between the arms of the frame is cut away. The lower inner edge of the frame is rebated to enable a 2 × $^{15}/_{16}$ in. coverslip (*C*) to be inserted and act as the floor of the chamber. The roof consists of two $^{7}/_{8}$ in. coverslips or a single 2 × $^{15}/_{16}$ in. coverslip these being lightly cemented in position with small spots of a lanolin-resin cement. Inoculation, final isolation, and other macromanipulation is carried out by sliding out the basal coverslip to the required distance.

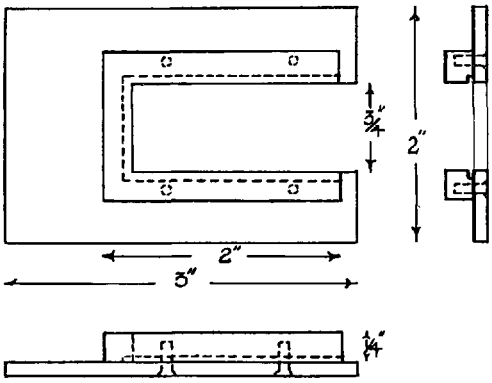

Fig. 2.

P. A. DIXON

Birkbeck College, University of London, March 19, 1957.

Résumé

Une chambre humide pour la micromanipulation a été construite en Perspex. Elle est munie d'un plancher inférieur mobile en verre, qui facilite l'inoculation initiale, la macromanipulation intermédiaire et l'isolement final.

Informations - Informationen - Informazioni - Notes

STUDIORUM PROGRESSUS

The Metabolic Products of *Penicillium patulum* and their Probable Interrelationship[1]

By E. W. BASSETT and S. W. TANENBAUM[2]

During the course of a study of the biosynthesis of patulin in *Penicillium urticae*, Bainier (syn. *P. patulum*) strain 2159A[3], several closely related compounds were found to accumulate in the growth filtrate. Many of the metabolic products had previously been identified, including gentisic acid, gentisaldehyde, and gentisyl alcohol by BIRKENSHAW *et al.*[4], and 6-methylsalicylic acid by EHRENSVÄRD[5]. In our experiments using this nonpigmented mutant strain, the following additional compounds have been identified: 6-formylsalicylic acid, 3-hydroxyphthalic acid, pyrogallol, *p*-hydroxybenzoic acid, anthranilic acid, and an aliphatic substance which we have tentatively named 'pre-patulin'.

Pathways for the biosynthesis of patulin have been proposed first by BIRKENSHAW[6] and later by EHRENSVÄRD[7]. Their salient features involve the oxidative rupture of gentisaldehyde followed by rearrangement and closure to patulin. EHRENSVÄRD's scheme places 6-methylsalicylic acid as the productof a side reaction and does not recognize a direct relationship between this C_8 metabolite and the C_7 aromatics postulated as patulin precursors. With the finding of 6-formylsalicylic acid and 3-hydroxyphthalic acid, heretofore unrecorded in biological systems, we are able to fit all of the known metabolic products of this mold into a logical sequence.

Results.—The identification of 6-formylsalicylic acid (6-FSA) was made by comparison with authentic[8] material synthesized by the method of ELIEL, RIVARD, and BURGSTAHLER[9]. A list of the physical and chemical characteristics of the isolated and authentic materials is given in the Table (p. 40). The ultraviolet absorption spectra are presented in the Figure. By comparison with synthetic material prepared from 3-aminophthalic acid, 3-hydroxyphthalic acid was identified. Both the synthesized and isolated compounds had the same properties, i.e., m.p. 154°, λ_{max} = 323 mμ, as well as identical paper chromatographic and color reactions. Further fractionation of the culture filtrates afforded pyrogallol in good yield (m.p. 134°, m.p. of triacetate 162°); while *p*-hydroxybenzoic acid and anthranilic acid were detected by paper chromatographic and spectrophotometric analysis. These last two acids have long been known to be intimately related to aromatic biosynthesis from shikimate both in *Aerobacter*[10] and in *Neurospora*[11]. To the authors' knowledge, pyrogallol, the classic example of the higher plant aglycones, has not previously been found among the fungi.

From cultures grown on glucose in the presence of $CaCO_3$ and which failed to show evidence of patulin formation by the usual criteria, a waxy material (λ_{max}

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[2] Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York, N.Y.

[3] We should like to thank Dr. C. W. HESSELTINE of the NRRL, Peoria, Ill., for sending us this culture.

[4] J. H. BIRKENSHAW, A. BRACKEN, S. A. MICHAEL, and H. RAISTRICK, Lancet *245*, 625 (1943). – J. H. BIRKENSHAW, A. BRACKEN, and H. RAISTRICK, Biochem. J. *37*, 726 (1943).

