JOURNAL OF BACTERIOLOGY, Dec. 1971, p. 1141-1146
Vol. 108, No. 3


# Structural Effects on *Arthrobacter* Methylene Hydroxylase Activity[1]

STEVEN HAYASAKA AND D. A. KLEIN

*Department of Microbiology, Oregon State University, Corvallis, Oregon 97331, and Department of Microbiology, Colorado State University, Fort Collins, Colorado 80521*

Received for publication 9 July 1971

*Arthrobacter* 4-44-2 (ATCC 25581), capable of subterminal oxidation of *n*-hexadecane to 2-, 3-, and 4-alcoholic and ketonic products, was examined for the ability of this methylene hydroxylase capability to be induced and repressed and for structural relationships influencing methylene function oxidation. Induction was best carried out by use of *n*-alkanes from 10 to 16 carbons in length and was especially strong with methylcyclohexane among cyclic compounds tested. Induction was not observed with several related alcohols, 1-unsaturated compounds, or methoxy and ethoxy compounds tested. After induction, *n*-alkanes 14 and 16 carbons in length were transformed to the corresponding internal oxidation products; however, no activity was observed with even-carbon alkanes of shorter chain length. Hexadecene-1 and all alcohols tested, including cyclododecanol, were transformed to corresponding ketonic or aldehydic products. Cyclic compounds tested, including cyclododecane, were not oxidized by induced cells, suggesting that a methyl group plays a role in orientation of the substrate for the methylene hydroxylation but that the methyl function was not as critical after completion of the hydroxylation step regardless of structural configuration. Acetate strongly repressed induction of *n*-hexadecane methylene hydroxylase activity. Inducibility of methylene hydroxylase activity was confirmed by use of cell-free systems with methylcyclohexane as an inducer. A stimulation of methylene hydroxylase activity by addition of reduced pyridine nucleotides and ferrous ion was indicated.

Although the subterminal oxidation of longer-chained normal alkanes (from 10 to 16 carbons) has been well documented in recent studies (3, 4, 6-9, 11), this work has primarily been involved with a description of products and of organisms capable of carrying out these reactions.

Studies of *n*-alkane inducer relationships have been carried out with organisms capable of terminal oxidations (12); however, to our knowledge, no work has been done regarding the role of inducers and structural relationships on methylene hydroxylase activity of longer-chained *n*-alkanes. In addition, there remains the problem of elucidating the original structure or "foreign molecule" (1) which first allowed induction of a certain enzymatic capability. Hopefully this type of study will aid in better understanding such structural relationships influencing microbial interactions with compounds which contain the methylene function.

## MATERIALS AND METHODS

**Culture conditions.** *Arthrobacter* 4-44-2 (ATCC 25581) (9) was specifically selected for its inability to emulsify *n*-hexadecane without yeast extract in a mineral salts solution, and to form 2-, 3- and 4-alcohols and related ketones from *n*-hexadecane. Cultures of the species were transferred monthly and maintained on veal infusion slants at refrigerator temperature.

To obtain an inoculum, except where indicated, cells were grown in 50 ml of basal medium A containing 1% (w/v) of the carbon source to be subsequently used. Basal medium A contained the following (per liter): $K_2HPO_4$, 1.6 g; $KH_2PO_4$, 0.8 g; $MgSO_4$, 0.2 g; $CaCl_2$, 0.002 g; $FeSO_4 \cdot 7H_2O$, 0.001 g; $NaNO_3$, 2.0 g; $NH_4Cl$, 1.0 g; and 1.0 ml of a filter-sterilized vitamin mixture consisting (per ml) of: biotin, 0.1 mg; vitamin $B_{12}$, 0.2 mg; thiamine hydrochloride 50.0 mg; calcium *D*-pantothenate, 50.0 mg; and nicotinic acid, 50.0 mg. This vitamin solution was adjusted to *p*H 6.8. For washing cell preparations, basal medium A without nitrogen was used.

