Studies in Penicillin Production by *Penicillium notatum* in Surface Culture

2. FURTHER STUDIES IN THE METABOLISM OF SULPHUR

By D. J. D. HOCKENHULL

The Department of Applied Chemistry, College of Technology, Manchester

(Received 24 March 1948)

An earlier report from these laboratories described the metabolism of carbon and nitrogen compounds by *Penicillium notatum* in surface culture (Hockenhull, 1946), and some references were made to the utilization of sulphur by this organism. Further work on the latter aspect seemed desirable, especially as penicillin is a sulphur-containing compound. It is the purpose of the present communication to describe these investigations.

Previous work on the sulphur metabolism of moulds is limited and is mainly concerned with *Aspergillus niger*. Rippel & Behr (1936) showed that this fungus, when supplied with sulphates, liberated considerable quantities of organic sulphur compounds into the medium. Möthes (1938) showed that when cystine, cysteine or thioglucose was supplied sulphates appeared in the medium. Möthes also showed that the reduction of sulphate to organic sulphur was greater in presence of ammonia than in presence of nitrate. Steinberg (1941) showed that many inorganic sulphur sources were available for the growth of *A. niger* (e.g. sulphate, thiosulphate, etc.) and concluded that the mould removed sulphur from organic compounds via sulphylic acids. Woolley & Peterson (1937) demonstrated the accumulation of 'cyclic' choline sulphate (β -sulphato-ethyltrimethylammonium betaine),

in the mycelium of *A. sydowi*.

Penicillium notatum is known to utilize cysteine, cystine or sulphate to produce optimal yields of penicillin. In our experiments we have examined the growth of this organism on a wide range of sulphur sources. The growth of two X-ray mutants, which are unable to grow on sulphate, was also examined. It was hoped that a study of these organisms would throw light on the metabolic stages used by the organism in the formation of organic sulphur compounds. Analytical studies were also made on the utilization of sulphur by the mould, and experiments similar to those described by Rippel & Behr (1936) and by Möthes (1938) were also carried out with

P. notatum in order to determine whether their behaviours were the same or different.

EXPERIMENTAL

Cultural methods and materials

Strains of the mould. Five strains were used in our experiments: (1) 1249.B21, which was received from the Northern Regional Research Laboratory, Peoria, Illinois; it has been used for the commercial production of penicillin and the culture used by us has been labelled by us *M2*; (2) *F4*, received from Sir Howard Florey; it was used at one time for the production of penicillin; (3) 832, received from Peoria, and used for early work in submerged culture; (4) *W-3* cyst, an X-ray mutant of Sir Alexander Fleming's strain, which, although white and powdery, also produces a yellow pigment freely on most media; (5) 832 *A*, an X-ray mutant of strain 832 morphologically similar to its parent strain, to which it tends to revert occasionally. The last two strains were received from Dr G. Pontecorvo of Glasgow University to whom our thanks are due. They both grow freely on media containing cystine but, unlike the others, do not grow on media containing sulphate as sole source of sulphur.

Growth conditions. All experiments with liquid media were carried out using 250 ml. conical flasks, with 100 ml. of medium in each. The medium was steam sterilized for 20 min. at 15 lb./sq.in. throughout.

The inoculum was a spore suspension containing 3×10^7 spores/ml., based on direct counts with a haemocytometer cell. It was prepared by washing off spores from cultures on glycerol-molasses-peptone (cf. Moyer & Coghill, 1946) with sterile water. 1 ml. of this suspension was pipetted into each culture vessel. All cultures were incubated, stationary, at $23.5^\circ \pm 0.5^\circ$.

Media. The media most commonly used were synthetic and were based on a 'standard basal' medium (hereafter referred to as *SB* medium) which had the following composition (as % w/v): lactose (B.P.), 3; glucose (B.P.), 1; citric acid (B.P.), 1.5; acetic acid, 0.25 (v/v); phenylacetic acid (recrystallized), 0.05; source of N (as text); source of S (as text); $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.05; FeCl_3 , 0.005; $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$, 0.002; ZnCl_2 (fused), 0.002; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.002; $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 0.002; NaH_2PO_4 , 0.1; NaF, 0.001.

