as a measure of exopenicillinase

Fig. 2. Effect of *p*-fluorophenylalanine on incorporation of valine into cell-protein fractions of cultures of *B. cereus* 569/H. Total cell protein + *p*-fluorophenylalanine, ○; total cell protein + *p*-fluorophenylalanine, △. Total exoprotein + *p*-fluorophenylalanine, ●; exopenicillinase-type protein + *p*-fluorophenylalanine, ▲. Exopenicillinase-type protein + *p*-fluorophenylalanine, □. Medium: 16AA(-Phe - Val) medium containing 50 μg. of L-[¹⁴C]valine/ml. (0.23 μC/μmole) ± 50 μg. of *p*-fluorophenylalanine.

protein synthesis. To test this possibility, the effect of *p*-fluorophenylalanine was studied on the incorporation of [14 C]valine into the total cell protein, total exocellular protein and exopenicillinase-type protein of cultures of *B. cereus* 569/H incubated in 16AA(-Phe-Val) medium $\pm$ 50 μ g. of *p*-fluorophenylalanine/ml. (Fig. 2). A culture of the inducible strain (569) was incubated under identical conditions (i.e. in the absence of inducer) and used as a control (see Materials and Methods section). The differential rate of [14 C]valine incorporation into the total cell-protein fraction was unaffected by the analogue (actually about 10 % stimulation, but see Materials and Methods section) the incorporation both into the total exocellular protein and exopenicillinase-type protein appeared slightly depressed. The depression in differential rate of valine incorporation into the exocellular proteins could either have been due to a fall in the total amount of exocellular protein synthesized per unit growth or to a specific effect of the analogue on valine incorporation. To decide between these possibilities, a culture was incubated in 16AA(-Phe-Val) medium containing *p*-fluorophenylalanine + [14 C]valine and the specific radioactivities (counts/min./ μ g. of protein) of the total cell protein and total exocellular protein were determined in the absence of carrier. Table 2 shows that the specific radioactivity of the protein fractions was approximately the same, despite a fall of about 10 % in the differential rate of total exocellular-protein synthesis. This implies that *p*-fluorophenylalanine has no specific effect upon the valine content of either protein fractions and it is reasonably certain therefore that it has no effect upon the incorporation of valine into the exopenicillinase-type protein.

In practice it was possible to use the appearance of 14 C from [14 C]valine in exopenicillinase-type protein as a measure of the valine content of that fraction. This depends upon the following conditions, which were found to be fulfilled:

(1) *B. cereus* requires an external supply of valine for normal growth and enzyme formation (M. H. Richmond, unpublished work).

(2) When organisms are grown in 17AA(-Val) medium containing [14 C]valine $\pm$ *p*-fluorophenylalanine, all but about 5 % of the radioactivity in the protein + cell-wall fraction is present as valine.

(3) Protein breakdown in this strain is about 8-10%/hr. in non-growing cultures. The rate of breakdown in growing cultures is usually markedly less (see Pollock & Kramer, 1958; Urbá, 1959). This means that at a culture density of 0.3 mg. dry wt. of organisms/ml. the supply of valine by protein breakdown is at the most 0.05-0.08 μ g. of L-valine/ml./hr. This is approx. 0.1 % of the external valine concentration at the beginning of

Table 2. Specific radioactivity of total cell- and exocellular-protein fractions from cultures of *Bacillus cereus* 569/H incubated in the presence of *p*-fluorophenylalanine and L-[14 C]valine

Organisms growing exponentially in 17AA(-Phe) medium were harvested and resuspended to a density of about 10 μg . dry wt. of bacteria/ml. in 16AA(-Phe-Val) medium containing 50 μg . of L-[^{14}C]valine/ml. (0.34 $\mu\text{Ci}/\mu\text{mole}$). After growth for 3 hr. in this medium *p*-fluorophenylalanine (50 μg /ml.) was added to the culture and samples of bacteria taken at intervals were treated to determine the specific radioactivity of the protein + cell wall and the total exocellular-protein fractions. Values in parentheses are approximate because of the inaccuracy of the protein estimations.

Interval from addition of analogue (hr.)	Interval from addition of tracer (hr.)	Culture density (mg./ml.)	Protein content (μg . of protein/mg. dry wt. of organisms)	Specific radioactivity in protein + cell-wall fraction (counts/min./ μg . of protein)	Total exocellular protein (μg . of protein/mg. dry wt. of organisms)	Specific radioactivity of exoprotein (counts/min./ μg . of protein)
0	3.0	0.09	395	26.7	10.3	(20)
0.5	3.5	0.15	415	25.4	11.3	(20)
1.0	4.0	0.23	420	26.1	10.4	21
1.5	4.5	0.32	395	28.0	10.6	21
2.0	5.0	0.405	375	27.6	9.8	23
3.0	6.0	0.52	415	26.8	9.3	24

Table 3. Valine content of normal exopenicillinase synthesized by *Bacillus cereus* 569/H in 17AA(-Val) medium with L-[¹⁴C]valine

A culture growing exponentially in 18AA medium was harvested and the organisms were transferred to 17AA(-Val) medium containing 50 μ g. of L-[¹⁴C]valine/ml. (0.23 μ C/ μ mole). Samples taken at intervals were treated to yield purified exopenicillinase as described in the text. e.u., Enzyme units.

