Effect of Chemical Structure on Microbial Degradation of Substituted Benzenes

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The rate of degradation of mono- and disubstituted benzenes by soil microorganisms was determined by a spectrophotometric technique. Chloro, sulfonate, and nitro groups retarded the rate of biodegradation whereas carboxyl and phenolic hydroxyl groups favored decomposition of the substituted benzenes. The meta isomer was commonly the most resistant to attack by soil microorganisms, but the ortho isomer was the most resistant for certain classes of compounds.

THE PERSISTENCE of synthetic chemicals in natural environments, particularly in soil and water, is a problem of considerable concern. Pesticides, detergents, packaging materials, and industrial wastes may reside in a particular ecosystem or move through a number of environments because of the inability of microorganisms to degrade the unnatural compound at significant rates, if at all. Despite the concern about the lack of rapid biodegradation of many compounds that are potential or actual soil or water pollutants, there is surprisingly little information about the influence of chemical structure upon the microbial degradation of classes of synthetic compounds which are appearing in significant amounts as environmental pollutants (*1*).

Considerable attention has been directed to determining the influence of the structure of unsubstituted hydrocarbons on their residence times in natural environments, but few studies have been concerned with the effect of the type, number, or position of substituents on the rate of decomposition of organic compounds by a mixed microflora. An influence of meta substitution on the rate of degradation of chlorophenols and chlorophenoxyalkanoic acids has been noted (*2, 3*), and benzoic acid metabolism by a soil population was observed to be delayed by the introduction on the ring of a halogen substituent (*11*). Biological attack on the triazine ring appears to be retarded by the addition of amino groups to the molecule (*6*), phenylurea destruction in soil is slowed down by insertion of chlorine on the aromatic moiety (*12*), and the position of the sulfonate group alters significantly the disappearance rate of alkylbenzene sulfonates (*13, 14*).

The present investigation was designed as a systematic study of the effect of type, position, and number of substituents on the rate of decomposition of the benzene ring. To date, few such systematic studies have been made, except for the work of Kameda, Toyoura, and Kimura (*9*) with pure cultures of Pseudomonas strains. To study the biological potential for the degradation of synthetic chemicals and to avoid idiosyncrasies and physiological limitations of individual microbial strains, a mixed population of soil microorganisms was selected as the appropriate assay system.

Methods

The procedure employed, essentially the same as that used earlier with the chlorophenols and chlorophenoxyalkanoic acids (*2*), relies upon the loss of ultraviolet absorbancy when the benzene ring is cleaved by microorganisms derived from a soil inoculum. The solution contained the test compound as the sole carbon source to support microbial proliferation and had, in addition to the aromatic compound, 1.6 grams of K_2HPO_4, 0.40 gram of KH_2PO_4, 0.50 gram of NH_4NO_3, 0.20 gram of $MgSO_4 \cdot 7H_2O$, 25 mg. of $CaCl_2 \cdot 2H_2O$, 2.3 mg. of $FeCl_3 \cdot 6H_2O$ and 1000 ml. of distilled water.

The absorption spectrum of each compound was determined by dissolving it in a solution containing 0.16% K_2HPO_4 and 0.04% KH_2PO_4 and recording the ultraviolet absorbancy obtained with the Beckman spectrophotometer, Model DB. The wavelength selected for measuring the rate of degradation was at or near the absorption maximum for each of the test substances, except where no distinct peak was noted; under such circumstances, the wavelength chosen was one at which the light absorption was sufficiently high for convenient use.

Forty-milliliter aliquots of the medium were placed in 4-oz. screw-cap bottles, 45 mm. diameter × 80 mm. high, and these were inoculated with 1.0 ml. of a 1% suspension of Niagara silt loam. A parallel series of reaction vessels was set up identical to the first except that each bottle also contained 8 mg. of $HgCl_2$ and $5 \times 10^{-7} M$ Tween 80. Readings were made on these flasks at the same time intervals. Another series identical to the first was set up to determine if the chemicals at the concentrations employed were toxic to the microflora; these vessels received glucose to a final concentration of 1%, and growth in the tubes was recorded visually. The bottles were incubated in the dark at 25° C.