The medium was made in tap water and was adjusted to pH 5.8–6.0 with 56% (w/v) KOH before sterilization. It was modified quantitatively or qualitatively by varying either the N source or the S source, or both. Unless

otherwise stated, all ingredients were A.R. quality. Other media employed are described in the text.

'SB agar' was similarly constituted except for the incorporation of 2.5% powdered agar.

Analytical procedure

Sampling. Five replicate flasks were removed at the appropriate times. Sterile samples were withdrawn and diluted twenty times with pH 6.5 phosphate buffer (0.05M) for penicillin assay. The mycelium was separated by filtration, and washed with five separate lots of 100 ml. distilled water. The pH and volume of first filtrate were measured. The washings, which were found adequate for complete removal of soluble matter and medium from the felt, were added, together with 10 ml. of conc. HCl, and the whole made up to 1 l. less an allowance for the medium used for penicillin assay. The solution was stored at 0°. In experiments with cystine and cysteine the filter paper was separated from the mycelial mat and was analyzed for precipitated S compounds.

The mycelium was dried to constant weight at 80°, weighed, powdered and stored for analysis. The composition of the mycelium with respect to % N or % S on dry wt. was found to be the same if the undried mycelium was homogenized with 20 vol. of distilled water, thus indicating that there was no loss of N or S on drying.

Total N was estimated by the micro-Kjeldahl method.

Total S was determined by the method of Luke (1943) but using a different mechanical procedure. Wet combustions of a sample containing 3–5 mg. of S were carried out in a silica crucible with a $\text{HNO}_3\text{-Br}_2$ solution containing dissolved ZnO: the subsequent reduction and distillation of H_2S was performed in a 50 ml. flask fitted to a spiral condenser. A stream of N_2 was passed through the apparatus and the H_2S was trapped in a spiral bubbler containing 10 ml. of the alkaline CdCl_2 reagent. After all the H_2S had been absorbed 10 ml. of 1.5N-HCl solution containing 3 ml. 0.1N- I_2 were added to the bubbler and quickly mixed. The H_2S was estimated by back titration with 0.01N- $\text{Na}_2\text{S}_2\text{O}_3$.

An alternative procedure used for checking purposes was the combustion of the same quantity of material using Benedict's reagent (Cole, 1933), followed by estimation of sulphate with benzidine. This method was found to be less accurate than the other.

Sulphate. Solutions containing 3–4 mg. of sulphate S were adjusted with HCl or NaOH until slightly acid to congo red paper. The sulphate was precipitated as benzidine sulphate (Cole, 1933). The precipitate was collected in a 30 mm. Gooch crucible containing asbestos wool. An alternative but more tedious method was precipitation with BaCl_2 in acid solution, followed by reduction of the BaSO_4 to H_2S as in the estimation of total S.

'Bound sulphate' in the mycelium was estimated by hydrolyzing about 300 mg. of dried mycelium at 15 lb./sq.in. steam pressure for 2 hr. with 2.5N-HCl. The liquor was made up to 25 ml. and filtered. 10 ml. were taken for estimation of sulphate by the BaCl_2 method.

Cystine. This was estimated by the procedure of Shinohara (1935). It was found that during medium preparation all cysteine was converted to cystine or oxidized further. Both cysteine and cystine media were therefore estimated by the cystine method.

Penicillin. Penicillin was estimated by the plate assay methods in current use in these laboratories using *Bacillus subtilis* as the test organism (cf. Foster & Woodruff, 1944), and expressed as Oxford units.

RESULTS

Three groups of experiments were carried out. In the first various strains, including the irradiated strains, were inoculated on to media containing a variety of sulphur sources, and growth was observed in each case. The availability of different forms of combined sulphur was determined in this way. Mixtures of the two irradiated strains were also inoculated on to sulphate media. In the second group synthetic media which yield penicillin were metabolized by strain 1249.B21 and the utilization of sulphur examined at intervals. In the third, some of the experiments carried out by earlier workers with *Aspergillus niger* were repeated using *Penicillium notatum* strain 1249.B21.

