The Metabolism of 6-Aminopenicillanic Acid and Related Compounds by *Penicillium chrysogenum* and its Possible Significance for Penicillin Biosynthesis

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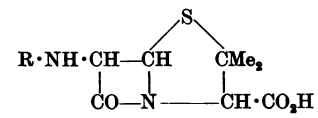
Several years ago it was reported that 6-aminopenicillanic acid (I) (then named penicin) is formed in cultures of *Penicillium chrysogenum* Q 176 when phenylacetate is omitted as side-chain precursor (Kato, 1953) or by enzymic hydrolysis of benzylpenicillin (II) by penicillin amidase (Sakaguchi & Murao, 1950). Until recently, however, compound (I) had not been properly characterized and its existence remained in doubt.



(I) R = H
(II) R = $C_6H_5 \cdot CH_2 \cdot CO$
(III) R = $HO_2C \cdot CH(NH_2) \cdot [CH_2]_3 \cdot CO$

The present search for (I) in mycelial extracts was prompted by the possibility that introduction of the side chain might represent the final step in penicillin biosynthesis (Arnstein, 1957) and by the report (Sheehan, 1958) that 6-aminopenicillanic acid had been synthesized, indicating that it was sufficiently stable to be capable of more than transitory existence. It was considered possible that conversion of (I) into (II) by reaction with phenylacetyl chloride (cf. Sheehan, 1958) might be followed by an increase in antibiotic activity, since acylations of this type are known to modify the biological activity of cephalosporin N (III) (Newton & Abraham, 1954).

Since at least some of the intermediates in penicillin biosynthesis may not be diffusible, as suggested by the work of Sermonti (1956) on heterokaryotic mutants (cf. Arnstein, 1957), it was decided to look for (I) in the mould mycelium rather than in the culture medium. Accordingly, we investigated the antibiotic activity of acetone extracts of mycelium and the effect of treating such extracts with phenylacetyl chloride. While this work was in progress, Batchelor, Doyle, Nayler & Rolinson (1959) published an account of their work on the isolation, characterization and properties of 6-aminopenicillanic acid (I), which had been isolated from the culture medium of *P. chrysogenum* grown in the absence of phenylacetate. Their work enabled us to identify by paper chromatography the major component of our 'phenylacetyl chloride-reacting substances' in mycelial extracts with (I). Subsequently, changes in the metabolism of these compounds in the presence and absence of phenylacetate were investigated by estimating the amounts present in both mycelium and culture medium and this aspect forms the main part of the present paper. Since, however, the increase in antibiotic activity obtained by reaction of crude extracts with phenylacetyl chloride was not correlated quantitatively with 6-aminopenicillanic acid, the term phenylacetyl chloride-reacting substances will be used throughout this paper.

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EXPERIMENTAL

Growth of organism and preparation of washed mycelium. *Penicillium chrysogenum*, WIS 51–20 F3, was obtained from Professor M. J. Johnson, Department of Biochemistry, College of Agriculture, University of Wisconsin. Subcultures were maintained on molasses–agar in medicinal flat bottles (area 36 in.^{2}). Mycelium were grown either in a fermenter (Arnstein & Grant, 1954) or in 250 ml. conical flasks on a rotary shaker (speed about 180 rev./min.) in the medium of Jarvis & Johnson (1950), usually as modified by Grau & Halliday (1958), without addition of phenylacetate unless specifically indicated.

In Expt. no. 1 (Table 1) spores from one flat bottle were used to inoculate four 100 ml. portions of unmodified medium (Jarvis & Johnson, 1950) and the flasks were incubated on the rotary shaker for 47 hr. The cultures were combined aseptically and one-half was used for preparing an acetone extract of the mycelium (see below). The remainder was divided into four 50 ml. portions, which were incubated further with or without 0·1% potassium phenylacetate as indicated in Table 1. The yields of mycelium (wet wt.) from these portions were 35 g. (47 hr.), 7·7 g. and 7·6 g. (72 hr.) and 5·2 g. and 8·2 g. (112 hr.). The dry wt. was assumed to be 10% of these values. In the absence of phenylacetate the antibiotic activity of the medium was 12·5, 60 and 50 i.u./ml. at 47, 72 and 112 hr. respectively; in the presence of phenylacetate the penicillin titres were 274 and 355 i.u./ml. at 72 and 112 hr. Expt. no. 2 was essentially similar except that the modified medium

