THE BIOSYNTHESIS OF PATULIN*

I. RELATED AROMATIC SUBSTANCES FROM *PENICILLIUM PATULUM*, STRAIN 2159A

S. W. TANENBAUM AND E. W. BASSETT

Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York (U.S.A.)

INTRODUCTION

Subsequent to the original isolation of patulin from the filtrate of a strain of *Penicillium urticae*, Bainier (synonym, *P. patulum*) by BIRKENSHAW *et al.*[1] and the characterization of gentisyl alcohol and gentisaldehyde as additional side products by this group[2], several other studies on the aromatic components of the mold have been made. The first of these by BRACK[3] noticed the presence of gentisic acid in the growth medium, and more important, stressed the striking effect of varied trace metal concentration on the ration of C_7 aromatics to patulin. EHRENSVÄRD[4] has confirmed BRACK's general observations with regard to the shift of balance of metabolic products engendered by the cationic composition of the medium, and has isolated 6-methylsalicylic acid from these fermentations.

In the course of a study of the comparative biochemistry of patulin formation among four strains of *P. patulum*,[5] it was noticed that the non-pigmented mutant strain 2159A, probably owing to the genetic block, accumulated a larger number and quantity of aromatic intermediates than did the others. Accordingly, it was selected for the most intensive investigation and from it have been isolated the aforementioned compounds plus the following: 6-formylsalicylic acid, 3-hydroxyphthalic acid, pyrogallol, *p*-hydroxybenzoic acid, and anthranilic acid. Several other not fully characterized components have also been obtained. This paper describes procedures for isolation of these additional substances from *P. patulum* broth, and tabulates their physical and chemical properties.

MATERIALS AND METHODS

Microbiological

P. urticae Bainier, strain 2159A was kindly made available by Dr. C. W. HESSELTINE, Peoria, Ill. A sterile suspension of material washed off Czapek-Dox slants was used to inoculate 2.8 l Fernbach flasks containing 500 ml of Czapek-Dox 4% dextrose solution. Unless mentioned otherwise, these were incubated at room temperature for 7–12 days. The growth medium was removed from the mycelial pads by decantation and was pooled for the isolation of metabolites.

Chemicals

Gentisaldehyde was synthesized by the method of NEUBAUER AND FLATOW[6], and gentisyl alcohol from this in turn by controlled catalytic hydrogenation[2] at atmospheric pressure over

* Supported by a grant (NSF-G2914) from the National Science Foundation.

References p. 30/31.

10% Pd on charcoal. Using the method of ELIEL *et al.*[7], 6-methylsalicylic acid was synthesized. We are indebted to Dr. ELIEL for his gift of 6-formylsalicylic acid. The preparation of 3-hydroxyphthalic acid (m.p. 154°) was effected by diazotization of 3-aminophthalic acid. The latter was obtained by the reduction of 3-nitrophthalic acid with stannous chloride in hydrochloric acid. By a similar series of reactions, 5-hydroxyphthalic acid was made. All other chemicals used were commercially available and were recrystallized several times.

Paper chromatography

The following procedures for preparation of samples and for paper chromatography of metabolites were used:

(a) A measured aliquot of the growth filtrate was placed directly on Whatman No. 1 or No. 3 MM filter paper. (b) An aliquot of the metabolism filtrate was acidified to pH 2 with HCl and was then extracted twice with equal volumes of ether. The ethereal extract was dried with sodium sulfate, evaporated, the residue was taken up in ethanol and applied to the paper. This procedure extracts the total ether-soluble material in the broth. (c) Acidic fraction: the ether extract from (b) was shaken with sodium bicarbonate, the aqueous layer was then acidified and re-extracted with ether. This, in turn, was dried, evaporated, taken up in ethanol and chromatographed. Three solvent systems were used routinely for the resolution of compounds by 18 h ascending chromatography. These were (d) *n*-butanol–0.5 *N* NH_4OH–ethanol[8] (70:20:10), which was effective with the acidic compounds. Patulin was decomposed to some extent by contact with this mixture. (e) benzene–propionic acid–water[9] (2:2:1). This system was very useful in separating patulin, pyrogallol, gentisic acid and 3-hydroxyphthalic acid from one another. (f) *n*-butanol–acetic acid–water[10] (4:1:1). This solvent was efficacious in separating "pre-patulin" from patulin. Indicating spray reagents used were: aniline hydrogen oxalate for reducing substances, *o*-dianisidine in glacial acetic acid for patulin, $FeCl_3$ for enols and phenols, and diazotized sulfanilic acid or "Napthanil" (du Pont 2,5-dichloro-benzenediazonium chloride) for phenols. A summary of R_F values in the various solvents and the color reactions of the metabolic products of *P. patulum* is presented in Table I.

