# The Effect of Nutrients on Formation of Penicillin by Washed Mycelium of *Penicillium chrysogenum*

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Many different substances are known to affect the yields of penicillin from growing cultures of *Penicillium chrysogenum*. Under the usual conditions, it is difficult to distinguish between the effect of a substance on growth of the mould (and hence the indirect effect on production of penicillin) and its direct effect on antibiotic synthesis. The use of washed mycelium (Halliday & Arnstein, 1956) was considered as a solution to the problem, since such mycelium can produce penicillin in a simple medium which would not permit growth.

Washed mycelium is known to require oxygen and phenylacetate to synthesize benzylpenicillin. The amino acids cystine and valine are incorporated if they are added, as can be shown by radioactive-tracer experiments (Halliday & Arnstein, 1956), but their effect on yields of penicillin from washed mycelium has not been examined critically. This report summarizes experiments which show that several amino acids and other nutrients cause significant changes in the yield of penicillin.

## EXPERIMENTAL

*Preparation and use of mycelium.* The culture of *P. chrysogenum* Wis. 51–20F3 was maintained in sterile soil. For the preparation of spores, this stock culture was streaked on molasses–agar (Perret, 1953) and incubated for 12 days at 24–25°. These cultures were stored at about 4°. The spores were washed from the surface of the medium with 0·02% Teepol (Shell Chemical Co.) in 0·1 M-potassium phosphate buffer (54 g. of $KH_2PO_4$/105 g. of $K_2HPO_4$) at pH 7. The density of this spore suspension was adjusted so that a 1 in 10 dilution had an optical density of 0·35, measured at 660 mμ in the Evelyn colorimeter. A volume (3 ml.) of the undiluted suspension was then used to inoculate each growth flask. The liquid medium for the growth of the mycelium was that of Jarvis & Johnson (1950), without phenylethylamine and with the ammonium lactate reduced from 6 g./l. to 3·5 g./l. The latter modification was found to give increased penicillin yields under the conditions used. Volumes of medium (100 ml. in 500 ml. conical flasks) were inoculated with spores as described above, and incubated on a rotary shaker at 250 rev./min. and at 24–25°. Mycelium was harvested on a sintered-glass filter and washed four times with the original volume of water (Halliday & Arnstein, 1956). In studies of production of penicillin, 1 g. amounts of wet mycelial pad were weighed into 100 ml. flasks, together with 9 ml. of 0·01 M-phosphate buffer, pH 7, containing potassium phenylacetate (final concn. 0·1 g./l., calc. as phenylacetic acid) and other substances as required. The flasks were incubated on the shaker as described above for 2 hr., then the contents were filtered through paper and the filtrates assayed for penicillin.

*Penicillin assays.* Penicillin was assayed by the method of Humphrey & Lightbown (1952) with *Bacillus subtilis* NCTC 8241 as test organism, and commercial sodium benzylpenicillin (4, 8 and 16 units/ml. in 0·1 M-phosphate buffer, pH 7) as standard. The dry weight of the mycelium was determined by heating 1 g. of the mycelial pad for 48 hr. at 85°. Yields of penicillin from washed mycelium were recorded as units/ml./2 hr., converted into 12 mg. dry wt. of mycelium/ml. if the actual dry weight varied from this figure.

*Effect of nutrients.* The effect of added nutrients (other than phenylacetate) was examined with mycelium which was harvested when 60–62 hr. old. In each experiment, seven replicate flasks were used for each of two treatments (i.e. with and without the specific nutrient), and the contents of each flask were assayed separately for penicillin. The mean penicillin yields were compared statistically by Student's $t$ test, after an $F$ test had shown the variances of the two treatments not to differ significantly. The 'endogenous' penicillin, produced in small amounts in the absence of added phenylacetate, was neglected in making these calculations.

