THE BIOSYNTHESIS OF PATULIN

II. THE GENERAL PHYSIOLOGY OF SEVERAL STRAINS OF *PENICILLIUM PATULUM**

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INTRODUCTION

A close relationship between the gentisic series of aromatic compounds and the pyrone derivative patulin was indicated by the RAISTRICK group[1,2], who discovered these substances in the growth filtrates of *Penicillium patulum*. Further evidence for an interplay between the aromatics and the C_7 pyrone arose when BRACK[3] discovered that the equilibrium of formation of gentisyl alcohol and patulin is a function of the trace-metal composition of the medium. Consideration of these facts led BIRKENSHAW[4] to propose a mechanism for the biosynthesis of patulin whereby gentisaldehyde, after oxidative fission and rearrangement, is transformed into patulin. The overall picture, however, was further complicated by the isolation of the C_8 compound 6-methylsalicylic acid by EHRENSVÄRD[5], and by our identification[6,7] in addition to this compound, of 6-formylsalicylic acid, 3-hydroxyphthalic acid, pyrogallol, anthranilic acid, and *p*-hydroxybenzoic acid in *P. patulum* growth broths.

Using both growing and replacement cultures, we have examined with several strains of *P. patulum* the effects of various carbon sources, trace metals, trapping agents and inhibitors on patulin and aromatic acid biosynthesis. The production of keto-acids during growth, and the conversion of various suspected aromatic precursors to patulin in replacements were also studied. It is possible to fit all of the above-mentioned metabolic products into a working sequence which is consistent with present-day biochemical concepts.

MATERIALS AND METHODS

Microorganisms

Four patulin-producing strains of *P. urticae*, Bainier were used. Strains NRRL 2159A, NRRL 1952, and NRRL 1953 were obtained from the Northern Regional Research Laboratory, Peoria, Ill. The fourth strain, ETH 815, was kindly provided by Dr. A. BRACK, Zurich, Switzerland. All cultures were maintained on Czapek-Dox (4% dextrose) agar slants at 30°, and were transferred every 15 days. Tap water was used throughout, except for those experiments with trace metals, where double-distilled water was employed. Details of large-scale growth of the fungi are given in the preceding paper[7].

Replacement experiments

Mycelial pads harvested at the 7–12th day of growth (at maximum patulin production)

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were drained free of growth medium and were then rinsed twice with tap water; a third portion of 500 ml of water was added, and the floated pad was allowed to stand for 1 h without agitation. This "endogenous" medium was drained off, the pad was again washed twice with water, and finally, Czapek-Dox medium containing the appropriate carbon source was infiltrated under the mycelium. Sterile technique was not employed in the replacement experiments. During several scores of these replacements, contamination (by other *Penicillia*) was detected only in a few isolated instances. Care was exercised during the above manipulations to avoid breaking or tearing the mycelial mat.

Paper chromatography

Separation, detection, and isolation of patulin, aromatic compounds and related metabolites are described in the accompanying paper[7]. The keto-acids were separated as their 2,4-dinitrophenylhydrazones following the methods of CAVALLINI AND FRONTALLI[8], using known derivatives as markers on the chromatogram. Where warranted, more rigorous identification was made by cochromatography with authentic samples, and by reduction of the 2-4-dinitrophenylhydrazones to their corresponding amino acids with hydrogen over 10% Pd black on charcoal. The concentration of keto-acids was estimated spectrophotometrically after elution of the respective dinitrophenylhydrazones from the paper[8,9].

Quantitative analysis of phenols

(*a*) *Growth experiments.* Following paper chromatography of small aliquots of the fermentation mixture, the chromatogram was air-dried and then examined with a U.V. Mineralite to detect patulin and 6-methylsalicylic acid. Knowns were usually run alongside on guide portions of the chromatogram. The spots were marked, cut out, eluted with warm alcohol, made up to specific volumes, and the concentration of each was determined by a measurement at the wavelength of maximum absorption in the Beckman spectrophotometer. Thus, the amounts of 6-methylsalicylic acid and of patulin were calculated directly, since their molar extinction coefficients at 307 mμ and 276 mμ, respectively, are known. The "other phenols" were then determined by measuring optical density at 242 mμ on an aliquot of the fermentation mixture. In these experiments, since gentisaldehyde was usually found in greatest concentration among the phenolic compounds produced, the "other phenols" were calculated in terms of gentisaldehyde by subtracting the sum of the molar contributions of patulin and of 6-methylsalicylic acid at this wavelength. When known mixtures of all of these substances were checked by the above procedure, reproducible and quantitative recoveries were obtained.

(*b*) *Replacement experiments.* Here, total phenols were estimated as 6-methyl-salicylic acid. The optical density of a suitable dilution of the filtrate was determined at 276 mμ and at 242 mμ. Using VIERORDT's method[10], the concentration of patulin and of total phenols was then calculated by solving the appropriate simultaneous equations.