[5] G. EHRENSVÄRD, Exp. Cell Res., Suppl. *3*, 102 (1955).

[6] H. J. BIRKENSHAW, Ann. Rev. Biochem. *22*, 371 (1953).

[7] J. H. BIRKENSHAW, A. BRACKEN, and H. RAISTRICK, Biochem. J. *37*, 726 (1943).

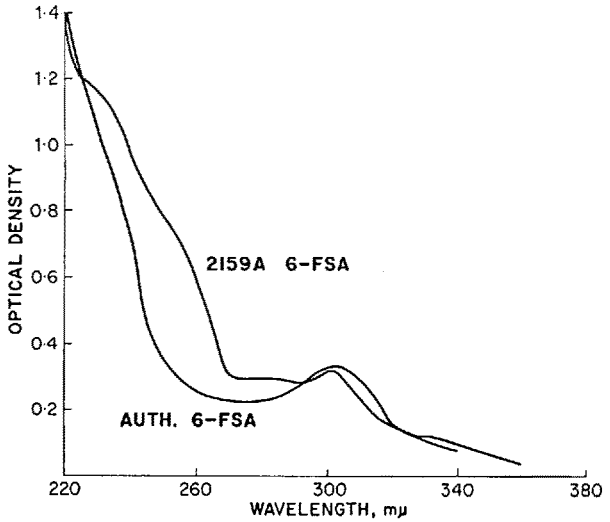
[8] We are indebted to Prof. E. L. ELIEL for a generous supply of this compound.

[9] E. L. ELIEL, D. E. RIVARD, and A. W. BURGSTAHLER, J. org. Chem. *18*, 1679 (1953).

[10] B. D. DAVIS, J. Bact. *64*, 729 (1952).

[11] E. L. TATUM, S. R. GROSS, G. EHRENSVÄRD, and L. GARNJOBST, Proc. nat. Acad. Sci., Wash. *40*, 271 (1954).

= 280 mμ) was obtained. This substance was free of aromatic phenolic acids and reacted readily with 2,4-dinitrophenylhydrazine to give a derivative with an R_f and melting point which differed from those for patulin-2,4-dinitrophenylhydrazone. Yet, this waxy substance could be converted to patulin by recrystallization in anhydrous solvents with a trace of acid. Conversely, patulin, by manipulation with mild base was transformed into the uncharacterized material. We have reason to believe therefore that this product of blocked metabolism is the open chain precursor of the antibiotic.

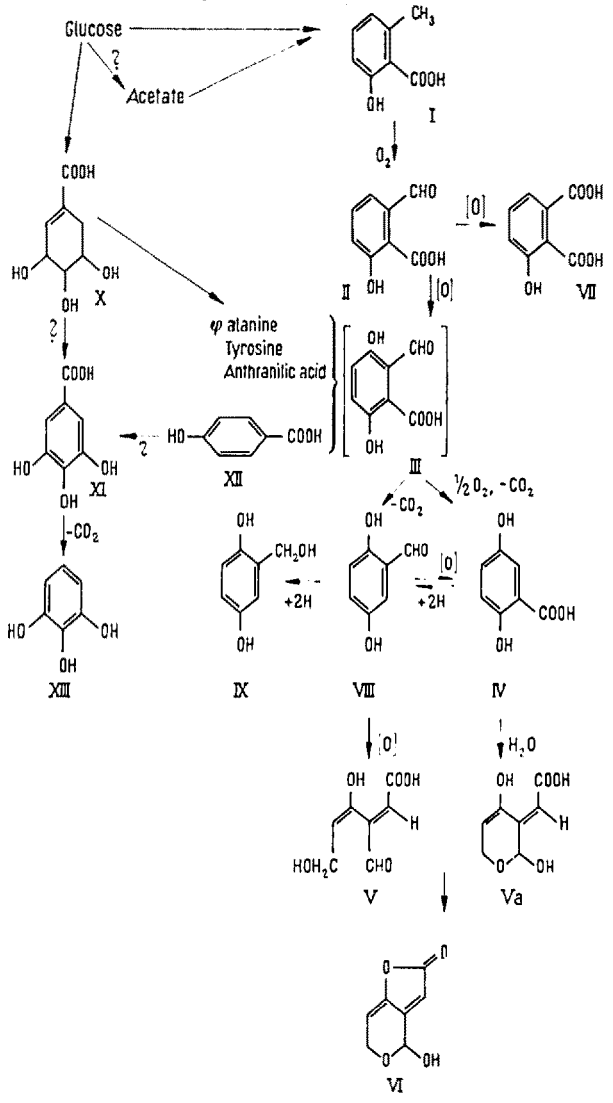


Finally, we wish to record at this time an interesting neutral substance (m.p. 218–219°, λ_{max} = 292 mμ) which has been isolated from *P. patulum* 2159A broth. This substance migrates as a single spot in several chromatographic systems, and upon acid hydrolysis gives rise to 6-methylsalicylic and 6-formylsalicylic acids. The molecular weight by the Rast method gives a value of 326, which is not inconsistent with values calculated for various combinations of these two moieties in depsidic, depsidone, or diester linkages. The exact chemical nature of this material is under investigation.

