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Toluene Induction and Uptake Kinetics and Their Inclusion in the Specific-Affinity Relationship for Describing Rates of Hydrocarbon Metabolism

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The kinetics of concentration-dependent toluene metabolism were examined by evaluating each term in the second-order rate equation. Marine and freshwater pseudomonads were used. Uptake for *Pseudomonas* sp. strain T2 was characterized by a completely saturatable system with small transport constant (K_t = 44 μg/liter) and large specific affinity. Kinetics for *Pseudomonas putida* PpF1 were similar. Induction had little effect on K_t, but it caused the specific affinity to increase from about 0.03 to 320 liters/g of cells per h. The level of induction depended on the time of exposure, the concentration of inducer, and the initial level of induction. If loss of the inducible system was not severe, toluene caused a linear increase in specific affinity with time, and the maximal value achieved at intermediate times (1 to 3 days) was hyperbolic with concentration when K_{ind} was 96 μg/liter (A. T. Law and D. K. Button, Appl. Environ. Microbiol. 51:469–476, 1986). As repression became complete, specific affinities were greatly reduced. Then induction required higher toluene concentrations and longer times, and the shape of the specific-affinity curve became sigmoidal with concentration. Cell yields (0.10 to 0.17 g of cells per g of toluene used) were low owing to liberation of organic products: 2-hydroxy-6-oxohepta-2,4-dienoic acid, toluene dihydrodiol, 3-methylcatechol, acetate, formate, and possibly pyruvate, which in turn caused lower rates of growth. Michaelis constants for the reaccumulation of products exceeded those for toluene, but specific affinities were lower and maximal velocities were higher, so that recycling was favored in cultures with high toluene concentration. Although these kinetics predict deviation from the linear relationship between uptake rate and biomass, we could detect none. Effects of saturation and induction were incorporated into the basic specific-affinity relationship. The result appears to be an improvement in the equation used for describing the kinetics of uptake and growth.

Microbial metabolism of hydrocarbons is a process that contributes to marine food chains (9), depuration of aquatic systems (2), and changes in water chemistry through the liberation of amphipathic products (1, 15–17, 43). Concentration-dependent kinetics often control the rate of substrate uptake by microorganisms in aquatic systems because concentrations are low. Such kinetic control is particularly applicable to the rates of metabolism of hydrocarbons because solubility (40), toxicity (26, 36), and enzymatic saturation (reported here) limit the concentration from which they can be collected. Usually the kinetics of substrate uptake by microorganisms are described by the Michaelis equation. However, a second-order relationship can reflect the process in greater detail. This relationship is as follows: uptake rate = specific affinity × substrate concentration × biomass, or

$$-dA_{out}/dt = v_A = a_A A_{out} X \quad (1)$$

Definitions and units are given in Table 1. Equation 1, together with the yield, gives the rate of growth when limited by a single substrate:

$$-dX/dt = \mu X = a_A A_{out}\, X\, Y_{XA} \quad (2)$$

or, in terms of the specific growth rate,

$$\mu = a_A A_{out}\, Y_{XA} \quad (3)$$

Equation 1 is identified as the specific-affinity relationship to distinguish it from the second-order model (47) in which a second-order rate constant is based on numbers of organisms. The present relationship accounts for variation in biomass and variation in rate-limiting enzyme per unit biomass, as well as saturation phenomena, and derives from the rate constants associated with those enzymes (8). Development of the equations has been reviewed (10), and we attempt to improve their validation here by presenting data which show that they can be used to describe an inducible, saturatable system that is associated with substantial formation of metabolic products.

Specific affinity is useful from a practical standpoint because it provides an absolute scale for evaluation of the nutrient accumulation ability of microorganisms (10). However, it may vary with culture history, be raised by induction, or be lowered from a maximal value a_A^0 by saturation of enzymes in Michaelian fashion. With low-K_t substrates such as hydrocarbons (10), particular attention must be paid to enzyme saturation, because at substrate concentrations above K_t there is a large effect on the saturation-dependent specific affinity a_A. Hydrocarbon metabolism kinetics are often observed only at substrate concentrations above saturation. In some experiments at lower concentrations this saturation has appeared to be incomplete (12). Since accurate determination of K_t is difficult for volatile hydrocarbons and the shape of the kinetic curve was in question, we examined the effects of saturation in some detail.

Induction is also an important aspect of hydrocarbon metabolism, since it directly affects the specific affinity. The oxidative enzymes are often inducible (3, 4, 6, 7, 24, 25, 26, 29, 32, 44, 56). However, data that specify the dependency of induction on external concentration are limited for all

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TABLE 1. Nomenclature

Symbol	Definition	Units
A	Substrate, toluene; A_{out}, intercellular substrate	Grams per liter
a	Specific affinity of organisms for substrate according to equation 1. Subscript gives the substrate, with 0 indicating that the value is at time zero; superscript gives its concentration (micrograms per liter) at the observed value; a_A^{max}, maximal value of the specific affinity for substrate A at prevailing conditions; superscript of 0 indicates that the value is computed from initial slope of v^s on time; and its absence (a_A) indicates the saturation-dependent value; $(a_A^0 - a_{A.0})_{max}$, maximal net increase in a_A^0 during induction; a_{PA}, a_{QA}, a_{XA}, values of partial specific affinity as apportioned to the formation of organic products, CO_2, and cell material from A (12)	Liters per gram of cells per hour
K	Saturation constant; K_t, Michaelis or substrate concentration at half-maximal transport rate; K_m, Michaelis concentration for an enzyme; K_{QA}, substrate concentration at half-maximal rate of CO_2 (Q) production from substrate A; K_{ind}, induction constant or concentration at $0.5(a_A^0 - a_{A.0})_{max}$; K_μ, growth constant at 0.5 μ_{max}	Grams per liter
P	Organic product group in units of substrate mass on a carbon basis; subscript identifies each product by number	Grams per liter
Q	Second product of substrate, CO_2 (See P)	Grams per liter
t	Time	Hours
μ	Specific rate of growth; μ_{max}, maximal rate	Per hour
v	Rate of material passage across the organism surface; subscript identifies the substrate; V_{max}, maximal rate of transport	Grams of substrate per liter per hour
v^S	Specific rates (v) normalized to biomass	Grams of substrate per g of cells per hour
X	Biomass (wet weight); subscript specifies that indicated biomass is composed only of the substrate specified	Grams per liter
Y	Yield; subscript specifies biomass formed from mass of particular substrate	Grams of cells per gram of substrate consumed

classes of substrates. Van Eyk and Bartels (57) reported a hyperbolic relationship between the concentration of inducer and the rate of induction, with a K_{ind} of 1.3 mM for diethoxymethane induction of heptane metabolism in *Pseudomonas aeruginosa*. Novick and Weiner (45) treated induction of the galactoside permease system of *Escherichia coli* as an all-or-none probability for each cell depending on inducer concentration. Fitzgerald and Franklin (28) reported that the rate of induction of alkylsulfatase in *P. aeruginosa* was hyperbolic and that K_{ind} decreased with increasing chain length and hydrogenation of the hydrocarbon moiety of the inducer. We found that the relationship between the final value of the specific affinity and inducer concentration was hyperbolic for toluene and that the associated induction constant, like K_t, was very small (11, 39). However data were limited to cultures that had retained a substantial level of induction at the beginning so we extended studies to cultures with a lower level of induction.

Leakage of organic products has a direct effect on growth rate through the yield of biomass according to equation 3, tends to reduce K_μ with increasing substrate concentration as a result of recycling, and contributes additional constituents to the environment. For hydrocarbons the liberation of partial degradation products is well known (3, 5, 41, 42, 60). However, the extent of efflux in the absence of recycling has not been examined, and it was necessary to evaluate this process to calculate hydrocarbon uptake from products formed.

Toluene is a useful model substrate for evaluating the kinetics of hydrocarbon utilization because it is metabolized by well studied systems (22, 33, 58), is a surprisingly common constituent of the environment (52), and is advantageous for evaluation of the concentration-dependent kinetics of induction rates, since simple aeration can remove it from cultures, thus avoiding the trauma of cell harvest. In our examinations we used pseudomonads, including a marine isolate capable of growth in very dilute nutrient solutions and a well studied soil organism. Experiments were designed to investigate each term of the specific-affinity model as the independent variable. Since both saturation and induction affect the specific affinity, these phenomena were examined both separately and in combination.

The effect of biomass on rate is formulated as linear (equation 1) in the usual way (48, 53). However, for hydrocarbon limitation, release and subsequent recycling of organic products could cause the specific affinity to become biomass dependent, and so we examined the concentration dependency of toluene metabolism kinetics at a range of cell populations.