RESULTS

Growth and patulin formation on various carbon sources

An experiment to determine which of twenty-six carbon sources would be best for the production of patulin was carried out using strain 2159A. The results are summarized in Table I. It can be seen that of the hexoses, and indeed of all the substrates tested, glucose was the best precursor of patulin in growing cultures. After a longer adaptive lag, fructose was utilized almost as well for both patulin synthesis and for growth on a dry weight basis. Not all substances which gave high yields of mycelium were used for concomitant patulin production, *e.g.*, L-arabinose, D-ribose, D-lactose, and D-cellobiose. An interesting preference for growth on L-arabinose as opposed to the D-stereoisomer by the mold was observed.

The data of Table I parallel in general the experiments of EHRENSVÄRD[5], who measured phenol formation in terms of gentisyl alcohol from various carbon sources with *P. patulum*, strain ETH 815. One notable exception is the case of D-ribose, which was reported to be a poor source of phenols, while giving good growth[5]. In our experiments, long-term adaptation to this substrate resulted in excellent patulin formation. Tartaric acid, which gave moderate growth and poor yields of patulin,

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was transformed into a host of carbonyl compounds (*vide infra*). In addition to the compounds cited in Table I, the following compounds, each tested singly, would not support growth: Na citrate, Na acetate, α-ketoglutaric, pyruvic, and phenylpyruvic acids, ethanol, ethylacetate, ethylacetoacetate, acetone, and tyrosine. As will be seen later, several of these compounds were used as precursors for patulin and for aromatic acids by fully formed mycelial mats.

TABLE I

PATULIN PRODUCTION FROM VARIOUS CARBON SOURCES BY STRAIN 2159.A

Substrates were 4% concn. in Czapek-Dox minimal medium. A uniform inoculum of conidia in sterile water was added to each of the 125 ml Erlenmeyer flasks with 40 ml of test solution. The flasks were incubated at 30°. Patulin concentrations were determined spectrophotometrically on dilutions of the fermentation medium. Dry weight determinations were made on the well-washed mycelial pads harvested on the 16th day.

Compound	*mmoles/l of Patulin on*				*Dry weight mg*
	4th day	*8th day*	*12th day*	*16th day*	
D-Glucose	3.12	10.10	8.16	5.75	255
D-Fructose	0.01	1.45	7.20	3.00	243
D-Mannose	0.00	0.10	5.38	1.70	318
L-Sorbose	0.00	1.42	1.10	0.04	108
D-Mannitol	0.00	1.38	7.80	6.10	269
L-Rhamnose	0.00	0.07	0.06	1.28	213
L-Arabinose	0.00	0.07	4.00	7.52	326
D-Arabinose	0.00	0.00	0.00	0.00	10
D-Ribose	0.00	0.00	2.96	15.20	329
D-Xylose	0.00	0.00	5.04	1.98	224
D-Maltose	0.00	7.26	8.32	4.40	255
D-Lactose	0.18	0.02	0.03	0.04	450
D-Cellobiose	0.00	1.22	0.06	3.62	315
i-Erythritol	0.00	0.00	0.00	0.00	10
DL-Tartrate	0.01	0.98	1.64	2.98	214
Glycerol	0.00	0.46	2.10	5.00	264

The general pattern found for all substrates which supported growth was a sharp increase in patulin concentration from the 4th to the 12th day, followed by a gradual decline at this point. As determined in a more careful analysis of this situation (Fig. 1), this is due to the fact that the glucose was practically exhausted from the medium on the 9th day, and after a lag period the mold entered into a stage of diauxic growth at the expense of patulin. Diminution of patulin concentration of the medium on or about the 10th day of growth was originally observed by BRACK[3] and again by SIMONART AND LATHOUWER[11], who recently found that the antibiotic is also a product of the metabolism of *P. griseofulvum* Dierckx.

Effect of calcium carbonate

With the addition of $CaCO_3$ to growing cultures of all four strains of *P. patulum*, the most striking effect was seen with strain 2159.A, where the concentration of patulin was reduced to one-third of the normal amount, and where gentisic acid, usually detected only in trace amounts on paper chromatograms, was found in

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approximately 0.5 mmole/l concentration (Table II). Although no quantitative data are presented for "pre-patulin", this substance was also present in considerable amount, and its isolation from $CaCO_3$ flasks has been described[7]. The initial repression of patulin synthesis in strain 815 appeared to be overcome by the 12th day of growth.

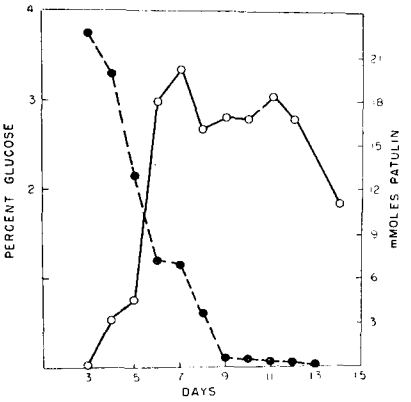

Fig. 1. Patulin production *vs.* glucose utilization by *P. patulum* strain 2159A. Glucose determinations were determined polarimetrically, while patulin was estimated spectrophotometrically on dilutions of the fermentation mixture. –●––●–, glucose concentration; –○–○–, patulin concentration.