RESULTS

Work-up of growth medium

The Fernbach flasks were spot checked for patulin and aromatic biosynthesis by paper chromatographic analysis and by taking optical density readings on 1/200 dilutions at 276 mμ. Peak production of these metabolites took place within the 7th to 12th day, depending upon room temperature. Care was taken not to disturb the mycelial mat, since agitation decreased the formation of patulin considerably. A portion of the pooled fermentation mixtures was saved for the isolation of oxygen-sensitive materials, while the bulk was filtered through Whatman No. 4 paper, and distilled under reduced pressure to around 1/20 of its original volume on the steam bath. Upon cooling, this concentrate was again filtered free of a precipitate of salts and proteinaceous material, acidified to Congo red with sulfuric acid, and was continuously extracted with ether for 48 h. Further fractionation of components was carried out as outlined in Fig. 1.

Patulin

The total ether extract was cooled and extracted with chilled saturated sodium bicarbonate in a separatory funnel. The ether layer was dried with Na_2SO_4 and evaporated to the cloud point, whereupon patulin crystallized out after cooling to 4°. By reducing the filtrate from this precipitation to 1/3 its volume, a second crop of patulin was obtained. The combined yield was then purified by recrystallization twice from benzene, m.p. 111–112°. A tabulation of the molar extinction coefficients and wavelengths of maximum absorption for this and subsequent compounds isolated is presented in Table II. The ultraviolet absorption spectrum for patulin is given in Fig. 2.

References p. 30/31.

TABLE I

SUMMARY OF CHROMATOGRAPHIC AND SPRAY REACTIONS OF COMPOUNDS

Compound	R_F *BuOH–EtOH–NH₃*	R_F *Benz–prop–H₂O*	R_F *BuOH–HAc–H₂O*	*U.V. color*	*FeCl₃*	*DSA* or DNap*	*o-Dis**	*AHO**
Patulin	0.80**	0.66	0.80	black			brown	gray
6-methylsalicylic acid	0.62	0.92	0.90	dk.blue	purple	orange	yellow	
6-formylsalicylic acid	0.60; 0.70	0.44; 0.95§		yellow; blue	red	blue		
Gentisaldehyde	0.85	0.85	0.90	yellow	blue§§§	yellow§§§		yellow§§
Gentisyl alcohol	0.76	0.80	0.88	dark	blue§§§	yellow§§§		
Gentisic acid	0.26	0.44		lt.blue	blue	white	gray	
3-HO-phthalic acid	0.08	0.34	0.40	lt.blue	red	yellow		
Pyrogallol	Streaks	0.22		dark	blue	brown		
p-HO-benzoic acid	0.24			dark	red	dk.yellow		
m-HO-benzoic acid	0.22			blue		orange		
o-HO-benzoic acid	0.32			blue		yellow		
Gallic acid	0.00	0.12		dark	blue-black	dk.yell.-brown		
5-HO-phthalic acid	0.00			blue	red	orange		
Anthranilic acid	0.55			lt.blue fluor		yellow		
"pre-patulin"	Streaks	0.60	0.68	dark			brown	gray§§
Shikimic acid	0.15			dark				
5-formylsalicylic acid	***			yellow	red	brown		yellow
"322 mμ" compound	0.58		0.94	dark	red-purple	yellow§§§		
"292 mμ" compound	0.92	0.88	0.90	blue		orange		

* Abbreviations used: DSA, diazotized sulfanilic acid; DNap, diazotized "Napthanil"; *o*-Dis, *o*-dianisidine; AHO, aniline hydrogen oxalate; U.V., color in ultraviolet light.

** Slight decomposition.

*** Obtained from Aldrich Chemical Co. Exhibits three spots, R_F 0.58, 0.64, 0.70 in this solvent.

§ 6-formylsalicylate gives two spots in all solvents tested.

§§ Color develops without heating.

§§§ Color fades on standing.