**Substrates.** Hydrocarbons were of the highest purity available and were purchased from the following firms: *n*-hexadecane, 1-hexadecene, *n*-decane, and *n*-octane (Humphrey Chemical Co., North Haven, Conn.); cyclododecanol, 2-decanol, 2-decanone, *n*-dodecane, 2-hexandecanol, 3-hexadecanone, 1-methylnaphthalene, and *n*-tetradecane (K & K Laboratories Inc., Hollywood, Calif.); benzene, cyclohexane, diethoxymethane, methylcyclohexane, and *p*-xylene (Eastman Kodak

[1] Technical paper no. 3142 of the Oregon Agricultural Experiment Station.

Co., Rochester, N.Y.); 1-decanol and 1-hexadecanol (Matheson Coleman and Bell Co., Los Angeles, Calif.); 2,3-butanediol, cyclodecane, cyclododecane, 1,3-diethoxy-2-propanol, 1,2-dimethoxyethane, dimethoxymethane, 4-heptanone, 1,5-hexadiene, 2-hexanone, 2-octanone, *trans*, *trans*, *cis*-1,5,9-cyclododecatriene, 1-undecanol, 2-undecanone, and 6-undecanone (Aldrich Chemical Co. Inc., Milwaukee, Wis.). Other substrates and coenzymes used in this study were obtained from additional commercial sources and were of at least analytical reagent grade or its equivalent.

**Growth experiments.** Inocula were grown on veal infusion agar slants for 24 hr. Growth was transferred to 2.0 ml of mineral salts solution (see Table 1) so that a slight turbidity was evident. One drop of inoculum was then added to test tubes containing 6 ml of basal medium A.

Carbon sources were sterilized separately from the basal medium either by autoclaving or by filtering through a fritted-glass filter. Water-soluble carbon sources were tested at 0.5% (w/v). For liquid and solid insoluble carbon sources, one drop or 0.025 g, respectively, was used.

All tubes were shaken at 30 C on a reciprocating shaker at 150 cycles/min. Growth was read after 5 days using a Spectronic 20 spectrophotometer (420 nm). An optical density of 1.0 was equivalent to 0.7 mg (dry weight) of cells per ml.

**Gas-liquid chromatography.** Gas chromatographic analyses were carried out by using an Aerograph Hy-Fi III model 1200 chromatograph incorporating a high-temperature flame-ionization detector. The unit was equipped with a ⅛ inch (3.2 mm) by 10 ft (305 cm) column containing 10% FFAP liquid phase (Varian Aerograph, Walnut Creek, Calif.) on 100 to 120 mesh Chromosorb-W (Varian Aerograph) to detect $C_{16}$ alcoholic and ketonic products (9).

Injector, column oven, and detector were set at 280, 210, and 270 C, respectively. Flow rates were: carrier gas, argon (25 ml/min); detector gases, hydrogen (25 ml/min); and air (250 ml/min).

**Preparation and assay of cell-free extracts.** The organism was grown on 1% (w/v) disodium succinate basal medium A for 3 days at 30 C. The cells were then centrifuged and suspended in 0.5% disodium succinate basal medium A with or without 0.3% methylcyclohexane as an inducer. After induction for 8 hr on the rotary shaker, cells were harvested by centrifugation at 5,000 × *g* for 20 min. The cells were washed three times with 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (*p*H 7.4) and resuspended in the same buffer. All subsequent operations were carried out at no greater than 10 C.

Cell-free extracts were prepared by using a sonifier cell disruptor (model W185; Heat Systems-Ultrasonic Inc., Plainview, N.Y.) with a power output of 90 w. After sonic treatment, extracts were centrifuged for 10 min at 15,000 × *g*. The cell-free extract supernatant fluid contained approximately 17 mg of protein per ml. Soluble protein was estimated by the biuret method (5) with bovine serum albumin as the standard.

