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On the Biosynthesis of the Pigments from *Penicillium islandicum* I

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The biochemical interrelationships of the pigments iridoskyrin, islandicin, rubroskyrin, luteoskyrin and skyrin in the mould *Penicillium islandicum* have been investigated. The specific radioactivity / time curves of the pigments have been determined after feeding the mould for a short time with ^{14}C-labelled acetate. Analysis of these curves indicates that the pigments are formed independently of each other.

The mould *Penicillium islandium* Sopp produces a set of various pigments of very close structural relationship to each other when grown on a medium with glucose as the sole carbon source. All the pigments are polycyclic compounds and have furthermore the anthracene structure in common. The dominant compounds are skyrin, rubroskyrin, luteoskyrin, iridoskyrin and islandicin. From the structures of these compounds (Fig. 1) one would anticipate a biological interconversion. Rubroskyrin and luteoskyrin are easily transformed *in vitro* by dehydrating agents or dithionite [1] to iridoskyrin so it is reasonable to assume them to be the biological precursors of iridoskyrin.

As shown in earlier papers [23] the actual pigments originate from acetate. In order to investigate their interconvertibility a series of experiments has been carried out on the incorporation of ^{14}C-labelled acetate into these pigments in addition to studying the production sequence of the pigments (expt. A). The variations in time of their specific radioactivities were followed (expt. B). In other experiments (C and D) the specific radioactivities were determined after replacement of the labelled substrate with normal Czapek-Dox medium.

EXPERIMENTAL

Experiment A. A Petri dish (5 × 15 cm) containing 100 ml of Czapek-Dox medium was sterilized and inoculated with a spore suspension of *Penicillium islandicum* Sopp, N.R.R.L. 1036. After two days the whole surface of the medium was covered by a white mycelium. At eight hourly intervals a piece of the mycelium (*ca.* 3 × 3 cm) was cut out and thoroughly extracted with acetone. A portion of the acetone solution was developed on a paper chromatogram in a solvent containing benzene-2 % formic acid (10:1). The

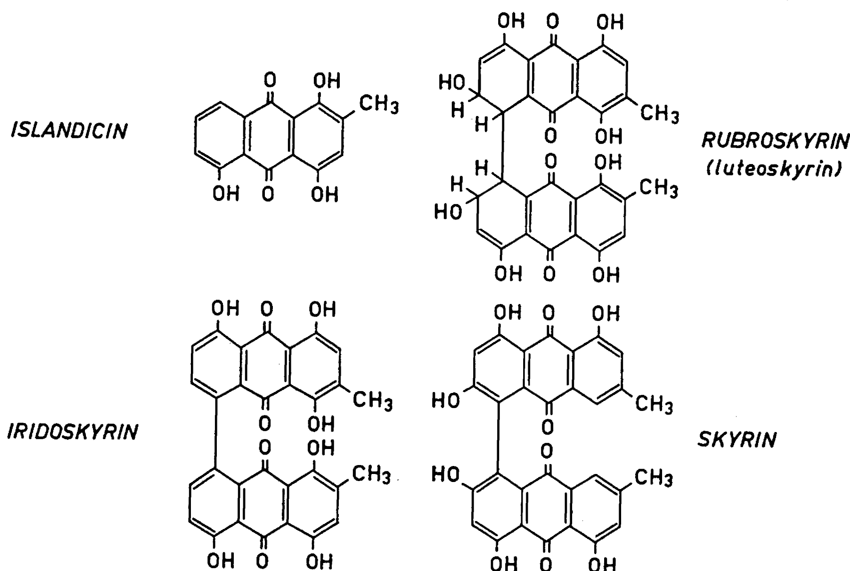

Fig. 1.

different pigments were identified by their R_F-values, color and color reactions with methanolic magnesium acetate solution (Table 1). The pigment that appeared first was skyrin. It was not possible to detect any other pigment until 24 h after the appearance of skyrin. The experiment was repeated with shorter intervals between the withdrawals of the test samples but even with one hourly intervals it was not possible to determine any production order in time of rubroskyrin, luteoskyrin, iridoskyrin and islandicin.