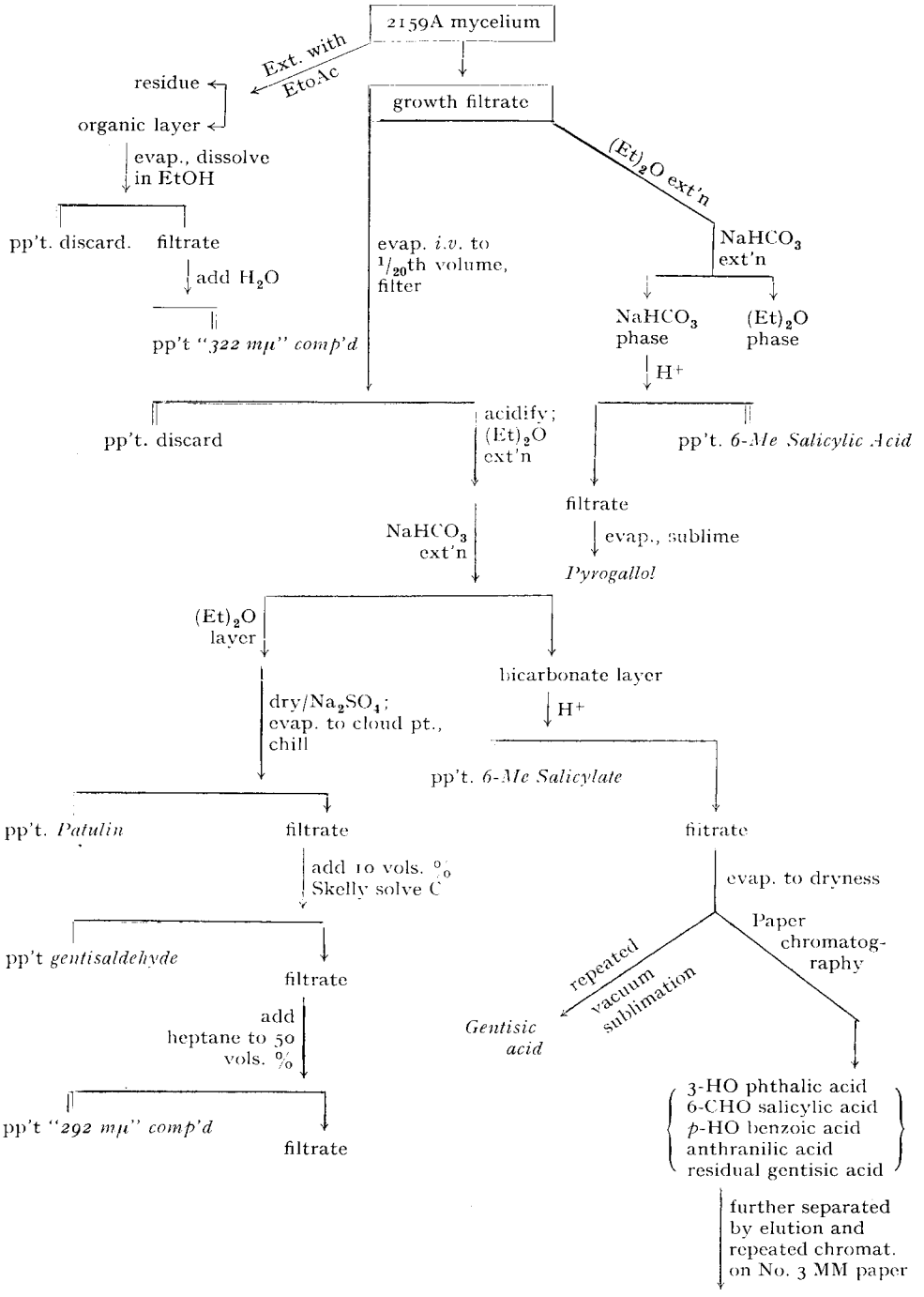

Fig. 1. Flow sheet of chemical operations for the separation of metabolic products of *P. patulum* 2159A.

Gentisaldehyde

Further concentration of the final ethereal filtrate from the patulin isolation and treatment with 10 vol. % Skellysolve C caused the deposition of a yellow-white precipitate. This was centrifuged off and recrystallized twice from chloroform. The pure gentisaldehyde melted at 97° and was identical with the synthetic compound in spectrophotometric (Fig. 2) and chromatographic behavior.

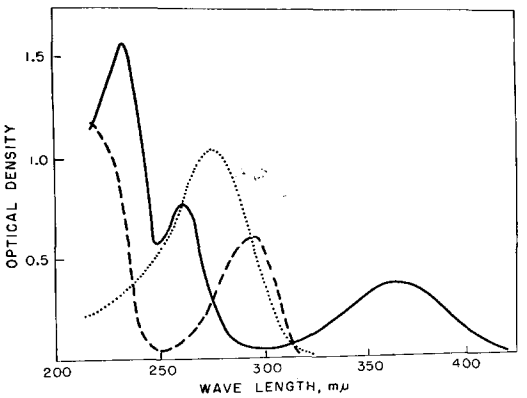

Fig. 2. Ultraviolet absorption spectra of isolated patulin, ·····; isolated gentisaldehyde, ———; and of synthetic gentisyl alcohol, – – – –. Solvents, for patulin, water; aromatics, 95 % ethanol.