The assay for formation of 2-hexadecanol from *n*-hexadecane in cell-free extracts was carried out in 125-ml Erlenmeyer flasks. The reaction mixture contained 20 ml of cell-free extract in 0.05 M Tris-hydrochloride buffer; *n*-hexadecane (20 μliters); nicotinamide adenine dinucleotide (NAD), reduced NAD (NADH), nicotinamide adenine dinucleotide phosphate (NADP), or reduced NADP (NADPH) (10 μmoles); and $FeSO_4 \cdot 7H_2O$ (2 μmoles). All flasks were shaken on a New Brunswick rotary shaker at 275 rev/min at 30 C. After 1 hr of incubation, 0.5 ml of concentrated HCl was added. A 50-ml separatory funnel was used to extract the mixture two times with a total volume of 8 ml of diethyl ether. After the ether was evaporated, 2-μliter samples of residual hydrocarbon plus oxidation products were analyzed directly by gas chromatography.

**Effects of alternate carbon sources on methylene hydroxylase induction.** Cells were grown for 72 hr either on 200 ml of 1% (w/v) "test carbon" or 2% (w/v) yeast extract-basal medium A. The test carbon sources —sodium acetate, disodium succinate, sodium pyruvate, sodium lactate, and glucose—were autoclaved separately before addition to the basal medium. The cells were then centrifuged and washed three times in phosphate buffer (*p*H 7). All centrifugations were done at 5,000 × *g* for 20 min unless otherwise stated. Each set of test carbon-grown cells was resuspended in 100 ml of phosphate buffer [1.3 mg (dry weight) of cells/ml] containing either 2 ml of hexadecane alone or 0.061 M of the same test carbon source used for growth plus 2 ml of hexadecane. Cells suspended with 0.061 M glucose, however, were previously grown up on 1% sodium acetate basal medium A because glucose could not be utilized as a sole carbon and energy source (D. A. Klein and R. A. Sokol, Bacteriol. Proc., p. 101, 1971). All flasks were then shaken on the rotary shaker, and 10-ml samples, removed after 4 and 8 hr, were extracted with 1 ml of diethyl ether, evaporated, and analyzed by gas-liquid chromatography.

**Quantitative estimation of ketones.** Quantitation of ketone formation was carried out by measuring 2-hexadecanone accumulation with 1-hexadecanol used as an internal standard. Hexadecane containing various concentrations of 1-hexadecanol was added to portions of the fermentation broth being assayed. After thorough shaking, a 10-ml sample of hydrocarbon plus mineral salts was removed and extracted with 1 ml of diethyl ether in a screw-top tube. A calibration curve was then derived by gas chromatography with product peak/area ratios plotted against internal standard molar ratios of 1-hexadecanol.

**Compounds effective as inducers in paraffin adaptation.** Cells grown for 48 hr on 1% disodium succinate basal medium A were centrifuged and suspended in 0.5% disodium succinate basal medium A containing $2.9 \times 10^{-4}$ or $5 \times 10^{-3}$ moles of inducer. After incubation for 8 hr on a rotary shaker, the cells were again centrifuged and washed three times with mineral salts solution. Upon adjustment of cell concentration to approximately 1.3 mg (dry weight)/ml, the cells were suspended in baffled flasks containing 2 ml of hexadecane and 100 ml of mineral salts solution. Cells were then shaken again on a rotary shaker at 200 rev/min at 30 C. After 4 hr of incubation, 2 ml of concentrated HCl and $4 \times 10^{-4}$ g of 1-hexadecanol (internal standard) were added to the flask. Samples (10 ml) were removed and extracted and assayed by the procedure

previously outlined; 2-hexadecanone concentration was measured in comparison with the 1-hexadecanol internal standard. Activity is expressed in nanomoles of 2-hexadecanone formed per milligram (dry weight) of cells over the 4-hr incubation period.

**Hexadecane transformation in induced resting cells.** After growth in 2% (w/v) yeast extract-basal medium A on a rotary shaker for 48 hr, the cells were centrifuged and washed three times in phosphate buffer (*p*H 7). The cells were then resuspended in 100 ml of phosphate buffer at approximately 2.6 mg (dry weight) of cells/ml. Liquid hydrocarbon (1.5 ml) and solid hydrocarbon (.65 g) dissolved in a minimal amount of 95% ethanol were added to the flasks. Controls consisted of flasks containing 100 ml of phosphate buffer with either a hydrocarbon sample or washed cells. After the flasks were shaken for 48 hr on a rotary shaker, the entire contents were extracted three times with 20-ml volumes of diethyl ether. Samples were analyzed by thin-layer chromatography.