TABLE II

EFFECT OF CALCIUM CARBONATE ON THE PRODUCTION OF PATULIN AND AROMATICS IN TWO STRAINS OF *P. patulum*

Stationary cultures at room temperature using 2.8 l Fernbach flasks containing 500 ml of Czapek-Dox 4% glucose medium. Where indicated, 10 g of sterile $CaCO_3$ was added before inoculation. All concentrations given are in mmoles/l.

Strain	*Metabolite analysed*	*Condition*	*Time in days*					
			4	*6*	*8*	*10*	*12*	*16*
2159A	Patulin	+ $CaCO_3$	3.8	6.1	7.0	5.8	5.6	
		Control	7.1	18.6	17.8	17.7	16.2	
	6-Methylsalicylic acid	+ $CaCO_3$	1.2	1.3	1.4	1.5	1.3	
		Control	0.1	0.4	0.5	0.6	0.8	
	Gentisic acid	+ $CaCO_3$	trace	0.1	0.1	0.3	0.4	0.6
		Control	trace	—	trace	—	—	trace
815	Patulin	+ $CaCO_3$	3.3	3.4	4.6	8.2	15.0	14.6
		Control	5.7	17.6	15.6	15.0	15.5	14.2

The presence of $CaCO_3$ did not inhibit the synthesis of mycelial protoplasm, on a dry weight basis. It was noted that strain 1953 failed to form its usual blue-green pigment under these conditions. Strain 1952, in addition to following the pattern set forth in Table II, accumulated a previously unknown aromatic substance, the nature of which has not yet been elucidated. Suppression of patulin biosynthesis due to $CaCO_3$ was also noticed with *P. griseofulvum*[11]. Our explanation of this phenomenon is that the aromatic acids and the open chain molecular species of patulin ("pre-patulin") are trapped as their calcium salts, and are thus made unavailable to the enzymes which would normally convert them to patulin.

Growth of *P. patulum* 2159A was neither inhibited by the presence of 0.02% 2-deoxyglucose nor by 10^{-6} *M* Na monofluoroacetate. The presence of 0.5% dehydroacetic acid inhibited growth completely.

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Sequence of appearance of metabolites

The appearance of patulin, 6-methylsalicylic acid, and other phenolic constituents (the latter measured in terms of gentisaldehyde) was examined in all four strains of *P. patulum* grown on glucose at room temperature. There were no large differences between these cultures insofar as the overall pattern is concerned, but several significant conclusions can be drawn from these data (Table III). The most important is the fact that there was a universal appearance of 6-methylsalicylate and of "gentisaldehyde" on the third day, much before any patulin was detected by paper chromatographic or spectrophotometric means. Thereafter, while patulin concentrations rose sharply, those of 6-methylsalicylic acid remained roughly constant, and the amounts of the other phenols underwent a gradual and slow rise. These findings can be taken to indicate that there is a genuine biogenetic relationship between these substances, and they further imply that the aromatic C_8 acid is a precursor of C_7 patulin via the C_7 phenols. The second point is the pronounced rise in pH of these cultures as growth proceeded. This, too, had been noted before with *P. patulum*[3] and again with *P. griseofulvum*[11]. The hypothesis is offered here that this is due, at least in part, to the utilization of nitrate nitrogen for protein synthesis, leaving Na ions, which become NaOH, in the fermentation mixture.

Effect of trace-metal concentration

By far the most significant observation[3] of earlier experiments on patulin biosynthesis was the trace-metal effect on distribution of metabolic products. We have repeated this type of experiment with the mutant strain 2159A, which is the best patulin producer, and which was thought likely to mirror in most exaggerated fashion the results of trace metal imbalance. The procedures of BRACK[3] were followed. Czapek-Dox medium contains $NaNO_3$, 3 g, KH_2PO_4, 1 g, KCl, 0.5 g, $MgSO_4 \cdot 7H_2O$, 0.5 g, $FeSO_4 \cdot 7H_2O$, 10 mg*, doubly distilled water, 1 l, and glucose 40 g. The initial pH of this mixture was 4.4. Results of growth on normal Czapek-Dox medium were compared to experiments in which the concentration of iron had been decreased tenfold, and to low iron media where zinc and manganese ions, respectively, were added to 10^{-6} *M* final concentration. These data are summarized in Table IV, where differences of environment show up best by comparing the figures for the 8th day of growth. As found by BRACK[3], with low iron concentration, patulin synthesis gave way to increased amounts of the gentisaldehyde phenols. The presence of zinc was especially effective in raising the phenolic and 6-methylsalicylate concentrations. With strain 815, EHRENSVÄRD[5] has reported that 10^{-8} *M* zinc influenced the shift towards 6-methylsalicylate more than did a concentration of 10^{-6} *M*. Manganese in the medium, as observed earlier, caused some diminution of patulin concentration, and raised the phenolic content. This effect was gradually overcome as time of incubation progressed, and by the 12th day the pattern for manganese approached that for normal growth conditions. This points up the fact that it is important not only to observe difference due to trace metals under comparable conditions at a given time interval, but that the changing pattern as the fermentation is allowed to proceed must also be taken into account.