At intervals of 3 to 6 hours and at 1, 2, 4, 8, 16, 32, and 64 days after inoculation, the solutions were mixed, an aliquot was removed, and the suspension was centrifuged 10 minutes at 825 × G. The absorbancy of the supernatant was read at the selected wavelength against the supernatant from the reaction vessel containing a soil-medium mixture free of the chemical but incubated in an identical fashion. The liquid and the soil residue were returned to the bottles and incubated further. Readings were made on the Beckman spectrophotometer, Model DU. All reaction vessels were set up in duplicate, and absorbancies measured on the replicates at each sampling period.

After selecting the appropriate wavelength for each of the test solutions, the concentration of chemical added to the soil-medium mixture was adjusted to give an absorbancy of 0.2 to 0.6 when read against the soil-medium mixture containing no aromatic compound. The wavelengths and chemical concentrations employed are presented in Table I.

Results

For the sake of brevity, the data on degradation rates are presented as the

Table I. Wavelength and Concentration of the Substrates Employed in Decomposition Studies

Second Substituent		First Substituent[a]							
Type	Position	COOH	OH	NO_2	NH_2	OCH_3	SO_3H	Cl	CH_3
None		250 (25)	269 (25)	266 (5)	283 (10)	270 (25)	264 (100)	...	...
COOH	*o*	250 (25)	300 (25)	267 (10)	311 (10)	280 (50)	269 (100)	250 (25)	245 (25)
	m	245 (20)	250 (25)	267 (10)	304 (10)	289 (25)	273 (200)	250 (25)	250 (25)
	p	260 (20)	270 (25)	274 (8)	240 (5)	253 (5)	275 (100)	260 (25)	260 (25)
OH	*o*		276 (25)	275 (15)	280 (25)	276 (15)	...	274 (25)	272 (10)
	m		275 (25)	272 (10)	285 (25)	274 (15)	...	274 (25)	273 (10)
	p		...	400 (5)	...	288 (15)	260 (75)	279 (25)	278 (10)
NO_2	*o*			263 (10)	285 (10)	282 (10)	250 (25)	260 (10)	265 (10)
	m			250 (5)	280 (10)	274 (10)	263 (10)	265 (10)	...
	p			268 (5)	380 (5)	318 (10)	267 (10)	283 (10)	285 (8)
NH_2	*o*				294 (10)	295 (20)	295 (25)	287 (15)	265 (25)
	m				293 (25)	285 (20)	294 (30)	287 (20)	275 (25)
	p				...	295 (25)	270 (25)	285 (25)	285 (30)
OCH_3	*o*					273 (15)	...	...	...
	m					273 (15)	...	...	...
	p					289 (15)	...	...	...
SO_3H	*m*						269 (200)	...	...
	p						...	266 (20)	263 (200)

[a] The first number represents the wavelength. The number in parenthesis indicates the substrate concentration in μg./ml.

Table II. Decomposition of Monosubstituted Benzenes by a Soil *Microflora*

Compound	Substituent	Decomposition Period, Days
Benzoate	COOH	1
Phenol	OH	1
Nitrobenzene	NO_2	>64
Aniline	NH_2	4
Anisole	OCH_3	8
Benzenesulfonate	SO_3H	16

time interval, in days, necessary for the absorbancy in the supernatant of the soil-medium mixture containing the chemical to fall essentially to the level found in the reaction vessels having none of the test substance. In most instances, a small quantity of ultraviolet-absorbing materials remained for some time after this period, possibly a result of the microbial formation of other ultraviolet-absorbing metabolites. The designation >64 indicates that significant ring cleavage was not detected even on the 64th day.