Interval after addition of tracer (min.)	Culture density (mg./ml.)	Total amount of crude exo- penicillinase (e.u.)	Total amount of purified exo- penicillinase (e.u.)	Recovery (%)	Exopenicil- linase protein equivalent of purified enzyme* (μ g.)	Specific radioactivity of purified exopenicillinase (μ C/ 10^3 e.u.)	Total L-valine content of exopenicillinase (μ g.)	Valine content of purified exopenicillinase (%, by wt.)
0	0.15	—	—	—	—	—	—	—
40	0.23	4 950	1 640	33	5.2	0.32	0.27	5.1
60	0.33	15 200	6 200	41	19.8	0.28	0.87	4.4
90	0.49	32 700	9 450	29	30.2	0.26	1.27	4.2
120	0.62	52 900	19 650	38	62.1	0.30	3.0	4.8
								Av. 4.6

* 1 μ g. of crystalline exopenicillinase $\equiv$ 312 enzyme units.

the experiment and about 0.5 % of that at the end. These quantities will not reduce, significantly, the specific activity (μ C/ μ mole) of the [¹⁴C]valine incorporated.

(4) The 'pool' concentration of [¹²C]valine at the beginning of the experiment does not dilute the exogenous [¹⁴C]valine to an appreciable extent. This can be seen from the result shown in Fig. 2, in which the differential rate of [¹⁴C]valine incorporation into the protein + cell wall is constant throughout the experiment and extrapolates through the origin.

Strong additional support for this method of estimating the valine content of a protein fraction is given by the experiments designed to measure the specific valine content of normal purified exopenicillinase. A culture growing exponentially in 18AA medium was centrifuged, the organisms were washed in 17AA(-Val) medium and then resuspended [to a density of about 0.1 mg. dry wt. of bacteria/ml.] in 17AA(-Val) medium containing L-[¹⁴C]valine. The culture was incubated under normal conditions and samples, removed at intervals, were treated to yield the purified exopenicillinase fraction. The specific radioactivity of the exopenicillinase on an activity basis (μ C/units of enzyme activity) was found to be approximately constant throughout the experiment (see Table 3). As the specific enzyme activity of crystalline exopenicillinase from this strain is known (Kogut, Pollock & Tridgell, 1956), it is possible to calculate the specific valine content of exopenicillinase. Table 3 shows that this value is approximately constant at an average of 4.6 %, which agrees reasonably well with the mean value of about 5.0 % obtained by chemical methods (S. Jacobs, personal communication). As *p*-fluorophenylalanine has no effect upon the differential rate of total cell protein, total exocellular protein, nor upon exopenicillinase-type protein synthesis, and as no effect of the analogue can be detected on the incorporation of [¹⁴C]valine into any protein fraction investigated, it follows that the incorporation of [¹⁴C]valine into exopenicillinase-type protein in these experiments provides a measure of the protein content of that fraction.

*Incorporation of β -(*p*-fluorophenyl)- [β -¹⁴C]alanine into cell fractions*

A culture growing exponentially in 18AA medium was centrifuged and the organisms, after washing in 17AA(-Phe) medium, were resuspended to a density of about 0.1 mg. dry wt. of bacteria/ml. in 17AA(-Phe) medium containing 50 μ g. of *p*-fluorophenyl[β -¹⁴C]alanine/ml. (0.17 μ C/ μ mole) and incubated under standard conditions. Samples removed at intervals were fractionated as

described in the Materials and Methods section to yield the internal 'pool', lipid, ethanol-soluble protein, total nucleic acid and protein+cell-wall fractions. The distribution of radioactivity in these fractions after incubation for 2 hr. in the presence of the analogue is shown in Table 4. More than 70% of the radioactivity was found in the protein+cell-wall fraction. Isolation of the bacterial cell walls from organisms grown under similar conditions showed that the amount of *p*-fluorophenylalanine incorporated was about 5% of that incorporated into an equal weight of protein+cell-wall fraction. Hydrolysis of the protein+cell-wall fraction obtained under these conditions in 12N-HCl at 35° for 36 hr. followed by ionophoresis of the product in collidine-acetate buffer, pH 7.0 (Newton & Abraham, 1954), showed the presence of radioactive material in the neutral and acidic bands of peptides obtained by this procedure. Further separation of the neutral peptides by ionophoresis in 2.0M-acetic acid at pH 2.4 showed the presence of a number of radioactive peptide components. It seemed very likely therefore that the incorporation of *p*-fluorophenylalanine into the

protein of *B. cereus* entailed the formation of peptide bonds. Hydrolysis of the protein+cell-wall fraction to liberate free amino acids showed, on subsequent chromatography, only one radioactive

Table 4. *Distribution of p-fluorophenyl[β-¹⁴C]-alanine in cell fractions of Bacillus cereus 569/H grown in the presence of the analogue*

Cell fraction	Percentage of total radioactivity incorporated
1 Internal 'pool'	0*
2 Mixed lipid and ethanol-soluble protein	7
2a Lipid (ether-soluble fraction)	2
2b Ethanol-soluble protein (water-soluble fraction)	5
3 Total nucleic acid	5
4 Protein+cell wall	74
Recovery (%) 93	

* Pool concentration of *p*-fluorophenylalanine is very low; the maximum level detected corresponded to 0.2 μmole/g. dry wt. of organisms.

Fig. 3. (a) Incorporation of *p*-fluorophenyl[β-¹⁴C]alanine into total cell protein and exopenicillinase-type protein. ○, Total cell protein, constitutive strain; ●, inducible strain uninduced. Δ, Exopenicillinase-type protein, constitutive strain; ▲, inducible strain uninduced (for details see text). *p*-Fluorophenyl[β-¹⁴C]alanine present was 50 μg./ml. (0.34 μg./μmole). (b) Effect of *p*-fluorophenylalanine on differential rate of appearance of exopenicillinase activity. ●, 17AA(-Phe) medium; ▲, 17AA(-Phe + *p*-fluorophenylalanine) medium.

spot corresponding to marker *p*-fluorophenylalanine. The specific radioactivity ($\mu\text{C}/\mu\text{mole}$) of the material isolated in this way was the same as that of the *p*-fluorophenylalanine added to the growth medium. It follows therefore that the radioactivity present in the protein+cell-wall fraction may be used as a direct measure of the amount of *p*-fluorophenylalanine incorporated into that fraction.