The presence of gentisyl alcohol *per se* has never been noted in these experiments

* This concentration of iron is $3.6 \cdot 10^{-5}$ molar. A misprint in the original publication stated it as $3.6 \cdot 10^{-3}$ molar.

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TABLE III

COMPARISON OF PATULIN, 6-METHYLSALICYLIC ACID AND "OTHER PHENOLS" FORMATION AMONG FOUR STRAINS OF *P. patulum*

Stationary cultures in Czapek-Dox medium at room temperature. 6-M = 6-methylsalicylic acid. "Other phenols" measured as gentisaldehyde. All concentrations expressed as mmoles/l. pH measured with the glass electrode.

P. patulum strains	*Strain 2159A*				*Strain 1952*				*Strain 1953*				*Strain 815*			
Days after inoculation	*pH*	*Patulin*	*6-M*	*Other phenols*	*pH*	*Patulin*	*6-M*	*Other phenols*	*pH*	*Patulin*	*6-M*	*Other phenols*	*pH*	*Patulin*	*6-M*	*Other phenols*
1	4.4	—	—	—	4.4	—	—	—	4.4	—	—	—	4.4	—	—	—
2	4.8	—	—	—	5.0	—	—	—	4.6	—	—	—	5.0	—	—	—
3	5.8	—	0.2	0.8	5.8	—	0.1	0.6	5.8	—	0.1	0.8	6.1	—	0.1	1.0
4	5.4	3.1	1.2	1.5	6.2	2.4	0.9	1.4	5.3	2.8	0.5	1.0	5.2	2.2	1.2	2.5
5	5.3	6.2	1.20	1.5	5.7	5.6	1.3	1.7	4.8	6.5	0.9	1.6	5.1	5.7	1.3	2.3
6	5.2	18.0	1.4	2.8	5.6	12.0	1.3	3.2	4.6	10.2	1.0	2.7	5.8	15.5	2.3	6.2
7	5.0	20.0	1.2	3.4	5.15	15.6	1.5	4.0	5.2	17.3	1.2	4.1	5.5	17.0	2.7	7.5
8	6.0	16.2	1.4	4.2	5.35	17.3	1.5	5.0	5.9	18.8	1.2	5.2	5.8	18.8	2.7	8.1
9	6.5	17.7	1.6	4.2	6.0	16.0	1.5	5.3	6.3	18.0	1.2	5.4	6.6	15.6	2.9	8.0

TABLE IV

EFFECT OF TRACE METALS ON BALANCE OF METABOLIC PRODUCTS FROM STRAIN 2159A

"Normal" flasks contained Czapek-Dox 4% glucose medium ($FeSO_4$ concentration = $3.5 \cdot 10^{-5}$ *M*). In the experiments with manganese and zinc the iron concentration was dropped to $3.5 \cdot 10^{-6}$ *M*. The final flask had no additions but the iron was dropped to the lower concentration. Metabolic product concentrations expressed as mmoles/l.

Medium	*Metabolites*	*Time in days*											
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
Normal	Patulin	0.0	0.0	0.0	0.4	1.0	2.4	7.1	*18.4*	18.6	17.6	17.7	16.4
	6-Methylsalicylic	0.0	0.0	0.2	0.2	0.4	1.2	1.2	*1.4*	1.4	1.4	1.4	1.4
	Other phenols	0.0	0.0	1.2	2.1	3.6	4.8	4.5	*4.8*	4.2	4.0	4.2	4.8
+ $MnSO_4$	Patulin	0.0	0.0	0.0	1.4	1.1	2.0	5.4	*11.2*	15.6	17.5	19.4	19.4
$1 \cdot 10^{-6}$ *M*	6-Methylsalicylic	0.0	0.0	0.0	0.3	0.4	0.8	0.6	*0.9*	0.8	0.8	0.7	0.7
	Other phenols	0.0	0.0	0.6	1.4	1.5	1.6	3.8	*3.4*	4.8	4.8	4.2	4.0
+ $ZnSO_4$	Patulin	0.0	0.0	0.0	1.4	1.8	2.5	3.2	*4.8*	6.5	11.2	11.6	10.8
$1 \cdot 10^{-6}$ *M*	6-Methylsalicylic	0.0	0.0	0.1	0.5	1.4	2.1	2.2	*2.0*	2.7	2.6	3.0	3.0
	Other phenols	0.0	0.0	1.0	1.5	1.5	4.4	8.1	*10.4*	10.6	10.8	10.0	10.0
$FeSO_4$	Patulin	←			none detectable		→	←			trace		→
$3.5 \cdot 10^{-6}$ *M*	6-Methylsalicylic	0.0	0.0	0.4	0.5	0.6	1.0	0.9	*0.8*	1.1	1.0	1.0	0.9
	Other phenols	0.0	0.0	1.2	1.8	4.0	5.2	5.5	*6.8*	6.4	6.2	6.0	6.2