A marked influence of chemical structure on biodegradation rates was noted with the monosubstituted benzenes, all of which were soluble in the medium at the concentration used (Table II). Phenol and benzoate were degraded rapidly, aniline and anisole were attacked less readily, and benzenesulfonate and nitrobenzene appeared to be the most resistant to decomposition by the mixed population. The observed loss of the compounds was considered to be a result of biological activity because the absorbancy was still high in the vessels containing $HgCl_2$ when aromatic ring cleavage was complete in flasks containing no inhibitor. Moreover, the long persistence of nitrobenzene or benzenesulfonate, or aromatic products formed from them, could not be attributed to any significant suppression of microbial activity, as suggested by the lack of inhibition of glucose breakdown by these compounds. Thus, it appears that the hydroxyl and carboxyl groups are the most favorable and the sulfonate and nitro substituents are least favorable to microbial degradation of benzene rings containing only a single substituent.

An effect of chemical structure on biodegradability is also evident among the disubstituted benzenes (Table III). An effect of type and position of the substituent is readily apparent. Not one of the 13 sulfonates and none of the 13 chloro-substituted compounds were destroyed in less than 2 weeks, and most persisted for periods in excess of 64 days; the only two of this group which had been metabolized in 16 days were para - substituted—namely, *p* - chlorophenol and *p*-chlorobenzenesulfonic acid. Nitro compounds were also quite difficult to degrade, and the ultraviolet absorbancy of all solutions was retained for periods in excess of 2 months, except for the compounds also containing a carboxyl or phenolic hydroxyl.

Most of the substituted anilines were not suitable substrates under the test conditions. The resistance of the anilines was rather surprising because amino compounds are universal cellular constituents, by contrast with nitro, chloro, and sulfonate compounds. Seven of the 15 anisoles were also largely inert when exposed to the mixed soil population, but six of the seven contained either a nitro or an amino substituent. Similarly, although four of the 13 toluenes had not been destroyed within a 64-day period, the four resistant molecules possessed either a nitro or a sulfonate group. Thus, as a first approximation, it seems that sulfonate, chloro, and nitro substitution tends to increase the resistance of these chemicals to biodegradation, as previously observed in two instances with the monosubstituted benzenes. On the other hand, the amino and methoxy groups either exert a retarding influence or, alternatively, they do not possess a marked enhancing effect on the rate of degradation.

In marked contrast are the phenols and benzoic acids. In each class of compounds except the chlorobenzenes, ring cleavage was most rapid when the molecule contained a carboxyl or phenolic hydroxyl. This generalization holds regardless of the second substituent, and is in agreement with the findings with the monosubstituted benzenes.

Not only is the type of substituent of importance in conferring resistance or susceptibility to the aromatic ring but also its position. Most commonly, it is the meta isomer which is associated with the greatest resistance—e.g., in the phthalates, nitrobenzoates, aminobenzoates, methoxybenzoates, methoxyphenols, dihydroxybenzenes, aminophenols, and dimethoxybenzenes. Such an effect was noted earlier for several chloro compounds (*2*), although the meta effect with chlorophenols is not evident here. Greater resistance is associated with the ortho isomer only in the toluic acids, nitrophenols, and toluidines.

Some changes in addition to the loss in ultraviolet absorbancy were noted during the incubation. For example, the *o*-phenylenediamine-containing solution was yellow at the eighth day, and a color change was noted with *p*- and *m*-toluidine on the fourth and sixteenth days, respectively.

Biodegradation of a particular material will be evident by the method herein described when the test compound is metabolized by a prototroph which is capable not only of degrading the compound but also of using it as a carbon and energy source to sustain growth. However, evidence exists that microorganisms may destroy readily compounds which they cannot apparently