Incorporation of p-fluorophenyl[β - ^{14}C]alanine into total cell protein and exopenicillinase-type protein

A culture was incubated in 17AA(–Phe) medium containing *p*-fluorophenyl[β - ^{14}C]alanine and samples, removed at intervals, were treated to yield the total cell-protein and exopenicillinase-type protein fractions. Fig. 3 (a) shows that the differential rate of incorporation of fluorophenylalanine into these fractions of the constitutive strain increases for about 1 hr. before becoming approximately constant. Similar increases are found to occur in the uninduced inducible strain except that no appreciable activity can be detected in the isolated exopenicillinase-type protein fractions. Fig. 3 (b) shows the differential rate of appearance of exopenicillinase (measured in terms of activity) in the same experiment. Comparison of the differential rate of *p*-fluorophenylalanine incorporation into the exopenicillinase-type protein fraction with the change in rate of appearance of exopenicillinase activity shows that the increase in rate of analogue incorporation is associated with a fall in enzyme activity in the culture. It is not possible, from these experiments, however, to determine the increase in exopenicillinase-type protein associated with these changes. Further, although the foregoing experiments show that *p*-fluorophenylalanine is incorporated into the total protein and exopenicillinase fractions of the organisms, they cannot show whether the unnatural amino acid is incorporated in place of phenylalanine, nor the extent of the replacement, if any.

As a result of the experiments reported in the Results section it is possible to use the net incorporation of [^{14}C]valine into the exopenicillinase-type protein as a measure of total protein content of that fraction. Further, the net *p*-fluorophenylalanine content of the exopenicillinase-type protein may be measured by following the incorporation of *p*-fluorophenyl[β - ^{14}C]alanine into the same fraction in a parallel experiment. To relate the amount of analogue incorporated into the exopenicillinase-type protein with the change in specific enzyme activity (enzyme units/ μg . of exopenicillinase-type protein), the following experiment was carried out. A culture of *B. cereus* 569/H growing exponentially in 18AA medium was centrifuged, the organisms

were washed in 16AA(–Phe–Val) medium and divided into three equal batches and resuspended to a density of 0.1 mg. dry wt. of bacteria/ml. under the following conditions. Batch 1 was resuspended in 16AA(–Phe–Val) medium containing 20 μg . of L-[^{14}C]valine. Samples taken from this culture at intervals were used to prepare normal [^{14}C]valine-labelled exopenicillinase. The amount of radioactivity incorporated into these samples, together with their enzymic activity, showed the specific valine content of normal exopenicillinase to be about 4.6 %, which is in good agreement with previous values (see Table 3). Batch 2 was resuspended in 16AA(–Val–Phe) medium containing L-[^{14}C]valine and unlabelled fluorophenylalanine. Samples were taken from this culture at the same intervals as culture 1 and treated to give the purified exopenicillinase fraction. The valine content of this preparation (by virtue of the arguments set out above) gave an estimate of the quantity of exopenicillinase-type protein, (column 4; Table 5, part i). Comparison of the enzyme activity (column 2; Table 5, part i) with the protein content of the purified samples (column 4) gave a measure of the specific enzyme activity (column 5). This was found to be normal at the beginning of the experiment but fell as incubation in the presence of the analogue continued and reached a value about 45 % of normal (column 6). Batch 3 was incubated in 16AA(–Val–Phe) medium containing unlabelled L-valine and *p*-fluorophenyl[β - ^{14}C]alanine. Samples were taken for this culture and treated exactly as with culture (2). In this case the radioactivity content is a measure of *p*-fluorophenylalanine incorporation and, since the growth curves for cultures 2 and 3 are almost identical, it is possible to relate the incorporation of the analogue to the [^{14}C]valine content of culture 2 and thus to the appearance of exopenicillinase-type protein and enzyme activity. The results of this part of the experiment are shown in Table 5, part ii. At the beginning of the experiment, when no effect of the analogue on specific enzyme activity could be detected in culture 2 (column 6), no *p*-fluorophenylalanine could be detected in the exopenicillinase-type protein of culture 3 (columns 9 and 11). As incubation in the presence of the analogue continued and the specific enzyme activity of the exopenicillinase-type protein fell, the incorporation of *p*-fluorophenylalanine increased. It is possible from this experiment to deduce that the exopenicillinase formed after incubation for 2½ hr. in the presence of *p*-fluorophenylalanine had 46 % of normal specific enzyme activity (Table 5, part i; column 6) and a *p*-fluorophenylalanine content of about 3 %, by wt. (see Table 5, part ii; column 11). Assuming that the molecular weight of the exopenicillinase is not

Table 5. Comparison of valine and p-fluorophenylalanine content of the exopenicillinase-type protein synthesized by *Bacillus cereus* 569/H in the presence of p-fluorophenylalanine

(i) (Culture 2): incorporation of [¹⁴C]valine into exopenicillinase-type protein from organisms growing in 16AA(-Phe - Val) medium + 25 µg. of unlabelled p-fluorophenylalanine/ml. + 20 µg. of [¹⁴C]valine/ml. (0.23 µC/µmole). Values in parentheses refer to non-radioactive carrier penicillinase added to control experiments carried out with the uninduced inducible strain (569) (see Materials and Methods section); e.u., enzyme units. For other details, see text.