(1) Availability of different sulphur sources to normal and irradiated strains of *Penicillium notatum*

Different sulphur sources

The five strains of the mould were grown on SB medium to which were added 0.5% of NH_4NO_3 and one of a large number of sulphur compounds. 1 mg./ml. of the sulphur compound was added. Three replicate flasks were used. The sulphur compound was dissolved in water and sterilized by autoclaving (or by Seitz filtration in the case of Na_2S and $\text{Na}_2\text{S}_2\text{O}_4$) and added to the sterile medium. Growth is shown as dry mycelial weight expressed as a percentage of the dry weight obtained using medium containing L-cystine. The results of this experiment are shown in Table 1. No growth was obtained unless a sulphur source was added to the basal medium.

Growth of mixed cultures of the irradiated strains

(a) **Liquid media.** Media containing as sulphur sources sulphate, thiosulphate, hyposulphite, 'cyclic' choline sulphate and acetone sodium bisulphite were inoculated with spore suspensions containing spores of the W-3 cyst and 832A strains or of a mixture of the two. There was no evidence of symbiosis between the two strains which grow satisfactorily only on the media containing hyposulphite and thiosulphate. The irradiated strains were also inoculated into Seitz-filtered SB medium containing sulphate, on which strain 1249.B21 had been growing for 4 days. There was no growth, showing that sufficient utilizable sulphur compounds had not been liberated into the medium by strain 1249.B21.

Table 1. *Effect of different sulphur sources on the growth of various strains of Penicillium notatum*(Medium: *SB* + S source + 0.5% NH_4NO_3 .)

Strain of mould ... Sulphur source (at 1 mg./ml.)	<i>M</i> 2		<i>F</i> 4		<i>W</i> -3 cyst (‘cystineless’)		832		832 <i>A</i> (‘cystineless’)	
	Wt.*	Pen.†	Wt.*	Pen.†	Wt.*	Pen.†	Wt.*	Pen.†	Wt.*	Pen.†
Na_2SO_4	100	70	100	12	0	0	102	0	0	0
NaSO_3NH_2	100	58	105	12	0	—	85	15	0	—
$\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$	60	66	100	10	0	—	148	26	20	0
$\text{Na}_2\text{S}_2\text{O}_4$	120	60	114	14	108	0	190	22	108	9
$\text{Na}_2\text{S}_2\text{O}_3 \cdot 10\text{H}_2\text{O}$	100	58	99	19	74	0	148	25	130	23
Na_2S	109	71	94	13	66	0	89	14	51	0
Na_2S_2	—	—	—	—	—	—	—	—	88	12
L-Cystine	100	52	100	14	100	5	100	24	100	20
L-Cysteine.HCl	100	58	105	13	98	3	97	22	73	21
Cysteic acid	91	6	98	5	100	0	92	5	92	—
Taurine	100	24	108	12	89	10	94	17	113	15
2-Mercaptoethylamine	80	21	88	—	40	—	124	—	134	—
DL-Dimethylcysteine	40	—	23	—	0	—	50	—	56	—
Mercaptosuccinic acid	50	—	29	—	0	—	55	—	59	—
Thioacetamide	135	—	67	—	56	—	80	—	76	—
Thiourea	33	—	27	—	0	—	57	—	26	—
‘Cyclic’ choline sulphate	125	76	100	16	0	—	94	24	0	—
Acetone sodium bisulphite	100	33	98	10	0	—	100	13	38	—
Ethyl xanthate	123	—	95	—	58	—	102	—	129	—
KCNS	115	—	93	—	40	—	150	—	100	4
Dry weight of felt grown with L-cystine (g./100 ml. medium)	0.65		1.31		1.22		0.57		0.91	

* Wt. = $\frac{\text{mycelial dry weight} \times 100}{\text{mycelial dry weight with cystine}}$.

† Pen. = Penicillin (units/ml.).

(b) *Solid media.* 0.1% of Na_2SO_4 was added to *SB* agar medium and 10 ml. portions were placed in Petri dishes. Strains *W*-3 cyst and 832 *A* were inoculated on this medium either separately or together.