Goals of this and related studies are to understand (i) how bacteria respond to particular concentrations of substrate in chemically complex aquatic systems, (ii) the mechanism by which bacteria accumulate nutrients from low concentrations, and (iii) how equilibrium concentrations of dissolved organics are set in the environment. The purpose of this communication is to improve understanding of the concentration dependency of toluene metabolism rates and report on the validity of expressing this understanding through the

specific-affinity relationship expanded to accommodate saturation and induction.

MATERIALS AND METHODS

Cultures. Two cultures isolated from oil tanker ballast water (13) were designated *Pseudomonas* sp. strain T2 and *Pseudomonas* sp. strain T3 according to *Bergey's Manual* (23). *Pseudomonas* sp. strain T2 was 0.35 to 1.3 μm^3 in volume depending on conditions. Chemical composition during exponential growth according to population and to mean cell volume of 1.0 μm^3 (Coulter Counter, model Z_{BI}), CHN analysis (model 240B; The Perkin-Elmer Corp.), and density (1.04 g/ml), 8% (dry weight) ash (34), and oxygen (equimolar to carbon) was (in picograms per cell) as follows: wet weight, 1.04; dry weight, 0.60; C, 0.22; N, 0.05; O, 0.30; and ash, 0.05. The organism grew on toluene, phenol, 2-methylphenol (*o*-cresol), 2-ethylphenol, 2-*n*-propylphenol, naphthalene, limonene, 3-methylcatechol, catechol, and benzoic acid. It oxidized but did not grow on benzene and differed from descriptions of known species.

Pseudomonas putida PpF1, which grows on toluene (33), and its mutant 39/D (32), which oxidizes toluene only as far as (+)-*cis*-3-methyl-3,5-cyclohexadiene-1,2-diol (toluene dihydrodiol), were kindly provided by D. T. Gibson.

Artificial seawater (35) was used, except that disodium EDTA was at 0.2 g/liter, $Fe(NH_4)_2(SO_4)_2 \cdot 2H_2O$ was at 0.1 g/liter, and silicate and sodium glycerol phosphate were omitted. The medium was autoclaved, and $NaHCO_3$ was brought to 0.17 g/liter, giving a pH of 8.3. The freshwater medium used for *P. putida* was similar, except that NH_4Cl was at 0.5 g/liter, $MgSO_4 \cdot 7H_2O$ was at 0.025 g/liter, and the NaCl was eliminated.

Stock cultures were stored on agar slants. *Pseudomonas* sp. strain T2 was maintained by weekly transfer into seawater medium containing 0.5 g of casein hydrolysate per liter.

The marine isolates were grown at 10°C; *P. putida* was grown at 20°C. Toluene was supplied to growing cultures as a vapor from a perforated bulb suspended above shaken flasks containing the medium (19), where it equilibrated at 35 mg/liter (10°C). Organisms for rate experiments were harvested by centrifugation (20 min, 1,500 × *g*), washed twice, and preincubated in basal medium for 3 h.

Chemicals. [U-^{14}C]toluene (1.04×10^5 dpm/μg), [U-*ring*-^{14}C]toluene (1.45×10^6 dpm/μg), and [*methyl*-^{14}C]toluene (7.1×10^5 dpm/μg) (New England Nuclear Corp.) and [4-^{3}H]toluene (1.81×10^6 dpm/μg; Amersham Corp.) were purified by transfer to a cold finger, dissolved in sterile distilled water, and stored frozen (14). The tritiated toluene was diluted with unlabeled reagent-grade toluene (1:300) to eliminate potential interference by a contaminant (probably ^3H_2O).

3-Methylcatechol (Pfaltz and Bauer Inc.) was purified by sublimation and transfer to a cold finger under vacuum and stored frozen.

[U-*ring*-^{14}C]toluene dihydrodiol was prepared by incubation of amino-acid grown, toluene-induced, washed *P. putida* 39/D (100 mg/liter) with [U-*ring*-^{14}C]toluene (0.4 mg/liter) in 5 ml of sterile basal medium for 80 min. The resulting filtrate was sparged with nitrogen to remove toluene to give a 0.29-mg/liter stock preparation, which was filter sterilized (pore size, 0.2 μm; Gelman Sciences, Inc.) and stored frozen.

Product identification. Spectra of the yellow filtrate were obtained by extracting with diethyl ether, partitioning into $NaHCO_3$ solution (68 mg/liter), and measuring the shift with pH on a Beckman DB spectrophotometer (19).

Filtrates from high-biomass cultures were examined for low-molecular-weight acids by ion chromatography with a Dionex model 2000i chromatograph fitted with an HPICE-ASI ion exclusion column. Filtrate (35 ml) was sparged to remove toluene, acidified to pH 3, and extracted with diethyl ether. The extract was concentrated over Na_2SO_4, and the ether was removed by evaporation to produce a 0.5-ml aqueous solution for analysis.

Hydrazones were produced from carbonyl compounds in sparged filtrate by reaction with 2,4-dinitrophenylhydrazine reagent as described by Gailiusis et al. (30), extracted with diethyl ether, concentrated 20-fold, and chromatographed.

Thin-layer chromatography was run on silica gel (ITLC-SA; Gelman) with hexane-ether (8:2, vol/vol) or chloroform-methanol (8:2, vol/vol). Sample filtrate was treated with 10 mg of $Na_2S_2O_4$ per ml at high pH to prevent oxidation and polymerization of hydroxylated compounds (D. T. Gibson, personal communication), sparged with nitrogen to remove [^{14}C]toluene, treated with 0.05 M $BaCl_2$ and 0.05 M NH_4Cl to precipitate the $^{14}CO_2$, and centrifuged. The supernatant was filtered (pore size, 0.2 μm; Gelman) and frozen for subsequent chromatography. All steps were done quickly to minimize loss of volatile products. Phenolic standards were located by their blue color following reaction with Gibbs (31) spray reagent (1 mg/100 ml of methanol), dinitrophenylhydrazones were located by their yellow color, and 2-hydroxy-6-oxohepta-2,4-dienoic acid was located by its yellow color following exposure to NH_4OH vapor.

Metabolism rates. Toluene utilization rates were determined from the amounts of radiolabeled CO_2, cell material and organic products formed over time from [^{14}C]toluene supplied. Following [^{14}C]substrate addition, subsamples of 5 to 20 ml were removed and filtered (pore size, 0.2 μm; Nuclepore) to stop the reaction. Incorporation into cell material was determined by washing the filters with 10 ml of CH_3OH and counting filter radioactivity. The subsample filtrate was quickly frozen (−20 to −50°C), thawed before analysis, acidified, and sparged and the $^{14}CO_2$ was collected in the air stream, purified, and counted (14). A 1-ml portion of the sparged filtrate was further aerated to remove any residual [^{14}C]toluene; the remaining radioactivity was taken as nonvolatile products.

Oxidation rates of toluene at high concentrations and of 3-methylcatechol in suspensions of washed cells (200 to 500 mg/liter) were determined from oxygen consumption by using an oxygen electrode (model 5/6 Oxygraph; Gilson Co., Inc.).

Affinities. Specific affinities were computed from equation 1. Initial toluene concentrations were adjusted according to the radioactivity, and the loss in radioactivity was measured after stripping with nitrogen or air. Biomass (wet weight) was determined from cell volume by using a Coulter Counter calibrated with an erythrocyte standard (49). Partial affinities which allocate metabolized substrates to various products (12) were determined from the appearance of metabolic products and reported in units of substrate mass on a carbon basis. For A → P + Q + X, which can specify conversion of toluene to organic products, CO_2, and cells, $a_A = a_{PA} + a_{QA} + a_{XA}$.

