AN ENZYME SYSTEM FOR ALIPHATIC METHYL KETONE OXIDATION[1]

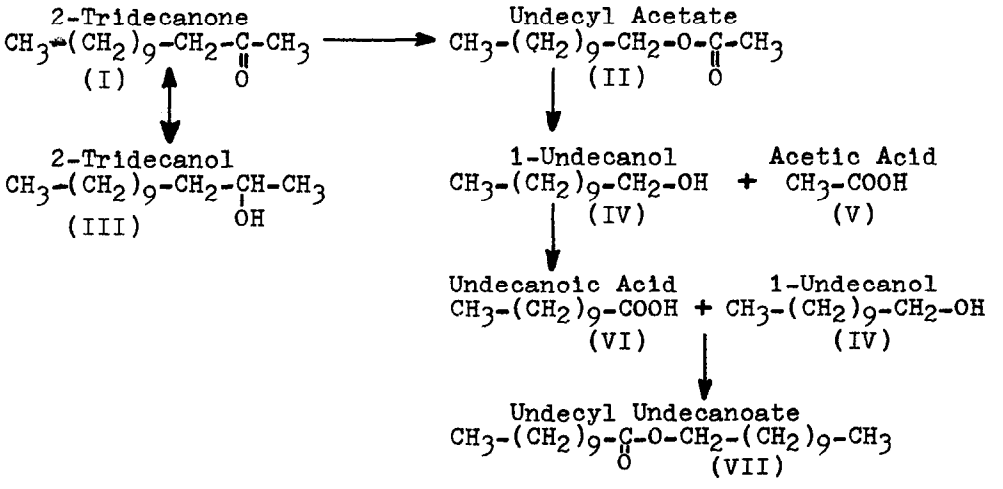
F. W. Forney and A. J. Markovetz
Department of Microbiology
University of Iowa, Iowa City

Received August 1, 1969

SUMMARY

Enzymatic conversion of 2-tridecanone to undecyl acetate has been achieved with a crude cell-free system prepared from Pseudomonas aeruginosa. The activities of several other enzymes present in unfractionated, unsupplemented supernatants of this bacterium also are reported.

2-Tridecanone biodegradation by whole cells of Pseudomonas multivorans and P. aeruginosa proceeds via oxidation to undecyl acetate which is cleaved to 1-undecanol and acetate; the former cleavage product accumulates whereas the latter furnishes carbon and energy for cellular metabolism (1,2). This preliminary communication provides evidence that cell-free enzymes obtained from methyl ketone-grown P. aeruginosa, when supplemented with NADH or NADPH in the presence of O_2, carry out a reaction sequence visualized as:



[1]This investigation was supported by grant GB-6875 from the National Science Foundation.

Cells were harvested from an 11-hour culture grown with aeration at 30 C in a mineral salts medium with 2-tridecanone as carbon source and were broken by sonic oscillation for 2 min at 120 watts in 0.05 M phosphate buffer, pH 7.5, containing 0.01 M ascorbic acid and mercaptoethanol. Centrifugation at 35,000 x g for 30 min yielded a supernatant which was employed as the enzyme source. The experimental system was constituted in the same buffer to a final volume of 50 ml and contained the following reagents: enzyme extract (95 mg protein), tridecan-2-one-3-C^{14} (0.2 mmole; specific activity, 0.088 mc/mmole), $FeSO_4 \cdot 7H_2O$ (0.025 mmole), glucose-6-phosphate (0.16 mmole), glucose-6-phosphate dehydrogenase (20 units) prepared according to DeMoss (3), and the following supplemental cofactors: either 0.5 µmole FMN and 0.03 mmole NAD (No. 1), 0.5 µmole FAD and 0.03 mmole NAD (No. 2), 0.5 µmole FMN and 0.03 mmole NADP (No. 3), 0.5 µmole FAD and 0.03 mmole NADP (No. 4), 0.03 mmole NAD (No. 5), 0.03 mmole NADH (No. 6), boiled extract and 0.03 mmole NAD (No. 7), enzyme extract only (No. 8), or substrate only (No. 9). Reaction mixtures were agitated gently for 12 hr at 30 C in an incubator-shaker; after incubation they were extracted with diethyl ether and the extracts were analyzed by routine thin-layer chromatographic (TLC) techniques.

