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Incorporation of ^{14}C-Acetate in the Phenolic Substances in *Penicillium Islandicum* Sopp

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The ^{14}C-distribution in 3-hydroxyphthalic acid, isolated from *P. islandicum*, grown on $CH_3{}^{14}COONa$, has been investigated. It was shown that the phthalic acid molecule is built up according to the acetate hypothesis. A new phenolic metabolite was studied and the anthraquinone content of the mycelium investigated which resulted in the identification of catenarin and emodin and the isolation of islandicin.

From previous studies [1,2] it is well known that acetate is the starting material for the formation of several aromatic compounds. The anthraquinone, emodin, belongs to those [3] and the present work will give evidence for an acetate coupling in other anthraquinones too.

In order to study the biosynthesis of anthraquinones a UV-mutant of *Penicillium islandicum*, strain N.R.R.L. 1036, was used, and the incorporation of ^{14}C-labelled acetate in the phenolic substances produced has been studied in the hope to find a precursor to the anthraquinone molecule among them. This special mutant shows a larger production of 3-hydroxyphthalic acid [4] and islandicin [5] than the wild type and it does not produce erythroskyrine [6] which in the wild type disturbs the localization of the anthraquinones on the paper chromatogram.

The culture medium contained very few appreciably labelled compounds. By developing an ether extract of the culture medium on paper chromatogram only three radioactive peaks could be detected by scanning the paper chromatogram with a G.M.-counter (Fig. 1). Two of these peaks were identified by chemical reagents as 3-hydroxyphthalic acid and a mixture of anthraquinones.

3-Hydroxyphthalic acid was isolated and its ^{14}C distribution investigated according to Fig. 2. The radioactive pattern of 3-hydroxyphthalic acid shows a strict conformity to the acetate theory.

The third radioactive peak contained a phenolic substance that has not previously been shown in *P. islandicum*. This substance which for the sake of brevity will be called compound A was isolated and its chemical properties

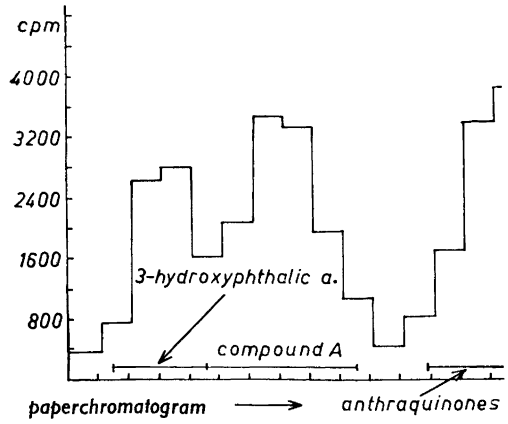

Fig. 1.

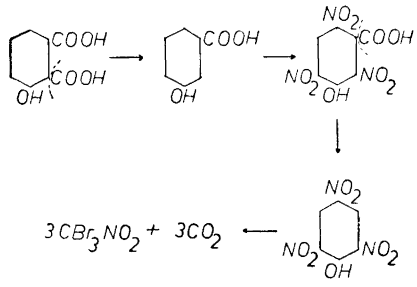

Fig. 2.

studied. It is very soluble in ethanol and acetone, slightly soluble in ether and water. It dissolves readily in 0.5 N $NaHCO_3$ with a slight yellow color and gives a gray brown coloration with ferric chloride solution. It has a strong reducing power indicating a hydroquinone structure. An interesting property of compound A is its behavior in concentrated sulfuric acid. By gentle warming it forms a new compound which shows all the reactions of an anthraquinone. It is orange in color and sublimes at 155—160°C in vacuum. It dissolves readily in 1 N NaOH and in 1 N Na_2CO_3 with a blue violet color. A pure blue color is obtained with alcoholic magnesium acetate. The anthraquinone has the following absorption maxima in ethanol, λ_{max} (mμ): 237.5, 258, 284, 301, 490, 515, 463—468 (inflexion). The solubility of the anthraquinone in 1 N Na_2CO_3 indicates that it has a β-hydroxyl group and the absorption spectrum points to a 1,4-position of two hydroxyl groups. The small amount of the sample has not allowed further analysis of the anthraquinone.

The compound A has a very sharp decomposition point at 212°C and shows no optical activity. Dithionite solution does not alter the compound and no anthraquinone is formed by thermal decomposition excluding any relationship to the hydrated anthraquinones rubroskyrin [6] and luteoskyrin [7].