Induction kinetics. *Pseudomonas* sp. strain T2, deprived of toluene for 2 years of growth on amino acids, was centrifuged, suspended in amino acid-free medium, washed and incubated in carbon-free medium for 3 h to give cells of decreased volume (from 1 to 0.4 μm^3), adjusted to a biomass of 1 mg/liter (10 mg/liter for the toluene-free control), amended with 0 to 10 mg of [^{3}H]toluene per liter, and

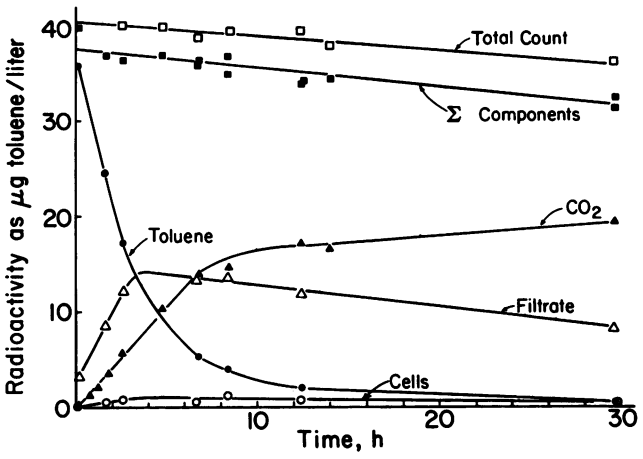

FIG. 1. Fate of [^{14}C]toluene (initial A_{out} = 40 μg/liter) exposed to toluene-grown *Pseudomonas* sp. strain T2 at 1 mg/liter. Radioactivity remaining after removing substrate, cells, and CO_2 is designated filtrate. The sum of the four fractions is designated Σ components.

incubated in 200-ml glass-stoppered bottles at 10°C. Subsamples (25 ml) were sparged with air for 15 min at 4°C to remove the inducing [^{3}H]toluene (loss rate constant, 2.3/min), amended with [^{14}C]toluene at 10 μg/liter, and incubated for 30 min. The formation rates of ^{14}C-labeled organic products, carbon dioxide, and cell material were measured and converted to partial specific affinities. When induction was low only the $^{14}CO_2$ determinations were reliable, and only the partial affinity a_{QA} is reported. Both the partial affinities and the specific affinity for toluene metabolism, as estimated from their sum, were related to the concentration of inducing toluene as well as to the duration of exposure to that concentration.

To measure induction kinetics in less severely repressed organisms, batch cultures were supplied with casein hydrolysate at 1 mg/mg of cells on a daily basis, while the biomass was maintained in a range from 73 to 400 mg/liter by dilution with fresh basal medium. Toluene was supplied as a vapor from the usual suspended glass bulb. The cultures were allowed to develop the desired specific affinity for toluene, harvested by centrifugation, washed and suspended in fresh carbon-free medium, preincubated, and amended with inducing [^{3}H]toluene as described above.

Metabolism kinetics. *Pseudomonas* sp. strain T2 was grown on toluene, harvested, washed, suspended in seawater medium at a biomass of 0.1 mg/liter (partly induced organisms, 1.0 mg/liter), and provided with [^{14}C]toluene at various concentrations. The time course of label appearance as organic products, carbon dioxide, and cell material was determined as detailed above.

Rate versus biomass. Organisms from a toluene-grown, washed culture of *Pseudomonas* sp. strain T2 were suspended in seawater medium containing 5.4 μg of toluene/liter and incubated for periods ranging from 12 min for 5 mg of biomass/liter to 5 h for 0.04 mg of biomass/liter. Five subsamples were collected, and P, Q, and X were measured as described above.

RESULTS

Marine isolates *Pseudomonas* sp. strain T2, *Pseudomonas* sp. strain T3, and *P. putida* grew on toluene at 35 mg/liter. For *Pseudomonas* sp. strain T2 the maximal growth rate was 0.06/h at 10°C. Liberated organics were the main product of toluene metabolism (Fig. 1), but were reaccumulated by the organisms, as shown by their subsequent decline. About 90% of the initial radioactivity was accounted for. The decrease in total radioactivity with time indicates a 10 to 20% evaporative loss. The time course for *Pseudomonas* sp. strain T3 (not shown) was similar.

Kinetics of toluene uptake. The dependency on toluene concentration of the rate of metabolism by *Pseudomonas* sp. strain T2 is given in Fig. 2. These cells were toluene grown and had maximal specific affinity for toluene a_A^{max} of 320 liters/g of cells per h. Like V_{max} (49), a_A^{max} was somewhat variable depending on culture conditions, measurement protocol, and the duration of culture growth on toluene. The highest a_A^{max} value regularly achieved after several weeks of exponential growth on toluene was 500 liters/g of cells per h. Changes in radioactivity over time for the cells, CO_2, and organic products formed from toluene are shown to indicate the uptake kinetics over the 8-h observation period used to determine the rates. Possible explanations for the intercepts of the product curves on the rate axis include residual substrate from incomplete sparging and abiotic oxidation products; traces of nonvolatile radioactivity sometimes occur in aqueous solutions of toluene alone. Kinetics in each component fraction responded to saturation effects (Fig. 2A to C). Michaelis plots are shown to present an undistorted view of the kinetics, while Eadie-Hofstee transformations, shown in the insets, spread the data at low concentrations and connect the whole concentration range examined. The sum of these rates at each concentration is shown in Fig. 2D as a measure of the kinetics of net toluene consumption, i.e., accumulation of toluene and recycled products less the toluene that penetrates the cell but escapes before oxidation. Specific affinities as obtained from the initial slopes of the v versus A plots, together with kinetic constants from a least-squares fit to a hyperbola (20), are summarized in Table 2.

To determine the completeness of saturation, that is, to establish the absence of apparent first-order kinetics caused by a low specific-affinity metabolic process with a high K_t, we measured toluene metabolism kinetics over a much larger concentration range (1 to 30 mg/liter) with an oxygen electrode. V^S_{max} was constant with concentration (not shown). The value, according to the stoichiometry shown in Fig. 8, was 14.7 μg of toluene per mg of cells per h, and rates agreed with those calculated from the sum of rates of toluene product formation at the lower concentrations of [^{14}C]toluene used. Data are consistent with metabolism primarily through a single system with a very low K_t (44 μg/liter) and high a_A^0 (320 liters/g of cells per h).

To determine whether the uptake kinetics were different in cells less completely induced, in which a constitutive system with different kinetics might prevail, we examined toluene metabolism in organisms induced to 3% of a_A^{max} (specific affinity a_A^0 = 16.5 liters/g of cells per h). The kinetics remained Michaelian as indicated by the small standard errors for K_t and V_{max} (Table 2). There was, however, a more complete diversion of toluene to organic products. The additional product accumulation in incompletely induced cells could be due to a metabolic system under coordinate control which is imperfect or to incomplete development of product retransport systems.

Kinetics in a soil pseudomonad. *Pseudomonas* sp. strain T2 is distinctive in its high sodium requirement. Also, the K_t observed for toluene is small compared with values for carbon sources in general (10). To decide whether the