Figure 1 presents typical chromatographic results of the qualitative assays described above. Undecyl acetate (II) was produced in several of the reaction mixtures, as shown in Analyses 1, 2, 3, and 4. Furthermore, Analyses 1 through 5 each show that these mixtures contained (VII) which provides indirect verification for enzymatic oxidation of (I) because (VII) could arise only by prior conversion of (I) to (IV) and (VI) and by subsequent esterification of the latter two products. The addi-

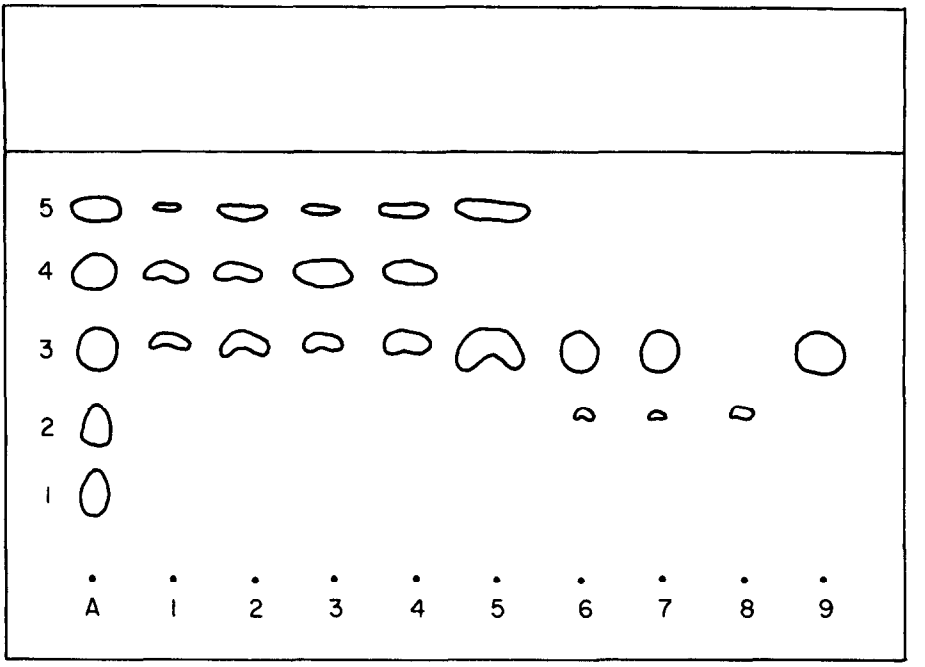


Figure 1. Thin-layer chromatogram of a qualitative assay for 2-tridecanone oxygenase in a crude cell-free preparation of P. aeruginosa. One-tenth mg of each reference compound was spotted at (A): undecyl undecanoate (5), undecyl acetate (4), 2-tridecanone (3), undecanoic acid (2), and 1-undecanol (1). Experimental extracts, without solvent, were spotted in 10-µliter amounts at positions corresponding to individual assay mixtures numbered and described in the text. The solvent system employed was 90:10:2 (v/v/v) hexane-diethyl ether-acetic acid and the spots were visualized under UV light after spraying with ethanolic 2',7'-dichlorofluorescein.

---

tion of a NADH- or NADPH- generating system is required to visualize oxidation of (I) because no products were detected in reaction mixture 6 (Analysis 6) which was provided with NADH in the absence of a generating system. Analysis 7, when compared with Analysis 5, illustrates that boiled protein did not substitute for native protein in the reactions observed. Analyses 8 and 9 serve to confirm that neither enzyme extract nor substrate was the source of the products obtained. Both (II)

and (VII) were verified to be present in the assay extracts by identifying them on an FFAP column employed for routine gas-liquid chromatographic (GLC) analyses during which the experimental compounds were compared with synthetic reference compounds, as previously described (2).

