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Cell-free C-Methylation in Relation to Aromatic Biosynthesis

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Feeding experiments with methyl-labelled methionine have demonstrated that methyl groups attached to aromatic nuclei in mould products originate from C-1 metabolism in many cases. Lederer and his collaborators are studying extensively the mechanism of C-methylation, and they have recently been able to synthesize menaquinone by methylation of demethylmenaquinone in a cell-free system using *S*-adenosylmethionine as the methyl donor.[1] So far, however, the enzymic introduction of a C-methyl group into an aromatic structure has not been reported.

The mould *Aspergillus flaviceps* produces a series of aromatic compounds,[2,3] *e.g.* 5-methylorsellinic acid, flavipin, and, in low yield, orsellinic acid. A conceivable biological relation between these compounds would be the following sequence: orsellinic acid ⟶ 5-methylorsellinic acid ⟶ flavipin.

In order to study the C-methylation of orsellinic acid on an enzymatic basis, a protein fraction was prepared from *A. flaviceps*. The mould was grown for 24 h at 28° on a shake table in Czapek-Dox medium. The mycelial growth was collected and washed with buffer solution (tris 0.2 M, Na phosphate 0.05 M, NaCl 0.2 M, pH 7.8) before homogenization in an X-press. From the supernatant obtained after centrifugation of the homogenate for 40 min at 20 000 *g* and +2°, a protein fraction was precipitated by ammonium sulfate (up to 35 % of saturation) and discarded. Continued precipitation with ammonium sulfate (60 % of saturation) yield the protein fraction used in this investigation.

This protein fraction, dissolved in buffer solution at pH 7.8, was incubated with *S*-adenosylmethionine-$^{14}CH_3$ with and without added orsellinic acid in the presence of dithiothreitol. Although no significant difference in radioactivity was observed in ether extracts from acidified incubation mixtures with and without added orsellinic acid, they were

both highly radioactive indicating a transfer of the methyl group from *S*-adenosylmethionine to a substance present in the protein fraction which was extractable into ether under these conditions. The methylated product obtained in the different experiments was identified as 5,6-dimethylresorcinol by recrystallisation to constant specific radioactivity from benzene, after the addition of nonlabelled 5,6-dimethylresorcinol to the sublimed residues of the ether extracts. Sublimation was performed to decarboxylate any 5-methylorsellinic acid formed to yield the commercially available 5,6-dimethylresorcinol thus simplifying our analytical problem. The substrate for methylation which is attached to the protein, could not be easily separated from the protein by dialysis or gel chromatography. However, acidification of the protein solution or treatment with urea released the substance, which could then be taken up in ether and reused as substrate.

By growing the mould in presence of acetate-1-^{14}C, a minute amount of substrate of very high specific radioactivity was obtained. Recrystallization of the substrate, labelled from acetate, in the presence of nonlabelled orsellinic acid (or orcinol after sublimation) resulted in a rapid disappearance of radioactivity in the crystallized product, showing that the substrate was not identical with orsellinic acid (or orcinol). However, after the labelled substrate had been refluxed for 30 min in an alcoholic solution of KOH, the presence of orcinol was demonstrated with the washing-out method used above. A similar conversion has been described by Bentley and Zwitkowits [4] in the transformation of tetraacetic lactone (TAL) into orcinol under the same conditions. Thin-layer chromatography in different solvents and recrystallizations to constant specific radioactivity with nonlabelled TAL, prepared from *Penicillium stipitatum*, as described by Bentley and Zwitkowits, confirmed the presence of TAL in the substrate released from the protein.

When TAL was added to the incubation mixture for enzymic methylation only a weak response was obtained which was not comparable to that of the endogenous substrate. Apparently TAL is a poor substrate, and it does not seem to be the precursor of C-methylated orsellinic acid. A possible precursor would then be the non-lactonized tetraacetic acid. This acid has been synthesized and obtained in a crude preparation by Harris and Carney [5] who also described its transformation into orsellinic acid under very mild conditions *i.e.* at pH 5 in aqueous buffer for 16 h at 25°. No further characteristics have been reported as yet for non-lactonized tetraacetic acid.