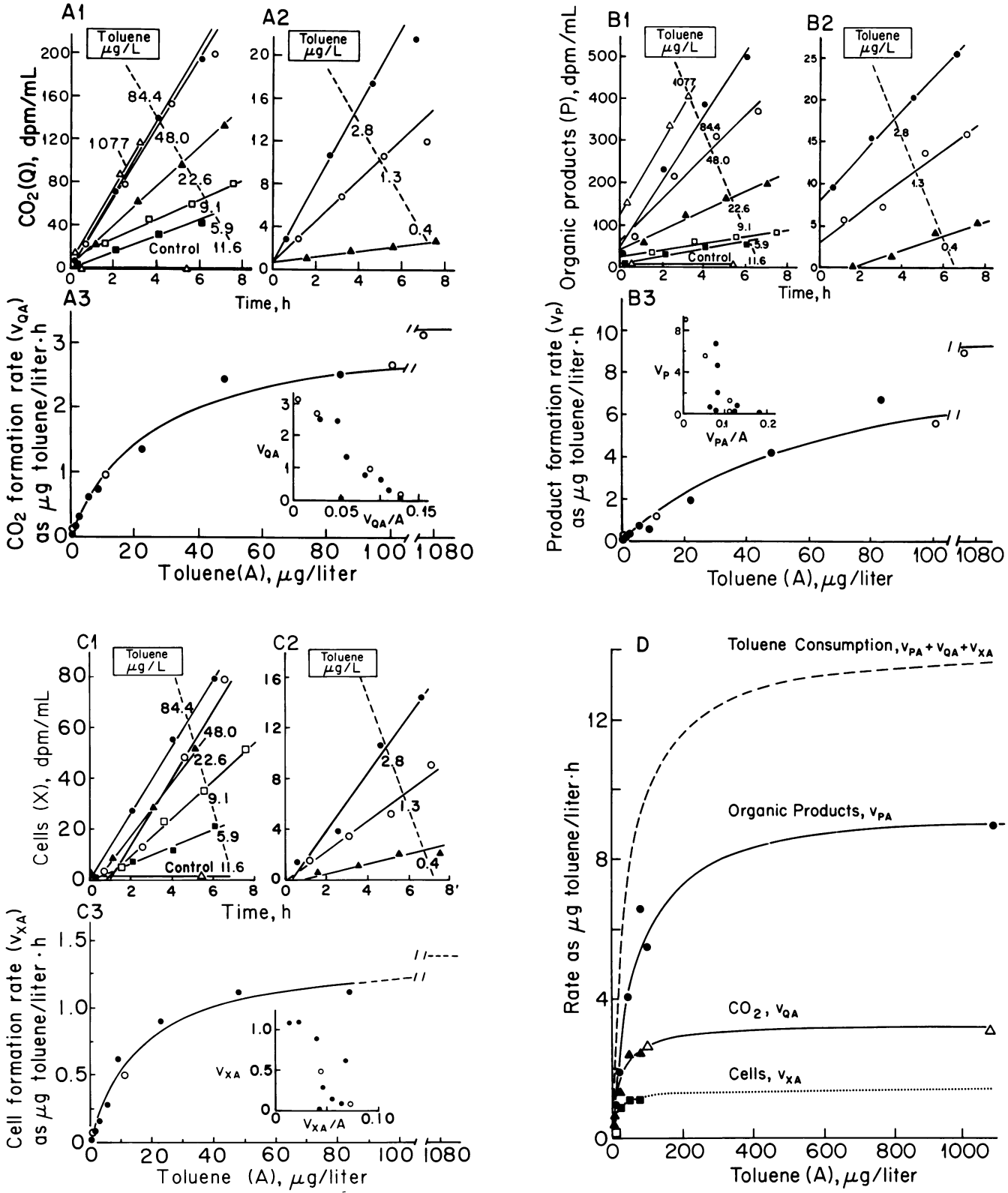

FIG. 2. Concentration-dependent kinetics of toluene metabolism by fully induced *Pseudomonas* sp. strain T2 at a biomass of 0.1 mg/liter (A1). $^{14}CO_2$ production versus time at [^{14}C]toluene concentrations from 11.6 to 1,077 μg/liter; control: cell-free medium amended with 11.6 μg of toluene/liter. (A2) Same as panel A1, except that toluene was 0.4 to 2.8 μg/liter. (A3) Rate of CO_2 production versus toluene concentration (notice break in scale). Rates are normalized to a biomass of 1.0 mg/liter. Symbols: ○, ●, data from different experiments with rates normalized to affinities measured at similar concentrations of toluene. The curve is the best hyperbola according to a least-squares fit (20) to the data. (B) Same as panel A, except that data are for the liberation of organic products. (C) Same as panel A, except for production of biomass. (D) Combined kinetic curves for all products measured in panels A3, B3, and C3 and for toluene consumption according to the sum of the products.

TABLE 2. Kinetic constants for the metabolism of toluene

Strain and conditions	Component rate	V^S_{max} (mg of toluene/g of cells per h)[a]	K_t (μg of toluene/liter)[a]	a_A^0 (liters/g of cells per h)[b]
Pseudomonas sp. strain T2, fully induced, 10°C	v_{PA}	9.7 ± 0.5	66 ± 10	147
	v_{QA}	3.3 ± 0.1	26 ± 3	126
	v_{XA}	1.4 ± 0.1	16 ± 3	87
	v (combined)	14.0 ± 0.5	44 ± 4	320
Pseudomonas sp. strain T2, incompletely induced, 10°C	v_{PA}	0.63 ± 0.28	118 ± 66	5.4
	v_{QA}	0.13 ± 0.02	30 ± 9	4.4
	v_{XA}	0.05 ± 0.10	10 ± 6	5.0
	v (combined)	0.57 ± 0.07	34 ± 9	16.5
P. putida PpF1, fully induced, 20°C	v_{QA}	20.0 ± 0.5	63 ± 8	316

[a] Results are given as mean ± standard error.
[b] Partial specific affinities and their sum: the specific affinity.

unusual kinetics observed were due to the nature of the substrate or the nature of the organism, we examined toluene metabolism kinetics in the terrestrial hydrocarbon oxidizer *P. putida*. This soil isolate grew poorly at 10°C. In previous experiments with mixed populations in stored seawater, K_t values at 10 and 20°C were the same, while V^S_{max} increased with temperature (12). We thus assumed that temperature would not greatly affect K_t, and experiments were conducted at the higher temperature. The kinetics of toluene oxidation to $^{14}CO_2$ were again hyperbolic (Fig. 3) and were characterized by a low-K_t system (Table 2) with $K_t = K_m$ (preliminary data). The specific affinity was very large, (1,055 liters/g of cells per h); it was twice that of *Pseudomonas* sp. strain T2, but V^S_{max} was three times higher at the higher temperature, and since a_A^0 is directly proportional to V^S_{max} at constant K_t, these transport constants were approximately the same in the two organisms. The specific affinity of both organisms for toluene was therefore high compared with those for other carbon source substrates. The proportion of toluene diverted to organic products was 56%, similar to that of *Pseudomonas* sp. strain T2. Therefore low K_t values for toluene and extensive liberation of metabolic products may be common characteristics for pseudomonads, both terrestrial and marine. In these two pure-culture systems and in some mixed-culture systems (12), toluene uptake was Michaelian and

$$v^S = \frac{V^S_{max} A_{out}}{K_t + A_{out}} \quad (4)$$

where the observed rate v varied with biomass according to $v = v^S/X$ as evaluated below.

Identification of organic products from toluene. At high population *Pseudomonas* sp. strain T2 gave a yellow solution with maximal absorbance at 388 nm (pH > 7) and 315 nm (pH 3) (Fig. 4A). The proximal extradiol or *meta*-cleavage product of 3-methylcatechol, 2-hydroxy-6-oxohepta-2,4-dienoic acid, has these characteristics (5). In a culture of 700 mg of biomass per liter the concentration of 2-hydroxy-6-oxohepta-2,4-dienoic acid (extinction coefficient, 13,800 liters/mol per cm at 388 nm and pH 7.6 [5]) reached 70 mg/liter. When the organism was grown on phenol another yellow metabolite appeared with maximal absorption at 375 nm (320 nm at pH 3), consistent with 2-hydroxymuconic semialdehyde. Although both compounds are produced by extradiol cleavage of the aromatic ring, only the keto acid is formed (if metabolism is through 3-methylcatechol rather than decarboxylation to catechol (37). A positive Gibbs reaction (31) indicated that 3-methylcatechol was formed. Other metabolic intermediates in high-density cultures were acetate at 20 g/liter and formate at 0.1 g/liter (Fig. 4B).

Products in dilute culture. Hydroxylated products of toluene metabolism can undergo abiotic oxidation and be reutilized (Fig. 1) or polymerize and turn the culture medium brown (33, 51). Products liberated during toluene metabolism, as opposed to those that accumulated, were observed when short incubation times (75 min) and dilute cell populations (10 mg/liter) were used to minimize recycling. Environmentally atypical leakage was minimized by supplying toluene at only 76 μg/liter to minimize formation of intracellular intermediates at rate-limiting metabolic steps and their appearance as leaked products.

Radiochromatograms of the dilute-culture filtrate are shown in Fig. 5. Toluene dihydrodiol was located by acid-catalyzed (pH 2) dehydration of the product to form *o*-cresol (33), which gave a new peak in Fig. 5B at the expense of one in Fig. 5A. Both the presence of 3-methylcatechol and its lability were indicated by peaks that only sometimes appeared. The traces of formate could indicate some metabo-

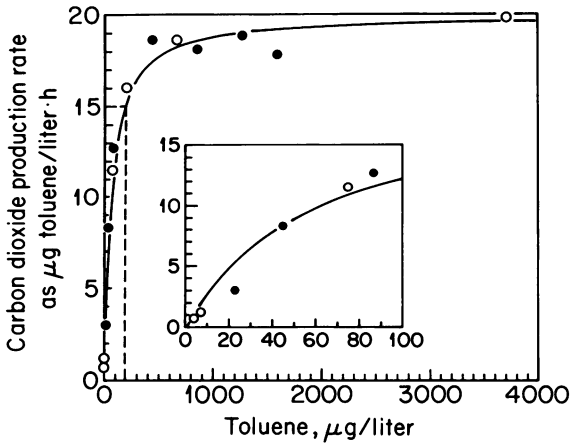

FIG. 3. Concentration-dependent kinetics of toluene metabolism by toluene-grown *P. putida* PpF1 at a biomass of 1 mg/liter. Rates v_{QA} were computed from $^{14}CO_2$ formation. Data were collected from different experiments (○, ●) and normalized to computed values of V^S_{max}.

