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

1. A PRELIMINARY STUDY OF THE METABOLISM OF CARBON, NITROGEN, SULPHUR AND PHOSPHORUS

By D. J. D. HOCKENHULL, *Imperial Chemical Industries Ltd., Research Laboratories, Blackley, Manchester*

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The following studies on *Penicillium notatum* were intended to prepare the way for a more detailed investigation of the method by which the mould produces penicillin, and furthermore to ascertain to what extent the production of penicillin could be increased by controlling the cultural environment.

It is important to note that the production of penicillin only represents a minute fraction of the total metabolic activity of the mould, in contrast to the production of citric acid by *Aspergillus niger*. Moreover, none of the common intermediate products of metabolism such as oxalic, fumaric and gluconic acids or glycerol, etc., could be detected in other than negligible quantities during the growth of *Penicillium notatum* under conditions favouring penicillin production. It thus appeared that an essential preliminary to investigations of the mechanism of penicillin production was to ascertain the fate of individual constituents of the culture medium. In the present investigation an attempt has been made to follow the changes undergone by some of the principal nutrient substances during the growth of the mould.

Existing methods of analysis have been modified to deal with the substances investigated.

EXPERIMENTAL

Cultural methods and materials

Composition of medium. The earlier part of the work was carried out using a modified Czapek-

Turley medium subsequently referred to as *casein medium* of which the composition was:

	%
Glucose (first grade commercial quality)	3
NaNO ₃ (analytical reagent quality)	0.3
KH ₂ PO ₄ (analytical reagent quality)	0.1
KCl (analytical reagent quality)	0.05
MgSO ₄ .7H ₂ O (analytical reagent quality)	0.025
FeSO ₄ .7H ₂ O (analytical reagent quality)	0.001
Casein, as 20% (v/v) HCl digest	0.5

This medium was made up in tap water and adjusted to pH 4.5 with 40% NaOH before sterilization.

It was communicated to us by Dr Coghill of Peoria, U.S.A., that penicillin production was greatly increased by the use of corn-steep liquor (30% total solids) in the medium. Accordingly a medium, known as *CSL medium* was adopted for commercial production of penicillin. This medium was consequently used in the investigation. Its composition was:

	%
Lactose B.P.	3
Glucose, commercial (as above)	1
Filtered corn-steep liquor	8
NaNO ₃ (analytical reagent quality)	0.3
KH ₂ PO ₄ (analytical reagent quality)	0.1
MgSO ₄ .7H ₂ O (analytical reagent quality)	0.025
ZnSO ₄ .7H ₂ O (analytical reagent quality)	0.001
MnSO ₄ .4H ₂ O (analytical reagent quality)	0.001
CuSO ₄ .5H ₂ O (analytical reagent quality)	0.001
FeSO ₄ .7H ₂ O (analytical reagent quality)	0.001

This medium was made up in tap water and adjusted to pH 4.5 with 40% (w/v) NaOH before sterilization.

Strain of organism. At the same time as the change to *CSL* medium, the original strain used on the casein medium and obtained from Sir Howard Florey and described here as 'strain F4', was supplanted by the more efficient penicillin-producing strain obtained from the Northern Regional Research Laboratories, Peoria, Illinois, and known as strain 1249 B21.

Growth conditions. Exp. 1 was carried out in earthenware culture vessels as described by Chain, Florey, Gardner, Heatley, Jennings, Orr-Ewing & Sanders (1941). One litre of medium giving a depth of 1 in. was used in each vessel.

were incubated at $23.5 \pm 0.5^\circ$ throughout. Unless otherwise stated all experimental bottles were set up in duplicate.

Methods of analysis

Total carbon by wet combustion. The sample, containing 5–8 mg. carbon, was dried down at 110° and oxidized by a chromic-sulphuric acid mixture and the CO_2 evolved collected in 0.05M-baryta in a special spiral bubbler after Pearce & Robertson (1936). The method is a modification of that of McCreedy & Hassid (1942).