From the mixed cultures a new type of quickly growing colony developed which could be readily subcultured on the same medium. When single spores from this culture were transferred to *SB* medium containing cystine, the two parental strains were obtained. When hyphal tips were transferred to malt-wort-agar, colonies were obtained which sectored, and from the sectors the parental strains were again recovered. There was thus the appearance of a symbiosis of an interesting type, but further work is necessary before more extensive discussion is possible.

(2) *Metabolism of sulphate in synthetic media on which penicillin is produced*

Reduction of sulphate in SB medium

(a) *SB* medium containing 0.5% $(\text{NH}_4)_2\text{SO}_4$ and 0.5% NaNO_3 was inoculated with strain *M* 2 and incubated. Analyses showed that only a very small fraction of the sulphate was utilized. The results of this experiment, which show the utilization of sulphate as well as of other compounds, are shown in Fig. 1.

(b) The above experiment was repeated, but in order to increase the proportion of sulphur which

would be utilized the initial sulphur content of the medium was decreased to 0.35 mg./ml. A small quantity of Na_2SO_4 supplied the sulphur and 0.5% NH_4NO_3 the nitrogen.

The results of the experiment are shown in Fig. 2.

About 0.12 mg./ml. of sulphate was reduced, of which 0.09 mg./ml. appeared in the mycelium and 0.004 mg./ml. as penicillin. No cystine or cysteine could be detected in the liquor.

Influence of nitrate and ammonia on sulphate reduction

SB medium was used in this experiment with the addition of 0.35 mg./ml. of sulphur, as sulphate. Three different nitrogen sources were used: (A) 0.3% NH_3 ; (B) 0.3% NH_3 plus 0.25% NaNO_3 ; and (C) 0.08% NH_3 plus 1.0% NaNO_3 . Although set (A) produced more mycelium as measured by dry weight, it contained only the same quantity of mycelial total sulphur as did the others. Maximal penicillin yields were: (A), 30 units/ml. (day 8); (B), 29 units/ml. (day 6); (C), 100 units/ml. (day 16). Set *B* gave 53 units/ml. on day 8.

Media containing cystine with different additional sulphur and nitrogen sources

(a) Three different media were made up using *SB* medium containing 0.5% NH_4NO_3 . To this were added (D) c. 0.4% cysteine hydrochloride;

Fig. 1. Sulphate metabolism of *Penicillium notatum* in relation to that of other medium components. Left-hand graph: ○, liquor total S (mg./ml.); □, liquor SO₄-S (mg./ml.); ○, penicillin (units/10 μl.); ●, acetic acid (mg./ml.); △, ammonia N (mg./ml.). Right-hand graph: ●, mycelial N (mg./ml.); ○, mycelial S (mg./10 ml.); □, mycelial dry weight (mg./100 μl.); +, pH (units ÷ 10).

Fig. 2.

Fig. 2. Sulphur metabolism of *Penicillium notatum*. Medium: SB + NH₄NO₃ + Na₂SO₄. ○, liquor total S (mg./ml.); □, liquor SO₄-S (mg./ml.); ◇, penicillin (units/ml.); ●, mycelial total S (mg./ml.); +, pH.

Fig. 3.

Fig. 3. The utilization of cystine and sulphate by *Penicillium notatum* showing the conversion of part of the cystine supplied into sulphate. Basal medium: SB medium + 0.5% NH₄NO₃. Sulphur source: 0.4% cystine (left-hand figure), 0.2% cystine + 0.2% Na₂SO₄ (centre figure) or 0.4% Na₂SO₄ only (right-hand figure). ○, liquor total S (mg./ml.); □, liquor SO₄-S (mg./ml.); ●, cystine S (mg./ml.); +, mycelial SO₄-S (mg./ml.); ×, mycelial dry weight (mg./100 μl.).

(E) c. 0.2% cysteine hydrochloride plus 0.2% Na_2SO_4 ; (F) 0.4% Na_2SO_4 .

After autoclaving the medium all the cysteine was found to have been converted to cystine and was estimated as such. As would be expected set F behaved as in the previous experiment.

In both D and E cystine fell steadily throughout. Total sulphur disappeared from the liquor until day 8 but subsequently rose again (Fig. 3). The conversion of organic sulphur to sulphate was first apparent on day 16 in both sets D and E. About 30% of the total sulphur in set D appeared as sulphate. About 15% of cystine in set E appeared as sulphate. About 5% of the total sulphur in D was detected as 'bound' (acid-hydrolyzable) sulphate in the mycelium.