The radioactive compound A was added to spores from *P. islandicum* suspended in a Czapek-Dox-agar medium. The culture was harvested after fourteen days. It was extracted with acetone and the extract put on a thick paper chromatogram. Two radioactive peaks were obtained by scanning the paper chromatogram with a G.M.-counter. One of them represented the unaltered compound A, the other one a yellow pigment with no anthraquinone properties. This pigment has not been further investigated.

The radioactive 3-hydroxyphthalic acid was tested in the same way as above in a spore suspension. No radioactive substance other than 3-hydroxyphthalic acid could be detected on the paper chromatogram under the experimental conditions employed.

The mycelium, cultivated on labelled acetate, was extracted with chloroform and the chloroform extract fractionated on a silica gel column. From one fraction, orange in UV-light, islandicin was isolated. The specific radioactivity of islandicin was compared with that of 3-hydroxyphthalic acid. The following values were obtained: islandicin, 10.1×10^4 counts min^{-1} $mmole^{-1}$ C-$atom^{-1}$; 3-hydroxyphthalic acid, 9.6×10^4 counts min^{-1} $mmole^{-1}$ C-$atom^{-1}$; *i. e.* they have about the same specific radioactivity indicating a similar acetate coupling in islandicin as there is in 3-hydroxyphthalic acid.

From a second fraction, violet in UV-light, small amounts of two further anthraquinones could be isolated after repeated running on thick paper chromatogram. One of them was catenarin which has recently been found by Shibata *et al.*[7] in *P. islandicum*. It was identified by the absorption spectrum and the melting point 246°C. The other anthraquinone was emodin, which was recognized by its R_F value on the paper chromatogram. Extraction of the emodin from the paper and adding unlabelled emodin as a carrier to the extract resulted in substantial amount of this compound. It could then be recrystallized to constant radioactivity. Emodin has earlier been found in higher plants and in higher fungi.

EXPERIMENTAL

Culture conditions. Four flasks, each containing 500 ml of Czapek-Dox medium, were cultivated according to Howard and Raistrick [5] for thirteen days. At that time a solution of 1 mC of $CH_3{}^{14}COONa$ was equally distributed to the four flasks and the growth was allowed to continue for further eight days. The mycelium was filtered off and washed with distilled water.

Culture medium. The culture medium was neutralized with solid Na_2CO_3 and concentrated in vacuum to about 600 ml. It was acidified with concentrated HCl and extracted four times with ether. The ether phases were extracted with 0.5 N $NaHCO_3$. The ether solution did not contain any radioactivity after the extraction. The hydrogen carbonate solution was acidified with HCl and extracted with ether. After drying the ether solution with anhydrous Na_2SO_4 it was evaporated to a small volume. The residue was placed on Whatman paper No. 3 and the chromatogram developed in a solvent containing chloroform-methanol-formic acid (4 %) (10 : 1 : 1). The 3-hydroxyphthalic acid (R_F about 0.15) was cut out and eluted with ethanol. The strongly colored ethanol extract was put on a silica gel column with water as the solvent. The 3-hydroxyphthalic acid fraction was collected and extracted with ether. The ether extract was then further purified by paper chromatography as described above. The ethanol solution was concentrated in vacuum and the concentrate evaporated to dryness at room temperature and atmospheric pressure. The substance isolated melted at 155—157°C and the melting point was not depressed on admixture with authe tic 3-hydroxyphthalic acid.

The compound A that was detected by its aborption in UV-light was cut out of the paper chromatogram and purified in the same way as 3-hydroxyphtalic acid.

Degradation of 3-hydroxyphthalic acid. (All radioactive measurements are listed in Table 1.) 311 mg of unlabelled, synthetic 3-hydroxyphthalic acid were added to the radioactive 3-hydroxyphthalic acid, isolated from the culture medium, and the mixture dissolved in acetone. The acetone solution was evaporated to dryness at room temperature. A portion of the substance was totally combusted according to van Slyke and Folch [8]. The carbon dioxide was trapped in a barium hydroxide solution. The barium carbonate was isolated and its radioactivity measured. Throughout the work the same amount of barium carbonate (15 mg) has been used for the measurements of the radioactivity and with the same apparatus as reported earlier.