Further evidence for the identity and origin of the enzymatic products observed by TLC assay was obtained in the following manner. Both (II) and (VII) were isolated by preparative TLC from the assay extracts. These isolated products were spotted on a thin-layer plate, the plate was developed to give visible spots, and the silica gel containing the compounds was scraped off, placed in 15 ml of toluene-based scintillator fluid and assayed for radioactivity by liquid scintillation spectrometry. The remainder of each product was hydrolyzed separately and the two hydrolytic mixtures were extracted with organic solvent. These extracts were analyzed by TLC and the resulting visible products were scraped off and counted. No effort was made to quantitate the radioactivity in any of the products.

Figure 2 presents the composite results of TLC and scintillation analyses. Not only were the esters radioactive which indicates they originated from tridecan-2-one-3-C^{14}, but they each yielded the expected hydrolytic products which were also radioactive. In addition, the identity of (IV) arising from (II), and the identities of (IV) and (VI) arising from (VII), were confirmed by GLC analysis.

In the course of GLC analyses, (III) was detected in low concentration in several of the enzyme assay extracts. In fact, assay mixture 5, which had been supplemented with NAD and a NADH-generating system, contained relatively more (III) than the other mixtures. This strongly implicates NADH as a stimulating

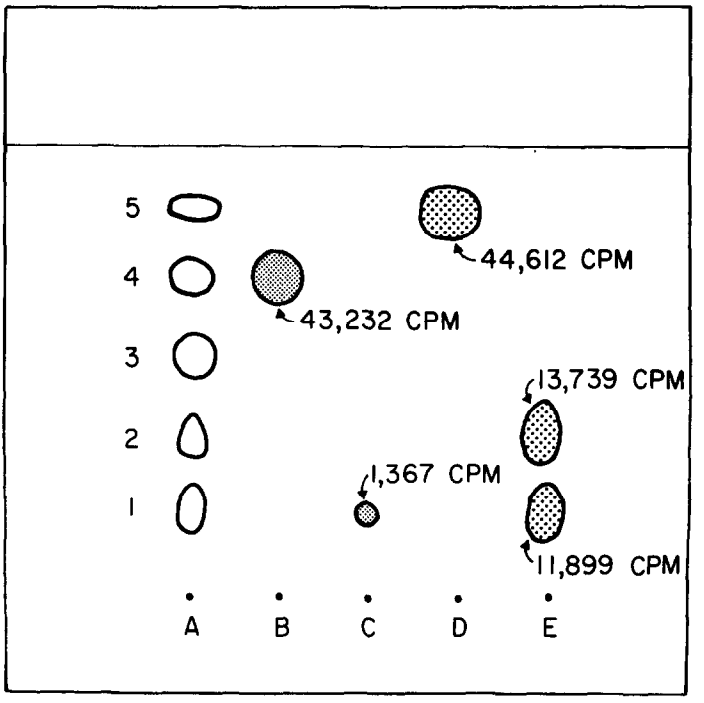


Figure 2. Thin-layer chromatogram of purified, radioactive esters produced by a crude cell-free preparation of P. aeruginosa, and of their hydrolytic products. Reference compounds were spotted at (A) as described in the legend of Figure 1. Experimental esters and their hydrolytic products, each dissolved in the noted volume of hexane, were spotted in 5-µliter amounts as follows: (B) undecyl acetate in 0.5 ml, (C) hydrolyzed (B) in 0.3 ml, (D) undecyl undecanoate in 0.5 ml, and (E) hydrolyzed (D) in 0.1 ml. Development and visualization of the spots was the same as for Figure 1; determination of their radioactivity is described in the text.

cofactor for secondary alcohol dehydrogenase activity and hints that this enzyme might be NAD-linked.

Unfractionated, unsupplemented supernatants also catalyzed the formation of (III) from (I), as well as (IV) and (VI) from (II) and, in addition, coupled (IV) with (VI) to form (VII). These same supernatants could neither couple (IV) with (V) to form (II), nor convert (I) to (II), but they could convert (III)

to (I). Further studies of the esterase(s) present in these unsupplemented supernatants were conducted in which undecyl acetate-2-C^{14} was employed as substrate to detect the conversion of (II) to (IV) and (V). This reaction was measured rapidly and quantitatively by an assay procedure that was based on detection of ^{14}C-acetate released enzymatically from ^{14}C-labeled (II). The procedure employs Bio-Rad AG-21K ion-exchange resin in the OH^- form to trap acetate-1-C^{14} which is counted in an appropriate scintillation cocktail. Details of the method appear elsewhere (4). Cells induced to catabolize (I) or (II) contained a potent undecyl acetate esterase whereas cells grown on succinic acid did not. Boiled extracts had no activity on (II) and this substrate did not hydrolyze when shaken in buffer under assay conditions.