Calc. as	Culture density (mg./ml.)	Total crude penicillinase (e.u.) (1)	Total purified penicillinase (e.u.) (2)	Total valine content of purified		Recovery (activity) (%) (2) × 100 (1)	Total amount of purified exopenicillinase-type protein (µg.)* (4)	Specific enzyme activity (e.u./µg. of exopenicillinase protein) (5)	Percentage of normal specific activity (6)
				purified exopenicillinase-type protein (µg.) (3)	exopenicillinase-type protein (µg.) (3)				
...	...	...	...	...	...	...	...	...	...
0.13	8 860	1 680	19	0.26	—	—	—	—	—
0.20	11 000	3 070	28	0.49	—	—	5.4	310	100
0.28	35 600	8 200	32	2.02	—	—	10.2	302	97
0.42	28 300	11 600	41	3.22	—	—	43.0	192	61
0.47	33 700	12 200	36	4.04	—	—	68.5	168	54
0.58	(44 000)	(16 700)	38	0.25	—	—	86.0	143	46
Control	...	...	...	...	...	...	...	...	...

* Assuming a value for valine content of purified exopenicillinase at 4.6% (see above and Table 3).

(ii) (Culture 3): incorporation of p-fluorophenyl[β-¹⁴C]alanine into exopenicillinase-type protein from organisms growing in 16AA(-Phe - Val)⁺medium + 25 µg. of p-fluorophenyl[β-¹⁴C]alanine/ml. (0.55 µC/µmole) + 20 µg. of unlabelled L-valine/ml. Values in parentheses refer to non-radioactive penicillinase added to control experiments carried out with the uninduced inducible strain (569); e.u., enzyme units. For other details, see text.

Calc. as	Culture density (mg./ml.)	Total crude penicillinase (e.u.) (7)	Total purified penicillinase (e.u.) (8)	Recovery (activity) (%) (8) × 100 (7)	Total p-fluoro-phenylalanine content of purified exopenicillinase-type protein (µg.) (9)	True total amount of exopenicillinase-type protein (µg.)† (10)	p-Fluorophenyl-alanine content of exopenicillinase-type protein (%) (11)		Net no. of residues of p-fluorophenyl-alanine in exopenicillinase-type molecules (12)	Possible percentage replacement of phenylalanine residues‡
							(9) × 100 (10)	(10) × 100 (11)		
...	...	...	...	...	...	...	...	...	...	...
0.13	8 750	2 350	27	—	—	—	—	—	—	—
0.20	11 100	2 380	21	0.11	—	—	0	0	—	Nil
0.285	24 500	6 860	28	0.86	—	—	1.4	1.4	1.9	28
0.42	27 400	8 500	31	1.56	—	—	2.4	2.4	4.3	61.5
0.47	32 000	12 500	39	2.8	—	—	3.1	3.1	5.4	77
0.56	(44 000)	(14 700)	33	0	—	—	3.2	3.2	5.6	80
Control	...	...	...	...	...	...	0	0	0	0

† Valine content of exopenicillinase-type protein at a given moment is assumed to be the same as that for culture 2; see (i) above.

‡ Assuming seven phenylalanine residues/mole of exopenicillinase (S. Jacobs, unpublished work).

affected by the incorporation of *p*-fluorophenylalanine, these results correspond to a net incorporation of between five and six *p*-fluorophenylalanine residues/molecule of exopenicillinase-type protein (Table 5, part ii; column 12).

It was not possible to know from these experiments whether the *p*-fluorophenylalanine was incorporated into the exopenicillinase-type protein at the expense of phenylalanine, nor was it possible to investigate this problem by studying the effect of *p*-fluorophenylalanine on the incorporation of [14 C]phenylalanine into the exopenicillinase-type protein in experiments analogous to those involving [14 C]valine. It was found that exogenous [14 C]-phenylalanine was used preferentially by the organisms and even an external ratio of 20 parts of *p*-fluorophenylalanine to 1 part of phenylalanine led to an uptake of phenylalanine in preference to the *p*-fluoro derivative. Further, it has not proved possible to use any specific precursor of phenylalanine which does not interfere with *p*-fluorophenylalanine assimilation. The only radioactive precursor available was [14 C]glucose, which was converted into phenylalanine in about 0.1% yield and in addition labelled most of the amino acids of the protein fraction to an appreciable extent, even in 17AA(-Phe) medium.

In these circumstances therefore it was decided to study the effect of *p*-fluorophenylalanine on the uptake of phenylalanine into various protein fractions that could be isolated readily from cultures of *B. cereus* 569/H, as this should give an indirect measure of phenylalanine replacement in the exopenicillinase-type protein.

Replacement of phenylalanine in total cell protein and total exocellular protein by p-fluorophenylalanine

The effect of *p*-fluorophenylalanine on the phenylalanine content of total cell protein was studied by following the appearance of phenylalanine, tyrosine and *p*-fluorophenylalanine in the protein + cell-wall fraction of organisms incubated in the presence of the analogue. The phenylalanine and tyrosine may each be estimated directly on the chromatograms but *p*-fluorophenylalanine does not separate from leucine + isoleucine in the solvent used in these experiments and must be estimated indirectly. This was done by carrying out the initial incubation in the presence of *p*-fluorophenyl- $[\beta\text{-}^{14}\text{C}]$ alanine. Estimation of the ^{14}C content of the combined leucine + isoleucine and *p*-fluorophenylalanine spot then gives a measure of the amount of analogue present. For this experiment, organisms growing exponentially in 17AA(-Phe) medium were centrifuged, washed in similar medium and resuspended to a density of 0.1 mg. dry wt. of bacteria/ml. in 17AA(-Phe) medium containing *p*-fluorophenyl- $[\beta\text{-}^{14}\text{C}]$ alanine. A culture growing in