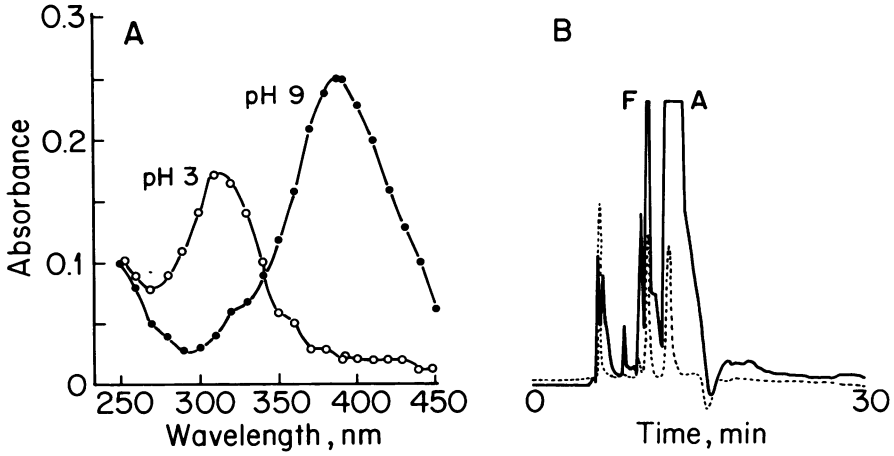

FIG. 4. Products of toluene metabolism in filtrates of high-population batch cultures of *Pseudomonas* sp. strain T2. (A) Absorption spectrum at pH 3 and 9. (B) Ion chromatogram of a concentrated diethyl ether extract of culture filtrate. Peaks labeled F and A correspond to retention times of authentic formate and acetate. – – –, Control with sterile toluene-amended medium.

lism via *meta* cleavage through the benzoate branch rather than the 3-methylcatechol branch of the *meta* pathway. Moderate movement of the dinitrophenylhydrazones (Fig. 5D) is consistent with the presence of highly polar *meta*-pathway carbonyl compounds such as 2-hydroxy-6-oxohepta-2,4-dienoic acid and pyruvate; 20% of the radioactivity was associated with standard pyruvate-dinitrophenylhydrazone. Recovery of radioactivity in the hexane-ether system was only about 80% at pH 9 owing to volatile compounds

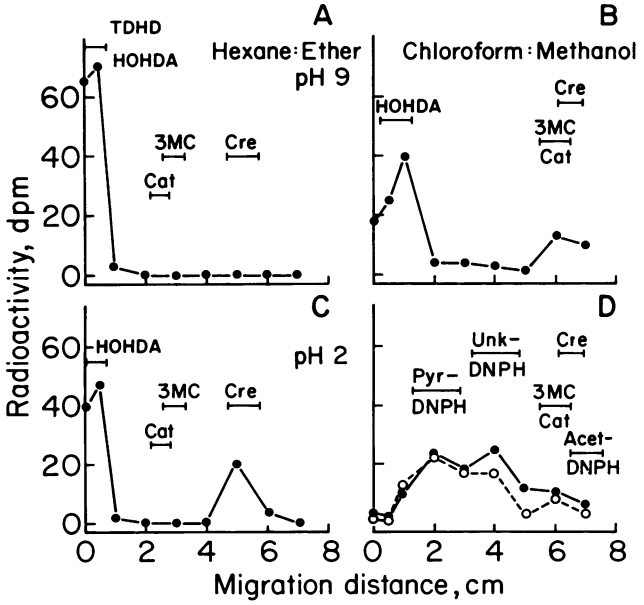

FIG. 5. Chromatographic separation of ^{14}C-labeled organic products from a dilute culture. Applied samples contained 170 dpm in 0.1 ng of material. Solvents were hexane-ether (left) and chloroform-methanol (right). (A and C) Migration of radioactivity from filtrate adjusted to pH 9 and 2, respectively. Chromatograms of acidified filtrate are shown before (B) and after (D) reaction with 2,4-dinitrophenylhydrazine. Horizontal bars show the location of standards: (TDHD, toluene dihydrodiol; HOHDA, 2-hydroxy-6-oxohepta-2,4-dienoic acid; Cat, catechol; 3MC, 3-methylcatechol; Cre, *o*-cresol). Dinitrophenylhydrazone standards: Pyr-DNPH, pyruvate; Acet-DNPH, acetaldehyde; Unk-DNPH, unknown.

such as 3-methylcatechol and acetaldehyde (lost before treatment with dinitrophenylhydrazine) and 70% at pH 2 owing to the additional loss of *o*-cresol formed from the dihydrodiol and the loss of acetate and formate.

This pseudomonad did not oxidize benzoate when grown on toluene unless specifically induced to do so, and radioactivity in the organic product fraction was similar whether the toluene supplied was ring labeled or methyl carbon labeled. Thus benzoate is an unlikely major intermediate of toluene metabolism, and substantial initial oxidation at the methylene carbon by *Pseudomonas* sp. strain T2 is absent. From these data the organic products liberated appear to be composed of 50% carbonyl compounds such as 2-hydroxy-6-oxohepta-2,4-dienoic acid and pyruvate, 28% toluene dihydrodiol, and 20% volatile products such as acetate, formate, 3-methylcatechol and acetaldehyde.

Organic-product reutilization. Efflux and reuse of organic products (Fig. 1) form a metabolic loop that extends outside the cells. Its significance depends on the relative toluene/product concentration and hence the biomass. An indirect influence is a reduction in K_μ for toluene with increased biomass as a result of increased yield. Low specific affinities and large K_t values for these products would favor their accumulation only at high concentration (see Discussion). For 3-methylcatechol the observed specific affinity was fairly low (8.5 liters/g of cells per h), with K_t of 3.2 mg/liter (data not shown). Preliminary radioactivity data indicated an even lower specific affinity (5 liters/g of cells per h) for toluene dihydrodiol, and preliminary spectrophotometric data gave a value of >0.013 liters/g of cells per h for 2-hydroxy-6-oxohepta-2,4-dienoic acid (at 70 mg/liter). Oxygen uptake rates were additive when toluene and 3-methylcatechol, both at 5 mg/liter, were present together (not shown), which demonstrated concomitant use of a substrate and its liberated product. V^S_{max} for 3-methylcatechol was at least 280 mg/g of cells per h (uncertain owing to substrate inhibition), which is a sufficiently large value to allow growth despite the low specific affinity.

Induction at high toluene concentration. The specific affinity depended on the level of induction. After growth on amino acids for 2 years, growth on toluene proceeded at a normal rate, but at 10°C, a 2-week period of induction was required. The partial affinity $a_{QA}{}^{10}$, from the rate of toluene use resulting in CO_2 liberation at 10 μg/liter, typically

TABLE 3. Induction of toluene metabolism in *Pseudomonas* sp. strain T2 at high toluene concentrations[a]

Time (days)	a_A^{10} (liters/g of cell per h)[b]
0	0.03
1	0.29
3	0.26
4	0.29
6	0.17
8	0.29
13	3.7
14	12.9

[a] Severely repressed *Pseudomonas* sp. strain T2 was supplied with casein hydrolysate (1 mg/mg of cells) and toluene (35 mg/liter). Biomass was maintained at 73 to 400 mg/liter by daily dilution with seawater medium.
[b] a_A is taken as $a_{QA}/0.35$.

increased 10-fold within 24 h after exposure to 35 mg of toluene/liter, plateaued (Table 3), and then gradually increased to 175 liters/g of cells per h after 2 to 3 weeks of exposure. This value is marginally sufficient for slow growth with low-K_t substrates (8). The induction time was shortened by yeast extract but not by amino acids and was reduced to 3 days when the temperature was raised from 10 to 25°C. Rates were reproducible, so that organisms with a particular specific affinity for toluene could be prepared.

Kinetics of induction. Kinetics relating induction rate to concentration are very limited and are restricted to hydrophilic (or at least amphiphilic) substrates. Since toluene is a nonpolar hydrocarbon, an exploration of induction kinetics by this compound was required to formulate the effect of induction on the specific-affinity term in the rate equation. In experiments with *Pseudomonas* sp. strain T2 there was a hyperbolic increase in specific affinity with concentration following induction for a standard period (39). The same relationship was observed for the organisms in raw seawater. In addition, a_A^{max}, the value of the specific affinity at saturating concentrations for the standard period, increased with induction time (11). We also observed that the specific affinity of induced organisms gradually decreased over a 2-year period and that the rate of reinduction (probably classical derepression) depended on the beginning value of the specific affinity $a_{A\,0}$.