**Thin-layer chromatography.** Thin-layer chromatography (TLC) plates were prepared by layering 0.250 mm of a 50% (w/v) water slurry of Silica Gel G. (no. 08075, American Optical Corp.) on glass plates. The plates were then allowed to dry overnight before being activated for 1 hr at 105 C.

Solvent systems used for separating the various reaction products consisted of benzene, benzene-chloroform (1:1), benzene-ethanol (98:2), and butyl alcohol saturated with 1.5 N $NH_4OH$.

Residual hydrocarbon products dissolved in a minimal amount of 95% ethanol were applied to the TLC plates (10 to 20 $\mu$liters per spot) and dried with forced air. The solvent front was allowed to traverse 10 cm before removal from the tank. After solvent evaporation, a 0.4% (w/v) solution of 2,4-dinitrophenylhydrazine in 2 N HCl was used for carbonyl or aldehydic function detection. A positive reaction indicated that at least $10^{-5}$ mole of ketonic or aldehydic product was formed with the described extractive and analytical procedures.

## RESULTS AND DISCUSSION

**Growth studies.** Of the various hydrocarbons tested (Table 1), only the $C_{14}$ and $C_{16}$ hydrocarbons and related unsaturated and oxidized compounds were utilized as sole carbon and energy sources by *Arthrobacter* ATCC 25581 in the nitrogen-containing mineral salts medium plus vitamin mixture used in this study. Earlier efforts (9) to obtain growth on 2-hexadecanol and 3-hexadecanone gave negative results due to the absence of thiamine in the test medium.

Under these test conditions, growth was not observed from *n*-hexane, *n*-octane, *n*-decane, 1-decanol, 2-decanone, *n*-dodecane, 1-undecanol, 2-undecanone, cyclododecane, cyclododecanol, cyclodecane, cyclohexane, and methylcyclohexane; in addition, no growth was observed from 15 sugars, 6 sugar alcohols, 20 additional amino acids, and 17 miscellaneous compounds including phenolics, purines, and pyrimidines. Both ammonium and nitrate ions were found to be utilized as nitrogen sources.

**Compounds effective as inducers in paraffin adaption.** A number of compounds were tested for their ability to induce paraffin oxidation in washed cells of *Arthrobacter* ATCC 25581. Typical kinetics for 2-hexadecanone formation with methylcyclohexane as an inducer are shown in Fig. 1. In subsequent experiments methylcyclohexane was not found to be transformed to detectable oxidation products or to be used as a

TABLE 1. *Suitable carbon and nitrogen sources for aerobic growth of Arthrobacter ATCC 25581*

| Sources of carbon | Growth at 5 days[a] |
|---|---|
| Hydrocarbons | |
| *n*-Hexadecane | 0.35 |
| 3-Hexadecanone | 0.37 |
| 2-Hexadecanol | 0.33 |
| 1-Hexadecanol | 0.55 |
| *n*-Tetradecane | 0.24 |
| 1-Hexadecene | 0.10 |
| Miscellaneous | |
| Disodium succinate | 2.5 |
| Sodium acetate | 0.76 |
| Sodium lactate | 3.0 |
| Sodium pyruvate | 2.6 |
| Amino acids | |
| Alanine | 0.40 |
| Histidine | 0.48 |

[a] Growth expressed as optical density at 420 nm.

