Paraffin Oxidation in *Pseudomonas aeruginosa*

I. Induction of Paraffin Oxidation

J. VAN EYK AND TRUDE J. BARTELS

Koninklijke/Shell-Laboratorium, Amsterdam, The Netherlands

Received for publication 4 March 1968

The induction of paraffin oxidation in intact cells of *Pseudomonas aeruginosa* was investigated. Oxidation of $^{14}$C-heptane by cell-free extracts of adapted cells showed that the activity of whole cells is a reliable reflection of the synthesis of the first enzyme in the degradation of *n*-alkanes. Induction was significantly affected by glucose and could be completely repressed by malate. The amino acids L-proline, L-alanine, L-arginine, and L-tyrosine exhibited a rather low repressor action. Malonate, a nonrepressive carbon source, allowed gratuitous enzyme synthesis. A number of compounds which did not sustain growth were found to be suitable substitutes for paraffins as an inducer. Among these were cyclopropane and diethoxymethane. The induction studied under conditions of gratuity with the latter compound as an inducer showed immediate linear kinetics only at saturating inducer concentrations. With *n*-hexane as the inducer, a lag time was always observed, even when high concentrations were used.

*n*-Alkanes can serve as sole sources of carbon and energy for the aerobic growth of many bacteria (5). Following the initial attack by a monoxygenase, the alcohol and aldehyde formed as the primary products can enter the pathways of cellular intermediary metabolism (15).

With a few possible exceptions (4, 8), the capacity to oxidize paraffins has proved to be inducible, this specific capacity being elicited by exposure of the cells to an *n*-alkane (2). During the past decade, a variety of inductive and repressive control mechanisms occurring in bacterial metabolic sequences have been analyzed (1). The adaptive enzyme system in paraffin oxidation, however, is largely unknown. This has prompted us to investigate the mechanism of paraffin adaptation in *Pseudomonas aeruginosa*.

The data reported here are mainly derived from studies in which intact cells were employed.

**Materials and Methods**

**Organism.** The experiments were carried out with *P. aeruginosa* laboratory strain 473.

**Maintenance and growth.** Cultures of *P. aeruginosa* were maintained on peptone-agar slopes. The organism was subcultured daily and grown at 30 C. For growth in liquid media, loopfuls of a suspension of bacteria grown for 24 hr on a slope were transferred to Erlenmeyer flasks (500 ml) containing 50 ml of basal medium and an appropriate oxidizable substrate. The basal medium was composed of a mineral medium (see 22) supplemented with 0.01% yeast extract and 0.01% peptone extract. Growth was measured in a colorimeter (Bausch & Lomb, Spectronic-20) at 600 nm. Cultures were incubated at 30 C unless otherwise specified, in a gyratory water-bath shaker.

**Induction in growing cultures.** The results given in Table 1 were obtained in the following manner. A culture grown overnight (see above), in which growth had stopped owing to substrate exhaustion, was diluted with fresh, prewarmed basal medium to 100 ml and supplemented with a carbon source (0.4%) and an inducer. Samples were taken at intervals, centrifuged, and suspended in a phosphate buffer at 0.6 mg (dry weight)/ml. Subsequently, the ability of whole cells to oxidize *n*-hexane was measured.

For experiments in which malonate served as the carbon and energy source, cultures grown overnight were diluted with a mineral medium only and supplemented with 0.4% malonate.

In kinetic experiments, the inducer was added after the culture had reached the exponential-growth phase.

**Oxygen uptake.** The rate of *n*-hexane oxidation was measured by the Warburg technique at 30 C, essentially as described by Thijsse et al. (23). Chloramphenicol was added to produce a final concentration of 100 μg/ml to prevent further induction.

Rates of *n*-hexane oxidation by whole cells are expressed as Q$_{O_{2}}$ (microliters of O$_{2}$ per hr per mg, dry weight) and have been corrected for endogenous respiration.