Experiment B. A culture was grown for seven days in the same way as in experiment A. After the addition of 0.25 mC $CH_3{}^{14}COONa$ (5 mC/mmole) samples were taken from the mycelium at different times as described above. Every sample was extracted with acetone, the acetone solution evaporated to dryness and the residue triturated several times with petroleum ether (b. p. 40—60°C) in order to remove the lipids. After dissolving the pigments in acetone, two equal portions were withdrawn, one of which was then developed on a paper chromatogram. The total radioactivity of the iridoskyrin-islandicin spot was measured directly on the paper. The pigments were extracted from the paper with 3 ml of HCl-acidified acetone. Their concentration was measured in a Hilger Uvispek spectrophotometer at 492 mμ. Iridoskyrin and islandicin have their absorption maxima at the same wavelengths but the molar extinction coefficient of iridoskyrin is twice that of islandicin (log ε_{492} 4.09). The molar specific radioactivity of the mixture iridoskyrin-islandicin was calculated, the amount being expressed as islandicin.

The other portion of the acetone solution was evaporated to dryness and 1 ml of concentrated H_2SO_4 added. After keeping at room temperature for 20 h it was diluted with water and exhaustively extracted with ether. The extract was concentrated to a small volume and developed on a paper chromatogram in the same solvent as above. The radioactivity and the amount of the mixture of the natural iridoskyrin-islandicin and the iridoskyrin obtained from rubroskyrin and luteoskyrin by the treatment with H_2SO_4 was measured as above. After subtraction of the radioactivity and the amount of natural iridoskyrin-islandicin, the specific radioactivity of rubroskyrin-luteoskyrin was calculated. The values are given in Fig. 2.

Table 1.

Compound	R_F-value (benzene-formic acid	Color	Color with meth. Mg-acetate
Islandicin	1.00	Pink	Purple
Iridoskyrin	0.95	Pink	Purple
Skyrin	0.15	Yellow	Violet
Rubroskyrin	0.20	Red	Green
Luteoskyrin	0.30	Yellow	—

It was also desirable to know the molar specific radioactivity of skyrin. In the solvent used, skyrin and rubroskyrin overlap on the paper chromatogram and no other solvent could be found which would separate them completely. In order to obtain a true value of the specific radioactivity, skyrin was cleaved to emodin which is easily separated from the other pigments by paper chromatography. The cleavage was performed according to Raistrick [4] by dissolving an aliquot of the acetone extract in 1 N NaOH and treating the solution with sodium dithionite for one hour at room temperature. After acidification with concentrated HCl the solution was extracted with ether and the ether phase developed on a paper chromatogram in benzene-formic acid. The radioactivity and the amount of emodin was then determined as above (log ε_{437} 4.10).

Experiment C. The mould was cultured in a Petri dish as described in experiment A. After seven days an aqueous solution of 0.25 mC of $CH_3{}^{14}COONa$ was added to the medium. The radioactive medium was withdrawn with a pipette after half an hour and fresh Czapek-Dox solution added after washing the mycelium three times with sterilized, distilled water by injecting the water beneath the mycelium. Test samples were taken from the mycelium over a period of 10 h. The pieces of mycelium were extracted with acetone, the lipids removed and the extract developed on a paper chromatogram as above. The molar specific radioactivity of iridoskyrin-islandicin was determined. The region on the paper containing rubroskyrin and luteoskyrin was cut out and the pigment extracted from the paper with acetone. The acetone solution was evaporated to dryness, treated with concentrated H_2SO_4 for 20 h and the molar specific radioactivity determined after further chromatographing as in expt. B. The values are given in Fig. 3.

Experiment D. Expt. C was repeated with the modification that islandicin was separated from iridoskyrin by sublimation in vacuum at 160°C. The residue from the sublimation consisting of iridoskyrin was resublimed in vacuum at 270°C. The molar specific radioactivities of islandicin and iridoskyrin were then determined separately. As the culture in this experiment produced unusally large amount of luteoskyrin this pigment was isolated by paper chromatography and its molar specific radioactivity determined without prior conversion to iridoskyrin. The concentration of luteoskyrin was measured at 447.5 mμ (log ε 4.46). The results of expt. D are shown in Fig. 4.