*Growth of washed mycelium.* Experiments to determine the effect of nutrients on growth were performed by incubating replicate samples of mycelium (of known dry weight) with various nutrients. After 2 hr. on the shaker, the mycelium was filtered off, washed, dried, weighed and the increase in dry weight calculated. It was found advantageous to use 5 g. samples of mycelium in 50 ml. of liquid, in order to detect more easily the small changes in dry weight. The experiment was done in three parts (see Table 2) because of limitations of shaker space.

*Cystine in mycelium.* Washed mycelium was examined for free cystine by the following method (cf. Schram, Moore & Bigwood, 1954): the mycelium was extracted with 75% (v/v) ethanol at about 40°, and the extract was evaporated to dryness. The residue was dissolved in 10% (v/v) propan-2-ol and the solution was passed through a small column of Zeo-Karb 225 resin (The Permutit Co. Ltd.) in the hydrogen form. The resin was washed with water, and then eluted with aq. N-$NH_3$ soln. This eluate was evaporated to dryness under reduced pressure, and the residue was oxidized with performic acid to convert

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cystine and cysteine into cysteic acid. After removal of the performic acid by evaporation, the oxidized material was again passed through a resin column as above, and the resin was washed with water. The aqueous effluent was concentrated by evaporation and fractionated either on paper chromatograms (developed with propan-2-ol, 70%, v/v) or on columns of Dowex-2 resin (10% cross-linked; The Dow Chemical Co.) in the chloride form (developed with 0·01 N-HCl). Cysteic acid and related substances were then detected by reaction with ninhydrin. A solution of cysteic acid was prepared for reference purposes by oxidizing L-cystine with performic acid.

*Chemicals.* The inorganic chemicals, lactose, and glycine were of A.R. quality. Phenylacetic acid, DL-valine, L-glutamic acid, L-cystine, DL-serine and L-leucine were laboratory reagents (British Drug Houses Ltd.). These substances, with the exception of the cystine, dissolved readily in the phosphate buffer which was used to suspend the mycelium. Cystine solutions were prepared by boiling the buffer with the required amount of cystine, then allowing the liquid to cool; nevertheless, some of the substance remained undissolved.

## RESULTS

### *Effect of age of mycelium*

Samples of mycelium, harvested at various times after inoculation of the growth medium, were washed and their ability to synthesize penicillin from phenylacetate was determined in duplicate. At the same time, determinations were made of the total dry weight of the growing mycelium, the penicillin concentration in the growth medium and the 'endogenous' production of penicillin by washed mycelium in the absence of phenylacetate. The results are shown in Fig. 1.

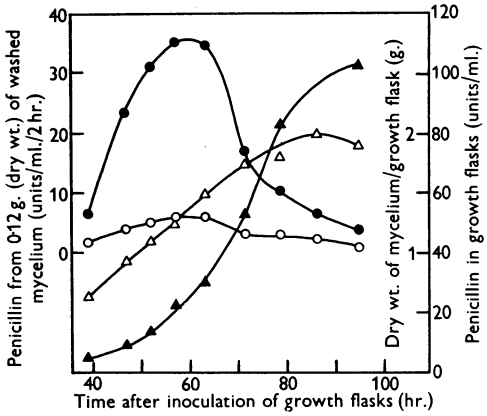


Fig. 1. Changes in total penicillin produced in growth flask (▲), dry weight of mycelium/growth flask (△), 'endogenous' penicillin from washed mycelium harvested at different ages (○) and penicillin from washed mycelium harvested at different ages and incubated with phenylacetate (●), for shake-flask cultures of *P. chrysogenum* Wis. 51–20 F 3.

In preliminary experiments, potassium phenylacetate (0·5 g./l.) was added to the growing cultures at 30–40 hr. after inoculation, in order to stimulate synthesis of penicillin. It was later found that this had no apparent beneficial effect on the ability of the washed mycelium to produce penicillin when exposed to phenylacetate. Indeed, cultures grown without phenylacetate reached the stage of maximum production of penicillin earlier, and showed more rapid growth than those with added phenylacetate, which is known to be slightly toxic to the mould (Singh & Johnson, 1948). Mycelium grown without phenylacetate was used in all subsequent work, and it was harvested at 60–62 hr., the age at which occurred the maximum rate of production of penicillin by washed mycelium (Fig. 1).