**Nonmetabolizable inducers.** The following organic compounds, screened for their ability to act as inducers without serving as substrates for growth, were obtained from commercial sources as indicated, or were synthesized in this laboratory (KSLA) according to standard methods: 1,2-dimethoxyethane,
Noury Baker; diethoxymethane, Fluka; 2,7-dithia-
octane, Ega Chemie; 2,6-diazahexane, Columbia
Organic Chemicals Co., Inc.; cyclopropane (purity,
99.9%), Fisher Scientific, Pittsburgh, Pa.; methyl-
cyclopropane (purity, 99%), API; cyclopentane
(purity, 99%), Philips; spiropentane, spirotexane,
and spiro[2.4]heptane, KSLA; dicyclopentymetha-

nol and dicyclopentymethane (purity, 99%), Aldrich
Chemical Co., Milwaukee, Wis.; 1,2-dicyclopropyl-
ethylene, bicyclo[4.1.0]heptane, tricyclo[5.1.0.02,4]-
octane, bicyclo[4.1.0]heptane, tricyclo[5.1.0.02,4]-
octane, bicyclo[4.1.0]heptane, tricyclo[5.1.0.02,4]-

heptane, KSLA. 

Chemicals and reagents. Sodium capronate, glu-
cose, and L-hydroxyproline were obtained from
Merck; disodium succinate, from Carlo Erba; citric
acid, from Noury-Baker; p-hydroxybenzoic acid
and malonic acid, from Fluka; disodium fumarate
(cry stalline), α-oxoglutaric acid (chromatographically
pure), and L-amino acids, from Calbiochem.; malic
acid, from British Drug Houses, Ltd.; and 14C-hept-
tane, from Philips-Duphar.

Enzymological methods. Enzyme extracts for assay
of the paraffin oxygenase were prepared from n-
hexane-grown cells. Glucose-grown cells were used
as controls.

Cells were always harvested from exponentially
growing cultures and centrifuged in a refrigerated
centrifuge operated at 3000 x g for 10 min. The
pellets were washed and chilled in 0.05 M tris(hydroxy-
methyl)aminomethane (Tris) buffer (pH 7.5) and
were then centrifuged. The cells were resuspended
in 0.05 M Tris buffer (pH 7.5), and extracts were
prepared by sonic oscillation (Branson Sonifier). After
sonic treatment, the extracts were centrifuged in the
cold for 10 min at 3000 x g to remove unbroken
cells. The supernatant fluid, which was used as an
enzyme extract, contained approximately 20 mg of
protein per ml. Soluble protein was measured by the
method of Lowry et al. (14) with bovine serum albumin
as a standard.

Radiochemical assay. For the assay of the paraffin
oxygenase, 1 μmole of 14C-heptane (specific activity
1.25 mc/m mole) in 0.05 M of ethyl alcohol and 0.2
μmole of reduced nicotinamide adenine dinucleotide
(NADH) were added to 1 ml of enzyme extract, and
the mixture was incubated for 3 hr at 30 C. Then, 50-
μlter samples were analyzed with radio-gas liquid
chromatography (GLC), using an isocytogenol-
opolyglycol ether (OPC 100) column operated at
130 C.

RESULTS

Adaptivity versus constitutivity. Before we per-
formed experiments with intact cells, we had to
establish whether in our induction experiments the
activity of whole cells was a reliable reflection of
synthesis of the first enzyme in the degradation of
the n-alkanes. To investigate this, we prepared
extracts from n-hexane-grown cells and from

The enzymatic assay was based on detection
with radio-GLC of conversion products from 14C-
labeled n-heptane. The radiochromatogram from
adapted cells showed, in addition to heptane, the

Fig. 1. Radio-GLC of an extract of P. aeruginosa
incubated for 3 hr in the presence of 14C-heptane. From
right to left: peaks are 14C-heptane, heptanal, and
heptanol.
dicarboxylic acids, much larger differences are evident in the degree of repression. By the use of appropriate concentrations, adaptation can be completely inhibited with the latter compounds. By contrast, with glucose as a repressor, a maximal repression of 80% is attained at a glucose concentration as low as 0.1%. However, there is a significant difference in degree of repression between acetate and malonate. Although both compounds accelerate the growth rate to the same extent, acetate shows an inhibition of 60% and malonate shows practically none. Since either acetate or succinate may be intermediates in the degradation of n-paraffins, the effect of these compounds is possibly due to end-product repression (7, 19). The very low repressor effect of malonate is rather surprising, since this compound is also converted to acetate (9). Since both $\alpha$-oxoglutarate and caproate, under the conditions stated, inhibit growth, no conclusions can be drawn from the inhibitory effects.