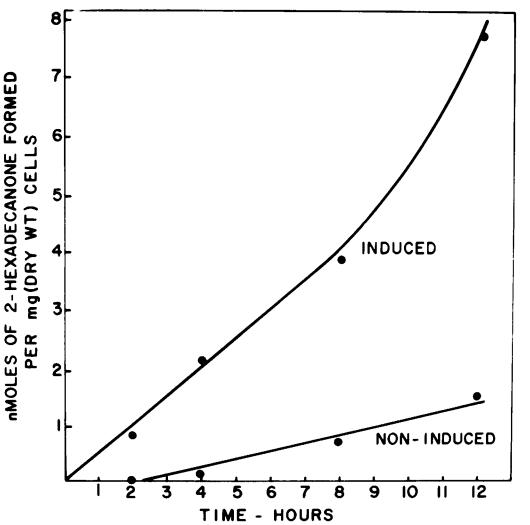


FIG. 1. *Formation of 2-hexadecanone by washed cells of induced and noninduced Arthrobacter ATCC 25581. Activity expressed in nanomoles of 2-hexadecanone formed per milligram (dry weight) of cells.*

growth substrate, indicating that methylcyclohexane, and not a subsequent oxidation product, was the actual inducer. It should be recognized that other putative inducers tested which were utilized for growth could have shown activity due to the presence of metabolic products resulting from transformation by the organism.

A 4-hr reading was chosen for quantitation of methylene hydroxylase-dehydrogenase enzyme induction rates, based on studies by Henning (Ph.D. thesis, Oregon State Univ., Corvallis, 1970) of 2-hexadecanone transformation by resting cells of this organism. Transformation of 2-hexadecanone to further products was found to be minimal during the first 4 hr of incubation. However, after 8 to 10 hr a shift to a steady-state relationship between formation and further metabolism was indicated.

Induction results are presented in Table 2. With *n*-alkanes and corresponding ketonic compounds, induction of paraffin oxidation began at $C_{10}$ straight-chained compounds. Hydrocarbon alcohols of $C_{11}$, $C_{10}$, and shorter chain lengths failed to give strong induction. However, 1-hexadecanol, 2-hexadecanol, and 3-hexadecanone, solid substrates found to support good growth (Table 1), were determined to be satisfactory inducers of methylene hydroxylase activity in a series of related experiments.

Surprisingly, 1-hexadecane was not found to be a strong inducer. With methoxy and ethoxy compounds tested, strong induction was not observed. In this connection, it is interesting that in another study (12), one of these compounds, 1,2-dimethoxymethane, was found to be an excellent *n*-alkane terminal hydroxylase inducer for *Pseudomonas aeruginosa*.

Among the various cycloalkanes and cycloalkenes tested, only methylcyclohexane was observed to give strong induction. This is in accordance with the view that a methyl group may be required for enzyme specificity in paraffin oxidation (9). In contrast, it should be noted that *p*-xylene and 1-methylnapthalene did not allow observable induction.

**Effects of alternate carbon sources on hexadecane transformation.** Glucose has been observed to stimulate hexadecane transformation by this organism in a basal salts medium (9). Therefore, studies were conducted to determine the possible effects of utilizable carbon sources on induction of *n*-hexadecane methylene hydroxylase activity.

As shown in Table 3, sodium lactate, sodium pyruvate, and glucose, stimulated, to various degrees, induction when present with *n*-hexadecane in a nongrowth medium. Sodium acetate strongly repressed induction to 20% of control induction levels, whereas only a slight repression was observed with sodium succinate, a substrate used for growth of uninduced cells in this study.

**Induced Arthrobacter ATCC 25581 nongrowth transformation activity.** Normal alkane oxidation to ketones by resting cells of induced *Arthrobacter* ATCC 25581 is observed with $C_{14}$ and $C_{16}$ carbon substrates; however, *n*-dodecane and *n*-decane transformation could not be detected (Table 4). Oxidation of residual *n*-dodecane and *n*-decane with $KMnO_4$ for conversion of alcohols possibly formed initially into ketones, followed by gas chromatographic analysis, also indicated that oxidation of these compounds had not taken place. Similarly, various cyclic compounds, including cyclohexane, methylcyclohexane (an excellent inducer), and cyclododecane, were not transformed to alcohols or ketonic products. Nevertheless, related straight-chain and cyclic compounds containing alcoholic functions tested were further oxidized.