was used, the original cultures were combined after 40 hr. growth and eight 50 ml. portions were incubated further. The wet wt. and dry wt. (in parentheses) of mycelium from successive 50 ml. samples (Table 1) were as follows (g.): 3·05 (0·266), 3·04 (0·344), 3·63 (0·429), 3·39 (0·432), 5·80 (0·547), 5·49 (0·530) and 6·19 (0·704). The ability of this mycelium to produce penicillin in the presence of phenylacetate was tested in the washed-mycelium experiment WM1 described in Table 2. It was found that after 60 hr. this fermentation was exceptional in that the penicillin-producing capacity of the mycelium fell precipitously. For Expt. no. 3 the organism was grown in a fermenter in 1·5 l. of the modified medium without phenylacetate, and portions (21–45 ml.) were withdrawn aseptically at the stated intervals. At 48 hr., three 40 ml. samples were transferred to conical flasks and incubated with 0·1% potassium phenylacetate on the rotary shaker for 19 hr. The volumes of culture medium, yields of mycelium (g. dry wt. in parentheses) and antibiotic activity of the medium from successive samples (Table 1) were as follows: 25 ml., 0·350 g. (0·034), $< 0·6$ i.u./ml.; 45 ml., 3·32 g. (0·274), 17 i.u./ml.; 13·5 ml., 2·42 g. (0·257), 25 i.u./ml.; 21 ml., 2·34 g. (0·181), 28 i.u./ml.; 1320 ml. (remaining contents of fermenter at 64 hr.), 180·0 g. (18·7), 32 i.u./ml.; 110 ml. (cultures incubated with phenylacetate from 48–67 hr.), 12·3 g. (1·45), 266 i.u./ml.

For washed mycelium experiments, portions of mycelium (0·5 g. or 1·0 g.) that had been harvested by filtration and washed with water or 0·01 M-potassium phosphate buffer, pH 7 (Halliday & Arnstein, 1956), were incubated in 100 ml. conical flasks containing 0·01 M-potassium phosphate buffer (1·36 g. of KH_2PO_4 and 0·40 g. of KOH/l.), pH 7 (10 vol.), with or without potassium phenylacetate (0·005 or 0·05%), on the rotary shaker at 24° for 1–3 hr.

Expt. WM1 was done with mycelium from Expt. no. 2 (Table 1). Duplicate flasks, each containing 0·5 g. of mycelium, were incubated with 5 ml. of medium for 1 hr. After bioassay of the medium (individual flasks differed from the mean, which is given in Table 2, by less than 5%), the mycelia were combined and extracted with acetone for estimation of antibiotic activity and phenylacetyl chloride-reacting material. Other details are given in Tables 1 and 2.

For Expt. WM2, mycelium was grown on the rotary shaker for 62 hr., in the modified medium without phenylacetate. Portions (1 g. wet wt.; dry wt. 10%) were incubated with or without 0·005% of potassium phenylacetate for 0, 1, 2 and 3 hr. in duplicate flasks. Acetone extracts were prepared (see below) from the separated mycelium without further washing it.

Preparation of acetone extracts and reaction with phenylacetyl chloride. Portions of washed mycelium were extracted by stirring in cold acetone (5 ml./g. wet wt.). After they had stood for at least 15 min. at 2°, centrifuging yielded a supernatant solution which contained approx. 80% of the total detectable phenylacetyl chloride-reacting material in the mycelium. Portions of the fermentation medium were diluted with acetone (2–4 vol.) before phenylacetylation.

The acetone extract of mycelium or medium was made up to 2·5 ml. with acetone and freshly prepared 0·5 M-$NaHCO_3$ (0·08 ml.), bromothymol blue indicator solution (British Drug Houses Ltd.; 0·02 ml.) and 0·1 M-potassium phosphate buffer, pH 7–7·5 (0·2–0·4 ml.), were added to bring the final volume of water, including that in the sample, to 0·5–0·7 ml., the ratio of water to acetone being approx. 1:3.