### *Penicillin yield with various nutrients*

Several substances were tested for their influence on yields of penicillin from washed mycelium, in the presence of phenylacetate.

*Cystine.* During some experiments on the synthesis of penicillin by washed mycelium of different ages, L-cystine (0·5 g./l.; 0·002 M) caused slight increases in yield, over the whole growth cycle. The significance of these increases was tested by the use of larger numbers of replicate samples of mycelium, with the results shown in Table 1. In two such trials (Expts. 1 and 3), the increase in yield of penicillin in the presence of cystine was significant at the 5% level, and in another trial (Expt. 2) the significance just failed to reach the 5% level. When these results were pooled and submitted to an analysis of variance, the stimulation by cystine was significant at the 0·1% level. That is, $P$, the probability that the effect was due to chance, was less than 0·001.

The absolute yields in Expt. 3 were much higher than normal, although cystine was still stimulatory. Mycelium for this experiment had shown poor growth before harvesting.

*Serine.* DL-Serine (0·004 M) had no effect on yield of penicillin under the conditions used (Table 1).

*Other amino acids.* Glycine, DL-valine, L-leucine and L-glutamic acid (all 0·004 M), added separately to washed mycelium with phenylacetate, caused significant decreases in yields of penicillin (Table 1). When valine was added together with cystine, the yield was significantly lower than with cystine alone (Expt. 11).

*Lactose and ammonium sulphate.* When either lactose (0·004 M) or ammonium sulphate (0·002 M) was added separately to washed mycelium with phenylacetate, no significant effect could be detected. When both were added, the yield of penicillin was decreased (Expt. 12 in Table 1). The

solution remained constant at pH 7 in all flasks of this series, throughout the incubation period.

It was thought possible that the added nutrients might have influenced the apparent yields of penicillin indirectly, by affecting the antibiotic assays. A comparison of the potency of a standard solution of penicillin (8 units/ml.), with similar solutions containing cystine (0·002 M) or valine (0·004 M), failed to show any significant differences when subjected to an analysis of variance.

*Effect of nutrients on growth of mycelium*

Washed mycelium. incubated in the presence of the same nutrients as above, showed significant changes in dry weight after 2 hr. The results are shown in Table 2. Most of the added substances

Table 1. *Effect of various substances on yields of penicillin*

Each flask (100 ml. vol.) contained 1 g. of wet washed mycelium and 9 ml. of 0·01 M-phosphate buffer, pH 7, with potassium phenylacetate (0·1 g./l.). Other substances were 0·004 M in concentration, with the exception of cystine and ammonium sulphate, which were 0·002 M. Penicillin yields are corrected for differences in dry wt. of mycelium from the standard 12 mg./ml., and each yield reported is the mean from seven replicate flasks.