**Cell-free activity of induced and noninduced cells.** To confirm that induction of whole cells used in this study accurately reflected induction of the paraffin transformation enzyme system, cell-free extracts were prepared and analyzed as described above.

In comparing 2-hexadecanol accumulation in cell-free extracts of methylcyclohexane-induced and uninduced (succinate-grown) cells, marked differences in 2-hexadecanol accumulation were observed. This activity appeared to be stimulated by the addition of reduced pyridine nucleotides and ferrous ion and was inactivated by heat treatment at 100 C for 2 min.

The unique characteristic of the *Arthrobacter* isolate used in this study is that its primary mode of *n*-alkane attack appears to be subterminal. Further, by the use of structurally related compounds it was found that a number of cyclic hydrocarbon compounds and *n*-alkanes were not transformed to either alcoholic or ketonic products. However, related substrates containing alcoholic functions could be transformed to the corresponding ketonic and aldehydic products. These studies therefore indicate that hydroxylation appears to be a rate-limiting step in cyclic hydrocarbon oxidation with this *Arthrobacter* isolate, as observed by Fonken et al. (2) in studies of monocyclic alcohol oxidation by a *Sporotrichum* species.

Although this study demonstrated *n*-alkane methylene hydroxylase induction, it should be emphasized that the differing solubilities and vapor pressures of the various hydrocarbons should be considered in further interpretation of these results. Compounds functioning as inducers which were solids (1-hexadecanol, 2-hexadeca-

TABLE 2. *Structural effects on induction of methylene hydroxylase activity*[a]

| Compound | Yield[b] | Utilized[c] for growth |
|---|---|---|
| Noninduced | 0.1 | |
| *n*-Hexadecane | 2.1 | + |
| *n*-Tetradecane | 1.7 | + |
| *n*-Dodecane | 2.1 | − |
| *n*-Decane | 1.7 | − |
| *n*-Octane* | 0.2 | − |
| *n*-Hexane* | <0.1 | − |
| 1-Hexadecane | 0.3 | + |
| 1,5-Hexadiene* | 0.3 | − |
| 6-Undecanone | 1.7 | − |
| 2-Undecanone | 1.6 | − |
| 2-Decanone | 1.5 | − |
| 2-Octanone* | 0.3 | − |
| 4-Heptanone | 0.4 | − |
| 2-Hexanone* | 0.4 | − |
| 2-Butanone* | 0.3 | − |
| 1-Undecanol | <0.1 | − |
| 1-Decanol | <0.1 | − |
| 2-Decanol | 0.5 | − |
| 2,3-Butanediol* | 0.2 | ND |
| Ethanol* | 0.2 | ND |
| Dimethoxymethane* | 0.3 | ND |
| 1,2-Dimethoxyethane* | 0.2 | ND |
| 1,3-Diethoxy-2-propanol* | 0.4 | ND |
| Diethoxymethane* | 0.2 | ND |
| *Trans, trans, cis*-1,5,9-cyclododecatriene | 0.2 | ND |
| Cyclohexane* | 0.3 | − |
| Methylcyclohexane* | 2.0 | − |
| 1-Methylnaphthalene | <0.1 | ND |
| *p*-Xylene | <0.1 | ND |

[a] Compounds tested at $2.9 \times 10^{-4}$ molar. Those marked with an asterisk were also tested at $5 \times 10^{-3}$ molar.

[b] Nanomoles of 2-hexadecanone formed per milligram (dry weight) of cells in 4 hr.

[c] Symbols: (+) growth observed, (−) no growth observed, (ND) not determined.

TABLE 3. *Effect of test repressor compounds on induction of methylene hydroxylase-dehydrogenase activity*

| Test repressor | Induction ratio[a] |
|---|---|
| Sodium acetate | 0.20 |
| Disodium succinate | 0.74 |
| Sodium lactate | 1.80 |
| Sodium pyruvate | 1.15 |
| Glucose | 1.13 |

[a] Ratio of the amount of 2-hexadecanone formed by cells suspended in a phosphate buffer (*p*H 7) containing hexadecane and a "test carbon" source to that formed when the cells were suspended in a phosphate buffer with only hexadecane.