Duplicate or triplicate tubes were prepared for each sample, a set containing sodium benzylpenicillin (4–10 units, potency 1670 i.u./mg.; Glaxo Laboratories Ltd.) was included as internal standards and all tubes were kept stoppered in an ice bath.

To one set was added 2·4% (v/v) of conc. HCl in acetone (0·15 ml.) in order to determine the effect of HCl liberated during reaction of the acid chloride; the duplicate sets received, with shaking, 2% (v/v) of phenylacetyl chloride in acetone (0·15 ml., 45 m-equiv.) prepared just before use. All tubes were left at 2°, with occasional shaking, for 20–25 min. Ether (4 vol.) was then added and, after mixing, the aqueous layer was allowed to separate and was frozen by cooling the tubes in solid CO_2. The ether–acetone supernatant layer, which contained unreacted phenylacetyl chloride, was decanted and the aqueous solutions were bioassayed for penicillin activity.

In the second washed-mycelium experiment (Expt. WM2) phenoxyacetyl chloride was used instead of phenylacetyl chloride. The mycelium (1 g. wet wt.) was extracted with acetone (5 ml.) at 0° and a portion of the extract (2 ml.) was added to 0·5 M-$NaHCO_3$ (0·25 ml.), 0·1 M-potassium phosphate buffer, pH 7 (0·25 ml.), and 10% (v/v) phenoxyacetyl chloride in acetone (0·1 ml.). After 1 hr. at 0°, the mixture was treated as described above and assayed for antibiotic activity. Control samples of the same extracts were treated identically except that phenoxyacetyl chloride was omitted. Incubation medium was bioassayed directly after filtration and suitable dilution where necessary. Portions (5 ml.) were similarly acylated with phenoxyacetyl chloride (0·1 ml. of the 10% solution in acetone) in the presence of 0·5 M-$NaHCO_3$ (0·5 ml.) and 0·1 M-phosphate buffer (0·5 ml.). A solution of sodium benzylpenicillin (5 ml., 103·5 i.u.) was treated in the same way as a control; the recovery of antibiotic activity was 77% and the results of this experiment have been corrected accordingly (see below).

Bioassay and calculation of results. Bioassay of the aqueous solutions from the phenylacetylation procedure on agar plates seeded with *Bacillus subtilis* by the method of Humphrey & Lightbown (1952) yielded results which were corrected for the percentage recovery of penicillin in the internal standards (average recovery in 28 cases was 89%) and are expressed as units/ml. of medium or units/g. wet or dry wt. of mycelium. Dry weights were obtained by extracting the mycelial residue a second and third time with acetone and drying at 110°; the dry wt. varied from 8·5 to 12% of the wet wt. Suitable controls, such as buffer solutions, fermentation medium obtained from cultures before the beginning of the penicillin-producing phase, and sodium benzylpenicilloate (equivalent to 30 units of sodium benzylpenicillin), showed no antibiotic activity before or after phenylacetylation. There was no significant difference (< 4%) in activity between standard penicillin solutions treated with HCl and those treated with phenylacetyl chloride.

Chromatography. Paper chromatograms were always run by the ascending technique at 2° with the upper layer of butan-1-ol–ethanol–water (4:1:5, by vol.) (Batchelor *et al.* 1959) and propan-1-ol–water (7:3, v/v) with 1 cm. strips of Whatman no. 3MM paper. After drying, the paper was cut in half longitudinally and one part was treated with

0·5 M-$NaHCO_3$ and 2% (v/v) phenylacetyl chloride in acetone. The two halves were then developed on an agar plate seeded with *B. subtilis*.

With other solvents, descending chromatography at room temperature was used, the composition (by vol.) of the various mixtures being as follows: (*a*) butan-1-ol–acetic acid–water (63:10:27); (*b*) pyridine–water (4:1); (*c*) pentan-1-ol–pyridine–water (7:7:6).