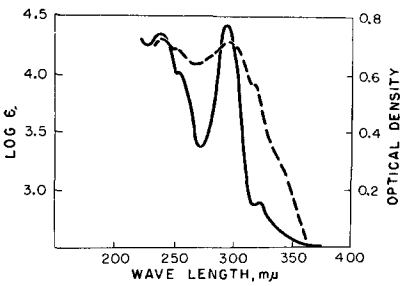

Fig. 3. Comparison of the ultraviolet absorption spectra of the "292 mμ" compound in ethanol, ———; and of griseofulvin, in methanol, – – – –. This last curve copied from GROVE *et al.*[11], absorption plotted as log ε.

TABLE II

SUMMARY OF ABSORPTION PROPERTIES OF COMPOUNDS ISOLATED FROM FILTRATE OF STRAIN 2159A

Compound *	*Primary*		*Secondary*	
	λ*max*, mμ	ε*max*	λ*max* mμ	ε*max*
Gentisic acid	335	4,700		
Gentisyl alcohol**	294			
Gentisaldehyde***	228	16,730	260	8,040
6-methylsalicylic acid	242	5,790	307	3,095
6-formylsalicylic acid	300	3,570		
3-HO-phthalic acid	323	1,665		
Patulin	276	13,350		
p-HO-benzoic acid	255			
Anthranilic acid	247		336	
Pyrogallol§	295			

* All in 95 % ethanol except for patulin values which were determined in water.
** Synthetic.
*** A third λ max at 370 mμ.
§ This peak is broad and variable, due to rapid oxidation in solution of compound.

"292 mμ" compound

After removal of gentisaldehyde, an increase in the volume of Skellysolve C in the solution to 50 vol. % precipitated the "292 mμ" compound. It was obtained in better yield from the first ether extract of the bulk medium by evaporation to dryness and subsequent treatment with boiling heptane. The hot heptane was filtered from the

References p. 30/31.

amorphous residue and after chilling deposited long yellow needles. These were collected and sublimed several times at 1 mm pressure with a bath temperature of 200°. The resulting white sublimate melted at 218°, and migrated as a single spot in several chromatographic solvents (Table I). Tests for nitrogen, halogens and sulfur were negative. The substance possessed optical activity, $[\alpha]_D^{27} = +625°$ ($c = 1.2\%$ in acetone). It gave a positive hydroxamic acid test. A molecular weight of 326 was obtained by the Rast method (camphor). Chromatographic analyses following partial acid hydrolysis with 2 *N* HCl revealed 6-formylsalicylic and 6-methylsalicylic acids as products. Alkaline hydrolysis with 10% NaOH for several hours gave rise to 6-methylsalicylate and five additional spots which were diazo positive and ultraviolet absorbing. The nature of these latter degradation products is not known. From a consideration of these properties, it may be tentatively concluded that the "292 mμ" compound is a dimer containing 6-methyl and 6-formyl salicylic acid moieties either in depside, diester or anhydride linkage or with a spirane linkage, such as found in griseofulvin[11]. Indeed, the high optical activity and the fairly close resemblance of the absorption spectrum of the "292 mμ" compound to that of griseofulvin (Fig. 3), weigh in favor of the spirocyclic possibility. A carbon and hydrogen analysis of C, 58.23%, H, 5.24% was obtained. Although this fits best a combination of both moieties as an anhydride, with one water of hydration, the last structure would not possess a center of asymmetry. The chemical nature of the "292 mμ" compound must be deferred until more material is available for further degradative experiments.

6-Methylsalicylic acid

The major acidic compound in the bicarbonate extract of the total ether-solubles from the fermentation mixture was 6-methylsalicylic acid. After acidification with HCl and cooling, crystals of this substance promptly precipitated. It was then recrystallized from water, and was finally sublimed under high vacuum. The sublimate melted at 164–165°. The acetyl derivative was prepared in the usual manner and melted at 130–132° after recrystallization from benzene. Both synthetic and isolated compounds had the same chromatographic and spectrophotometric (Fig. 4) characteristics.