18AA medium provided a control. These cultures were incubated under standard conditions and samples taken at intervals were treated to yield the protein + cell-wall fraction, which was then hydrolysed, as described in the Materials and Methods section, to yield free amino acids. The hydrolysates were transferred quantitatively to chromatograms and separated to isolate phenylalanine, tyrosine and leucine + isoleucine (+ *p*-fluorophenylalanine, if present). The phenylalanine and tyrosine were estimated quantitatively and the *p*-fluorophenylalanine by the ^{14}C content of the combined leucine + isoleucine and *p*-fluorophenylalanine spot (see Materials and Methods section). Fig. 4 shows the

Fig. 4. Effect of *p*-fluorophenylalanine on appearance of amino acids in total cell protein. 17AA(-Phe) medium: ▲, phenylalanine; ●, tyrosine. 17AA(-Phe + *p*-fluorophenylalanine) medium: △, phenylalanine; ○, tyrosine; ■, *p*-fluorophenylalanine. L- $[\beta\text{-}^{14}\text{C}]$ Valine, 0.23 $\mu\text{C}/\mu\text{mole}$; *p*-fluorophenyl- $[\beta\text{-}^{14}\text{C}]$ alanine, 0.55 $\mu\text{C}/\mu\text{mole}$.

Table 6. Replacement by p-fluorophenylalanine of phenylalanine residues in total cell protein formed by cultures of *Bacillus cereus* 569/H growing in the presence of p-fluorophenylalanine

Organisms growing exponentially in 17AA(–Phe) medium were harvested by centrifuging and resuspended in 17AA(–Phe) medium or 17AA(–Phe) medium containing 50 µg. of p-fluorophenyl[β-¹⁴C]alanine/ml. (0.55 µC/µmole). Samples of bacteria taken at intervals were treated to yield the protein + cell-wall fraction. The phenylalanine, tyrosine and p-fluorophenylalanine content of the cell protein was estimated as described in the text.

17AA(–Phe) medium				
	Culture density (mg./ml.)	Tyrosine content of protein + cell wall (µg./mg. dry wt. of organisms) (1)	Phenylalanine content of protein + cell wall (µg./mg. dry wt. of organisms) (2)	Phenylalanine/ tyrosine ratio (3)
Calc. as	...	—	—	(2)/(1)
Sample no.				
1	0.12	41	68	1.68
2	0.215	39	68	1.75
3	0.265	40	63	1.59
4	0.35	38	62	1.61
5	0.43	40	61	1.64
6	0.60	42	60	1.64
17AA(–Phe) medium + p-fluorophenylalanine				
	Culture density (mg./ml.)	Tyrosine content of protein + cell wall (µg./mg. dry wt. of organisms) (4)	Observed phenylalanine content of protein + cell wall (µg./mg. dry wt. of organisms) (5)	Theoretical phenylalanine content of protein + cell wall (µg./mg. dry wt. of organisms) (6)
Calc. as	...	—	—	(4) × (3)
Sample no.				
1	0.12	38	63	64
2	0.185	41	59	66
3	0.22	39	52	65
4	0.265	41	48	66
5	0.29	38	44	64
6	0.34	41	38	65
17AA(–Phe) medium + p-fluorophenylalanine (cont.)				
	Culture density (mg./ml.)	Difference between theoretical and observed phenylalanine content of protein + cell wall (µg./mg. dry wt. of organisms) (7)	p-Fluorophenyl[β- ¹⁴ C]- alanine content of protein + cell wall (µg./mg. dry wt. of organisms) (8)	Replacement of phenylalanine by p-fluorophenylalanine (%) (9)
Calc. as	...	(6)–(5)	—	(8) × 100 (7)
Sample no.				
1	0.12	0	0	—
2	0.185	7	4.3	61
3	0.22	13	8.6	66
4	0.265	18	12	66
5	0.29	20	17.2	86
6	0.34	27	21	78

differential effect of p-fluorophenylalanine on the appearance of these amino acids in the protein + cell-wall fraction. The analogue has no effect upon the differential rate of incorporation of tyrosine. The incorporation of phenylalanine, however, is markedly depressed. After incubation for 60 min. in the presence of the analogue, the differential rate of incorporation is about 40 % of normal, and after

incubation for 2 hr. the flow of phenylalanine to protein has practically ceased. On the other hand, no p-fluorophenylalanine is detected in the cell protein at the beginning of the experiment. Subsequently, however, the differential rate of p-fluorophenylalanine incorporation increases for about 2 hr., after which the rate remains constant. To calculate the degree of 'replacement' of phenyl-

alanine by *p*-fluorophenylalanine, it is necessary to know how much phenylalanine would have been incorporated if the *p*-fluoro derivative had no differential effect upon the incorporation of natural amino acid. This may be deduced from the tyrosine content of the protein under these conditions (see Table 6). As shown in Fig. 4, *p*-fluorophenylalanine has no effect on tyrosine uptake into protein. As the phenylalanine-tyrosine ratio of normal protein is about 1.6:1 (see Table 6, columns 1-3) it is possible to calculate the theoretical phenylalanine content of protein synthesized in the presence of the analogue (Table 6, column 6). For complete 'replacement' of phenylalanine by the *p*-fluoro derivative, the difference between the theoretical and observed phenylalanine contents of protein (Table 6, columns 5-7) would have to be the same as the *p*-fluorophenylalanine content observed at the same time (Table 6, column 8). In fact the *p*-fluorophenylalanine content accounts for about 65-85% of the displaced phenylalanine. This could amount to complete replacement within the error of the method.

This experiment shows therefore that after growth for about 2 hr. in the presence of *p*-fluorophenylalanine, the flow of phenylalanine to protein is almost completely suppressed in favour of the *p*-fluoro derivative. In separate experiments, similar effects were obtained with *p*-fluorophenylalanine on the total exocellular-protein fraction and therefore under these conditions a high degree of replacement of phenylalanine in exopenicillinase-type protein by *p*-fluorophenylalanine might be expected, particularly in the later stages of inhibition.