RESULTS AND DISCUSSION

The values in Fig. 2 show that the molar specific radioactivity of naturally occurring iridoskyrin-islandicin is definitely higher than that of rubroskyrin-luteoskyrin. If rubroskyrin-luteoskyrin were precursors of iridoskyrin-islandicin they should have a higher specific radioactivity than iridoskyrin-islandicin or at least the same. While the results of expt. B immediately exclude this possibility they do not argue against the reverse mechanism.

Although expt. C shows a rather high incorporation of labelled acetate after half an hour, experiment A and repeated experiments of the same type indicate that the radioactivity of the pigments has not reached the steady state within this time.

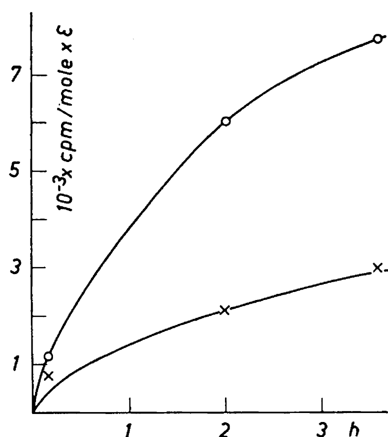

Fig. 2. Specific radioactivity/time curves, iridoskyrin-islandicin O——O, rubroskyrin-luteoskyrin ×——×.

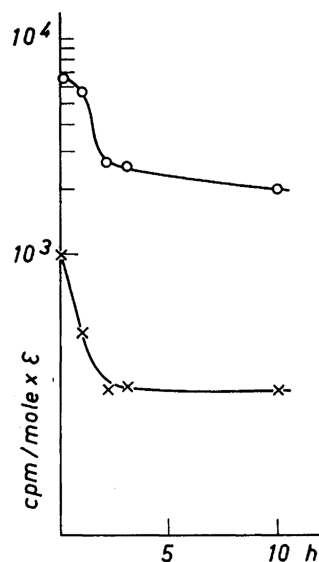

Fig. 3. Specific radioactivity/time curves, iridoskyrin-islandicin O——O, rubroskyrin-luteoskyrin ×——×.

If the iridoskyrin-islandicin mixture was transformed to rubroskyrin-luteoskyrin by a hydration reaction, the specific radioactivity of the former should decrease relative to that of the latter. As seen from Fig. 3 the specific radioactivities of iridoskyrin-islandicin and rubroskyrin-luteoskyrin decrease almost at the same rate and this result has been obtained in several repeated

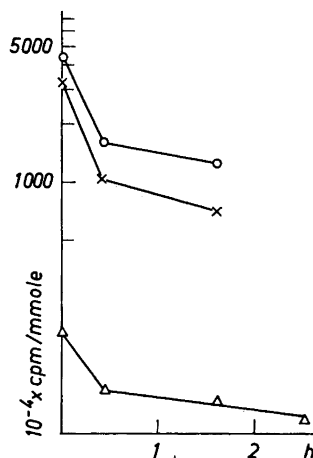

Fig. 4. Specific radioactivity/time curves, islandicin O——O, iridoskyrin ×——×, luteoskyrin Δ——Δ.

experiments. This is in accord with the independent formation of these pigments.

Expt. D confirms the conclusions derived from expt. C that there is no interconvertibility of the anthraquinones and their hydrated forms. Furthermore, expt. D gives the surprising result that islandicin is not the immediate precursor of its dimer, iridoskyrin. The parallel specific radioactivity/time curves of these compounds in Fig. 4 indicate that the biosynthesis of one pigment is wholly independent of the production of the other one.

Emodin, which is equivalent to skyrin, has about the same specific radioactivity as rubroskyrin-luteoskyrin and is consequently not a precursor of iridoskyrin-islandicin. The early formation of skyrin indicates that it has a separate position in the biosynthesis of the pigments. As skyrin occurs in other strains of *Penicillium islandicum*[4] and in other moulds, *e.g. P. rugulosum*[5], *P. wortmanni*[5], *Endothia parasitica* and *E. fluens*[6], which do not produce rubroskyrin or luteoskyrin, it is assumed, that skyrin has no connection with these pigments.

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