Pyrogallol

Since it was found that vacuum distillation caused its destruction, this compound was isolated from an aliquot of the original growth filtrate. The work-up closely paralleled the procedure given above for the isolation of 6-methylsalicylate from the concentrated growth medium (Fig. 1). After removal of the fairly insoluble 6-methylsalicylate by precipitation, the filtrate was again extracted with ether, the ethereal extract was evaporated to dryness, and pyrogallol was obtained from the resultant mixed residue by sublimation at a bath temperature of 60° at 1 mm pressure. After further purification by resublimation, it melted at 134° mixed m.p. with a commercial sample, 134°. The triacetate, m.p. 162°, mixed m.p. with an authentic derivative, 160–162°, was prepared by acetylation with acetic anhydride in pyridine. The ultraviolet absorption spectrum for the isolated pyrogallol is presented in Fig. 4.

Gentisic acid

This compound could be recovered in small yield from the 6-methylsalicylic acid filtrates by repeated fractional vacuum sublimation, which gradually separated

References p. 30/31.

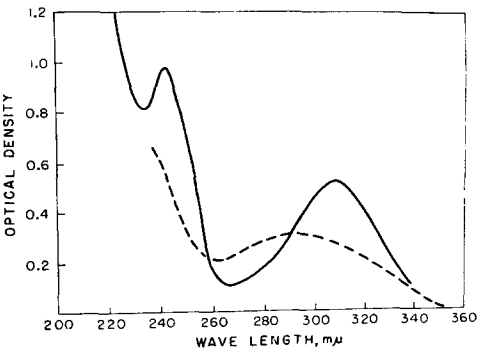

Fig. 4. Ultraviolet absorption spectra of 6-methylsalicylic acid, ———; and of pyrogallol, – – – –; isolated from strain 2159A. Solvent, 95% ethanol.

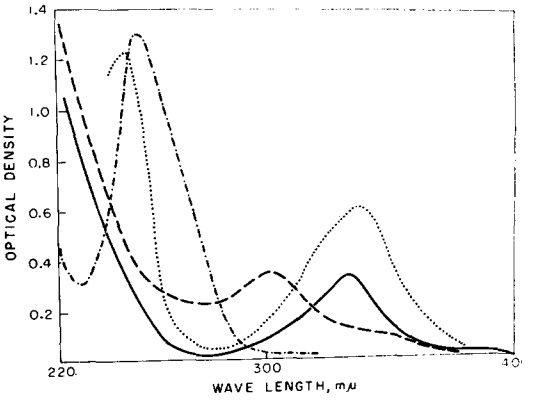

Fig. 5. Ultraviolet absorption spectra of isolated gentisic, ———; and 6-formylsalicylic, – – – – acids. Anthranilic acid, ·····; and *p*-hydroxybenzoic acid, ·—·—·, were eluted directly off paper chromatograms. Solvent, ethanol.

it from other minor constituents. It was better isolated from a fermentation with strain 2159A to which $CaCO_3$ had been initially added. The presence of $CaCO_3$ in growing cultures caused a marked diminution in the yield of patulin, and 6-methylsalicylate, gentisic acid, and "pre-patulin" were present in high concentrations. After removal of the 6-methylsalicylate by precipitation, gentisic acid was crystallized from the mother liquors. Upon recrystallization it melted at 201° and exhibited its characteristic absorption spectrum in the ultraviolet region (Fig. 5).

6-formylsalicylic acid

Although not enough of this material has yet been isolated for a microchemical analysis, the following properties indicate that it is indeed an intermediate in the interconversion of aromatic compounds by *P. patulum* 2159A. 6-Formylsalicylate was first detected on paper chromatograms of the fraction from which 6-methylsalicylate had been removed, and which contained gentisic acid. It was separated from gentisate and the other minor aromatic components by repeated large-scale paper chromatography in the butanol–ethanol–ammonia solvent. The final chromatogram was marked under the ultraviolet lamp, and the 6-formylsalicylic acid area was eluted with ethanol. This extract was placed into the lower half of a vacuum sublimation apparatus and was concentrated on a water bath. Previous experience had shown that vacuum distillation of the ethanolic extract or reduction in volume in an air stream at this point in the procedure caused destruction of the 6-formylsalicylate. The residue was then sublimed at high vacuum and collected on the cold finger. The resultant white crystals had the same absorption spectrum (presented elsewhere[12]; see also Fig. 5) and melting point as did an authentic sample. It readily formed a 2,4-dinitrophenylhydrazone with Brady's solution. This derivative was then reduced in a microhydrogenation apparatus to give the presumptive 6-aminomethylsalicylic acid. A comparison of the properties of the isolated and authentic 6-formylsalicylic acids and their derivatives is presented in Table III.

References p. 30/31.