In addition to malonate, the amino acids L-arginine, L-alanine, L-proline, and L-tyrosine have a low repressive effect on hydrocarbon adaptation. For various reasons, which will not be explained here, we preferred the use of malonate for further experiments.

TABLE 1. Effect of supplemental energy sources on paraffin adaptation in P. aeruginosa

<table>
<thead>
<tr>
<th>Supplement to basal medium</th>
<th>$\omega$</th>
<th>$Q_{02}$</th>
<th>Repression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.28</td>
<td>265</td>
<td>—</td>
</tr>
<tr>
<td>Yeast-peptone extract</td>
<td>0.82</td>
<td>11</td>
<td>96</td>
</tr>
<tr>
<td>Fumarate</td>
<td>0.70</td>
<td>19</td>
<td>93</td>
</tr>
<tr>
<td>Malate</td>
<td>0.69</td>
<td>8</td>
<td>97</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.66</td>
<td>33</td>
<td>88</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.66</td>
<td>52</td>
<td>80</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.57</td>
<td>84</td>
<td>68</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.56</td>
<td>62</td>
<td>76</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.55</td>
<td>99</td>
<td>63</td>
</tr>
<tr>
<td>Malonate</td>
<td>0.46</td>
<td>223</td>
<td>16</td>
</tr>
<tr>
<td>Acetate</td>
<td>0.45</td>
<td>101</td>
<td>62</td>
</tr>
<tr>
<td>$p$-Hydroxybenzoic acid</td>
<td>0.41</td>
<td>124</td>
<td>53</td>
</tr>
<tr>
<td>Caproate</td>
<td>0.25</td>
<td>128</td>
<td>52</td>
</tr>
<tr>
<td>$\alpha$-Oxoglutarate</td>
<td>0.11</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>0.61</td>
<td>122</td>
<td>54</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>0.60</td>
<td>122</td>
<td>54</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>0.51</td>
<td>200</td>
<td>24</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>0.48</td>
<td>198</td>
<td>25</td>
</tr>
<tr>
<td>L-Proline</td>
<td>0.45</td>
<td>195</td>
<td>26</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>0.35</td>
<td>150</td>
<td>43</td>
</tr>
<tr>
<td>L-Hydroxyproline</td>
<td>0.33</td>
<td>187</td>
<td>29</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>0.32</td>
<td>209</td>
<td>21</td>
</tr>
</tbody>
</table>

* After incubation for 3 hr in the presence of n-hexane, paraffin oxidation was assayed manometrically.

* Number of divisions per hour.

Induction in nongrowing cells. Cells in exponential growth in glucose or malonate-mineral medium were harvested, washed, suspended in a phosphate buffer with hexane only, 0.2 ml (○), or with glucose, 0.2% (△), or malonate, 0.2% (△), in addition. Suspensions were shaken at 30°C and samples were removed. The ability of whole cells to oxidize n-hexane was measured.

**Fig. 2. Effect of glucose and malonate on paraffin adaptation in nongrowing cells.** Cells were suspended in a phosphate buffer with hexane only, 0.2 ml (○), or with glucose, 0.2% (△), or malonate, 0.2% (△), in addition. Suspensions were shaken at 30°C and samples were removed. The ability of whole cells to oxidize n-hexane was measured.
however, induced paraffin in grown as energy upon uptake for tested without metabolized compounds could summarized in (i) being: experiments, an of in produce

<table>
<thead>
<tr>
<th>Name of compound</th>
<th>Specific activity*</th>
<th>Utilized for growth</th>
<th>Metabolizability, Q_{10}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethane</td>
<td>0*</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Propane</td>
<td>0*</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>n-Butane</td>
<td>186*</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>n-Pentane</td>
<td>166</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>n-Hexane</td>
<td>236</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>n-Heptane</td>
<td>136</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>n-Octane</td>
<td>165</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>1,2-Dimethoxyethane</td>
<td>103*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Diethoxymethane</td>
<td>279</td>
<td>-</td>
<td>19</td>
</tr>
<tr>
<td>2,7-Dithiaoctane</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2,6-Diazahéptane</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Cyclopropane</td>
<td>216*</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Methylcyclopropane</td>
<td>0*</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cylobutane</td>
<td>52*</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cylobutene</td>
<td>0*</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cyclopentane</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Dicyclopropylmethane</td>
<td>232</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1,2-Dicyclopropylethane</td>
<td>220</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Dicyclopropylmethanol</td>
<td>224</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>Spiropentane</td>
<td>3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Spirohexane</td>
<td>81</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Spiro[2,4]heptane</td>
<td>132</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Bicyclo[4.1.0]heptane</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Tricyclo[5.10.0]octane</td>
<td>28</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Compounds were tested at 5 × 10^{-2} M (0.5 mmole) except for those marked with an asterisk, which were tested at 5 × 10^{-2} M. ND = not determined.