Penicillin yields were high in all three sets and peak titres (12 or 16 days) were 102, 117 and 92 units/ml. respectively.

(b) A second experiment was carried out using L-cystine (c. 0.25%) as sulphur source together with (G) 0.25% NaNO_3 and 0.3% NH_3 or (H) 1% NaNO_3 and 0.25% NH_4NO_3 . The concentration of sulphur in the mycelium was the same with both media

Fig. 4. The effect of nitrogen source on the utilization of cystine by *Penicillium notatum*. Medium: SB containing (G) 0.25% NaNO_3 + 0.3% NH_3 ; and (H) 1.0% NaNO_3 + 0.25% NH_4NO_3 . Cystine (c. 0.25%) as S source. Liquor total S (mg./ml.) in G, $\circ$; in H, $\square$. Cystine S (mg./ml.) in G, $\square$; in H, $\blacksquare$. Mycelial S (mg./ml.) in G, $\circ$; in H, $\bullet$. Precipitated S (mg./ml.) in G, $\square$; in H, $\blacksquare$.

but transference was greater with (G) because the mycelium was heavier (Fig. 4). No sulphate could be detected in the medium in either case. Penicillin yields were low with both media (maxima 32 and 42 units/ml. respectively) possibly because the concentrations of ammonia supplied were unsuitable for this purpose.

Utilization of sulphur in enriched media

In this experiment two media were used which gave thicker growth of the mould and relatively higher yields of penicillin than did SB medium. The first of these (A) containing lactose and corn-steep liquor is similar to that used previously by Hockenhull (1946). This medium contains no sulphate. The results are similar to those obtained before. The second medium (B) consisted of the SB medium with the addition of 1.5% soluble starch, 0.6% NH_4NO_3 , 0.24% Na_2SO_4 and 0.3% monoethylamine. The result with this medium is shown in Fig. 5 which shows that 40% of the sulphate was

Fig. 5. Sulphate metabolism in special media. Medium A contained lactose and corn-steep liquor as used by Hockenhull (1946). Medium B: SB + 1.5% soluble starch, 0.6% NH_4NO_3 , 0.24% Na_2SO_4 , 0.3% monoethylamine. Liquor total S (mg./ml.) in A, $\circ$; in B, $\square$. Liquor $\text{SO}_4\text{-S}$ (mg./ml.) in A, $\bullet$; in B, $\blacksquare$. Mycelial total S (mg./ml.) in A, $\circ$; in B, $\square$. Mycelial $\text{SO}_4\text{-S}$ (mg./ml.) in A, $\bullet$; in B, $\blacksquare$. No liquor $\text{SO}_4\text{-S}$ was detectable in series A.

reduced to a soluble sulphur fraction which was not identified. Although some sulphur reappeared in the liquor from the felt, this was not in the form of sulphate. The results of this experiment are shown in Table 2.

Table 2. Development of *Penicillium notatum* on enriched media

(Media: as legend to Fig. 5.)

Media	...	Penicillin (units/ml.)		Mycelial dry weight (mg./ml.)		Mycelial N (mg./ml.)	
		A	B	A	B	A	B
Day 8		107	86	12.0	11.3	0.85	0.88
12		107	112	14.6	11.4	0.94	0.83
16		70	80	9.7	8.0	0.55	0.52

(3) *Metabolism of sulphur with simpler media*

A number of media were used which were similar to, but not quite identical with, those used by Rippel & Behr (1936) and Möthes (1938) for metabolic work with *Aspergillus niger*.

Sucrose medium with ammonia or nitrate

The basal medium contained 5% sucrose, mineral salts and 0.5% Na_2SO_4 . Nitrogen sources were either 0.3% NH_3 or 1.3% NaNO_3 . The former gave much heavier felds than the latter, and on corresponding days gave higher mycelial sulphur and sulphate than the other. Changes in sulphate and total sulphur in the medium corresponded with these figures, but were only a small fraction of the total sulphur available (Fig. 6).