TABLE III

COMPARISON OF PROPERTIES OF ISOLATED AND AUTHENTIC 6-FORMYLSALICYLIC ACIDS

Determination	*Isolated*	*Authentic*
M.p.	134°	134–137° (mixed, 134°)
$FeCl_3$ color in solution	Red	Red
R_F (U.V. color)*	0.60 (yellow) 0.82 (blue)	0.60 (yellow) 0.82 (blue)
Diazo spray	Light brown	Light brown
M.p. 2,4-dinitrophenylhydrazone	265°	272° (mixed, 265°)
R_F of [6-aminomethylsalicylic acid]	0.66	0.66
Ninhydrin color of above	Orange	Orange

* 6-Formylsalicylate consistently gives two spots in all solvents tested.

p-*Hydroxybenzoic and anthranilic acids*

These well-known mold metabolites[13] were separated from gentisic and 6-formylsalicylic acids by paper chromatography. They were provisionally identified by their characteristic appearance under the U.V. Mineralite and from their R_F values and color reactions (Table I). Final identification was made by co-chromatography with known samples, as well as from their absorption spectra (Fig. 5) after elution from the paper with ethanol. It is interesting to note that growth of *P. patulum* at 37° resulted in increased yields of *p*-hydroxybenzoate in the medium.

3-hydroxyphthalic acid

This is another instance of a substance whose presence was initially suspected from examination of paper chromatograms of the gentisic acid fraction. Due to its low concentration it could never be isolated from the growth medium. However, from replacement experiments with mycelial mats of strain 2159A, using 6-methylsalicylic acid as substrate, enough of the material accumulated so that it could be isolated[12] after repeated chromatographic separation on Whatman No. 3 MM paper. A comparison of the properties of the substance thus obtained and of the synthetic compound is made in Table IV. Their absorption spectra are likewise compared in Fig. 6. GATENBECK[14] has recently detected this metabolite by chromatographic means in *P. islandicum*.

"*Pre-patulin*"

As mentioned earlier, this product accumulated in relatively large amounts when cultures of *P. patulum* 2159A were grown with added calcium carbonate. Continuous ether extraction of the concentrated bulk medium, followed by partition into $NaHCO_3$ and then acidification, removed most of the 6-methylsalicylic acid. The filtrate was then concentrated to yield a reddish oil, which contained "pre-patulin," residual traces of 6-methylsalicylate, and gentisic acid. This oil was taken up in water, and was extracted once more with ether in a separatory funnel to remove the more ether-soluble aromatic acids. The aqueous phase was evaporated to dryness

References p. 30/31.

TABLE IV

COMPARISON OF PROPERTIES OF ISOLATED AND AUTHENTIC 3-HYDROXYPHTHALIC ACIDS

Determination	*Isolated*	*Authentic*
M.p.	148°, 154°	154° (155–159°)[18]
$FeCl_3$ color in solution	red-brown	red-brown
R_F (butanol–NH_3–ethanol)	0.08	0.08
R_F (benzene–propionic acid–H_2O)	0.44	0.44
U.V. color	light blue	light blue
Diazo spray	yellow	yellow

and was then repeatedly taken up in boiling anhydrous ether, cooled and evaporated again. The final hot ether extract when cooled deposited first a reddish sticky material (m.p. softens at 45–50°), followed by a crop of white crystals which turned out to be patulin. The red amorphous material, or "pre-patulin," has an absorption maximum in the ultraviolet at 280 mμ with a low molar extinction coefficient as opposed to patulin, which has a peak of maximum absorption at 276 mμ with a high molar absorbance. "Pre-patulin" can be catalytically reduced over Pd to give an acidic substance which chromatographs as a single spot (R_F = 0.15, propanol–ammonia[15] (90:10); bromphenol blue indicator). It reacts immediately with Brady's solution at room temperature to give an acidic 2,4-dinitrophenylhydrazone, m.p. 156°, (λ max. ethanol, 372 mμ; λ max. Na_2CO_3, 380 and 400 mμ). "Pre-patulin" reacts immediately on paper chromatograms with aniline hydrogen oxalate. Patulin does not react with Brady's solution readily; it must be allowed to stand for a period of time[1], or else be heated, before a visible precipitate is seen. It also must be heated on paper to 105° in order to be detected with aniline oxalate. When, however, patulin was dissolved in 95% alcohol and allowed to stand for several days at room temperature, it assumed the characteristics of "pre-patulin" with regard to all of the above recorded