Table 2. Compounds effective as inducer in paraffin adaptation

reveal paraffin oxidation in P. aeruginosa. In these experiments, cells were harvested after 3 hr of incubation in malonate medium in the presence of an inducer (0.5 mmole) and were subsequently tested for paraffin oxidation. The results are summarized in Table 2, which also states whether the compounds could be utilized for growth or metabolized without growth, the respective criteria being: (i) growth on mineral agar to which the compounds had been added as sole carbon and energy source, and (ii) stimulation of oxygen uptake upon addition to suspensions of cells grown in the presence of the compound.

As is shown in Table 2, a variety of compounds induced paraffin oxidation. Except for the paraffins, however, none of these seemed to support growth, and, as far as was determined, only two were metabolized to any significant extent. Before trying to make some generalizations concerning molecular specificity in the induction of paraffin-oxidizing enzymes, we wish to point out that it is difficult to compare the activities of the various compounds directly, in view of the different solubilities and vapor pressures. For instance, cyclopropane is a gas at ambient temperatures and 1,2-dicyclopropylethane boils at 128 C.

n-Alkanes. Table 2 shows that in the n-alkane series induction of paraffin oxidation started with n-butane. Surprisingly, growth on n-butane could not be detected. In P. aeruginosa, however, both n-butyl alcohol and butyric acid sustained growth.

Analogues of n-alkanes. Of the analogues of n-alkanes which contain sulfur, nitrogen, or oxygen instead of a methane group, only 1,2-dimethoxyethane induced paraffin oxidation. Diethoxymethane, likewise, was an excellent inducer, giving high derepression, but, in contrast to the latter, it was metabolizable.

Monocyclic and dicyclic cycloalkanes. Among the compounds that do not serve as a growth substrate, cyclopropane, in contrast to n-propane,
was a surprisingly good inducer, although it lacks a methyl group. Introduction of a methyl group completely destroyed activity (methylcyclopropane). Dicyclopentylmethane and dicyclopentanylmethanol were fully active, but only the latter was metabolized. The size of the ring seemed to be critical. Cyclobutane had only a low activity and cyclopentane lacked activity altogether.

Spiro compounds. The smallest possible spiro compound (spiroheptane) had a very low activity. Activity increased with the size of the spiro compound, spiroheptane being quite as active as n-heptane.

Bicyclanes and tricyclanes. The only bicyclane tested (bicycloheptane) was completely inactive. By contrast, tricyclooctane did show activity.

In summary, we can say that molecular specificity in paraffin adaptation is not very stringent, a methyl group apparently is not needed, and an oxygen atom in the chain does not affect function.

Kinetics of induction. From the foregoing, it is evident that diethoxymethane is the inducer of choice for studies on the kinetics of paraffin adaptation, especially in view of its high water solubility.

We studied the rate of induction as a function of inducer concentration in the exponential phase of growth (see Materials and Methods). The effect of inducer concentration is shown in Fig. 3. Total activity is plotted against the time of incubation in the presence of inducer.

At $10^{-4}$ M, no induction occurred. As the concentration of inducer was increased, the capacity to oxidize $n$-hexane increased. The highest concentration of inducer shown on the graph is $5 \times 10^{-3}$ M, and at this concentration the rate of paraffin adaptation was maximal and was linear from the time the inducer was added. No measurable increase in activity was obtained when the concentration was increased beyond this value.

In Fig. 4, the total activity obtained after a 60-min incubation period in the presence of various concentrations of inducer is plotted against the concentration of inducer. The same relationship has been found and described for some other induction systems (6). However, the mechanism of the induction of paraffin oxidation can be discussed only after completion of further experiments.