Fig. 6. Sulphate metabolism on simpler media. Basal medium: 5% sucrose, mineral salts, and 0.5% Na_2SO_4 . Nitrogen source either 0.3% NH_3 or 1.3% NaNO_3 . Liquor sulphate S (mg./ml.) in medium containing ammonia, $\square$; nitrate, $\circ$. Mycelial $\text{SO}_4\text{-S}$ (mg./ml.) in medium containing ammonia, $\blacksquare$; nitrate, $\bullet$. Mycelial total S (mg./ml.) in medium containing ammonia, $\square$; nitrate, $\circ$.

These results indicate that *Penicillium notatum* behaves quite differently from *Aspergillus niger* in that there was no tendency for organic sulphur compounds to accumulate in the medium.

Sucrose medium with urea and cystine

A medium was prepared containing 1% sucrose, 0.5% urea, mineral salts and 0.2% cystine, and inoculated with *Penicillium notatum* 1249.B21. Up to and including the twenty-third day of fermentation neither sulphate nor penicillin could be detected in the medium.

DISCUSSION

The results with *P. notatum* on the availability of various forms of sulphur are in almost complete agreement with those described by Steinberg (1936, 1941) for *Aspergillus niger*. Steinberg found that sulphate, sulphite, hyposulphite, thiosulphate and sulphamate gave maximal growth of *A. niger*, while freshly prepared sulphide or disulphide gave poor growth. Further work with organic compounds showed that L-cysteine or methionine and methoxysulphonic or n-butylsulphonic acids gave good growth, while mercaptans and disulphides did not. Steinberg also found that sulphate was put out of action by the addition of excess barium salt to the medium, but none of the reduced sulphur compounds was affected in this way. From these results Steinberg concluded that sulphate is not the substance through which sulphur is incorporated into the mould, but that the substance concerned is more oxidized than the sulphides. Steinberg did not consider that this compound could be H_2SO_3 in view of the stability of the S—C link in sulphonic acids (ignoring ketone-bisulphite-type compounds). He therefore thought it likely that the lower thio acids played the intermediate roles in the process of sulphur assimilation.

The present work, especially with the mutant strains, appears to bear out this hypothesis, for neither of these strains gave good growth with sulphate or sulphite, whereas hyposulphite and thiosulphate were utilized readily. It would therefore appear that in each of the mutants one of the links in the chain of reactions involved in sulphur metabolism is broken, and that the chain involves the reduction of sulphate to thio acids before conversion to organic sulphur compounds which are built into the mycelium. The nature of this symbiosis is as yet not fully understood. If the results so far obtained can be confirmed by a fuller investigation they should be of considerable interest. The number of steps in this process is unknown, but it would appear that the breaks occur at different places in the two mutants for they are able to grow symbiotically on sulphate. Examples of the breaking of links in metabolic chains by mutation are provided by the classical work which has been done with *Neurospora* on the formation of tryptophan and other compounds (cf. Beadle, 1945). Of more particular interest is the recent work of Lampen, Roepke & Jones (1947), in which they report the isolation of two mutant strains of *Escherichia coli* unable to utilize sulphate as sole source of sulphur but able to grow in presence of sulphite as sulphur source. One strain able to utilize sulphide only and several strains unable to use either are also reported. Similar 'parathiotropic' mutants of *Ophiostoma multianellatum* have been obtained by Fries (1945). The author

(Hockenhull, 1948) also reports mustard-gas mutants of *Aspergillus nidulans* which behave in a similar manner.

The metabolic experiments show that when sulphur is utilized by *Penicillium notatum* in simple media, it is nearly all transferred directly to the mycelium, and that there is little tendency for the new sulphur compounds to accumulate in the medium. When sulphur was supplied as sulphate, cystine was rarely formed in the medium; with cystine, sulphate did not usually appear in the medium. In this the mould differs from *Aspergillus niger* which has been found to produce organic sulphur from sulphate and sulphate from cystine. The metabolism of sulphur by *A. niger* is also influenced by the nature of the nitrogen source, but this effect does not seem to be so marked with *Penicillium notatum*.