Decarboxylation of 3-hydroxyphtalic acid. 100.9 mg of 3-hydroxyphthalic acid were dissolved in 20 ml of diluted H_2SO_4 (1:1). The solution was refluxed for 25 min. The

Table 1. ^{14}C-Distribution in 3-hydroxyphthalic acid.

Material	Number of C-atoms from 3-hydroxy-phthalic acid	Radio-activity counts/min	Total radio-activity of the material counts/min.	Numbers of COOH-groups according to the acetate theory	
				Found	Calculated
1	2	3	4	5	6
3-Hydroxyphthalic acid (total comb.)	8	739	5 912	3.96	4
COOH-Group (2-position)	1	1 495	1 495	1	1
m-Hydroxybenzoic acid (total comb.)	7	644	4 508	3.01	3
COOH-Group (1-position)	1	79	79	0.05	0
Picric acid (total) comb.)	6	734	4 404	2.95	3
Bromopicrin (C-atoms from 2,4,6-positions)	3	2	6	0	0
CO_2 from the hypo-bromite degradation (C-atoms from 1,3,5-positions)	3	1 361	4 083	2.74	3

The numbers of the COOH-groups in column 5 are obtained by taking the radioactivity of the COOH-group from 2-position as the basis for the calculations. This COOH-group is supposed to derive directly from the COOH-group in acetic acid.

top of the condensor was connected with a barium hydroxide solution. A stream of carbon dioxide free nitrogen was flushed through the system to force the evolved carbon dioxide into the barium hydroxide solution. The barium carbonate was isolated and its radioactivity measured. The sulfuric acid solution was diluted with water and then extracted four times with ether. The residue from the ether extract was recrystallized twice from water yielding 52.7 mg of *m*-hydroxybenzoic acid, m. p. 200°C. A part of the *m*-hydroxybenzoic acid was combusted as above while the remaining acid was diluted 5.22 times with nonradioactive *m*-hydroxybenzoic acid by recrystallization from 3.5 ml of water. All radioactive values listed have been corrected to correspond to the most diluted state.

Nitration of m-hydroxybenzoic acid. The nitration was made according to Wolffenstein and Paar [9]. 0.78 ml of HNO_3 (*d* 1.52) was added to 208 mg of *m*-hydroxybenzoic acid and the mixture evaporated to dryness on a steam bath. This procedure was repeated with the same amount of HNO_3. After extracting the residue three times with boiling benzene, 2,4,6-trinitro-*m*-hydroxybenzoic acid crystallized from the benzene solution. Yield 203 mg, m. p. 180°C.

Decarboxylation of 2,4,6-trinitro-m-*hydroxybenzoic acid.* 203 mg of trinitro-*m*-hydroxybenzoic acid were suspended in 5 ml of glycerol and the mixture refluxed for 20 min. The carbon dioxide was trapped in barium hydroxide solution and the radioactivity measured as above. The glycerol solution was diluted with water, acidified with HCl and extracted five times with ether. The ether solution was evaporated to dryness and the picric acid recrystallized from diluted sulfuric acid. Yield 130 mg, m. p. 120°C. About 10 mg of the picric acid were combusted and the radioactivity measured.

Hypobromite degradation of picric acid. 70 mg of picric acid were added at 0°C to a carbonate free solution of barium hypobromite (from 2.8 mg of barium hydroxide and 0.38 ml of bromine in 60 ml of water). After keeping the mixture at room temperature for 30 min, the bromopicrin was isolated by steam distillation, washed with diluted HCl and water, combusted as above and the radioactivity of the barium carbonate measured. The barium carbonate formed during the reaction was filtered off and washed with water. It was purified by recycling the carbon dioxide into a fresh solution of barium hydroxide. The barium carbonate was isolated and the radioactivity measured.

The mycelium. The mycelium was air dried and extracted with chloroform in a Soxhlet apparatus. The chloroform solution was concentrated to a small volume on the steam bath and then dried at room temperature. Islandicin, catenarin and emodin were isolated as described above. After sublimation and recrystallization from glacial acetic acid the yield of islandicin was 600 mg, m. p. 218°C.

The fractions from the silica gel column containing catenarin and emodin were developed on paper chromatogram (Whatman No. 3) in a solvent consisting of benzene-formic acid (2 %) (10 : 1). Catenarin has R_F 0.45 and emodin R_F 0.50. The anthraquinones were extracted from the paper with acetone and identified as already mentioned in this paper.

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