TABLE 4. *Induced Arthrobacter ATCC 25581 nongrowth transformation activity*

| Compound | Ketones detected |
|---|---|
| *n*-Hexadecane | + |
| *n*-Tetradecane | + |
| *n*-Dodecane | − |
| *n*-Decane | − |
| *n*-Octane | − |
| *n*-Hexane | − |
| 1-Hexadecene | + |
| 1-Hexadecanol | + |
| 2-Hexadecanol | + |
| Cyclododecanol | + |
| Cyclodecanol | + |
| 1-Decanol | + |
| 2-Decanol | + |
| Cyclohexane | − |
| Cyclododecane | − |
| Methylcyclohexane | − |
| *p*-Xylene | − |
| Benzene | − |

none, 3-hexadecanone) did not give the induction kinetics shown in Fig. 1.

The rather narrow range of hydrocarbons which were oxidized or used for growth by this bacterium would indicate that molecular specificity for paraffin oxidation is rather stringent. This can be contrasted with the *Pseudomonas* species used by Ooyama and Foster (10) which not only oxidized *n*-alkanes ranging from $C_1$ to $C_{22}$, but also transformed branch-chain and cyclic hydrocarbons.

Hopefully, this study will provide an initial insight into factors influencing long-chained alkane methylene hydroxylase activity in this *Arthrobacter* species and lead to a better understanding of the role which this type of microorganism might play in the wide range of environments in which it can be postulated to occur.


## ACKNOWLEDGMENTS

This investigation was supported by Environmental Protection Agency research grant R-EP-00278-03 and by the Oregon Agricultural Experiment Station, Corvallis, Ore.


## LITERATURE CITED


1. Billings, R. E., H. R. Sullivan, and R. E. McMahon. 1970. Oxidation of indan to indanol and indanone by the hepatic microsomal system. Biochemistry **9**:1256–1260.
2. Fonken, G. S., M. E. Herr, H. C. Murray, and L. M. Reineke. 1967. Microbial hydroxylation of monocyclic alcohols. J. Amer. Chem. Soc. **89**:672–675.
3. Forney, F. W., and A. J. Markovetz. 1970. Subterminal oxidation of aliphatic hydrocarbons. J. Bacteriol. **102**: 281–282.
4. Fredricks, K. M. 1967. Products of the oxidation of *n*-decane by *Pseudomonas aeruginosa* and *Mycobacterium*

*rhodochrous*. Antonie Van Leeuwenhoek J. Microbiol. Serol. **33**:41-48.
5. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177**:751-766.
6. Jones, D. F., and R. Howe. 1968. Microbiological oxidation of long-chain aliphatic compounds. Part I. Alkanes and alk-1-enes. J. Chem. Soc. (C):2801-2808.
7. Killinger, A. 1970. Der Abbau von Undecan durch ein marines Bakterium. Arch. Mikrobiol. **73**:160-176.
8. Klein, D. A., J. A. Davis, and L. E. Casida, Jr. 1968. Oxidation of *n*-alkane to ketones by an *Arthrobacter* species. Antonie Van Leeuwenhoek J. Microbiol. Serol. **34**: 495-503.
9. Klein, D. A., and F. A. Henning. 1969. Role of alcoholic intermediates in formation of isomeric ketones from *n*-hexadecane by a soil *Arthrobacter*. Appl. Microbiol. **17**: 676-681.
10. Ooyama, J., and J. W. Foster. 1965. Bacterial oxidation of cycloparaffinic hydrocarbons. Antonie van Leeuwenhoek J. Microbiol. Serol. **31**:45-65.
11. Schnable, H., and H. J. Rehm. 1971. Oxidation von tetradekan durch pseudomonadeceae. Naturwissenschaften **58**:55.
12. Van Eyk, J., and T. J. Bartles. 1968. Paraffin oxidation in *Pseudomonas aeruginosa*. I. Induction of paraffin oxidation. J. Bacteriol. **96**:706-712.