Following growth of strain 2159A on Czapek-Dox medium with 4% glucose at room temperature, maximum patulin accumulation occurred on the seventh to ninth day. The following acidic carbonyl compounds could be detected in the filtrate and were separated and identified as their 2,4-dinitrophenylhydrazones: pyruvic, glyoxylic, α-ketoglutaric, and oxaloacetic acids, and 'pre-patulin'. The first component to appear on the second day of growth in cultures with $CaCO_3$ and glucose was 6-methylsalicylic acid; this substance increased steadily in concentration until the sixth day, at which time gentisic acid began to accumulate and 6-methylsalicylate diminished progressively. At this point gentisaldehyde and 'pre-patulin' were also present in high concentration, probably owing to the trapping of these C_7 acids as their calcium salts.

In replacement experiments with eight-day-old exhaustively washed mycelial pads, glucose readily gave rise to patulin within 48 h. Glucose-grown pads converted acetate exclusively to 6-methylsalicylic acid in this interval. In turn, replacements with 6-methyl-salicylate as substrate formed gentisic acid, gentisaldehyde, 3-hydroxyphthalic acid, and patulin. The replacement experiments with gentisaldehyde produced patulin and gentisic acid in small yield, with most of the substrate decomposing to dark colored oxidation products. However, gentisic acid was converted promptly to patulin by this technique. With shikimic acid as supplement, *p*-hydroxybenzoic acid was the sole detectable aromatic substance.

The first indication of the effect of trace metal concentration on the proportion of metabolic products from *P. patulum* came from the experiments of BRACK[12]. Similar experiments in this laboratory with strain 2159A have shown that cobalt and manganese at 10^{-6} *M* favor patulin formation, whereas zinc in this concentration causes the equilibrium to shift towards 6-methylsalicylic acid accumulation. Again, when the iron concentration in Czapek-Dox medium is reduced to 1 mg $FeSO_4 \cdot 7\,H_2O$/l, reduction in yield of patulin occurs with concurrent increase of the C_7 and C_8 aromatic acid concentration. Contrary to the results of BRACK[12] and of EHRENSVÄRD[5], neither gentisyl alcohol nor toluquinol was detected in any of the above experiments.



Discussion. – By consideration of the chemical nature of the substances found in the growth filtrate and from the sequence in which they have appeared in growing and in replacement cultures, the following scheme is proposed for their biogenetic interrelationship (Formula).

[12] A. BRACK, Helv. chim. Acta *30*, 1 (1947).

Comparison of 6-FSA Properties

	Isolated	Authentic
m.p.	134°	134–137° (134° mixed)
$FeCl_3$ Test	red	red
*R_f (Uvl. Color)	(*a*) 0·60 (yel)	(*a*) 0·60 (yel)
	(*b*) 0·82 (bl)	(*b*) 0·82 (bl)
Diazo Spray	Light brown	Light brown
2,4-dnp m.p.	265°	272° (265° mixed)
*R_f 2,4-dnp	0·75	0·75

* BuOH–0·5 *N* NH_4OH–EtOH 70/20/10

Thus, we envision that aromatization takes place first either from glucose or from activated acetate to give 6-methylsalicylic acid (I), which in turn by a two step oxidation at the methyl group becomes 6-formylsalicylic acid (II). The finding of 3-hydroxyphthalic acid (VII) in filtrates and in replacement media is taken simply as a result of further oxidation of (II) at the formyl group, which yields an additional carboxyl. The hypothetical 5-hydroxy-6-formylsalicylic acid (III) is postulated to arise by nuclear hydroxylation[13] of (II). Although this intermediate has yet to be detected, its structure is not unlike the 'orsellinic units' found in the lichen depsides[14]. Decarboxylation of (III) affords gentisaldehyde (VIII) directly, whereas oxidative decarboxylation would give rise to gentisic acid (IV). The mechanism of the conversion of (II) to the gentisic series by way of (III) is subject to experimental examination with isotopic carbon, since in each of the foregoing pathways a different carboxyl can be lost.

It is not yet clear whether gentisaldehyde or gentisic acid is closer to the open chain 'pre-patulin' (V or Va). From the replacement experiments it would appear that gentisic acid is more directly related to patulin (VI) than is the aldehyde, as might well be expected from the fact that the acid and patulin have the same empirical formula. Certainly, however, BIRKENSHAW's attractive hypothesis[6] or some variation thereof seems at this point to have promise of explaining the actual mechanism of this interconversion.