Enzymatic conversion of (I) to (II) suggests that primary attack on the substrate occurs by subterminal oxidation. The enzyme mediating this conversion may be an oxygenase which belongs to the class of mono-oxygenases (5), and the reaction could occur by insertion of a linking or bridging atom of oxygen into the carbon chain between carbon atoms 2 and 3 of (I) by a mechanism analogous to that of the chemical Baeyer-Villiger oxidation of carbonyl compounds by peracids (6); biologically, this would require molecular oxygen and would result in the formation of (II). Although a Baeyer-Villiger type of reaction has not been reported to occur biologically with any simple, unbranched or unsubstituted aliphatic methyl ketone, reports of enzymatic modification of alicyclic compounds such as various microbial transformations of steroids (7, 8), and camphor (9), or carbohydrates such as inositol (10), indicate that a Baeyer-Villiger oxidation mechanism may be responsible for either ring or side-

chain cleavage during biological degradation of these complex compounds. Bacterial oxidative decarboxylation of lactic acid (11) and decarboxylations of alpha-keto and alpha-hydroxy acids that occur in animal cells (12) may also be reactions of this type. So far most investigations concerning Baeyer-Villiger oxygenases have not dealt with enzymes derived from bacteria; however, one enzyme, camphor ketolactonase, was purified from an aerobic pseudomonad (9). This report is offered as preliminary evidence for the existence of methyl ketone oxygenases in cells of aerobic pseudomonads induced to catabolize these substrates.

It should be emphasized that no conclusions can be drawn from these experiments with crude enzyme preparations as to the absolute cofactor requirements of 2-tridecanone oxygenase. Undecyl acetate did not form in the absence of reduced pyridine nucleotides which had to be generated in the reaction mixtures in order to overcome the leakage of reducing power into competitive reactions. Both NADH and NADPH seemed to serve as effective coenzymes. This merely indicates an essential requirement of the oxygenase for hydrogen donors; however, this requirement does conform to what is known about the essentiality of reduced pyridine nucleotide cofactors for enzymes of this class. Likewise, it is premature to conjecture about the necessity for the other cofactors included in the assays. Further work is necessary to purify 2-tridecanone oxygenase, to determine the essential cofactors which enable it to convert 2-tridecanone to undecyl acetate, and to elucidate directly the mechanism by which this enzyme functions.

REFERENCES

1. Forney, F. W., Markovetz, A. J., and Kallio, R. E. J. Bacteriol. 93, 649 (1967).

2. Forney, F. W., and Markovetz, A. J. J. Bacteriol. 96, 1055 (1968).

3. DeMoss, R. D. In Methods in Enzymology, Vol. I, p. 328, Academic Press, New York, 1955.

4. Forney, F. W., Ph.D. Dissertation, Univ. of Iowa, 1969.

5. Hayaishi, O., Proc. Plenary Sessions, 6th Intern. Congr. Biochem., IUB Vol. 33, p. 31, Federation of American Societies for Experimental Biology, Washington, D.C., 1964.

6. House, H. O., Modern Synthetic Reactions, p. 123-129, W. A. Benjamin, Inc., New York, 1965.

7. Charney, W., and Herzog, H. L., Microbial Transformations of Steroids, p. 46-47, Academic Press, New York, 1967.

8. Rahim, M. A., and Sih, C. J., J. Biol. Chem., 241, 3615 (1966).

9. Conrad, H. E., DuBus, R., Namtvedt, M. J., and Gunsalus, I. C., J. Biol. Chem., 240, 495 (1965).

10. Charalampous, F. C., J. Biol. Chem., 235, 1286 (1960).

11. Hayaishi, O., and Sutton, W. B., J. Am. Chem. Soc., 79, 4809 (1957).

12. MacDonald, R. C., and Mead, J. F., Lipids 3, 275 (1968).