Immunological properties of exopenicillinase in relation to incorporation of p-fluorophenylalanine

Experiments (Richmond, 1960) have shown that exopenicillinase synthesized in the presence of *p*-fluorophenylalanine has abnormal neutralization characteristics when titrated with anti-exopenicillinase γ -globulin. As the abnormality of the enzyme increased during the initial incubation for 2 hr. in the presence of the analogue, it seemed possible that this was related to the increasing content of *p*-fluorophenylalanine and the concomitant fall in specific enzyme activity (enzyme units/ μ g. of exopenicillinase-type protein).

To relate these processes more closely, the previous experiment was repeated and samples of exopenicillinase were tested for their 'abnormality' in the neutralization reaction. Table 7 compares the level of incorporation of analogue with the specific enzyme activity and the immunological properties of the enzyme, measured in terms of the plateau zone, the neutralization slope and the residual enzyme activity. The analogue content of the exopenicillinase rises during the initial incubation for

Table 7. *Relationship between incorporation of p-fluorophenylalanine and immunological properties of exopenicillinase synthesized in the presence of the analogue*

Interval after addition of the analogue (min.)	Culture density (mg./ml.)	<i>p</i> -Fluorophenyl- $[\beta\text{-}^{14}\text{C}]$ alanine content of purified exopenicillinase (% by wt.)	Possible degree of replacement of phenylalanine by <i>p</i> -fluorophenylalanine (%)	Specific enzyme activity (e.u./ μ g. of enzyme protein)	Percentage of normal specific enzyme activity	Immunological properties*		
						Plateau zone (n.u.)	Neutralization slope (n.u./0.1 ml.)	Residual activity (%)
0	0.13	—	—	—	—	—	—	—
45	0.20	0	0	307	98	8 $\pm$ 1	35	5.8
60	0.275	1.7	43	245	78	12 $\pm$ 1	26	5.8
80	0.34	1.9	48	220	70	13 $\pm$ 1	23	8.8
100	0.39	2.8	71	165	53	18 $\pm$ 2	15	8.7
120	0.48	3.0	76	150	48	25 $\pm$ 3	14	14.2
180	0.68	2.7	68	155	49	27 $\pm$ 3	14	12.0
Normal enzyme	—	Nil	Nil	312	100	7 $\pm$ 1	36	5.4

* Tested at 30 e.u./ml.

Bacteria growing exponentially in 17AA(-Phe) medium were centrifuged and the organisms resuspended in 17AA(-Phe) medium containing 50 μ g. of *p*-fluorophenyl- $[\beta\text{-}^{14}\text{C}]$ alanine/ml. (0.55 μ C/ μ mole). Duplicate samples taken at intervals were treated: (a) to determine the *p*-fluorophenylalanine content of the exopenicillinase-type protein; (b) to determine the neutralization characteristics of the exopenicillinase-type protein synthesized in the presence of the analogue. The neutralization characteristics of a normal exopenicillinase preparation are included for comparison; e.u., exopenicillinase units; n.u., neutralization units.

2 hr. and this is accompanied by a fall in specific enzyme activity and increased immunological abnormality. Subsequently the steady differential rate of *p*-fluorophenylalanine incorporation is associated with approximately constant enzymic and immunological properties. As discussed by Richmond (1960), the abnormal properties of the exopenicillinase synthesized under these conditions could be partly explained on a basis of lowered specific enzyme activity. In fact, the fall in specific activity found in this experiment (50–60%: cf. Table 6, part i) is about sufficient to account for the abnormal neutralization slope exhibited in the neutralization test.

DISCUSSION

The overall pattern of changes after the addition of *p*-fluorophenylalanine to cultures of *B. cereus* are similar to those reported for *E. coli* (Munier & Cohen, 1956, 1959) and yeast (Cohen, Halvorson & Spiegelman, 1959). There are, however, certain differences, the effects of the analogue being neither so immediate nor so clear-cut. Growth of *B. cereus* is markedly inhibited by the analogue, but linear growth does not commence immediately (Richmond, 1960). For about 45 min. after the addition of the analogue to cultures growing exponentially, the growth rate is slowed but still greater than linear. Subsequently a period of 'linear' growth occurs, during which the rate of increase in dry weight of the culture slowly falls over a period of 3–4 hr. If the analogue were causing inhibition of the division process but the mass of the culture were increasing at a linear rate, enlarged forms of the organisms would be expected. In fact, the mean length of organisms was only slightly increased even after 8 hr. in the presence of the analogue (M. H. Richmond & J. Collins, unpublished work) and therefore division of *B. cereus* must occur in the presence of *p*-fluorophenylalanine (cf. Cohen *et al.* 1959). The differential rate of *p*-fluorophenyl[β - 14 C]alanine incorporation into the protein fraction of *B. cereus* increases over the first 45 min. of incubation and then becomes constant. This time course of incorporation is distinctly different from that obtained in *E. coli* (Munier & Cohen, 1956, 1959), where the differential rate of incorporation is constant from the moment of addition of the analogue to the system. It is possible that the delayed effect of the analogue in *B. cereus* is due to a high internal 'pool' concentration of phenylalanine. This amino acid is known to compete effectively with *p*-fluoro derivative when the amino acids are present together in the growth medium. However, the 'pool' concentration of phenylalanine in *B. cereus* is relatively low (approx. 0.8 μ mole/g. dry wt. of organisms; cf. glutamic acid at 8 μ moles/g.) and this quantity of phenylalanine

would suppress *p*-fluorophenylalanine incorporation into protein for only about 15 min. at the observed growth rates.