| Expt. no. | Substances added | Penicillin yield (units/ml./2 hr.) | Standard deviation (units/ml./2 hr.) | Result and significance |
|---|---|---|---|---|
| 1 | None | 25·2 | 4·3 | Increase; |
| | L-Cystine | 31·3 | 4·3 | $P<0·05$ |
| 2 | None | 32·4 | 4·4 | Increase; |
| | L-Cystine | 38·0 | 5·3 | $P\sim0·05$ |
| 3 | None | 62·1 | 5·6 | Increase; |
| | L-Cystine | 69·3 | 6·1 | $P<0·05$ |
| 4 | None | 37·0 | 4·2 | No effect |
| | DL-Serine | 35·3 | 3·0 | |
| 5 | None | 38·8 | 4·3 | Decrease; |
| | Glycine | 32·6 | 2·9 | $P<0·01$ |
| 6 | None | 35·3 | 3·7 | Decrease; |
| | DL-Valine | 23·1 | 2·5 | $P<0·001$ |
| 7 | None | 36·8 | 5·4 | Decrease; |
| | L-Leucine | 22·4 | 3·2 | $P<0·001$ |
| 8 | None | 24·9 | 2·9 | Decrease; |
| | L-Glutamic acid | 19·2 | 2·3 | $P<0·001$ |
| 9 | None | 38·6 | 3·6 | No effect |
| | Lactose | 40·9 | 5·6 | |
| 10 | None | 35·1 | 3·7 | No effect |
| | Ammonium sulphate | 36·0 | 3·9 | |
| 11 | L-Cystine | 37·6 | 4·1 | Decrease; |
| | L-Cystine + DL-valine | 22·5 | 2·7 | $P<0·001$ |
| 12 | None | 20·0 | 2·1 | Decrease; |
| | Lactose + ammonium sulphate | 15·3 | 1·5 | $P<0·001$ |

Table 2. *Effect of nutrients on growth of washed mycelium*

Wet mycelium (about 5 g.) was accurately weighed, suspended in 45 ml. of 0·01 M-phosphate buffer, pH 7, containing potassium phenylacetate (0·1 g./l.) and nutrients under investigation, and shaken for 2 hr. Final dry weights were determined after filtration and washing of the mycelium. Initial dry weights were determined on separate samples of mycelium dried without further treatment.

$$\frac{\text{Final dry wt. of mycelium}}{\text{Initial dry wt. of mycelium}} \times 100$$

| Added nutrient | Expt. *A* | Expt. *B* | Expt. *C* | Change in mean ratio (%) |
|---|---|---|---|---|
| None | 96·0, 95·9, 96·6 | 94·7, 95·3, 95·5 | 94·0, 93·3, 94·4 | — |
| L-Cystine (0·002 M) | 93·0, 94·0, 94·2 | — | — | −2·4 |
| DL-Serine (0·004 M) | — | 96·0, 95·8 | — | +0·8 |
| Glycine (0·004 M) | — | 96·4, 95·2 | — | +0·6 |
| DL-Valine (0·004 M) | 97·0, 98·9, 97·5 | — | — | +1·8 |
| L-Leucine (0·004 M) | — | — | 96·7, 97·1, 94·5 | +2·4 |
| L-Glutamic acid (0·004 M) | — | — | 97·2, 95·3, 95·3 | +2·1 |
| Lactose (0·014 M) | 100·4, 99·3 | — | — | +4·0 |
| Ammonium sulphate (0·007 M) | — | 92·5, 90·7 | — | −3·8 |
| Lactose + ammonium sulphate | 98·9, 99·3, 100·3 | 100·1 | — | +3·6; +5·2 |

supported growth, as shown by increases in dry weight above that of the control. Cystine and ammonium sulphate did not support growth, the mycelium decreasing in weight with these nutrients.

*Presence of cystine in washed mycelium*

Paper chromatograms, prepared from oxidized extracts of washed mycelium, showed five well-defined spots. The most pronounced of these had the same $R_F$ value as cysteic acid. A fraction containing cysteic acid was also detected by column chromatography on Dowex-2 resin. Thus washed mycelium of *P. chrysogenum* contains free cystine (or cysteine). The quantity of cystine in the mycelium was not determined.