with strain 2159A, although it was exhaustively sought for by paper chromatographic analysis, using synthetic material as a marker. This is strange in light of the facts that this substance was first found in the growth medium of a patulin forming *Penicillium*[1,2] and that it was isolated in g/l amounts by BRACK[3] from strain 815 under iron deficiency.

An obvious explanation for the accumulation of the gentisic and related phenols in the iron-deficient cultures can be offered, based upon the fact that homogentisic acid oxidase[12] and protocatechuic acid oxidase[13] are known to be iron-containing enzymes. By analogy, it is to be expected, if splitting open of the aromatic ring of one of the gentisic series is an obligatory step prior to rearrangement and closure to patulin, that, under conditions of low iron, the phenolic compounds would accumulate. EHRENSVÄRD[5] has already speculated on the effect of zinc ions as the metal prosthetic group of decarboxylase enzymes in *P. patulum*. If zinc functions as a co-decarboxylase, one would have expected higher yields of patulin in its presence, rather than the pile-up of C_7 phenols which resulted.

Keto-acids

P. patulum grown on glucose forms the following acidic carbonyl compounds in the medium: α-ketoglutaric, oxaloacetic, and pyruvic acids, "pre-patulin," and traces of glyoxal and of glyoxylic acid. Several other neutral carbonyls were also

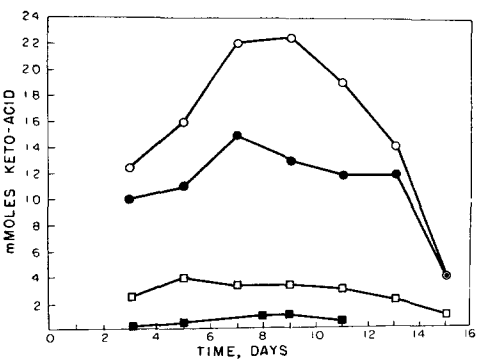

Fig. 2. Analysis of 4 keto-acids in the medium of strain 2159A grown on Czapek-Dox at 30°. Pyruvic acid, O—O; "pre-patulin," ●—●; α-keto-glutaric acid, □—□; and oxaloacetic acid, ■—■.

detected but were not further investigated. As indicated earlier, growth on DL-tartaric acid produced a spectrum of keto-acids similar to those reported by KUN AND HERNANDEZ[14] for the action of liver homogenate on this substrate. High concentrations of pyruvic, α-ketoglutaric, oxalacetic, and glyoxylic acids were noted, and the presence of considerable quantities of diketosuccinic acid (checked by co-chromatography with known material) was also noted. The finding of glyoxylate, both in glucose and in tartrate-grown cultures, along with the usual acids of the tricarboxylic acid cycle, is indicative that the "glyoxylate bypass"[15], recently advanced as an alternative pathway of terminal oxidation for fungi and bacteria, is probably operative in the *Penicillia*.

In order to gain an insight into the relationship between metabolites of the TCA cycle, and patulin biosynthesis, if any, the timewise production of three of these substances plus that for "pre-patulin" is shown in Fig. 2. These data are from the same cultures used in following the timewise course of glucose disappearance with

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patulin formation (Fig. 1). Examination of these Figures shows that there appears to be a close parallel between the shape of the curve, and, indeed, between quantities in terms of mmoles/l of the presumptive open chain molecular species of patulin, "pre-patulin", and the cyclized antibiotic itself. Further experimental work on the nature of this relationship is in progress.