With more complex media, such as those containing organic acids, there was some production of sulphate from cystine in the later stages, perhaps due to autolysis of the mycelium, but there was no accumulation of organic sulphur from sulphate. With the richest media used, containing corn-steep liquor or organic acids together with starch and ethylamine as supplementary sources of carbon and nitrogen there was a marked tendency for sulphur compounds, possibly organic, to appear in the medium. These media also gave the highest yields of penicillin. Evidence that sulphate may arise from autolysis of the mycelium is provided by the fact that as much as 40% of the total mycelial sulphur was in the form of ethereal sulphate. This finding is in agreement with that of Woolley & Peterson (1937) who isolated 'cyclic' choline sulphate from the mycelium of *Aspergillus sydowi*. This sulphate may act as a reservoir of sulphur for the organism. The work with the mutants also supports the view that sulphate is not likely to be an intermediate in the metabolism of cystine. These results contrast with those obtained with *A. niger*, and also indicate that the composition of the medium has a considerable influence on the metabolism of sulphur by the organism.

Our experiments do not throw any particular light on the mechanism of the formation of penicillin.

This is not altogether surprising, since the quantity of penicillin produced is so small that the amount of sulphur involved is scarcely measurable. Since, however, considerable yields of penicillin can be obtained when other sulphur compounds are not accumulating in the mycelium it seems reasonable to suppose that penicillin is formed in the mycelium and subsequently split off by some enzymic process. The presence of cysteine, cystine or other specific sulphur compound does not seem to be a limiting factor in this process.

SUMMARY

1. The growth of normal and mutant strains of *Penicillium notatum* on different sources of sulphur has been studied, and the metabolism of some of them followed analytically. Normal strains can utilize many inorganic substances, such as sulphate, sulphite, hyposulphite and thiosulphate, as well as other compounds, and high yields of penicillin and mycelium can be obtained with a considerable variety of sulphur sources.

2. Two mutant strains which were unable to grow on sulphate, 'cyclic' choline sulphate, sodium acetone bisulphite, sulphite, or sulphamate grow well on other sulphur compounds such as cystine, thiosulphate or hyposulphite.

3. When mixed the two mutant strains could be grown together on a medium containing sulphate as sole source of sulphur. Isolates from the mixed colonies produced 'sector' colonies on malt medium, and from the sectors strains similar to the originals were isolated.

4. With the normal strains the utilization of sulphur, through the reduction of sulphate or disappearance of cystine, is mainly linked with the formation of mycelium. Only with certain media do non-sulphate compounds accumulate in the medium. Ethereal sulphate was detected in the mycelium.

5. The metabolic route adopted for sulphur by *P. notatum* appears to be similar to that used by *Aspergillus niger*, namely that inorganic sulphur compounds are incorporated into the molecule via a chain of reduced thio acids. On the other hand, the two moulds react differently to the presence of ammonia or nitrate in the medium.

REFERENCES

- Beadle, G. W. (1945). *Physiol. Rev.* **25**, 643.
 Cole, S. W. (1933). *Practical Physiological Chemistry*. Cambridge: Heffer.
 Foster, J. W. & Woodruff, H. B. (1944). *J. Bact.* **47**, 43.
 Fries, N. (1945). *Nature, Lond.*, **155**, 757.
 Hockenhull, D. J. D. (1946). *Biochem. J.* **40**, 337.
 Hockenhull, D. J. D. (1948). Unpublished work.
 Lampen, J. O., Roepke, R. R. & Jones, M. J. (1947). *Arch. Biochem.* **13**, 55.
 Luke, C. I. (1943). *Industr. Engng Chem. (Anal. ed.)*, **15**, 602.
 Möthes, K. (1938). *Planta*, **29**, 67.
 Moyer, A. J. & Coghill, R. D. (1946). *J. Bact.* **51**, 57.
 Rippel, A. & Behr, G. (1936). *Arch. Mikrobiol.* **7**, 584.
 Shinohara, K. (1935). *J. biol. Chem.* **112**, 671.
 Steinberg, R. A. (1936). *Bot. Gaz.* **97**, 666.
 Steinberg, R. A. (1941). *J. agric. Res.* **63**, 109.
 Woolley, D. W. & Peterson, W. H. (1937). *J. biol. Chem.* **122**, 213.