ACTA CHEMICA SCANDINAVICA 13 (1959) 1561–1564

Acetate Carboxyl Oxygen (^{18}O) as Donor for Phenolic Hydroxy Groups of Orsellinic Acid Produced by Fungi

STEN GATENBECK and KLAUS MOSBACH

Institute of Biochemistry, University of Lund, Lund, Sweden

Oxygen-18 labelled sodium acetate was administered to the culture medium of the orsellinic acid producing fungus *Chaetomium cochliodes* Pall. The ^{18}O content in the carboxyl group and the hydroxy groups of orsellinic acid was determined. A high degree of incorporation was found, where the ^{18}O content of the carboxyl group was half that of each hydroxy group. These results confirm the hypothesis of a β-polyketoacid as an intermediate in the biosynthesis of orsellinic acid.

During the last five years it has been shown by several investigators [1-4] that the biogenesis of some aromatic substances proceeds by head to tail coupling of acetate units. However, from these results nothing can be said with any certainty about the intermediate steps in the course of the biosynthesis of the final aromatic substance from acetate.

Structural analysis of naturally occurring phenolic compounds proposes an intermediate of β-polyketo character [5,6]. The distribution of the oxygen atoms in for example orsellinic acid corresponds exactly to the sequence of the carbonyl groups in an unbranched β-polyketo acid (Fig. 1).

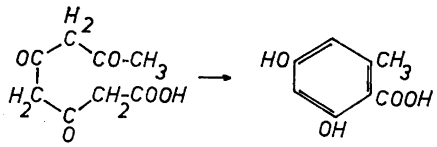

Fig. 1.

If there is a direct condensation of acetate units to the postulated β-polyketo acid, administration of ^{18}O labelled acetate to the orsellinic acid [7] producing fungus *Chaetomium cochliodes* should result in ^{18}O-labelling of the orsellinic acid.

EXPERIMENTAL

Preparation of $CH_3-{}^{14}CO^{18}ONa$. Oxygen-18 labelled water (344 mg) with 90 atom% of ^{18}O was slowly added to a slight excess of the equivalent amount of icecold acetyl chloride. The acetic acid and the hydrochloric acid formed were neutralized with 1 N NaOH by titration. To this solution 1 mC (17 mg) of ^{14}C-carboxyl labelled CH_3COONa was added. A representative sample of the mixture was evaporated to dryness and its isotope content measured.

Culture conditions. Four culture flasks, each containing 500 ml of Czapek-Dox medium, were inocculated with perithecia from *Chaetomium cochliodes* Pall. The culture medium was removed by suction after 20 days incubation at 28°C in the dark. Care was taken not to rupture the mycelium. Fresh Czapek-Dox medium and the labelled acetate were added after rinsing the mycelium twice with distilled water. The incubation was continued for further 8 days.

Isolation of orsellinic acid. The mycelium was filtered off. The culture medium after taking out a small volume for ^{18}O analysis, was acidified with HCl and extracted three times with ether. The residue (300 mg) of the evaporated ether extract was recrystallized from diluted acetic acid yielding orsellinic acid (150 mg), m.p. 176°C.

Determination of ^{14}C. A part of the dry sample of the sodium acetate mixture was combusted to CO_2 according to van Slyke and Folch [8]. The carbon dioxide was trapped in carbonate-free barium hydroxide solution. The barium carbonate formed was isolated and its radioactivity measured with a G.M.-counter. The found radioactivity was 52 600 cpm/mg C. Thus the radioactivity of the carboxyl group will be 2 × 52 600 cpm/mg C.

The same procedure was undertaken with orsellinic acid. The found radioactivity after the total combustion was 2 200 cpm/mg C. As orsellinic acid is built up by four acetate units, which has been shown by one of us (M)[9], the labelled C atoms in the molecule will contain 2 × 2 200 cpm/mg C.

Determination of ^{18}O. The analytical data of all the ^{18}O measurements are listed in Table 1.

The oxygen of the sodium acetate was isolated as carbon dioxide which was obtained after cracking the substance in the presence of heated (1 100°C) carbon and oxidation of the formed carbon monoxide with iodine pentoxide to carbon dioxide according to Unterzaucher [10]. One of the oxygen atoms in the resulting carbon dioxide will thus be derived from the test sample. The liberated iodine was fixed on a slightly heated silber gauze and the carbon dioxide flushed with nitrogen into a clear solution of octadecylamine in petroleum ether (b.p. 40–60°C). The precipitate of octadecyl carbamate was isolated by centrifugation and dried in vacuum over solid paraffine. The dry octadecyl carbamate was used directly for measurement in a masspectrometer whereby the carbon dioxide was released by heating the carbamate to about 100°C. The same procedure of isolating the oxygen as carbon dioxide has been applied throughout this work. The use of octadecylamine as absorbing agent for carbon dioxide has been introduced by Ehrensvärd [18]. This method has turned out to be most convenient for ^{18}O determinations.

The spared test sample of the culture medium was distilled. A few milligrams of the water were taken out, transformed to carbon dioxide in the same way as described above and the ^{18}O content measured.

The average ^{18}O content of orsellinic acid was determined from 5 mg of the substance.

For decarboxylation of the orsellinic acid, 30 mg were heated in a stream of nitrogen in a quartz tube at 180°C for 20 min. The formed carbon dioxide was driven over into a solution of octadecylamine in petroleum ether and its ^{18}O content measured.