Replacement experiments

Our attention was next drawn to the replacement culture technique with fully grown mycelial pads, as an approach for obtaining more information on the sequence of metabolites leading to patulin. It was qualitatively noticed that glucose-grown pads replaced with glucose formed 6-methylsalicylate as the first detectable aromatic metabolite, again even before patulin could be found. Subsequent to this, 6-formylsalicylic acid and gentisic acids, in turn, made their appearance and finally patulin was seen. These findings were made by hourly chromatography of the culture filtrate from flasks kept at 25°. In Table V are presented quantitative data for patulin and total phenols (measured in terms of 6-methylsalicylic acid) on a day-by-day basis in replacement flasks kept at approximately 30°. As glucose concentration was raised from 1 to 4%, patulin formation continued to increase for a longer period of time. The total amount formed in these replacements in three days on 4% glucose approached quantities found after 8 to 12 days of growth with this substrate. This suggests that, in many instances, future isotopic incorporation experiments may best be done in replacements rather than during growth. Mycelial pads which were grown on a mixture of acetate and glucose, when replaced with glucose, formed a higher ratio of 6-methylsalicylate to patulin than did the glucose-grown pads. Presumably, then, these mycela were "pre-adapted" to form 6-methylsalicylate from C_2 fragments. Glucose-grown pads on a mixture of sodium acetate and glucose also exhibited a higher ratio of the C_7 acid to patulin. Sodium acetate itself when used as a substrate with glucose-grown pads gave 6-methylsalicylate exclusively within one day. This experiment argues for the "head to tail" condensation theory of BIRCH[16], which postulates condensation of activated acetate directly to aromatic compounds. As sodium acetate was used for synthesis of 6-methylsalicylic acid, the pH of the medium went up sharply, as would be expected from the fact that four molecules of this aliphatic acid must be used for the synthesis of one of the aromatic by this mechanism. Replacements with glucose as carbon source containing 10^{-6} *M* zinc also had a relatively high pH, with a concurrent decrease in patulin and a higher phenolic content.

The time course of formation of patulin from aromatic substances and from several aliphatic esters in replacements of 2159A is plotted in Fig. 3. Because of the relative scarcity of 6-methylsalicylate, this substance was added in 0.01% concentration, while the other suspected precursors were tested at 0.1%. "Endogenous" formation was ruled out by running a control mycelial pad in minimal medium and subtracting the contributions of this source from the values plotted in Fig. 3. This experiment demonstrated that 6-methylsalicylate and gentisate were transformed into patulin. A conclusion as to which of these substrates is a more direct precursor cannot be drawn since permeability factors which attend the entrance of substrates into the mycelium are largely unknown. Gentisaldehyde too was transformed into patulin; in this instance there was also concomitant formation of a large amount

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TABLE V

BIOSYNTHESIS OF METABOLITES IN REPLACEMENT CULTURE

Mycelial pads from eight day glucose-grown cultures of strain 2159.A were held at 30° in 2.8 l Fernbach flasks containing Czapek-Dox minimal plus the following additions: "Endogenous" production of metabolites was determined in a blank flask with no carbon source. pH determinations were taken with the glass electrode. Metabolic product concentrations expressed as mmoles/l. Abbreviations, 6-M = 6-methylsalicylic acid; Pat. = patulin.

Additions	*1 Hour*			*24 Hours*			*48 Hours*			*72 Hours*			*96 Hours*		
	pH	*Pat.*	*6-M*	*pH*	*Pat.*	*6-M*	*pH*	*Pat.*	*6-M*	*pH*	*Pat.*	*6-M*	*pH*	*Pat.*	*6-M*
1 % Glucose	4.6	0.0	0.0	5.9	4.3	2.2	5.4	3.2	2.4	6.2	3.0	1.3	6.2	3.1	1.3
2 % Glucose	4.6	0.0	0.0	4.7	4.7	2.8	4.5	6.2	2.8	4.5	7.5	2.5	4.6	7.8	2.0
4 % Glucose	4.6	0.0	0.0	4.6	5.8	3.0	4.5	7.2	4.6	4.4	10.4	4.6	5.8	10.0	5.8
4 % Glucose (a)	4.9	0.0	0.0	4.6	3.0	4.1	4.2	3.8	4.8	3.8	14.3	5.2	4.2	14.2	8.0
4 % Glucose + $CaCO_3$	6.4	0.0	0.0	6.5	1.9	1.6	5.8	2.8	4.5	5.4	5.7	4.4	5.8	5.2	5.8
2 % Glucose + 10^{-6} *M* zinc	4.1	0.0	0.0	5.0	2.5	2.8	5.4	1.9	2.1	5.8	10.6	4.4	6.3	9.5	7.6
2 % Glucose + 10^{-6} *M* Mn	4.1	0.0	0.0	5.0	2.0	1.5	4.8	4.2	1.4	4.7	7.6	2.8	6.3	7.1	4.5
1 % Na acetate	5.8	0.0	0.0	8.3	0.0	0.2	8.4	0.0	0.5	8.4	0.0	0.6	8.4	0.0	0.7
2 % Glucose 2 % Na acetate	6.3	0.0	0.0	6.5	0.1	2.0	6.1	2.4	3.3	6.1	4.9	4.2	6.8	4.8	4.8

(a) This mycelium was grown on 2 % glucose–2 % Na acetate mixture.

of dark oxidation products, which did not arise when gentisate was used as substrate. The esters tested also served as precursors, and ethylacetoacetate seemed to be the best aliphatic substance yet found for patulin formation by these procedures. Ethanol, acetate plus ethanol, acetone, and pyruvate gave essentially negative