Reaction of an acetone extract of mycelium with labelled phenylacetyl chloride. [*carboxy*-^{14}C]Phenylacetic acid (16·6 mg., 150 μc, 1·23 mc/m-mole; obtained from The Radiochemical Centre, Amersham, Bucks) was warmed at 100° with an excess of thionyl chloride (0·30 ml.) for 30 min. After cooling to room temperature, excess of thionyl chloride was removed *in vacuo* (water pump). The residue was dissolved in acetone (2 ml.), 0·1 M-phosphate buffer, pH 7 (2 ml.), was added followed by an acetone extract (16 ml.) prepared from 6·16 g. (wet wt.) of mycelium. Solid $NaHCO_3$ (125 mg.) was added and, as the pH was still slightly acid, more $NaHCO_3$ was added at intervals whilst the mixture was shaken at 2° for 30 min. Ether (50 ml.) was added, the solution was mixed and the aqueous layer was frozen by cooling in solid CO_2. The ether layer was separated and the aqueous layer was re-extracted in a similar way with ether (30 ml.). The combined ether layers were re-extracted with 0·1 M-phosphate buffer, pH 7 (5 ml.). Bioassay showed that the first aqueous solution contained 17·3 i.u./ml., whereas the second contained only 0·2 i.u. of penicillin/ml. The total amount of penicillin activity was approx. 38 units (equivalent to 23 μg. of sodium benzylpenicillin). Carrier sodium benzylpenicillin (100 mg.) was added, the penicillin was extracted with ether at pH 2 and converted into the *N*-ethylpiperidine salt, which was recrystallized four times from dry chloroform–acetone (1:4, v/v). This product was degraded to penilloaldehyde (Arnstein & Clubb, 1957) which was isolated as the 2:4-dinitrophenylhydrazone. After crystallization from ethanol it was chromatographed first on paper with pentan-1-ol–pyridine–water as solvent and then on an alumina column with ethyl acetate as eluent.

The mother liquors from the first recrystallization of the *N*-ethylpiperidine salt of benzylpenicillin were chromatographed (Leigh, 1949) on a column (38 cm. × 1·2 cm. diameter) of silica gel (10 g.) prepared by suspending the silica gel in ethyl acetate (50 ml.) and adding water (7 ml.) and *N*-ethylpiperidine (0·5 ml.). Ethyl acetate containing 0·1% of *N*-ethylpiperidine was used as eluent and 5 ml. fractions were collected.

Radioactivity determinations. Measurements were made with a thin end-window Geiger–Müller tube (type EHM 2/s, General Electric Co. Ltd.) with samples of infinite thickness on 1 cm.^{2} polythene or 0·3 cm.^{2} Perspex disks (Popják, 1950).

RESULTS

Evidence for the presence of 6-aminopenicillanic acid in phenylacetyl chloride-reacting material

When extracts of mycelia or media from fermentations from which phenylacetate had been omitted were treated with phenylacetyl chloride at pH 7, a consistent increase in antibiotic activity was observed. This activity was sensitive to penicillinase and was presumed to be due to a penicillin. After the paper by Batchelor *et al.* (1959) had been published, paper chromatography by their method revealed the presence in such extracts of a major component which showed antibacterial activity after phenylacetylation. This compound has R_F 0·14 in butanol–ethanol–water, which is identical with that reported by Batchelor *et al.* (1959) for 6-aminopenicillanic acid (I). The R_F values of this substance in other solvents are: 0·50 in propanol–water, 0·34 in butanol–acetic acid–water, 0·60 in pyridine–water and 0·24 in pentan-1-ol–pyridine–water. In addition to this major component, varying amounts of other compounds have been detected in any one mycelial extract. These compounds had R_F values in butanol–ethanol–water of 0–0·05, 0·26 and 0·09.

The material before phenylacetylation was not extractable by ether at pH 2, but, like the product, was labile to alkali. The penicillinase-sensitive, major product of phenylacetylation was extractable with ether at acid pH and its behaviour on paper chromatography in butanol–ethanol–water and propanol–water was identical with that of benzylpenicillin.