The effects of trace metals in altering the ratios of metabolic products are probably exerted at the following points: either the two step oxidation of (I) to (II), blocking of which can result in the accumulation of 6-methylsalicylate in the medium, or at the oxidative *vs* straight decarboxylation of (III), which results in the primary accumulation of either gentisaldehyde or of gentisic acid. The accumulation of the last mentioned metabolites under low iron concentration may be related to the finding that the analogous homogentisic acid oxidase of animal tissues[15] is an iron requiring enzyme. Reduction of gentisaldehyde by glycolytic fragments or enzymatic dismutation of this compound will produce gentisyl alcohol (IX), which was originally found as a metabolite in *P. patulum*[16].

The second group of compounds, pyrogallol (XIII), *p*-hydroxybenzoic acid (XII), and anthranilic acid are more closely allied to the well characterized components of the aromatic amino acid pathway from shikimic acid[17]. Accordingly, we hypothesize that pyrogallol may arise from shikimate via gallic acid (XI). The latter can be conceived of coming either by some direct aromatization of shikimic acid, or more likely from oxidation of *p*-hydroxybenzoate. Indeed, preliminary evidence by paper chromatographic and color tests indicated that gallate and shikimate can be found in the broth of strain 2159A, and the conversion of shikimic acid to *p*-hydroxybenzoate in replacements has already been mentioned.

In this connection, it must be pointed out that BIRCH *et al.*[18], working with the related *P. griseofulvum*, conclude that 6-methylsalicylic acid arises from the direct 'head to tail' condensation of acetate. This is to a measure borne out by our experiments with acetate in replacement media. However the acetate hypothesis cannot be directly invoked to explain pyrogallol formation since condensation followed by cyclization of C_2 units results in meta orientated polyphenolic compounds. Nor does the conversion to pyrogallol from any of the products related to 6-methylsalicylic acid appear any the more likely. An outside alternative is that pyrogallol is formed from nuclear hydroxylation of resorcinol. The possibility of such ortho oxidations in nature has been considered by SESHADRI[14] in a discussion of the C_8 lichen substances. A search for the presence of resorcinol or for meta hydroxylated acids in *P. patulum* has failed to uncover evidence for this type of substance.

We are faced therefore with the consideration that within the same microorganism two pathways toward aromatization might co-exist: the one from hexose to shikimic acid and thence to pyrogallol, *p*-hydroxybenzoic and the aromatic amino acids, and the other from hexose, possibly through acetate to 6-methylsalicylic acid and to patulin. If both pathways are present we might also expect to find that there are one or more points of convergence. We are planning to resolve some of these questions by means of mutant, isotopic tracer, and enzymological techniques.

Zusammenfassung

Ausser Patulin, Gentisinsäure und 6-Methylsalicylsäure wurden die folgenden Verbindungen als Metaboliten von *P. patulum* Stamm 2159A gefunden: 6-Formylsalicylsäure, 3-Oxyphthalsäure, Pyrogallol, *p*-Oxybenzoesäure und Anthranilsäure. Eine aliphatische Vorstufe von Patulin und eine Substanz vom Depsidtypus konnten noch nicht näher identifiziert werden. Die möglichen Stoffwechselzusammenhänge zwischen diesen Verbindungen wurden diskutiert.

[13] J. H. WEISBURGER, E. K. WEISBURGER, and H. P. MORRIS, Science *125*, 503 (1957).

[14] T. R. SESHADRI, Exper. Suppl. II, 258 (1955).

[15] D. I. CRANDALL, *A Symposium on Amino Acid Metabolism* (Baltimore 1955), p. 867.

[16] J. H. BIRKENSHAW, A. BRACKEN, S. A. MICHAEL, and H. RAISTRICK, Lancet *245*, 625 (1943). – A. BRACK, Helv. chim. Acta *30*, 1 (1947).

[17] B. D. DAVIS, *A Symposium on Amino Acid Metabolism* (Baltimore 1955), p. 799. – D. B. SPRINSON, *A Symposium on Amino Acid Metabolism* (Baltimore 1955), p. 817.

[18] A. J. BIRCH, R. A. MASSEY-WESTROPP, and C. J. MOYE, Austral. J. Chem. *9*, 539 (1955).

CONGRESSUS
GREAT BRITAIN

Symposium on the Ecology of Soil Fungi

Liverpool, 19th to 21st of August 1958

A symposium on the Ecology of Soil Fungi will be held in the Hartley Botanical Laboratories, The University, Liverpool, England, on the 19th to 21st of August 1958. Details of the symposium may be obtained from Dr. D. PARKINSON, The Hartley Botanical Laboratories, The University, Liverpool, England.