To extend this understanding to the environmentally significant region of very low concentrations, we examined the effect of $a_{A\,0}$ on a_A as a function of time. Beginning with washed cultures of *Pseudomonas* sp. strain T2 exposed to [^{3}H]toluene at low (<34-μg/liter) concentrations, the partial specific affinity remained constant at a very low basal value of a_{QA}^{10}, 0.01 liters/g of cells per h, for 11 days. Induction was first observed in the culture exposed to toluene at 132 μg/liter for 1 week (not shown). After 11 days of exposure the minimum apparent concentration for induction was 55 μg/liter and the value of the specific affinity achieved was proportional to the concentration of toluene (Fig. 6A, inset). The value of the specific affinity obtained (9.5 liters/g of cells per h) remained well below the maximal value of a_A^0 which can be achieved. The intercept indicated a threshold concentration for induction. Loss of the inducing [^{3}H]toluene was only about 15%. A control in which [^{14}C]toluene replaced the inducing [^{3}H]toluene gave about the same $^{14}CO_2$ evolution rate and helped confirm the results. The cell population remained constant during induction, but the initial 5.4 mg of biomass per liter decreased by half as the cells shrank from 0.97 to 0.37 μm^3 per cell during this period of slow toluene metabolism.

Induction kinetics of organisms that were initially at a slightly higher state of induction were examined by bringing the specific affinity of the stock culture up to an a_{QA}^0 of 0.18 liters/g of cells per h or an a_A of ca. 0.04% of a_A^{max}. The concentration dependency of induction (increase in specific affinity following additional exposure to toluene for a standard period), $a_A - a_{A\,0}$ is shown in Fig. 6B and C. After 43 h (Fig. 6B) there was an increase in the rate of $^{14}CO_2$ production at only 2.5 μg/liter compared with the unamended control. After 93 h, induction progressed suffi-

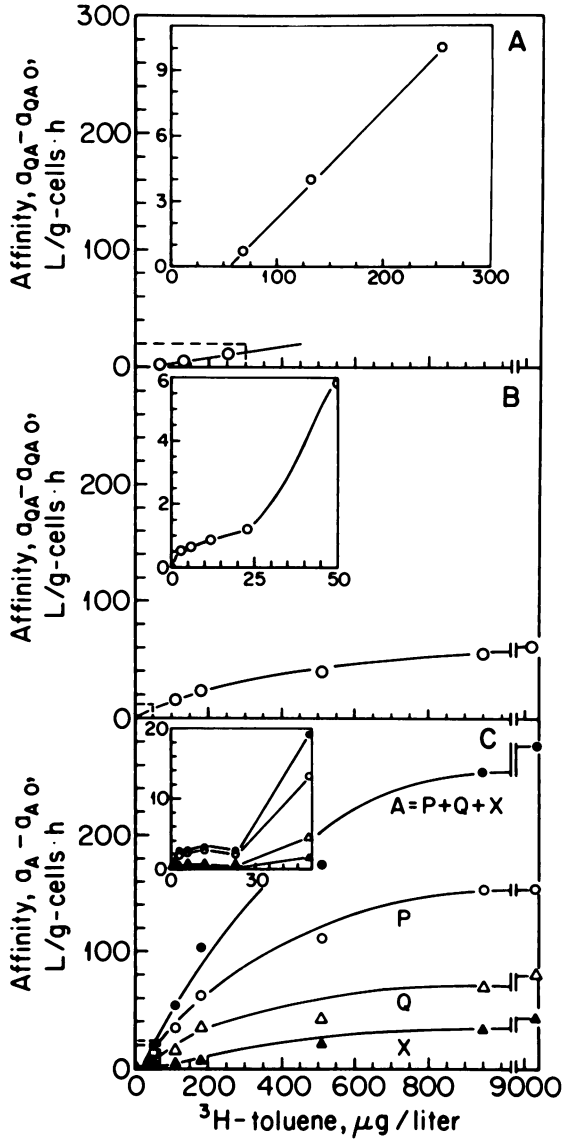

FIG. 6. Concentration dependency of toluene induction according to the net increase in specific affinity. (A) Increase in the partial specific affinity $a_{QA} - a_{QA\,0}$ ($a_{QA\,0}$ = 0.01 liter/g of cells per h) of *Pseudomonas* sp. strain T2 following extensive toluene deprivation as measured by $^{14}CO_2$ formation from [^{14}C]toluene following exposure to [^{3}H]toluene at the concentrations shown for 260 h. (B) Same as panel A after 43 h of exposure to inducing [^{3}H]toluene, but beginning with partially induced ($a_{QA\,0}$ = 1.8 liters/g of cells per h) organisms. (C) Same as panel B but after 93 h of exposure to [^{3}H]toluene and also giving partial affinity associated with products (P), CO_2 (Q), cells (X) and the sum as toluene (A); $a_{PA\,0}$ = 0.44, $a_{QA\,0}$ = 0.1, $a_{XA\,0}$ = 0.02, and $a_{A\,0}$ = 0.56 liter/g of cells per h.

ciently well that toluene incorporation into organic products and cell material, as well as carbon dioxide, could be detected and the increase in specific affinity a_A (rather than the partial specific affinity a_{PA}) could be evaluated directly (Fig. 6C). Metabolism rates were corrected to 10 μg of toluene per liter to give affinities a_A^{10}. With K_t at 44 μg/liter, saturation reduces the rate by 18% or less, so that these values are near the specific affinity a_A^0. Since the base level of induction $a_{A\,0}$ is low, the increase in specific affinity over its base value ($a_A - a_{A\,0}$) is approximately the same as the value measured from rate/concentration a_A (see legend to Fig. 6). Insets in Fig. 6B and C show increased induction at a minimum of 2.5 μg/liter, and the apparent threshold for induction (Fig. 6A) becomes the foot (inducing concentration at the upward inflection point in rate) of an approximately sigmoidal curve with a half-maximal inducing concentration K_{ind} of 290 μg/liter and a foot of 23 μg of toluene per liter.

Growth was absent in this 4-day experiment at toluene concentrations of <12 μg/liter and was negligible at intermediate concentrations. At higher concentrations (9.1 mg of toluene per liter), cells grew from 7 to 9.7 mg/liter over 4 days and the medium turned yellow with 2-hydroxy-6-oxohepta-2,4-dienoic acid. At the lower inducing concentrations, toluene was converted principally (>80% of the toluene used) to organic products, while at >25 μg of toluene per liter more carbon dioxide (35%) and cell material (15%) was produced. Beginning with partly induced cells, the induction level after 4 days was about 50% of the maximal value, 275 versus 500 liters /g of cells per h. The concentration required to give this incomplete level of induction, K_{ind}, was 320 μg of toluene per liter, and the foot remained constant at 23 μg/liter; full induction followed later.

Beginning with cells at an even greater initial level of induction (a_{QA}^{10} = 3 liters/g of cells per h), the sigmoidal curve became hyperbolic (39), as it did with the organisms in raw seawater (12). For such systems the increase in affinity with respect to concentration for mildly repressed organisms following exposure to inducing substrate for some time (t) during the induction process is

$$(a_A^0)_t = \left(\frac{a_A^{max} A_{out}}{K_{ind} + A_{out}}\right)_t \quad (5)$$

where K_{ind} expresses saturation of the induction process (11, 39) as the metabolic capacity, expressed as a_A^{max}, increases with time. When the initial stage of induction was very low and sigmoidal kinetics were observed, exponents according to the Hill equation could be added to improve the data fit. However, cause of the upturn is not seen as interactions among sites, so that until data are sufficient for appropriate theory we will simply express this phenomenon according to the size of the foot in the induction curve (when present), the inducer concentration at the first inflection point, and the induction constant K_{ind}. These constants, as they varied with the initial state of induction, are summarized in Table 4.

TABLE 4. Kinetic constants for induction of toluene metabolism

Initial state of induction $a_{A\,0}$ (liters/g of cells per h)	Exposure time (h)	Foot (μg/liter)	$(a_A^0 - a_{A\,0})_{max}$ (liters/g of cells per h)[a]	K_{ind} (μg/liter)
0.03[b]	260	55	>10	>135
1.8[b]	43	23	170	321
2.5	93	23	268	380
10[c,d]	68	0	314	96
1.4[c,e]	48	0	15	1.9

[a] Asymptote (maximal value) of net change in specific affinity.
[b] Affinities obtained from carbon dioxide production alone, a_{QA}, were divided by 0.35 to account for organic product and cell formation.
[c] $a_{A\,0}$ was calculated by using a correction for saturation from Michaelis-Menten, $(a_A^0 - a_{A\,0})_{max}$, K_{ind}, and the toluene intercept.
[d] From reference 39.
[e] From reference 11.