In the experiment with [^{14}C]phenylacetyl chloride, the product (23 μg. of benzylpenicillin by bioassay) was diluted with carrier and isolated as the *N*-ethylpiperidine salt, which had specific radioactivies of 3·23, 2·80, 2·96 and 3·02 μc/g. upon successive recrystallizations. The average specific activity of this product (3·00 μc/m-mole) was, however, considerably greater than that calculated from the original specific radioactivity of the labelled phenylacetyl chloride, the yield of penicillin after phenylacetylation and the amount of carrier (0·28 μc/mole). The penicillin was degraded to penilloaldehyde, which was converted into the 2:4-dinitrophenylhydrazone. After recrystallization from ethanol, 10 mg. with a specific radioactivity of 2·21 μc/g. (0·790 μc/mole) was obtained. The colour and radioactivity of the dinitrophenylhydrazone chromatographed as a single substance on paper and on alumina and the material obtained after these procedures (4 mg.) had almost the same specific radioactivity (1·87 μc/g., 0·67 μc/m-mole) as before. The difference in radioactivity of penicillin and the penilloaldehyde 2:4-dinitrophenylhydrazone obtained by degradation suggested the presence of a highly radioactive impurity in the penicillin despite the constant specific radioactivity.

Chromatography of the concentrated mother liquors from the first recrystallization of the *N*-ethylpiperidine salt on silica gel revealed a major peak of radioactivity (0·5 μc) associated with antibiotic activity in fractions nos. 37–100. When chromatographed on paper with butanol–ethanol–water or propanol–water as solvents, this material

Table 1. *Antibiotic activity in mycelial extracts of* Penicillium chrysogenum *before and after reaction with phenylacetyl chloride*

Mycelium was harvested at the stated times and extracts were prepared and bioassayed as described in the Experimental section. For details of fermentations see text.

Expt. no.	Time (hr.)	Phenylacetate present (+) or absent (−)	Antibiotic activity in mycelium: Before reaction with phenylacetyl chloride (i.u./g. dry wt.)	Increase after reaction with phenylacetyl chloride (i.u./g. dry wt.)	Increase after reaction with phenylacetyl chloride (%)
1	47	−	29·6	91·8	310
	72	−	89·0	61·3	69
	72	+	28·0	20·0	72
	112	−	66·8	21·2	32
	112	+	54·5	2·0	4
2	40	−	37·0	141	381
	46·5	−	67·8	195	228
	56·5	−	81·0	234	289
	56·5	−	63·3	239	377
	61	−	73·0	403	552
	61	−	77·4	340	434
	68	−	53·5	256	478
3	26	−	⩽10	⩽5	—
	43	−	45·3	108	238
	48	−	99·1	104	105
	55	−	66·4	276	415
	64	−	35·0	273	780
	67	+	51·3	4·3	8

Table 2. *Effect of incubating washed mycelium with or without phenylacetate on the amount of mycelial phenylacetyl chloride-reacting material* (*Expt.* WM 1)

Portions (0·5 g. wet wt.) of washed mycelium, obtained from Expt. no. 2 (Table 1) and harvested at various times, were incubated with or without phenylacetate in duplicate flasks. The amount of phenylacetyl chloride-reacting substances in the mycelium was determined by measuring the increase in antibiotic activity after the mycelial extracts had reacted with phenylacetyl chloride as described in the Experimental section. Zero-time values have been calculated from the results given in Table 1. For experimental details of the fermentation see text. All results are expressed on the basis of 10 ml. of medium and 1 g. (wet wt.) of mycelium.

Age of mycelium (hr.)	Incubation conditions: Time (min.)	Potassium phenylacetate added (%)	Mycelial dry wt. (mg.)	Antibiotic activity (i.u.): In medium	In mycelium: Initial	In mycelium: Increase after phenylacetylation
46·5	0	—	110	—	7·5	21·5
	60	0·05	102	206	12·5	6·9
56·5	0	—	118	—	9·6	27·6
		—	127	—	8·0	30·4
	60	0·05	115	142	10·8	9·4
		0·05	120	107	12·7	9·2
61	0	—	94	—	6·9	37·9
		—	97	—	7·5	33·0
	60	None	104	3·0	6·8	27·2
		None	103	None	6·8	29·4
	60	0·05	96	112	7·3	12·0
		0·05	93	102	10·2	10·9
68	0	—	112	—	6·0	28·6
	60	None	113	None	7·5	17·5
		0·05	109	None	9·6	6·8