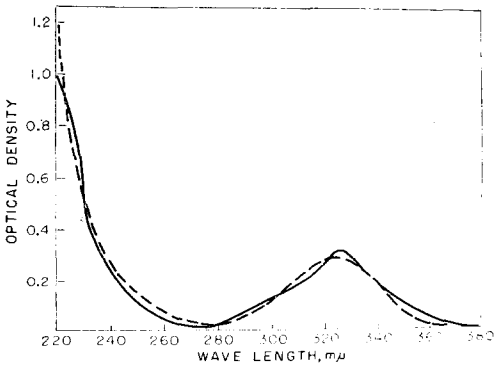

Fig. 6. Comparison of ultraviolet spectra of synthetic 3-hydroxyphthalic, — · — · —; and isolated 3-hydroxyphthalic, – – – –, acids in ethanol.

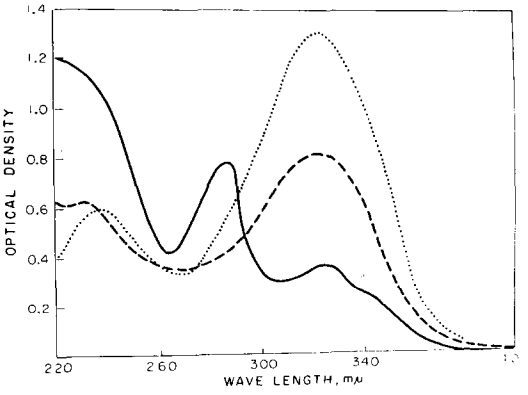

Fig. 7. Ultraviolet absorption spectrum of "322 mμ" compound. ———, in cyclohexane; — · — · —, in ethanol; ·····, in 50% 0.1 *N* HCl–50% ethanol.

References p. 30/31.

properties. The dinitrophenylhydrazone of this alcohol-aged patulin was acidic, with an R_F of 0.78 (identical for that of the dinitrophenylhydrazone of "pre-patulin") in butanol–ethanol–NH_3 solvent, as opposed to one of 0.92 for the dinitrophenylhydrazone from the freshly precipitated species. The aged patulin dinitrophenylhydrazone was furthermore hydrogenated to give the same amino acid (R_F 0.40, butanol–acetic acid–water) which resulted from reduction of "pre-patulin" dinitrophenylhydrazone. All of this evidence supports the contention that "pre-patulin" and the mishandled patulin are the same. This substance may correspond to the completely open chain configuration for patulin described by WOODWARD AND SINGH[16]. That "pre-patulin" is not an artefact arising from the steps used in the isolation of patulin is supported by the repeated observations that it did not arise after the same chemical manipulations of the medium from normally grown strain 2159A. Because it is held as its calcium salt, "pre-patulin" must be made unavailable to the lactonizing and hemi-acetal forming enzymes which usually transform it into patulin.

"322 mμ" compound

This material has been isolated by treating the washed mycelial pads of strain 2159A with ethyl acetate. This "skinning" resulted in the removal of all of the white or non-pigmented material from the top of the mat. The ethyl acetate layer was then dried with Na_2SO_4, evaporated to dryness, and the residue was taken up in ethanol, chilled, and then filtered. The insoluble portion was discarded, and addition of water to the clear filtrate precipitated the "322 mμ" compound. It melted at 142–144°, dissolved in alkali to give a yellow color, reacted positive in the iodoform test, negative in the fuchsin aldehyde test, and formed a 2,4-dinitrophenylhydrazone, m.p. 124–128°. It possesses optical activity. Ultraviolet absorption spectra in ethanol, ethanolic hydrochloric acid, and in cyclohexane are presented in Fig. 7. The shift in absorption maximum from 230 mμ in ethanol to 286 mμ in cyclohexane is usually indicative of keto-enol tautomerism. From the above chemical and physical characteristics, coupled to the fact that this substance reacts on chromatograms (Table I) with diazo sprays, we have drawn the conclusion that the "322 mμ" compound contains the aromatic nucleus and a methyl ketone group. The presence of aromatic methyl ketones in the closely related *P. brevi-compactum* was shown by OXFORD AND RAISTRICK[17]. Further work on the structure of the "322 mμ" compound is in progress.

SUMMARY

A non-pigmented mutant strain of *Penicillium patulum* excretes a variety of aromatic substances into the fermentation medium. In addition to gentisic acid, gentisaldehyde, 6-methylsalicylic acid, and patulin, already known in this species, this microorganism forms 6-formylsalicylic acid, 3-hydroxyphthalic acid and pyrogallol. Anthranilic acid and *p*-hydroxybenzoic acid were also detected chromatographically. The properties of several other isolated but as yet uncharacterized materials designated as "pre-patulin", "292 mμ", and "322 mμ" compounds have been described.