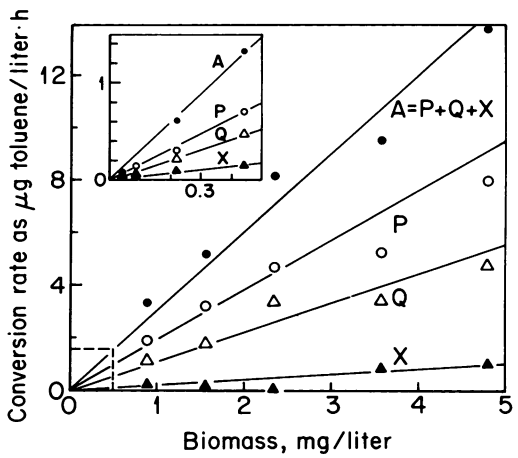

FIG. 7. Change in toluene metabolism rate with biomass of toluene-grown *Pseudomonas* sp. strain T2. Production rates of organic products v_{PA} (P), carbon dioxide v_{QA} (Q), and cell material v_{XA} (X) were measured at a [^{14}C]toluene concentration of 5.7 μg/liter. Toluene (A) consumption v_A was computed from $v_{PA} + v_{QA} + v_{XA}$.

Nature of the inducer. Mutant *P. putida* 39/D lacks toluene dihydrodiol dehydrogenase (50). It produces large quantities of toluene dihydrodiol from toluene, but only after induction, which can be achieved by exposure to high concentrations of toluene. Since little $^{14}CO_2$ was produced from [14C]toluene, formation of subsequent *meta*-pathway intermediates which may serve as inducers is unlikely. We presume that toluene cannot be concentrated in the cytoplasm by membrane transport because of its high partition coefficient into lipid (40), causing high permeability (38), so that it would simply diffuse back out. That induction is due to a metabolic product is consistent with the appearance of these products at low inducer concentration followed by more complete diversion to CO_2 and cells at higher inducer concentrations (Fig. 6C). Since toluene dihydrodiol is the only product formed, it is probably one of the inducers for toluene metabolism in this organism.

Rate versus biomass. Equations 1 and 2 state that the specific rates of substrate uptake and growth are independent of biomass. However, numerical simulations of steady-state systems in which metabolic products capable of recycling are liberated indicate that overall metabolic rates can increase with biomass at a constant and rate-limiting concentration of a single substrate and that the increased metabolic rates are reflected by a decrease in K_μ. Thus leakage potentially complicates the relationship between growth rate and biomass. To explore the significance of leakage on the rate equation, we kept toluene at a constant subsaturating concentration, manipulated biomass concentration, and measured the rates of toluene uptake (Fig. 7). Errors were approximately 10% due to biomass loss from endogenous metabolism, 5% due to subsample timing during short incubations at high biomass, 5% due to substrate

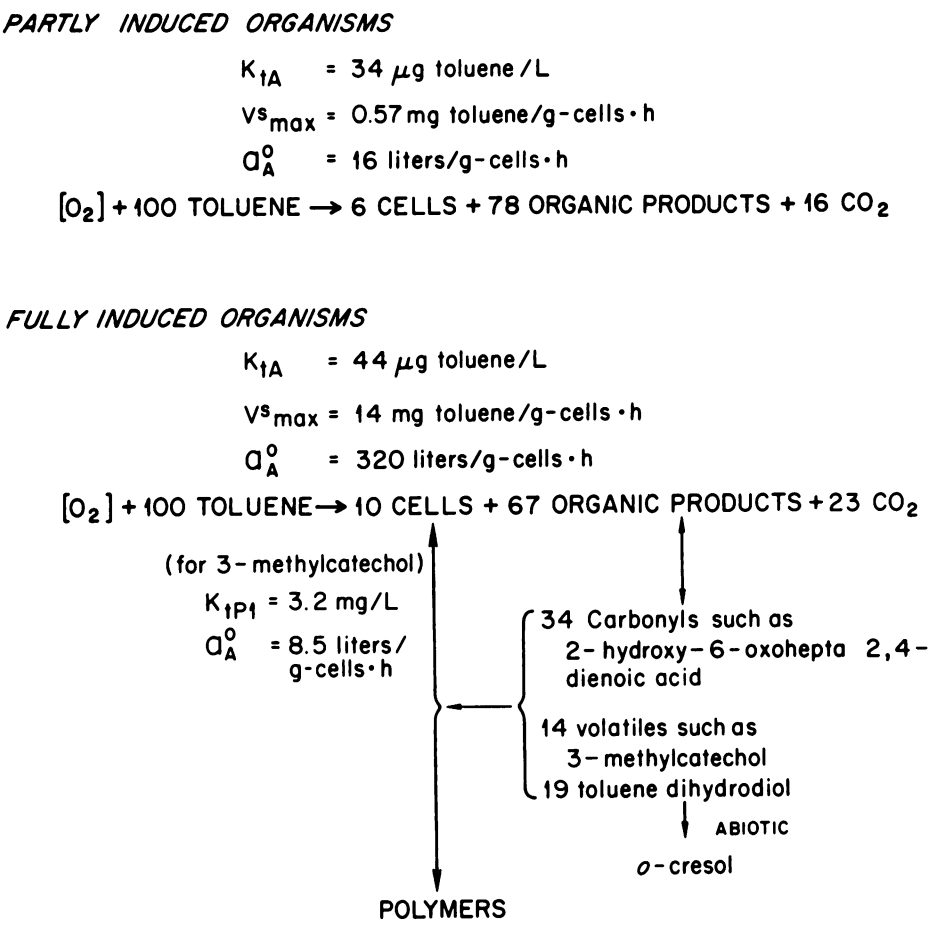

FIG. 8. Toluene metabolism by *Pseudomonas* sp. strain T2 showing kinetics and distribution of products formed by both partly and completely induced cells along with recycling of products.

depletion, and another 5% due to counting error, or about 20% overall. Within these constraints, data show that the kinetics were indistinguishable from first-order in biomass over a large range of cell populations and that there was no apparent change in the distribution among the products of toluene metabolism with rate. Thus the influence of biomass on growth rate is adequately expressed by a second-order rate equation of the type given by equation 2.

Overall kinetics of toluene metabolism. Material balances and kinetics for toluene metabolism by *Pseudomonas* sp. strain T2 are shown in Fig. 8 for both the partly (3%) and the fully induced organisms. They emphasize the enhanced diversion of toluene to metabolic products and low yields associated with incompletely induced organisms, indicate the importance of recycling, and summarize the kinetic constants available.

Values for cell yields from toluene-derived radioactivity and Coulter Counter determinations of biomass required data on the elemental composition of the organisms. Using carbon-nitrogen analysis together with cell volumes, we obtained values of 19 and 22% carbon for cells in stationary and exponential phases of growth with a mass of 0.57 and 1.04 pg per cell, respectively. This gave values of 0.10 and 0.17 for the yields of biomass from toluene in partly and completely induced cells to complete evaluation of all terms in equations 2 and 3.

DISCUSSION

Kinetics of toluene metabolism. Results show that toluene is accumulated by two pseudomonads with Michaelian kinetics. Complete saturation was achieved, which rules out other important processes characterized by secondary diffusion limitation (59) or high K_t values (8). These values were atypically small for a biochemical serving as the carbon (and energy) source (10), but they could be usual for hydrocarbons, since except for methane (46), toluene is the first hydrocarbon for which the kinetics of transport have been extensively examined. The value of 44 μg/liter is remarkably low if it reflects a concentration at the site of a metabolic enzyme before accumulation by a transport system. K_t data reflect extracellular concentrations, and accumulation of toluene to higher concentrations in the cytoplasm is prevented by high permeability of the cell membrane to it. Possibly toluene is metabolized within the cell membrane, where concentrations are higher owing to partitioning. Even lower K_t values, 2 μg/liter (12), have been obtained with the indigenous microflora of seawater as corroborated by Shimp and Pfaender (54, 55). The higher K_t values reported here, compared with those for raw seawater, indicate that many marine bacteria sequester substrate with kinetic characteristics different from those reported for laboratory cultures, characteristics which remain undefined because of the diffi-

culty in growing most of those organisms. These low K_t values must be associated with enzymes that are either very active or very abundant, because relatively high specific affinities were attained. High specific affinities are required for growth on toluene alone to avoid the truncation of rate by enzymatic saturation, since K_t sets the maximal growth rate according to $\mu_{max} = a_A^0 Y_{XA} K_t$ (10).