## DISCUSSION

The changes occurring in growing cultures of *P. chrysogenum* have been studied intensively by several groups of workers (e.g., Gailey, Stefaniak, Olson & Johnson, 1946). The present determinations of dry weight of mycelium and penicillin concentrations, as summarized in Fig. 1, show only that the culture was behaving normally under our conditions. Estimations of the penicillin-producing ability of washed mycelium, harvested at various stages of growth, provide new information in that the optimum time for harvesting the mycelium was established. In addition, it was noted that the presence or absence in the culture of the side-chain precursor, phenylacetate, had very little effect on the subsequent ability of the mycelium to convert phenylacetate into penicillin. It appeared that the mechanism necessary for this reaction was always present at a certain stage of mycelial growth, this mechanism probably being the same as that concerned in the conversion of natural precursors (aliphatic acids, etc.) into natural penicillins. The nature and proportions of the different penicillins produced under different conditions were not determined, although mycelium without phenylacetate probably produced no benzylpenicillin, and washed mycelium incubated with phenylacetate probably produced largely benzylpenicillin, as may be inferred from studies of complete fermentations (Singh & Johnson, 1948). Since benzylpenicillin was used as the assay standard, the absolute yields of penicillin reported are reliable only for solutions in which benzylpenicillin predominated.

Attempts by several investigators to increase yields of penicillin from *P. chrysogenum* by the use of specific nutrients, especially amino acids, were initiated by the discovery that part of the stimulation of penicillin production by corn-steep liquor could be ascribed to its content of amino acids. Thus it was found that a mixture of histidine, arginine and glutamic acid (White, Krampitz & Werkman, 1945), or proline and glutamic acid (Halpern, Siminovitch & McFarlane, 1945), stimulated formation of penicillin in synthetic media. Stone & Farrell (1946) obtained increased yields, from a medium containing added leucine or cystine but the results were inconsistent. In all of these experiments the added nutrients were available to the organism over a period of several days, and were presumably utilized for both growth and penicillin synthesis. Wolf (1949) tested *P. chrysogenum* Q-176 for its ability to use single amino acids as the sole sources of nitrogen for growth and production of penicillin. He found that some amino acids, such as cystine, would not support growth and for this reason did not give rise to penicillin, although cystine is now known to be a direct precursor of the antibiotic (Arnstein & Grant, 1954*b*).

The use of mature mycelium, grown under standardized conditions, then washed and exposed briefly to specific nutrients, promised to give less ambiguous results. Cystine was the first nutrient examined, both because it is a precursor of penicillin and because exogenous cystine is incorporated into penicillin by washed mycelium with only slight dilution by endogenous cystine (Halliday & Arnstein, 1956). It was expected, on this basis, that the supply of cystine might become a rate-limiting factor in synthesis of penicillin if other known requirements were met. In the presence of adequate oxygen and phenylacetate, cystine was shown to stimulate the rate of production of penicillin (Table 1).

Washed mycelium has also been used by Demain (1956) to test the effect of amino acids on yields of penicillin. He found that L-cystine and L-valine stimulated synthesis of penicillin and reversed the inhibition caused by analogues or isomers of these amino acids. Long-term (44–48 hr.) experiments were conducted, in media containing both lactose and phenylacetate, and the results may therefore not be comparable with those reported here. Similarly, the yields of penicillin in complex nutrient media (Halliday & Arnstein, 1956) cannot be compared with the present findings.

The substances tested for their effects on yields of penicillin (with the exception of lactose and ammonium sulphate when used separately) are potential sources of carbon, nitrogen and energy for the growth of the mould. Cystine increased the yield of antibiotic, presumably by acting as a direct precursor of penicillin. It was thought that the inhibitory effects of glycine, valine, leucine, glutamic acid and lactose plus ammonium sulphate may have been caused by their ability to support growth, and thus to upset the balance between mycelial development and degeneration which

leads to synthesis of penicillin (Davey & Johnson, 1953). More specifically, the fresh nutrient may have allowed synthesis of protein to proceed, and to compete for the available cystine whose supply was already limiting the rate of synthesis of penicillin.