Reducing sugar as glucose. It was not found necessary to clear the filtered medium before analysis. Medium was hydrolyzed for 1 hr. with an equal volume of 2N-HCl,

Fig. 1. Aerated-culture apparatus with gas-sampling device. A, soda-lime tube; B, air humidifier; C, pressure regulator; D, release valve; E, air sterilizer (cotton-wool); F, culture flask; G, manometric flowmeter; H, gas-sampling vessel; T₁, air inlet tube; T₂, air exit tube; T₃, sampling tube; K, 25 ml. flask, hermetic seal for T₃; P, cotton-wool plug in T₃.

All subsequent experiments were carried out in glass culture vessels as described by Clayton, Hanes, Robinson, Andrews & Hunwicke (1944); 500 ml. of medium/vessel gave c. $\frac{3}{4}$ in. layer.

Steam sterilization was carried on for 20 min. at 15 lb./sq.in. in all experiments.

The *inoculum* was prepared as follows: A culture of the mould on minced potato (twice sterilized at 120 lb./sq.in. for 20 min.) grown for 5 days and then shaken with a 0.1% solution of a wetting agent 'Calsolene oil HS' (I.C.I., Ltd.). The resultant spore suspension was sprayed into the culture vessels by means of a sterile 'Aerograph' spray gun. This procedure gave rapid and even coverage. All cultures

neutralized and diluted so as to contain the equivalent of 1–1.5 mg. glucose in a sample of 5 ml. The glucose equivalent of the solution was determined by a modification of the method of Somogyi (1937) due to Hanes (Hockenhull & Herbert, 1945).

Carbon dioxide. Samples of gas from continuously aerated bottles fitted with flowmeter (Fig. 1) were analyzed by absorbing the CO_2 in 0.05N-baryta. A spiral bubbler similar to that used in the method for total carbon was used.

Total nitrogen. Samples of medium or mycelium containing about 2 mg. N, were dried at 110° and analyzed by the modified Kjeldahl procedure of Pepkowitz & Shive (1942).

Nitrate. A filtered solution of the medium containing 3–5 mg. of nitrogen as nitrate was diluted to 20 ml., acidified

Fig. 2.

Fig. 3.

Fig. 2. Carbon metabolism of *P. notatum*; earthenware culture vessels. Exp. 1, strain F4, *casein medium*, 1 l./vessel. ○ = total carbon in medium; ● = carbon as sugar (glucose equivalents); □ = carbon in mycelium; ■ = penicillin titre.

Fig. 3. Carbon metabolism of *P. notatum*; in glass culture-flasks. Exp. 2, strain F4, *casein medium*, 500 ml./vessel. ○ = total carbon in medium; ● = carbon as sugar (glucose equivalents); □ = carbon lost as CO₂; ■ = penicillin titre.

Fig. 4.

Fig. 4. Changes in pH values by two strains of *P. notatum*. Exp. 1, strain F2, *casein medium*; Exp. 3, strain 1249, *CSL medium*. ○ = pH values, strain F4; ● = penicillin titres, strain F4; □ = pH values, strain 1249; ■ = penicillin titres, strain 1249.

Fig. 5.

Fig. 5. Carbon metabolism of *P. notatum*. Exp. 3, strain 1249, *CSL medium*. ○ = total carbon in medium; ● = carbon as sugar glucose equivalents; ■ = penicillin titre.

with 0.5 ml. conc. H_2SO_4 and the nitrate precipitated with excess of a 10% solution of 'nitron reagent', and determined gravimetrically after washing with ice-cold water and drying at 110° .

Amino-nitrogen. The medium was analyzed for amino-nitrogen by the method of Pope & Stevens (1939). Neither of the media used contained interfering substances.

Volatile-base nitrogen. A sample of medium containing 0.4 mg. of nitrogen as volatile base was steam distilled from 10 ml. sat. solution of Na_2CO_3 and the distillate collected in 0.1N-HCl, which was back-titrated as in the method above for Kjeldahl nitrogen.