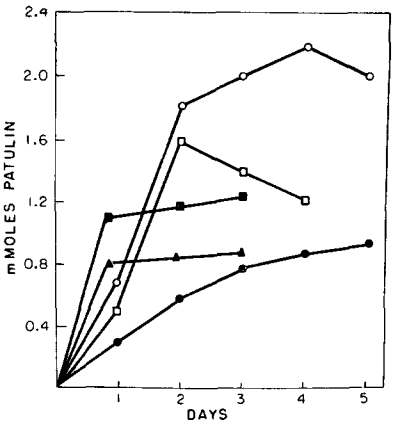

Fig. 3. Patulin formation in replacement experiments with strain 2159A. The following were tested as substrates: ethylacetoacetate, O—O; ethylacetate, □—□; gentisic acid, ■—■; gentisaldehyde, ▲—▲; and 6-methylsalicylic acid, ●—●.

results for patulin biosynthesis in replacement cultures examined up to 72 h. Either the esters are more permeable to the mycelial wall, or else the mold has mechanisms which more readily utilize the energy of the ester bond for the formation of active C_2 units. Also tested as possible substrates for patulin synthesis with essentially negative results were: ribose, pyrogallol, phenylpyruvate, tyrosine, α-ketoglutarate, dehydroacetic acid, and glyoxylic acid. It will be recalled that only after very long term adaptation did growth and patulin synthesis occur on ribose. Shikimic acid in replacement gave rise to *p*-hydroxybenzoate in the medium. No other metabolite besides this single product was detected by paper chromatography.

Effect of inhibitors in replacements

Since CRAMER AND WOODWARD[17] demonstrated the inhibition of glucose fermentation by intact cells of yeast with 2-deoxyglucose, and WICK *et al.*[18] have concluded that the site of this metabolic block in the rat is on the enzyme phosphoglucoisomerase, this analogue was among the first tested for its effect on patulin synthesis. When added in 0.02% concentration to 1% glucose, deoxyglucose caused no change in the amount or in the rate of patulin formation. In another series, 1% 2-deoxyglucose as substrate was converted both to patulin and 6-methylsalicylic acid. These metabolites were found to be present in amounts comparable to experiments where 1% glucose was substrate. This result indicates not only that 2-deoxyglucose fails as an inhibitory agent in this system, but suggests that the half of the molecule resembling glucose was probably broken down to triose and to active acetate. Low concentrations of Na monofluoroacetate (10^{-6} *M*) were without inhibitory action either on patulin formation from glucose or on 6-methylsalicylate production from acetate. As shown previously by EHRENSVÄRD[5], 0.04% 2,4-pentanedione (acetylacetone) stopped aromatic and patulin synthesis completely from glucose, and 0.04% dehydroacetic caused an accumulation of 6-methylsalicylate.

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DISCUSSION

If one examines the chemical structures of the compounds which have been isolated from *P. patulum* strain 2159A, and considers the sequence of appearance of these metabolites during growth of all of the strains tested, a tentative biochemical pathway for their interrelationship can be set forth. This pathway receives further support from an analysis of the results of the replacement experiments, wherein it was shown not only that the C_8 and C_7 aromatics gave rise to patulin, but that 6-methylsalicylate was transformed into 6-formylsalicylate, and thence to gentisic acid. These data, when coupled to BIRKENSHAW's suggestion[4], that oxidative cleavage of the aromatic ring of gentisaldehyde and rearrangement to patulin is the next step, can now be used to explain the conversion of hexose to the pyrone antibiotic. The additional findings in replacements that shikimic acid goes to *p*-hydroxybenzoate rather than to the gentisate series or to patulin; that acetate is a direct precursor of 6-methylsalicylate; and the discovery of pyrogallol in the growth filtrate, indicate moreover that two pathways toward aromatization coexist in this microorganism. These relationships are summarized in Fig. 4.

Here, glucose is pictured as undergoing two dissimilations, one through the well-proven[19] sequence of reactions to sedoheptulose diphosphate and thence to shikimate (VIII); and also to active acetate which would then condense to 6-methylsalicylic acid (I), as proposed by BIRCH and co-workers[16]. A two-step oxidation of 6-methylsalicylate to 6-formylsalicylate (II) is then envisaged. Precedence for a preferential oxidation at the methyl group of a hydroxylated aromatic structure, leaving the ring intact, was demonstrated by DAGLEY AND PATEL[13], who found in *Pseudomonas* that 4-methylcatechol and xylenol are oxidized via their formyl analogs to protocatechuic and 4-hydroxy, 2-methylbenzoic acids, respectively. Enzymic