Product formation. Toluene metabolism led mainly to organic products. When the level of induction was low, the concentration of organic products increased as the level of induction decreased. However, trace amounts of CO_2 were always produced. More complete metabolism to CO_2 and cell material was observed following extended exposure to toluene (Fig. 6C). As induction neared completion, product leakage increased with toluene concentration, indicated by the high K_t for organic products compared with CO_2 (Table 2); K_t would be constant if product yields remained constant at various rates of toluene uptake. Leakage from the toluene-deprived organisms could be due to the presence of only small amounts of the first enzymes for toluene metabolism and even less of those required further along the metabolic pathway. More of both of these, together with remaining pathway enzymes, are then produced when products begin to appear. In the more induced cells, product liberation could again be due to insufficient enzymatic capacity, but from enzymes farther downstream. However, simultaneous addition of both toluene and 3-methylcatechol, a likely intermediate of toluene metabolism, gave additive rates of oxygen consumption at saturating toluene concentrations (not shown), and so additional enzymatic capacity downstream must exist. Another explanation might be product formation within the cell membrane, where initial oxidation may occur but complete retention is physically impossible. However, leaked organics contain components from farther down the enzymatic pathway as well. Whatever the reasons for product leakage, the process is significant, giving depressed cell yields, new additions to environment, and changes in K_μ.

Recycling. The disappearance of products depends on their formation, abiotic conversions, and recycling. Specific affinities of *Pseudomonas* sp. strain T2 for organic products such as 2-hydroxy-6-oxohepta-2,4-dienoic acid are much lower than for toluene, so that when substrate concentrations are low these products accumulate. For 3-methylcatechol, a_P is only 8.5 liters/g of cells per h, but growth still occurs owing to the fairly high K_t (3,200 μg/liter). At low biomass levels, product concentrations are low and recycling is further minimized by low a_P values. In high-biomass systems with a high toluene concentration, metabolism remains restricted by the low K_t, and much of the toluene concentration is in kinetic excess. However, the rate of product reaccumulation increases with concentration over a much larger range. Tightly coupled recycling is therefore a phenomenon of high population and high substrate concentration, which are difficult to extrapolate to the environment.

Kinetics of induction. Induction usually requires only minutes (43) to hours (18, 29), compatible with our findings, considering the low temperature. We found that both the time and concentration dependency of induction changed with the initial level of induction, and the process could take as long as 11 days. Production of cells with a very low level of induction required deprivation of inducer for extended periods. A return to specific affinities characteristic of the induced state was adequately expressed by equation 5. Kinetic constants for induction (K_{ind}) and transport (K_t) were similar, as expected if the rate of induction is proportional to

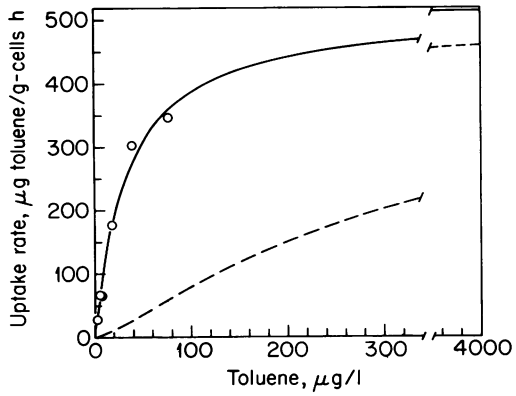

FIG. 9. Combined effects of saturation and induction on the kinetics of uptake as computed from equation 6. ——, Toluene uptake data for incompletely induced (a_A^0 = 16.5 liters/g of cells per h) organisms used to obtain the kinetic constants in Table 1. The curve is computed with K_{ind} = 0, i.e., from equation 4. – – –, Uptake by organisms after 93 h at concentrations for both induction and uptake which were the same. Hypothetical organisms had a sufficient base level of induction $a_{A\,0}$ to attain a maximal increase in specific affinity ($a_A^0 - a_{A\,0}$) of 16.5 liters/g of cells per h in 93 h at saturating toluene concentrations. At lower inducing concentrations (abscissa), the specific affinity was less and was given by equation 5 with an induction constant of 380 μg of toluene per liter (Table 4).

intracellular product concentrations and if those concentrations depend on rates of toluene uptake, rates that saturate at concentrations defined by K_t. Deprivation of substrate appears to result in the nearly complete loss of the ability of the organism to form toluene products, which are apparently used in the induction process. Leakage and endogenous processes consume some of the products formed, and so the higher toluene concentrations required to induce severely repressed cells, as well as the sigmoidal kinetics, are expected. As in complex enzymatic reactions, the shape of the induction curve depends on numerous rate constants. K_{ind} then is simply taken as the substrate concentration when the rate of induction is half-maximal.

New proteins in electrophoresis gels from both membrane and cytoplasmic fractions appeared in parallel with an increase in the specific affinity following induction (not shown). Although plasmids can carry genes for degradation of hydrocarbons (43) and other constituents of the environment, induction here appears to involve chromosomal genes. The *P. putida* PpF1 used in uptake experiments by soil organisms contains no detectable plasmids (27). Other strains of *P. putida* such as BG1 can contain a TOL plasmid, but they metabolize toluene differently, through the catechol branch of the *meta* pathway (58). Our seawater isolate leaves a signature of products which indicates metabolism by the chromosomally encoded 3-methylcatechol branch. Moreover, plasmid production, as well as changes due to mutation, seems unlikely in our nongrowing systems, in which cell numbers remained constant while the biomass level decreased by half. The kinetics of induction here were the same as those in natural systems with populations of only 10^6/ml, for which plasmid transfer frequencies of $<10^{-6}$/h would be expected even if all bacteria were receptive (21). Finally, the capacity to use toluene was retained in *Pseudomonas* sp. strain T2 following attempts to remove the plasmids by curing (K. Craig, preliminary data), so that the kinetics probably reflect rates of enzyme induction. The low rate of induction in noninduced cells is expected here, since

required levels of inducing substrate must be collected to start the process, but that rate is only $a_A A_{out}$, or about 1 μg of toluene per g of cells per h.

Use of the rate equation. The specific affinity relationship is general for uptake and can be used with equations for saturation and induction to give a biomass-specific description of uptake. For example, in the toluene-*Pseudomonas* sp. strain T2 system, because uptake is Michaelian, the specific affinity a_A in equation 1 can be replaced by $V^S_{max}/(K_t + A_{out})$ from equation 4 to express saturation of uptake. A computer trace of equation 4 fit to uptake rates for partly (3%) induced cells is shown in Fig. 9. Induction further modulates the capacity for uptake. Since the uptake curve is hyperbolic, the initial slope of the curve V^S_{max}/K_t ($= a_A^0$) is the upper limit of the specific affinity with respect to saturation. a_A^0 is also affected by induction, as expressed by equation 5. Substituting $a_A^0 K_t$ for V^S_{max} into equation 4, equation 5 into the result and that into equation 1 gives the rate of uptake at concentration A_{out} following exposure to substrate at that concentration for some standard time t:

$$(v)_t = \left[\left(\frac{a_A^{max} A_{out} K_t}{K_{ind} + A_{out}}\right)\left(\frac{A_{out} X}{K_t + A_{out}}\right)\right]_t \quad (6)$$

Plots of equation 6 (Fig. 9) are sigmoidal. Curvature is initially concave up owing to second-order kinetics, since rate changes in proportion to both substrate and enzyme concentration and limiting enzyme per unit biomass increases with induction. At higher concentrations saturation dominates the kinetics and rates become maximal. Separation between induction-dependent and induction-independent curves of Fig. 9 increases with both the size of the induction constant and the size of the foot in the induction curve. The foot of the induction curve was neglected in equation 5 because it represents a complication peculiar to severely repressed cells, which take a rather long time to develop. Both the foot and the first-order portions are related to $a_{A,0}$, the initial level of the specific affinity, so that as $a_{A,0}$ increases above approximately 3% of the fully induced level (68-h data in Table 4) the curves approach an identical hyperbola. Since the effects of saturation and induction are specified separately in the derivation of equation 6, the relationship can be tuned to accommodate more complex situations such as the sigmoidal kinetics of Fig. 6. In aquatic systems, in which simultaneous use of several organics is the rule, the contribution of toluene to growth would be given by its contribution a_A to the total affinity $a = a_A + a_B + a_C + \ldots$ (12) and associated yield at the prevailing rate of growth from equation 3. Since concentrations are low ($<K_t$), values of the various concentration-dependent affinities can be approximated as their upper limit a^0, which in turn can be approximated by a derivative of equation 4, V^S_{max}/K_t. These formulations allow one to anticipate the behavior of populations in systems in which substrate concentrations are dilute.

We have found specific affinity useful for anticipating mechanisms of transport (8), comparing the abilities of both organisms and substrates to participate in uptake (10), and anticipating unexpected carbon sources in the environment (9). Recent data (D. K. Button and F. Jüttner, manuscript in preparation) indicated that both the anticipation of compound types (terpenes) and their concentrations (micrograms per liter range) was correct. That correct predictions were made on the basis of theory and data presented here suggests the power of kinetics as one tool for helping understand aquatic microbial process.

ACKNOWLEDGMENTS

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