Phosphate. Free orthophosphate was estimated by the method of Youngberg & Youngberg (1930). Colour comparison was made in a Hilger 'Spekker' absorptiometer using an orange filter.

Total sulphur. The sample, containing c. 0.5 mg. of sulphur, was dried down at 110° and oxidized with a nitric acid-bromine mixture in which had been dissolved zinc oxide. The completely oxidized sulphur, as zinc sulphate, was reduced with a hydriodic-hypophosphorous acid mixture to H_2S , which was distilled off and collected in NaOH. Standard iodine was added and the solution acidified, the unused iodine being titrated with thiosulphate. The method and apparatus are modified from those used by Luke (1943).

Penicillin. The penicillin titres of the media were determined by my colleague, Mrs I. F. Hoggarth, by a method similar to that of Foster & Woodruff (1943). Figures quoted are in Oxford units (o.u.; Abraham, Chain, Fletcher, Florey, Gardner, Heatley & Jennings (1941)). Aberrant points occur on certain of the figures. These are due to a breakdown of the assay system on the days concerned.

Carbon metabolism

Using strain F4. In Exp. 1, sixty replicate earthenware bottles were used as described above. Bottles were removed at 24 hr. intervals for determination of the reducing sugar, total carbon and penicillin concentrations in the medium and the total carbon in the mycelium. After an initial lag phase both the carbon and the sugar fell steadily as shown in Fig. 2. The mycelium carbon after an initial lag phase rose to a maximum on the 10th day and then fell gradually (Fig. 2). There was a steady rise in pH value throughout (Fig. 4).

Exp. 2 was carried out in glass culture vessels as described above. The vessels were fitted up so that the rate of aeration and of CO_2 production could be measured (Fig. 1). Determination of reducing sugar, total carbon and penicillin concentrations in the medium, and the rate of evolution of CO_2 were made daily (Fig. 3). The mycelial carbon was determined at the end of the fermentation; 98% of the carbon was accounted for.

Using strain 1249. As mentioned above, CSL medium was used throughout with strain 1249. On this medium, which contained more carbon and nitrogen than did the casein medium, penicillin yields were much greater than those obtained with strain F4 (80–120 o.u./ml. as compared with 20–25 o.u./ml. with the latter).

In Exp. 3, total carbon and reducing sugar were determined (Fig. 5) and showed the same gross effects as with strain F4; the pH changes were also very similar (Fig. 4).

In Exp. 3, the CO_2 output was recorded as in Exp. 2. It was shown that the peak of respiratory activity preceded the peak of penicillin concentration by about 2 days (Fig. 6).

Fig. 6. Respiration of *P. notatum*. Exp. 4, strain 1249, CSL medium. o = rate of CO_2 evolution; ● = penicillin titre.

Nitrogen metabolism

Using strain F4. No experiments on amino-nitrogen utilization or volatile base production were carried out with this strain. In Exp. 1, total nitrogen and nitrate nitrogen in the medium, total mycelial nitrogen and penicillin were determined, at 24 hr. intervals (Fig. 7). After a very short lag phase, both total nitrogen and nitrate nitrogen fell rapidly. The total nitrogen fell, by the 11th day, to a minimum value of about 30% of its initial level; the nitrate nitrogen fell, asymptotically, to zero on the 14th day. Penicillin production lagged behind these curves by about 3 days. After the 11th day, total nitrogen in the medium increased by the 14th day to about 55% of its initial value. The uptake of nitrogen from the medium was reflected by the increase in mycelial nitrogen which reached its maximum at about the same time and then decreased, reflecting the reappearance of nitrogen in the medium. The discrepancy between the two series was due to the method of replicate sampling.

Using strain 1249. In Exp. 15, six replicate bottles were examined. CSL medium was employed. Total nitrogen and amino-nitrogen in the medium and penicillin were determined at 24 hr. intervals

(Fig. 8). After initial lags the amino-nitrogen concentration fell steadily throughout the metabolism period. The total nitrogen curve tended to flatten out by the 10th day; it did not, however, show a marked minimum like that observed in Exp. 1 (Fig. 7), although nitrogen was utilized to about the same extent.