With $n$-hexane as an inducer, different induction kinetics were observed. In contrast to diethoxymethane, $n$-hexane did not provoke immediate linear expression of paraffin adaptation (Fig. 5). However, the rate of dissolution of $n$-hexane in water might be low, and the time span elasping
before the rate of induction becomes linear (20 min) might be due to the attainment of equilibrium. Therefore, log-phase malonate cells were centrifuged and injected into an equilibrated system (Fig. 5). Under these conditions, the acceleration phase was indeed shortened (8 min) but still persistent (12 min).

**Discussion**

Repression of paraffin adaptation. The so-called glucose effect on paraffin adaptation is in line with the general occurrence of this effect in nature. Glucose is known to inhibit not only enzyme synthesis in related pathways, such as in lactose degradation, but also the synthesis of enzymes in quite distinct pathways, such as in tyrosine degradation, where glucose represses both p-hydroxyphenylpyruvate hydroxylase and homogentisate oxygenase (12). Apparently, there are exceptions, for Mandelstam showed (19) that the induction of the mandelate enzymes in *P. fluorescens* is in fact stimulated by glucose.

In paraffin adaptation, we do not yet know on what level glucose exerts its repressive effect. Preliminary experiments showed that addition of glucose to cells 20 min after addition of the inducer completely nullified the repressive effect (Fig. 6). From this, we conclude that either a supposed transport system is primarily affected or the system that produces the "internal" inducer is affected (see below). Some systems are known in which both an inducer and a repressor are involved in the regulation of the synthesis of an enzyme (7, 19). Whether such a mechanism operates in paraffin oxidation remains to be shown. In this connection, the strong repression in paraffin adaptation of the C4-dicarboxylic acids and acetate (as compared to malonate, Table 1) is quite intriguing.

Nonsubstrate inducers. *n*-Butane induces paraffin oxidation, but does not sustain growth. Since *P. aeruginosa* grows on *n*-butyl alcohol, oxidation of *n*-butane apparently is so slow as to prohibit subsequent growth. Alternatively, oxidation might not be terminal, but might lead to methyl-ethylketone (15), which cannot be further metabolized. Experiments carried out with cell-free extracts from *n*-hexane-grown cells showed, however, that *n*-butane is not oxidized to methyl-ethylketone but to *n*-butyric acid. However, the rate of oxidation is only one-third of that of *n*-heptane by the same extracts. Thus, absence of visible growth may be due either to (i) suboptimal induction of the paraffin oxygenase by *n*-butane, or (ii) to slow turnover of *n*-butane by this enzyme system, or possibly both.

Of the analogues of the *n*-alkanes containing sulfur, nitrogen, or oxygen, only the oxygen com-

![Fig. 6. Catabolite repression in the induction of paraffin oxidation in *P. aeruginosa*. Diethoxymethane (5 × 10^-3 M) was added to cells growing exponentially in a malonate medium. At the time indicated by the arrow, the culture was divided into two parts, and the following addition was made: O, 0.2% glucose; △, control.](http://jb.asm.org/)
growth. Whether dicyclopentylethane, which behaves similarly, is indeed not metabolized will be investigated by using the 14C-labeled compound.

Kinetics of induction. n-Hexane is a much stronger inducer in paraffin adaptation than diethoxymethane, since the oxygen compound is inactive at 10^{-4} M, the maximal solubility of n-hexane. In addition, the final rate of enzyme synthesis with n-hexane as an inducer is higher than with diethoxymethane at optimal concentrations (5 \times 10^{-4} M; Fig. 3). Still, n-hexane, not diethoxymethane, shows a definite lag of 12 min before the maximal rate of enzyme synthesis is obtained.

As to the origin of this acceleration phase, there are two possibilities: (i) maximal expression of paraffin oxidation is initially limited by the gradual development of a transport system (11) due to the low solubility of n-hexane; or (ii) n-hexane must first be converted by an inducible enzyme into another compound, which in turn acts as the real inducer (3). Considering the facts outlined above, it is tempting to believe that the adaptation with n-hexane as an inducer is triggered by an internal inducer and not by the development of a transport system.

At present, we do not know what this internal inducer might be, but it seems likely that it may be an oxygenated derivative. In this respect, cyclopropane, if proved definitely nonmetabolizable, suggests an epoxide as an intriguing intermediate.

**Literature Cited**