The ^{14}C-labelled endogenous substrate was extracted with ether after urea treatment of the protein fraction dissolved in phosphate buffer at pH 5. The labelled material was then reextracted into the pH 5 buffer, and the mixture was left for 16 h at room temperature. In an ether extract from this solution the formation of orcinol was demonstrated as described earlier. This result suggests the presence of tetraacetic acid in the protein fraction. According to Bentley and Zwitkowits [4] TAL is not converted into orsellinic acid under these conditions. In trying to obtain further evidence for the identity of tetraacetic acid, a sample of the labelled protein fraction was heated in 6 M HCl for 30 min. The acid solution was neutralized with $NaHCO_3$ and then thoroughly extracted with ethyl acetate after addition of 2,6-dimethylpyrone. Sublimation and recrystallizations from ligroin demonstrated that a substantial conversion of the labelled material into 2,6-dimethylpyrone had taken place during the treatment with acid. When TAL is treated in the same way, orcinol is the major product formed but 2,6-dimethylpyrone also appears.

As aromatic amines are known to catalyze the decarboxylation of β-keto acids, the released labelled substrate was dissolved together with oxalacetic acid as carrier in an aniline citrate solution at pH 5 and then treated for 30 min at room temperature. The CO_2 evolved was trapped in aqueous $Ba(OH)_2$. The $BaCO_3$ formed contained radioactivity indicating the presence of a carboxyl-labelled β-keto acid. TAL is not decarboxylated under these conditions.

The results obtained suggest that no C-methylation of an aromatic nucleus is involved in the formation of 5-methylorsellinic acid but that the methylation occurs on a tetraacetic acid structure bound to a protein prior to cyclization. It is possible that the synthetase participates in the formation of the polyketide intermediates from acetate and malonate residues. The occurrence of TAL in the released endogenous substrate may be an artefact of the preparation procedure.

Considering some experimental data reported from studies on whole cells, this type of mechanism for the formation of a C-methylated aromatic product may have a broader applicability. To be mentioned are the mutant studies in the tetracycline series [6] where the wild type cannot transform the nonmethylated cyclized inter-

mediate into tetracyclines and the failure to introduce methyl groups into resacetophenone for the formation of clavatol in *Aspergillus clavatus*.[7] Recently Steward and Packter [8] reported similar conclusions from their studies on gliorosein formation in *Gliocladium roseum*.

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Corrigendum to "4-Methylsulphonylbutylglucosinolate Ion, the Natural Thioglucoside Precursor of Erysolin"*

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Through a regrettable oversight, formula (II), p. 2875 is erroneously drawn: the hydroxy-substituents at C-2, C-3, and C-4 in the pyranose-ring should be reversed.

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Hydrothermal Preparation of Tellurium Compounds

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The tellurates M_3TeO_6 containing the metals Cu, Zn, and Hg, the silver tellurate, Ag_6TeO_6, and the lead tellurate, $Pb_2H_2TeO_6$, were prepared by Jander and Kienbaum [1] using precipitation from aqueous solutions. The precipitation of the compounds is mostly initiated by the formation of amorphous tellurates of varying compositions, which on aging yielded crystalline products. The tellurates M_3TeO_6 containing the metals Mg, Mn, Ni, and Cu were prepared by Bayer [2] using solid-state reactions from corresponding reagent-grade oxides. The tellurates M_2TeO_6 of Sc, In, Y, and the rare-earth elements were prepared by Natansohn [3] by reacting the sesquioxides and orthotelluric acid at elevated temperatures.

Strontium tellurite, $SrTeO_3$, H_2O, was prepared by Ivankova, Samplavskaya, and Karapet'yants [4] by precipitating a sodium tellurite solution with a strontium nitrate solution. The product obtained showed an endothermic effect in the temperature range 250—400°C, associated with the loss of water.

Only a limited number of tellurium compounds have been prepared by using hydrothermal technique. Compounds of the composition $MTeO_2(OH)_3$ and $MTeO_3OH$ containing K and Rb were hydrothermally prepared by Lammers.[5] Two modifications of tellurium oxide, TeO_3, and polymetatelluric acid $(TeO_3,H_2O)_n$ were prepared by Maurin and Moret [6] using hydrothermal technique.

We wish to report the hydrothermal synthesis of the tellurium compounds listed in Table 1.

Expts. Nos. 1—5. A solution of 0.01 M orthotelluric acid and solutions of 0.03 M of the respective metal ions were used. 20 ml of the metal ion solution was mixed with 20 ml of the orthotelluric acid solution in a silver ampoule and the precipitate was heated with the mother liquid in a 100 ml pressure bomb, as indicated in Table 1. The crystalline prod-