Fig. 7. Nitrogen metabolism of *P. notatum*. Exp. 1, strain F4, casein medium, 1 l./vessel. ○ = total nitrogen in medium; ● = nitrate nitrogen in medium; □ = nitrogen in mycelium; ■ = penicillin titre.

In Exp. 6, two sets, A and B, ten replicate bottles were set up, sown and grown in the normal way until the 8th day of growth. At this point, 4.7 g. $(\text{NH}_4)_2\text{SO}_4$, A.R. dissolved in 20 ml. of water and sterilized were carefully added to the medium in each bottle in set B; this addition corresponded to 2 mg. N/ml. as ammonium salt. 20 ml. of sterile water was added to each bottle of the control set A.

Fig. 8. Nitrogen metabolism of *P. notatum*. Exp. 5, strain 1249, CSL medium. ○ = total nitrogen in medium; ● = amino nitrogen in medium; ■ = penicillin titre.

Fig. 9. Volatile-base production and penicillin yield of *P. notatum*. Exp. 6, strain 1249, CSL medium. ○ = volatile-base nitrogen in medium; ■ = penicillin titre.

Table 1. The effect of the addition of ammonium ion to cultures of *Penicillium notatum*

Day	Penicillin titre (mean of 10) o.u./ml.		Standard deviations from means		Difference A-B	pH	
	A	2 mg. N/ml.	A	B		A	B
	No added [NH ₄] ⁺	added as [NH ₄] ⁺					
9	83.9	78.3	5.5	5.1	5.6	7.26	7.29
10	75.2	79.2	4.4	3.7	4.0	7.31	7.41
11	92.8	68.8	4.4	3.6	24.0	7.76	7.78
12	90.0	56.5	6.3	3.2	33.5	7.99	8.05
13	76.1	57.5	4.5	4.8	18.6	8.03	7.98
16	36.3	11.6	2.6	0.9	14.7	—	—

In the control set A, the volatile base nitrogen remained low and steady around 0.25 mg. N/ml. until the 8th day when it rose steadily to the level of 1.05 mg. N/ml. on the 16th day. This was accompanied by a decline in penicillin titre (Fig. 9). The decline in penicillin titre was, however, far greater in series B which contained added ammonium ion (Table 1). Since the pH values recorded for each series were the same, it seems likely that the $[\text{NH}_4]^+$ ion causes this acceleration in the destruction of penicillin at pH values above 7.5.

Phosphorus metabolism

Strain 1249 and CSL medium were used for this series of experiments.

In Exp. 7, which was done in six replicate bottles, the phosphate and penicillin in the medium were determined at 24 hr. intervals. The phosphate level

Fig. 10. Phosphate metabolism of *P. notatum*. Exp. 5, strain 1249, CSL medium. ○ = phosphate phosphorus in medium; ■ = penicillin titre.

showed considerable changes. It fell to a minimum on the 4th day, by which time a thick mycelial mat had formed. After this it rose almost to its initial value by the 8th day, when it again fell to a minimum (Fig. 10). At this second minimum, penicillin concentration reached its peak.

In Exp. 8 a series of modified media were made up containing from 0.04 to 1.00 mg. P/ml. These media differed from the usual CSL medium in that they were made up with specially treated corn-steep liquor from which most of the phosphate had been removed by precipitating with precipitated chalk. Graded amounts of phosphate as KH_2PO_4 were added to give values between the above extremes of phosphate content.

Between these limits there was no connexion between phosphate content of the medium and penicillin yield. All yields of penicillin were normal

(90–100 o.u./ml.). The phosphate metabolism followed a course similar to that of Exp. 7.

In Exp. 9 a medium containing only 0.04 mg. P/ml. was used. This was prepared by the method used in Exp. 8. When the mycelial felt had been fully established (5 days), sterile additions of sodium iodoacetate, sodium fluoride or sodium arsenate at 0.1 mg./ml. of medium or sodium selenite at 0.1 and 1.0 mg./ml. of medium were made. None of these inhibitors at 0.1 mg./ml. had any effect on penicillin yield. However, 1.0 mg./ml. of sodium selenite cut down penicillin yield to 50% of the normal.