REFERENCES

1 J. H. BIRKENSHAW, A. BRACKEN, S. E. MICHAEL AND H. RAISTRICK, *Lancet*, 245 (1943) 625.
2 J. H. BIRKENSHAW, A. BRACKEN AND H. RAISTRICK, *Biochem. J.*, 37 (1943) 726.
3 A. BRACK, *Helv. Chim. Acta*, 30 (1947) 1.
4 G. EHRENSVÄRD, *Exptl. Cell Research, Suppl.*, 3 (1955) 102.
5 E. W. BASSETT AND S. W. TANENBAUM, *Biochim. Biophys. Acta*, 28 (1958), in the press.

[6] O. NEUBAUER AND L. FLATOW, *Z. physiol. Chem. Hoppe-Seyler's*, 52 (1907) 375.
[7] E. L. ELIEL, D. E. RIVARD AND A. W. BURGSTAHLER, *J. Org. Chem.*, 18 (1953) 1679.
[8] M. F. S. EL HAWARY AND R. H. S. THOMPSON, *Biochem. J.*, 53 (1953) 340.
[9] M. D. ARMSTRONG, K. N. F. SHAW AND P. E. WALLS, *J. Biol. Chem.*, 218 (1956) 293.
[10] H. K. BERRY, H. E. SUTTON, L. CAIN AND J. S. BERRY, *Univ. Texas Publ.*, No. 5109, (1951) 22.
[11] J. F. GROVE, J. MACMILLAN, T. C. P. MULHOLLAND AND M. A. T. ROGERS, *J. Chem. Soc.*, (1952) 3949.
[12] E. W. BASSETT AND S. W. TANENBAUM, *Experientia*, in the press.
[13] E. L. TATUM, S. R. GROSS, G. EHRENSVÄRD AND L. GARNJOBST, *Proc. Natl. Acad. Sci. U.S.*, 40 (1954) 271.
[14] S. GATENBECK, *Acta Chem. Scand.*, 11 (1957) 555.
[15] F. A. ISHERWOOD AND C. S. HANES, *Biochem. J.*, 55 (1953) 824.
[16] R. B. WOODWARD AND G. SINGH, *Experientia*, 6 (1950) 238.
[17] A. E. OXFORD AND H. RAISTRICK, *Biochem. J.*, 27 (1933) 634.
[18] E. D. AMSTUTZ, E. A. FEHNEL AND C. R. NEUMOYER, *J. Am. Chem. Soc.*, 68 (1946) 352.

Received October 17th, 1957

THE SIGNIFICANCE OF POTASSIUM AND SODIUM FOR THE SYNTHESIS OF GLYCOGEN IN THE ISOLATED RAT DIAPHRAGM

Z. DRAHOTA, M. KLICPERA AND R. ŽÁK

Institute of Physiology, Czechoslovak Academy of Sciences, Prague (Czechoslovakia)

The synthesis of glycogen at various K/Na ratios has been studied extensively by many authors, especially on slices of liver[1–5]. All the reports indicate that the optimal synthesis occurs in a medium containing a high concentration of potassium. A decrease of potassium ions in the medium leads in these experiments to a decrease in the synthesis of glycogen. TUERKISCHER AND WERTHEIMER[6] and STADIE AND ZAPP[7] have shown, however, that in the isolated diaphragm, optimal synthesis of glycogen takes place when there is a low concentration of potassium in the medium or when the medium is completely free from potassium.

In the experiments performed both on the liver slices and on the diaphragm, changes in the synthesis of glycogen were only determined in relation to the ion composition of the medium. The relationship between glycogen synthesis and the potassium or sodium content of the tissue was not studied. For this reason our attention was directed to the problem of the relation of glycogen synthesis to changes of potassium content in the tissue.

METHODS

Rats 80–100 g were used and the animals fasted for 24 hours prior to the experiment. The rats were decapitated and isolation of the diaphragm was performed according to RIESER[8]. In order to determine the glycogen synthesis in the same rat in a normal and modified medium, experiments were carried out with quarters of the diaphragm. It has been shown in a previous paper[9] that the diaphragm synthesizes glycogen to the same extent, irrespectively of whether halves or quarters were used. The ventral (thicker) quarter of the diaphragm was always used for the determination of the initial values of glycogen, while the dorsal (thinner) quarter was always used for incubation. It is necessary to adhere to this standard procedure, because the dorsal halves

References p. 35.