A more likely explanation of the delayed effect of the analogue lies in the effect of this compound on the biosynthetic pathway of phenylalanine. It is known (Richmond, 1960) that phenylalanine is an efficient competitor for the *p*-fluoro derivative in the assimilation system when the two amino acids are present together in the growth medium. It seems possible therefore that, once inside the cell, the analogue may inhibit the endogenous synthesis of phenylalanine and only after this has occurred may the analogue act fully in the protein-biosynthetic system. Inhibition of the function of biosynthetic pathways has been reported for a number of amino acid analogues [e.g. 4-methyl-tryptophan inhibition of tryptophan synthetase, Trudinger & Cohen (1956)] and Munier & Cohen (1959) tentatively suggest that *p*-fluorophenylalanine inhibits the biosynthesis of phenylalanine in *E. coli*. Certainly *p*-fluorophenylalanine does not inhibit the biosynthesis of phenylalanine in *B. cereus* immediately since it can be shown that phenylalanine continues to be synthesized for more than 10 min. after addition of the analogue and is then incorporated into cell protein (M. H. Richmond, unpublished work). A delayed inhibition by *p*-fluorophenylalanine of the biosynthetic supply of phenylalanine could therefore be responsible for the delayed effect of the analogue on the protein-synthesizing system.

In the experiments reported above, the rate of incorporation of *p*-fluorophenylalanine into all the protein fractions studied increased and reached a maximum value after incubation for 1–2 hr. In this way the incorporation of the analogue into purified exopenicillinase increased to a maximum rate, at which the incorporation of *p*-fluorophenylalanine was equivalent to the majority of the seven available phenylalanine residues. Although it has not been proved that the incorporation of the analogue into exopenicillinase is at the expense of the natural amino acid, indirect evidence points strongly to this conclusion. The analogue does not seem to have a specific inhibitory effect on any single fraction of protein (see also Cowie, Cohen, Bolton & de Robichon-Szulmajster, 1959). Replacement of a large proportion of the phenylalanine by the *p*-fluoro derivative can be shown to occur both with the total cell-protein fraction and also with the total exocellular protein. If the same is not true of the exopenicillinase, it is necessary to invoke a specific effect of the analogue on this protein, a possibility for which there is no experimental support.

The previous paper (Richmond, 1960) has described the immunological properties of exo-

penicillinase synthesized in the presence of *p*-fluorophenylalanine. An attempt has been made therefore to explain the immunological findings on the basis of the abnormal chemical properties of the exopenicillinase-type protein.

As crystalline exopenicillinase from this strain of *B. cereus* contains seven phenylalanine residues (S. Jacobs, personal communication), and as the majority can be replaced by the analogue after incubation for 2 hr., it is important to know whether this represents a random replacement of residues or specific replacement of the same residues. The experiments reported in this paper favour random replacement. However, even if incubation in the presence of the analogue is continued for long periods it has not proved possible to show complete 'replacement'. The situation is complicated by the fact that the exopenicillinase fraction normally contains molecules, made soon after the addition of the analogue, which are known to have a low degree of replacement and this means that complete replacement can never be attained. However, after 4 hr. in the presence of the analogue, the degree of replacement should be greater than a net value of six residues. It is possible, however, that complete 'replacement' does occur for the following reasons. First the methods used to purify the abnormal enzymes would not detect an 'exopenicillinase' protein which had no enzyme activity and which did not react with the specific anti-exopenicillinase antiserum. Complete replacement could lead to such a protein. Secondly, the method used for measuring the degree of replacement is subject to very large errors and a value of five to six could easily represent complete replacement. Certainly, very little phenylalanine seems to appear in the total cell-protein fraction after 2 hr. in the presence of *p*-fluorophenylalanine, whereas the analogue continues to be incorporated (see Table 6).

It is certain that the addition of *p*-fluorophenylalanine to cultures of *B. cereus* leads to the formation of at least one type of abnormal exopenicillinase molecule with impaired specific enzyme activity.

If five to six residues of phenylalanine are randomly replaced this would lead to the formation of about 50 different types of 'penicillinase' molecule and it is not possible to predict anything about the enzymic or immunological properties of these proteins. Experimentally, it can be shown (Richmond, 1960) that the net immunological reaction of the exopenicillinase-type protein is impaired at low antibody-enzyme ratios; the neutralization slope with normal antiserum is lowered and the residual enzyme activity of the preparation, on addition of three equivalent doses of antiserum, is slightly higher than normal. As

has been shown above (see Table 7), the low neutralization slope and high residual activity can be accounted for on the basis of the low net specific enzyme activity of the abnormal exopenicillinase preparation. It is not possible, however, to account for the broad 'plateau zone' found on neutralizing abnormal enzyme preparations. Normal enzyme shows a slight plateau and some of the increase found with the abnormal enzyme could be due to a lowered affinity of some or all the components of the mixture for the antiserum. As discussed by Richmond (1960), neutralization of a complex mixture of related but differing proteins with a complex mixture of antibody molecules is difficult to analyse.

These findings suggest that it might be possible to separate a number of 'abnormal' exopenicillinase molecules by physicochemical methods. However, so far the abnormal mixture has been found to behave identically with the normal enzyme in immune electrophoresis by the method of Grabar (1959) and Grabar & Williams (1955), and also on attempted separation in the gradient-electrophoresis apparatus (Charlwood & Gordon, 1958). This may reflect the similar physicochemical properties of phenylalanine and its *p*-fluoro derivative when combined in proteins.