Sulphur metabolism

Strain 1249 was used in Exp. 10. The medium was a normal CSL medium except that the corn-steep liquor had been pretreated by extraction with hot butanol continuously for 12 hr. at pH 3.7–3.9;

Fig. 11. Sulphur metabolism of *P. notatum*. Exp. 10, strain 1249, modified CSL medium. ○ = total sulphur in medium; ■ = penicillin titre.

the butanol soluble fraction being rejected. The aqueous residue contained all the inorganic constituents of normal CSL, so there was no necessity to supplement the medium with metallic salts.

The total sulphur and penicillin contents of the medium were determined at 24 hr. intervals (Fig. 11). Sulphur disappeared from the medium rapidly until the 8th day, when it began to reappear and almost reached its initial concentration by the 14th day.

DISCUSSION

No close connexion could be established between the general metabolism of the metabolites investigated

and penicillin production, except insofar as the disappearance of these fractions gave an indication of the general state of mould development. The output of penicillin by the mould followed the uptake of carbon, nitrogen and sulphur by the mycelium with a lag of 2-3 days. The reappearance of sulphur and nitrogen in the medium and the decline in mycelial carbon is probably related to senescence in the older parts of the mycelium, but further investigation is necessary. The peculiar fluctuations in phosphate content of the medium are as yet inexplicable. This phenomenon may be connected with the observation made by the author that the addition of phosphate buffer to growing cultures of *Penicillium notatum* produces only a transient change in the pH value which rapidly reverts to that of the parallel control culture. The inhibition experiment may be contrasted with results obtained by Mann (1943). This worker found that sodium iodoacetate ($M \times 10^{-3}$), sodium azide ($M \times 10^{-3}$) or sodium fluoride ($5M \times 10^{-3}$), when added to cultures of *Aspergillus niger* on medium containing 10% glucose and inorganic salts, caused a complete inhibition of the phosphorus metabolism and brought respiration to a standstill.

SUMMARY

1. The following metabolic fractions have been determined throughout the growth of *Penicillium notatum*: total carbon, reducing sugar, total nitrogen, amino-nitrogen, nitrate nitrogen, volatile base, phosphate phosphorus and total sulphur of the medium, total carbon and nitrogen of the mycelium, and the rate and quantity of CO_2 evolved by the growing culture.

2. Penicillin production by *P. notatum* followed the general absorption of carbon, nitrogen and sulphur into the mould mycelium.

3. No sharp discontinuities in the metabolism of *P. notatum* have been observed.

4. The exchange of phosphorus between the mould mycelium and the medium followed a fluctuating course which is as yet not understood.

5. The decline in the penicillin activity of the cultures was accelerated by the addition of ammonium ion although no pH change was produced by this treatment.

My thanks are due to my assistants, Mr M. Fisher and Miss F. Gilder, for carrying out much of the chemical analysis.

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Studies on the Specificity of Choline Esterase in *Helix pomatia*

By K.-B. AUGUSTINSSON, *Department of Chemistry, Royal Veterinary Institute, Stockholm*

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Since 1940 the problem of the specificity of cholinesterase has been placed in a new light, when it was shown by Alles & Hawes (1940) and Hawes & Alles (1941) that the enzymes in human blood serum and red blood cells had different properties. Among other things, these authors found that excess acetylcholine depressed the activity of the erythrocyte enzyme but not that of the serum enzyme. These observations were subsequently confirmed in

different quarters. According to Richter & Croft (1942) cholinesterase in the erythrocytes is different from that in the blood serum, being exclusively specific toward choline esters and being affected differently by certain substances. Similar results were also obtained by Mendel & Rudney (1943*a*), according to whom it is 'clear beyond doubt that two distinct cholinesterases exist in the animal body' (1944). Zeller & Bissegger (1943) found their