The residue consisting of orcinol, shown to be pure by paper chromatography, was analyzed for the ^{18}O amount in the two remaining hydroxy groups.

RESULT AND DISCUSSION

The data in Table 1 show that the ^{18}O from the administered acetate has been incorporated to a high degree in orsellinic acid.

This confirms the hypothesis of direct condensation of activated acetate units to orsellinic acid. Furthermore, a condensation of the acetate *via* acetyl-

Table 1. ^{18}O and ^{14}C analysis.

Samples analyzed	Average ^{14}C- radio-activity	Radio-activity of labelled C-atoms	^{18}O-content	^{18}O-content	$^{18}O/^{14}C \times 10^4$	Dilution ^{18}O *versus* ^{14}C
	cpm/mg C	cpm/mg C	atom %	atom % excess		
Standard			0.20	0.00		
Sodium acetate	52 600	105 200	33.20	33.00	3.13	1
H_2O (culture medium)			0.20 (3)	0.00 (3)		
Orsellinic acid	2 200	4 400	0.72	0.52		
Carboxyl group		4 400	0.59	0.39	0.89	3.6
Orcinol		4 400	0.92	0.72	1.64	1.9

coenzyme A should end up in a hydrolysis of the terminal thiolester. The atom % excess of ^{18}O in the carboxyl group should then be half that of the hydroxy groups in the orsellinic acid. This is also confirmed by our experiment (COOH: 0.39 atom % excess; OH: 0.72 atom % excess ^{18}O).

By comparing the radioactivity in the carboxyl group of the original sodium acetate with that in orcinol a dilution of 24 times is observed whereas the ^{18}O excess of the carboxyl group is diluted 46 times. The ratio $^{14}C/^{18}O$ dilution is almost 1:2 giving place for speculations since the activation of the acetate through the adenosine triphosphate-coenzyme A system would not give rise to dilution of the acetyl oxygen unless there is hydrolysis of the intermediates. Of course there is the possibility of exchange of the oxygen with water before activation of the acetate. Nevertheless, the ratio 1:2 invites one to think of an organized exchange of the acetyl oxygen. It could be explained by a hydratization of one of the intermediates or there could be an hitherto unknown intermediate step in the course of acetate activation.

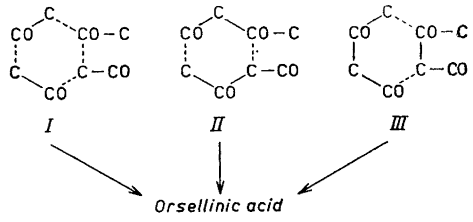

Fig. 2. Alternative pathways of the condensation of acetate to orsellinic acid. I. Successive condensation of acetyl-coenzyme A. II and III. Two different ways of formation of orsellinic acid from acetoacetyl-coenzyme A.

The condensation of the acetate units to orsellinic acid may proceed in one of two possible ways, either by successive condensation of four C-2 units presumably acetyl-coenzyme A or by coupling of two C-4 compounds like acetoacetyl-coenzyme A (Fig. 2). The alternative that a β-polyketo unit underlying the synthesis of orsellinic acid is formed by an oxidative mechanism acting upon a long chain fatty acid could be excluded, since in that case the oxygen atoms entering in successive β-positions should be nonlabelled in view of the very low ^{18}O content of the water in the culture medium (0.003 atom % excess).

It is reasonable to draw a parallel between orsellinic acid and fatty acid synthesis. In the first case the acetate condensation stops at the keto level whereas in the fatty acid synthesis the keto stage is further reduced *via* a DPN-system.

The high degree of incorporation of the ^{18}O from the acetate into the phenolic hydroxy groups of orsellinic acid seems to be promising for a further use of this procedure as a common method of distinguishing between oxidatively and non-oxidatively formed hydroxy groups in compounds derived from acetic acid.

The authors are much indebted to Prof. G. Ehrensvärd for valuable discussions and to Dr. C. Karrman for putting the resources of his analytical department at our disposal.

REFERENCES

1. Birch, A. J. *et al. Austr. J. Chem.* **8** (1955) 539; *J. Chem. Soc.* **1958** 360.
2. Thomas, R. *Proc. Chem. Soc.* **1959** 88.
3. Gatenbeck, S. *Acta Chem. Scand.* **12** (1958) 1211, 1985.
4. Schwenk, E., Alexander, G. J., Gold, A. M. and Stevens, D. F. *J. Biol. Chem.* **233** (1958) 1211.
5. Robinson, R. *Structural Relations of Natural Products*, Clarendon Press 1955.
6. Birch, A. J. and Donovan, F. W. *Austr. J. Chem.* **6** (1953) 360.
7. Mosbach, K. *Z. Naturforsch.* **14 b** (1959) 69.
8. *Cf.* Calvin, Heidelberg, Reid, Tolbert and Yankwich, *Isotopic Carbon* Wiley, New York 1949, p. 92.
9. Mosbach, K. *To be published.*
10. Unterzaucher, J. *Ber.* **73** (1940) 391; *Mikrochemie ver. Mikrochim. Acta* **36/37** (1951) 706.
11. Ehrensvärd, G. *In press.*

Received June 9, 1959.