behaved identically with benzylpenicillin. Two radioactive contaminants appeared in earlier fractions: one was phenylacetic acid of high specific radioactivity (total approx. 2·6 μc in fractions nos. 9–25), the other was an unknown compound which was eluted in fractions nos. 7 and 8 and contained both antibiotic activity and radioactivity (approx. 0·05 μc), as shown by coincidence on a two-dimensional chromatogram (butanol–ethanol–water, R_F 0·93, and propanol–water, R_F 0·87). It was clearly different from benzylpenicillin (R_F values 0·48 and 0·78 respectively).

Distribution of phenylacetyl chloride-reacting material in mycelium and culture medium and changes in its concentration during penicillin production

Although a small amount of phenylacetyl chloride-reacting material was observed in mycelial extracts from *Penicillium chrysogenum* grown for 72–118 hr. under the usual conditions with phenylacetate, with penicillin titres of 200–300 units/ml. a great deal more of these compounds was found in preparations grown for shorter times in the absence of phenylacetate (Table 1). Under these conditions some increase in penicillin titre after phenylacetylation was also found in the medium, representing a larger total amount, and sometimes also a higher concentration, than in the mycelium. In the absence of phenylacetate as side-chain precursor very little antibiotic activity (max. 60 i.u./ml. at 72 hr., Expt. no. 1) was found in the medium, whereas addition of phenylacetate gave titres of 270 and 360 i.u./ml. at 72 and 112 hr. respectively. The increase in antibiotic activity after treatment of medium with phenylacetyl chloride was usually between 5 and 25 i.u./ml. with cultures grown without phenylacetate, but was not detectable when phenylacetate had been added. No significant antibiotic activity was found at 26 hr. in either medium or mycelium before or after treatment with phenylacetyl chloride.

The maximum concentration in the mycelium appears to occur at about 60 hr. of growth. This time corresponds to that reported by Grau & Halliday (1958) for maximum penicillin-producing