Determinations of changes in dry weight of mycelium during incubation (Table 2) supported this hypothesis. The addition of cystine did not permit growth, the dry weight of the mycelium actually decreasing during the incubation period, as it did also with ammonium sulphate. Serine and glycine, amino acids related metabolically to cystine, supported growth, although serine had no effect on yields of penicillin. Possibly the conversion of serine into cystine, and thence into penicillin, balanced the tendency of the serine to deplete the cystine supply by promoting growth. Glycine, being less readily converted into cystine, inhibited synthesis of penicillin. Although valine is utilized for biosynthesis of penicillin (Arnstein & Grant, 1954*a*; Stevens, Vohra & DeLong, 1954), and thus might have been expected to increase the rate of production of penicillin under certain conditions, it showed a marked inhibitory effect, even when cystine was also supplied. This may be connected with the ready utilization of valine for growth. Neither lactose nor ammonium sulphate alone would be expected to promote synthesis of protein, and the increase in dry weight caused by lactose was probably an increase in stored carbohydrate or fat. Lactose and ammonium sulphate together constituted an excellent substrate for growth, and at the same time inhibited synthesis of penicillin markedly.

Leucine and glutamic acid, substances previously claimed to increase yields of penicillin in complete fermentations (see above), decreased yields significantly under the present conditions and at the same time permitted mycelial growth.

Free cystine is known to occur in cultures of *P. chrysogenum* grown in simple media (Rao & Venkataraman, 1952), and cystine was detected in washed mycelium during the work reported here. Apparently the intracellular concentration of cystine is suboptimum for synthesis of penicillin, additional amounts being able to stimulate the rate of production.

## SUMMARY

1. Of several substances tested for their effect on the rate of synthesis of penicillin by washed mycelium of *Penicillium chrysogenum* Wis. 51–20F3, only L-cystine was stimulatory. This amino acid did not support further growth of the mycelium.

2. Glycine, DL-valine, L-leucine, L-glutamic acid and lactose plus ammonium sulphate inhibited synthesis of penicillin. These substances supported further growth of the mycelium.

3. It is suggested that substances which promote synthesis of protein in the mycelium do so at the expense of synthesis of penicillin, the two processes competing for the available cystine.

4. Free cystine was detected in ethanolic extracts of washed mycelium.

5. The ability of washed mycelium to produce penicillin from phenylacetate was maximal 60–62 hr. after inoculation, and did not depend on previous exposure to phenylacetate.

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## REFERENCES

Arnstein, H. R. V. & Grant, P. T. (1954*a*). *Biochem. J.* **57**, 353.
Arnstein, H. R. V. & Grant, P. T. (1954*b*). *Biochem. J.* **57**, 360.
Davey, V. F. & Johnson, M. J. (1953). *Appl. Microbiol.* **1**, 208.
Demain, A. L. (1956). *Arch. Biochem. Biophys.* **64**, 74.
Gailey, F. B., Stefaniak, J. J., Olson, B. H. & Johnson, M. J. (1946). *J. Bact.* **52**, 129.
Halliday, W. J. & Arnstein, H. R. V. (1956). *Biochem. J.* **64**, 380.
Halpern, P. E., Siminovitch, D. & McFarlane, W. D. (1945). *Science*, **102**, 230.
Humphrey, J. H. & Lightbown, J. W. (1952). *J. gen. Microbiol.* **7**, 129.
Jarvis, F. G. & Johnson, M. J. (1950). *J. Bact.* **59**, 51.
Perret, C. J. (1953). *J. gen. Microbiol.* **8**, 195.
Rao, P. L. N. & Venkataraman, R. (1952). *Experientia*, **8**, 350.
Schram, E., Moore, S. & Bigwood, E. J. (1954). *Biochem. J.* **57**, 33.
Singh, K. & Johnson, M. J. (1948). *J. Bact.* **56**, 339.
Stevens, C. M., Vohra, P. & DeLong, C. W. (1954). *J. biol. Chem.* **211**, 297.
Stone, R. W. & Farrell, M. A. (1946). *Science*, **104**, 445.
White, A. G. C., Krampitz, L. O. & Werkman, C. H. (1945). *Arch. Biochem.* **8**, 303.
Wolf, F. T. (1949). *Mycologia*, **41**, 403.