SUMMARY

1. Cultures of *Bacillus cereus* 569/H incubated in fully synthetic amino acid medium lacking phenylalanine but containing *p*-fluorophenylalanine incorporate the unnatural amino acid into total cell protein, total exocellular protein and exopenicillinase of the organisms.
2. The incorporation of *p*-fluorophenylalanine into the total cell-protein and total exocellular-protein fractions of the organisms is associated with a high degree of replacement of the phenylalanine normally appearing in these fractions by the *p*-fluoro derivative. The degree of replacement on a differential basis increases for about 90 min. before reaching a maximum rate.
3. Incorporation of *p*-fluorophenylalanine into the exopenicillinase prepared from the organisms leads to the formation of at least one species of molecule with impaired specific enzyme activity and abnormal neutralization characteristics.
4. The relationship between the abnormal chemical and immunological properties of the exopenicillinase-type protein formed in the presence of *p*-fluorophenylalanine is discussed.

I would like to express my indebtedness to Dr M. R. Pollock for providing the anti-penicillinase sera and for much stimulating discussion in connexion with the work. I also wish to thank Mr Norman Dickens and Mr Rex Pyke for valuable technical assistance.

REFERENCES

- Boyd, W. C. (1956). *Fundamentals of Immunology*, 2nd ed. New York: Interscience Publishers Inc.
- Charlwood, P. A. & Gordon, A. H. (1958). *Biochem. J.* **70**, 433.
- Cohen, G. N., Halvorson, H. O. & Spiegelman, S. (1959). In *Microsomal Particles and Protein Synthesis*, p. 100. Ed. by Roberts, R. B. New York: Pergamon Press Ltd.
- Cohen, G. N. & Munier, R. (1959). *Biochim. biophys. Acta*, **31**, 347.
- Cowie, D., Cohen, G. N., Bolton, E. T. & de Robichon-Szulmajster, H. (1959). *Biochim. biophys. Acta*, **34**, 39.
- Grabar, P. (1959). *Meth. biochem. Anal.* **7**, 1.
- Grabar, P. & Williams, C. A., jun. (1955). *Biochim. biophys. Acta*, **17**, 67.
- Halvorson, H. O. & Spiegelman, S. (1952). *J. Bact.* **64**, 207.
- Kogut, M., Pollock, M. R. & Tridgell, E. J. (1956). *Biochem. J.* **62**, 391.
- Kruh, J. & Rosa, J. (1959). *Biochim. biophys. Acta*, **34**, 561.
- Kunkel, H. G. (1954). *Meth. biochem. Anal.* **1**, 141.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. S. (1951). *J. biol. Chem.* **193**, 265.
- McQuillen, K. & Roberts, R. B. (1954). *J. biol. Chem.* **207**, 81.
- Mandelstam, J. & Rogers, H. J. (1959). *Biochem. J.* **72**, 654.
- Mitchell, P. D. (1949). *J. gen. Microbiol.* **4**, 399.
- Munier, R. & Cohen, G. N. (1956). *Biochim. biophys. Acta*, **21**, 592.
- Munier, R. & Cohen, G. N. (1959). *Biochim. biophys. Acta*, **31**, 378.
- Newton, G. G. F. & Abraham, E. P. (1954). *Biochem. J.* **58**, 103.
- Pollock, M. R. & Kramer, M. (1958). *Biochem. J.* **70**, 665.
- Richmond, M. H. (1959). *Biochem. J.* **73**, 155.
- Richmond, M. H. (1960). *Biochem. J.* **77**, 112.
- Trudinger, P. A. & Cohen, G. N. (1956). *Biochem. J.* **62**, 488.
- Urbá, R. C. (1959). *Biochem. J.* **71**, 513.
- Vaughan, M. & Steinberg, D. (1958). *Fed. Proc.* **17**, 328.

Biochem. J. (1960) **77**, 135

Argininosuccinic Aciduria: Identification and Reactions of the Abnormal Metabolite in a Newly Described Form of Mental Disease, with Some Preliminary Metabolic Studies

By R. G. WESTALL

Medical Unit, University College Hospital Medical School, London, W.C. 1

(Received 22 February 1960)

Allan, Cusworth, Dent & Wilson (1958) have reported a new form of severe mental disease which they believe to be due to an inborn error of metabolism. The authors described a family with four children in which two of the sibs were normal and the other two were seriously mentally retarded. Paper-chromatographic examination of the urine of the family showed that the two affected children were excreting large amounts of a ninhydrin-reacting substance which was absent in the urine of the rest of the family. Moreover, further tests on the affected children showed a relatively high concentration of the abnormal metabolite in the cerebrospinal fluid and a somewhat lower concentration in the plasma. The unknown substance could not be identified as any of the substances usually found in normal urine, nor with any of the compounds found only in trace amounts (Westall, 1955). Hence at the time when the full report of the case was published the particular metabolite was unidentified but the authors were of the opinion that it was probably a peptide of a type resistant to acid hydrolysis.

Further work on this substance (reported briefly by Westall, 1958) has now established that it is argininosuccinic acid. This acid was first postulated on theoretical grounds to be an intermediate in the pathway of synthesis of arginine from citrulline and aspartic acid in the presence of liver enzymes (Ratner & Pappas, 1949). It was later synthesized, isolated and characterized from a similar system (Ratner, Petrack & Rochovansky, 1953). These authors demonstrated that the reaction takes place in two stages. In the first, argininosuccinic acid is formed from citrulline and aspartic acid under the action of a 'condensing enzyme' present in mammalian liver and kidney; this requires adenosine triphosphate. The second stage requires the presence of a 'splitting enzyme', which catalyses the breakdown of argininosuccinic acid to form arginine and fumaric acid. They were able to prepare argininosuccinic acid from both of these systems since the splitting-enzyme system is reversible. The natural accumulation of argininosuccinic acid in mammalian tissues or body fluids has not been demonstrated before and it may be