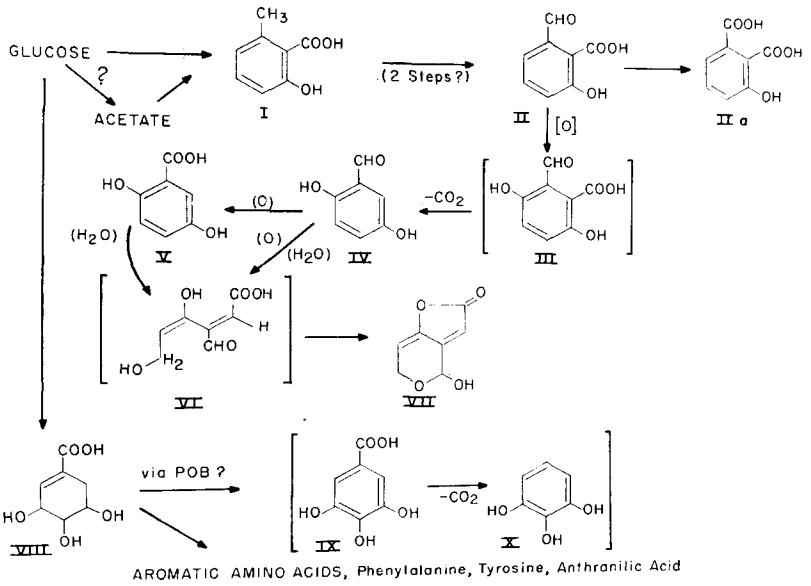

Fig. 4. Proposed interrelationship between aromatic compounds and patulin in *P. patulum*.

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nuclear hydroxylation of 6-formylsalicylic acid (II), possibly by an enzyme of the phenolase type[20] to the hypothetical 2,5-dihydroxyphthaldehydic acid (III), followed by straight decarboxylation of the latter will yield gentisaldehyde (IV). It is noteworthy that a compound closely related to (III), 3,5-dihydroxyphthalic acid, was isolated from *P. brevi-compactum*[21]. This substance differs from (III) only in the orientation of its hydroxyl groups, and in its higher oxidation state. Furthermore, compound (III) is not unlike the orsellinic acid units of which the lichen aromatic dimers are presumed to be made[22]. We explain the finding of 3-hydroxyphthalate (IIa) in the medium as the result of a simple biological oxidation of 6-formylsalicylate. It is placed on a side pathway in the chain of events leading to patulin (VII), since any conversion of 3-hydroxyphthalate to the gentisic series must involve a decarboxylation step, and the two requisite intermediary phenolic acids which might have arisen have not been detected in the medium. Oxidation of gentisaldehyde to gentisic acid (V), followed by splitting of the aromatic ring mediated by a presumptive iron-requiring enzyme will give rise to the open-chain aliphatic "pre-patulin" (VI). Whether fission of the aromatic ring occurs with gentisaldehyde or with gentisic acid is not yet known. The facts that gentisate yields patulin in replacement, and that its empirical formula is the same as for patulin, makes it appear a more likely precursor. The possibility of a combined oxidative fission at the aldehyde stage, as first outlined by BIRKENSHAW[4], may still prove to be the actual mechanism of this interconversion.

Some added support for this scheme comes from the work of SIMONART AND LATHOUWER[11] with *P. griseofulvum*. Upon recently re-examining the filtrates from this microorganism, these authors have found that patulin is also made. ANSLOW AND RAISTRICK[23] originally discovered 6-methylsalicylate as the major compound formed by this mold. From the metabolic sequence presented in Fig. 4, it might have been predicted that patulin should have been detectable, since an implied corollary to this pathway is that any mold capable of synthesizing 6-methylsalicylate or gentisate[24] will gradually sequentially induce enzymes to transform these substances to patulin. It also appears from the data presented here that patulin itself eventually becomes converted to as yet unknown substances, which are probably related to terminal respiratory pathways of the mold.

It is not possible to account for the formation of pyrogallol by BIRCH's "head to tail" acetate theory, since this mechanism always leads to materials with *meta*-orientated hydroxyls, and, at best, would involve a large number of hypothetical steps from 6-methylsalicylate if it were initially formed. Rather, pyrogallol (X) is taken at arising from shikimic acid via gallic acid (IX). BRÜCKER[25] has already shown with *Phycomyces blakesleeanus* that gallate can arise from sugars or from tyrosine. If such is the case with *P. patulum*, then it is conceivable that both aromatization mechanisms are functioning simultaneously. The recent findings of the NEISH group[26], of GEISSMAN AND SWAIN[27], and of GRISEBACH[28], that in the quercetin molecule ring A comes from acetate condensation and ring B arises via the shikimate route, have shown that such a situation can exist even within a single molecule. Whether an analogous situation actually exists among the *Penicillia* is being investigated by the isotopic-tracer technique and the use of cell-free enzyme systems.

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SUMMARY

The sequence of appearance of aromatic intermediates on the pathway toward patulin biosynthesis in several closely related strains of *Penicillium patulum* has been examined. Both growing cultures and the replacement method have been used in these experiments. A scheme whereby glucose gives rise to patulin via 6-methylsalicylic acid, 6-formylsalicylic acid, the gentisic series, and "pre-patulin" has been proposed. Based upon the finding of pyrogallol in the growth media, and on the fact that shikimic acid yields *p*-hydroxybenzoate in replacement cultures, it is suggested that two pathways toward aromatization coexist in these microorganisms.

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