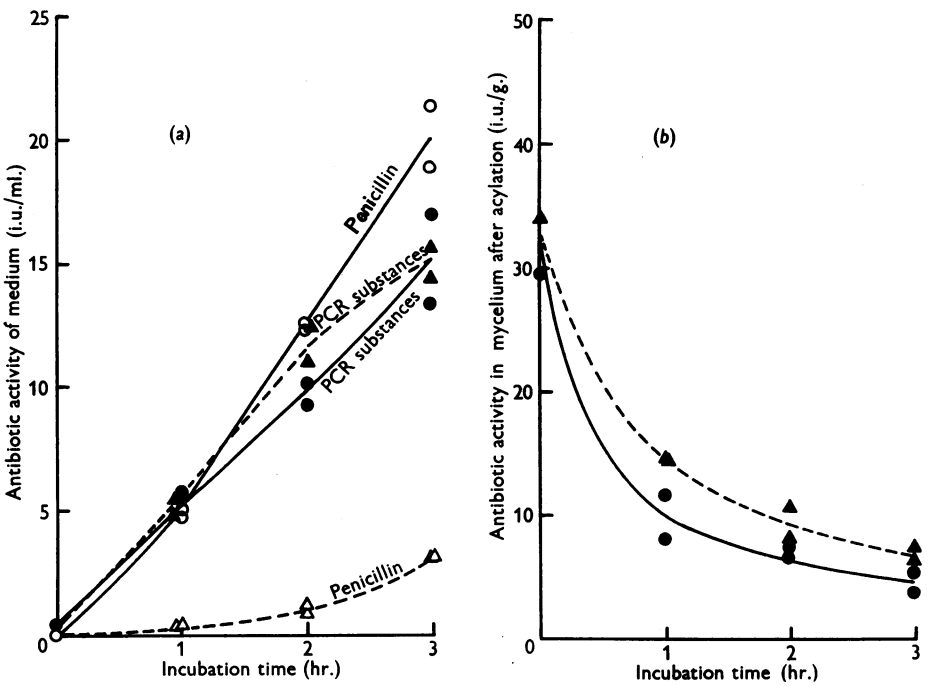

Fig. 1. Metabolism of phenylacetyl chloride-reacting substances by washed mycelium incubated with or without 0·005% potassium phenylacetate (Expt. WM2). Phenylacetyl chloride-reacting substances were estimated by the increase in antibiotic activity after acylation with phenoxyacetyl chloride (see text). (*a*) Activity of medium after incubation with (○) or without (△) phenylacetate and biosynthesis of phenylacetyl chloride-reacting substances in the presence (●) or absence (▲) of phenylacetate. (*b*) Antibiotic activity of mycelium, calculated/g. wet wt., after incubation with (●) or without (▲) phenylacetate. The values refer to the antibiotic activity after acylation; the activity of extracts before acylation was too low for accurate determination (< 2·5 i.u./g. wet wt.).

ability as measured in incubations of washed mycelium with 0·01 % potassium phenylacetate.

It has also been found that storage of mycelium at 0° in oxygen for 24 hr. resulted in a marked increase of phenylacetyl chloride-reacting substances as well as of antibiotic activity before phenylacetylation (350 % and 670 % respectively). Storage at room temperature for 1 hr. led to similar increases (386 % in phenylacetyl chloride-reacting substances and 564 % in antibiotic activity before phenylacetylation).

Washed-mycelium experiments. Measurements of the changes in phenylacetyl chloride-reacting substrate when portions of washed mycelium were incubated with and without phenylacetate yielded results (Table 2 and Fig. 1) which were quantitatively somewhat variable. In all experiments, however, phenylacetyl chloride-reacting material in the mycelium decreased on incubation with phenylacetate even after only 1 hr. In three out of four experiments, this decrease was greater than that observed in the absence of phenylacetate, but in one case there was no significant difference.

Phenylacetyl chloride-reacting substances were also found in the medium and are biosynthesized by washed mycelium (Fig. 1*a*). Surprisingly, there was little difference in the amount of phenylacetyl chloride-reacting substances found in the medium on incubating with or without phenylacetate, even though the antibiotic activity before acylation, presumably due to benzylpenicillin, was much greater when phenylacetate was added. The decrease in phenylacetyl chloride-reacting substances in the mycelium (Fig. 1*b*) after incubating for 1 hr. in the presence of phenylacetate was similar to that observed in Expt. WM 1 (Table 2). Omission of phenylacetate had, however, a much less marked effect than previously, although the decrease in mycelial phenylacetyl chloride-reacting substances was consistently less in the absence of phenylacetate throughout the 3 hr. incubation period.

In all cases examined, the total amount of phenylacetyl chloride-reacting material found in the medium was greater than that lost from the mycelium. Since the amount of penicillin formed was approximately ten times the total decrease in phenylacetyl chloride-reacting substances, there must be a rapid turnover of phenylacetyl chloride-reacting material if it is a penicillin precursor.

DISCUSSION

A comparison of our results with those of Batchelor *et al.* (1959), which include the complete characterization of 6-aminopenicillanic acid (I), suggests that the main component of phenylacetyl chloride-reacting material in the mycelium of *Penicillium chrysogenum* is in fact (I), but that in addition there exists one or more perhaps closely related compounds which also give rise to increased antibiotic activity against *B. subtilis* on treatment with phenylacetyl chloride. Estimation of these compounds after chromatographic separation on paper indicates that their relative amounts vary; at most these compounds together may equal the amount of (I) although usually they appear to be present in less than half that quantity.

The results on the production of phenylacetyl chloride-reacting material in fermentations and washed mycelium incubations are not inconsistent with but do not prove a precursor–product relationship between (I) and penicillin (II). The most significant effect of phenylacetate seems to be an increase in penicillin production, whereas the amount of phenylacetyl chloride-reacting substances is not always correspondingly decreased (see Expt. WM 2). It is possible therefore that (I) and (II) are synthesized independently, but further work with labelled precursors would be required to decide this point. The presence of considerable amounts of δ-(α-aminoadipyl)cyst(e)inylvaline, the open-chain tripeptide corresponding to cephalosporin N (III), in the mycelium of *P. chrysogenum* (Arnstein & Morris, 1960) suggests that the common precursor of the penicillins and 6-aminopenicillanic acid may be cephalosporin N. Whether cephalosporin N is one of the minor constituents of phenylacetyl chloride-reacting material in our mycelial extracts remains to be investigated.

The observation that addition of phenylacetate results in increased penicillin titres but in either no change or a decrease in phenylacetyl chloride-reacting substances and hence presumably in (I) appears to favour one of the foregoing explanations rather than the possibility of a conversion of benzylpenicillin into 6-aminopenicillanic acid by the action of penicillin amidase (Sakaguchi & Murao, 1950).

SUMMARY

1. The mycelium of *Penicillium chrysogenum* WIS 51–20 F 3 and the culture medium contain substances (phenylacetyl chloride-reacting substances) which give rise to enhanced antibiotic activity on acylation with phenylacetyl or phenoxyacetyl chloride.

2. One of these compounds appears to be identical with 6-aminopenicillanic acid.

3. The biosynthesis of phenylacetyl chloride-reacting substances has been studied with cultures or washed mycelium of *P. chrysogenum*.

4. Mycelia from cultures to which phenylacetate had been added contained little or no phenylacetyl chloride-reacting substances. Incubation of washed

mycelium in the presence of phenylacetate resulted in a greater decrease in mycelial phenylacetyl chloride-reacting substances than was found in the absence of phenylacetate.

5. No quantitative correlation was found, however, between the increased penicillin production resulting from the addition of phenylacetate and the decrease in phenylacetyl chloride-reacting substances.

6. The metabolic inter-relationship between 6-aminopenicillanic acid and penicillin is discussed.

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The Ultraviolet Fluorescence of Proteins in Neutral Solution

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The fluorescence characteristic of aqueous solutions of tyrosine, tryptophan and phenylalanine was described in detail by Teale & Weber (1957). The fluorescence of these aromatic amino acids when combined in the polypeptide chains of globular proteins is now reported and some structural implications of the results are discussed.

EXPERIMENTAL

Methods

Fluorescence spectra and fluorescence-excitation spectra. These were determined by means of the apparatus described by Teale & Weber (1957). Protein solutions contained in a quartz cell were irradiated by radiation of 2537 Å wavelength isolated from a low-pressure mercury arc by a combination of a Chance OX7 glass filter and *p*-nitrophenol solution in a silica cuvette. The fluorescence emitted at right angles to the direction of excitation was analysed by a Bausch and Lomb grating monochromator and detected by an E.M.I. 6255B quartz-window photomultiplier. The corrections for varying monochromator transmission and detector response with wavelength are described elsewhere (Teale & Weber, 1957). In certain cases a band of continuous radiation derived from a xenon-arc lamp by means of an aqueous solution of nickel sulphate and *p*-nitrophenol was used to excite over the whole absorption band of the protein.

Fluorescence-excitation spectra were obtained with a hydrogen- or xenon-arc lamp as the continuous source. A filter of Perspex 4 mm. thick or a Chance OX1 glass filter was used to separate exciting light from fluorescence. Cuvettes of extremely pure fused quartz (Thermal Syndicate Ltd., Wallsend, Northumberland, England) were used to reduce cell fluorescence over the 240 mμ excitation range. In the determination of both excitation and fluorescence spectra, experimental points were obtained at wavelength intervals of 5 mμ. Additional points were interpolated where the readings changed rapidly with wavelength.

Fluorescence quantum yields. These were measured by comparing the total emission from protein solutions with that of pure tyrosine or tryptophan solutions with the same excitation absorption. Absolute yields were then calculated by correction for differences in detector response to the fluorescence spectra, assuming the published values for the absolute quantum yields of tyrosine and tryptophan (Weber & Teale, 1957). For accurate measurements of the quantum yield in the wavelength region from 290 to 310 mμ, where the molecular extinction changes rapidly, monochromatic lines of the high-pressure mercury arc were employed to excite fluorescence.

Absorption spectra. These were determined with a Uvispek spectrophotometer at wavelength intervals of 5 mμ, or with an Optika recording spectrophotometer. The extinction coefficients of proteins at wavelengths where the absorption is small were obtained with concentrated