Table III. Decomposition of Disubstituted Benzenes by a Soil Microflora

Second Substituent		First Substituent[a]							
Type	Position	COOH	OH	NO_2	NH_2	OCH_3	SO_3H	Cl	CH_3
COOH	*o*	2	2	8	2	4	>64	>64	16
	m	8	2	>64	>64	16	>64	32	2
	p	2	1	4	8	2	>64	64	8
OH	*o*		1	>64	4	4	...	>64	1
	m		8	4	>64	16	...	>64	1
	p		...	16	...	8	32	16	1
NO_2	*o*			>64	>64	>64	>64	>64	>64
	m			>64	>64	>64	>64	>64	>64
	p			>64	>64	>64	>64	>64	>64
NH_2	*o*				>64	>64	>64	>64	64
	m				>64	>64	>64	>64	8
	p				...	64	>64	>64	4
OCH_3	*o*					8	...	...	...
	m					>32	...	...	...
	p					8	...	...	...
SO_3H	*m*						>64	...	...
	p						...	16	>64

[a] Values reflect days for total loss of ultraviolet absorbancy at the designated wavelength.

utilize as carbon and energy sources for growth—e.g., certain phenoxy compounds (*10*), aliphatic hydrocarbons (*5*), and benzoates (*8*). Supplemental available carbon will be required to permit proliferation of such organisms. To determine whether available carbon would enhance the decomposition of an apparently resistant disubstituted benzene, *o*-anisidine was selected because either it or an aromatic compound derived from it persists for more than 2 months. When glucose was added to the *o*-anisidine-containing solution, the ultraviolet absorption resulting from the benzene ring disappeared within 4 days. Presumably, the sugar served as an energy source for the population which was responsible for ring cleavage.

When $HgCl_2$ was included in most of the test solutions, there was little or no loss of ultraviolet light absorbancy at the designated wavelengths in the time interval required for biological destruction. With a few compounds, however, a slow loss of absorbancy was noted in the presence of the microbial inhibitor. Disappearance of these compounds may have resulted from their volatilization, chemical degradation, or from the development of organisms resistant to the mercury salt. For all compounds except one, however, either the rate of loss in the mercury-containing reaction mixtures was appreciably slower than in samples free of the inhibitor or no loss was detectable until some time after the ultraviolet absorbancy had disappeared from the mercury-free solutions. The sole exception was *o*-aminophenol, soil-inoculated solutions containing this compound losing their absorbancy at similar rates in the presence or absence of $HgCl_2$. However, if a second increment of *o*-aminophenol was added to solutions previously incubated 8 days with the same compound in the absence of the germicide, the absorbancy disappeared in about 4 hours, suggesting that microorganisms are capable of metabolizing the chemical and cleaving the benzene ring.

At the levels used, the disubstituted benzenes did not appear to alter the rate of microbial development in solutions containing glucose as the major carbon source. Although it is possible that the less readily degraded chemicals were selectively toxic to those species capable of destroying them, it is more likely that the failure to observe degradation is attributable to the inherent resistance to biodegradation of either the compounds themselves or aromatic products formed from them.

Discussion

The present report constitutes a systematic examination of the effect of structure of certain simple aromatic compounds on their susceptibility to degradation by a mixed population of soil microorganisms. The limitations of the techniques employed should be cited, however. A chief shortcoming is the possible unsuitability of the test conditions. For example, the active organisms may require growth factors, anaerobic conditions, solutions of different pH, or the presence of an organic compound suitable as an energy and/or carbon source. Moreover, the present assay technique makes use of a small soil inoculum, so selected to minimize interference by soluble aromatic substances derived from the soil, but inocula of larger size or those obtained from other soils or other ecosystems might contain biochemically active species degrading substrates herein found to be refractory. The method also neither reveals conversions of the parent compound to aromatic products nor indicates the specific aromatic structures which are recalcitrant, and the use of different substrate concentrations might have biased the results.

Despite these shortcomings, however, the data reveal a marked favorable effect of certain substituents, notably the carboxyl and phenolic hydroxyl groups, on microbial decomposition. The significance of position of substituent is also pronounced. Likewise, although larger soil inocula might have revealed the presence of microorganisms degrading some of the compounds, such prototrophs must be relatively rare by comparison with the strains degrading the short-lived chemicals such as the carboxylic acids and phenols.

The results also are in agreement with the few studies of pure cultures and mixed or natural populations. For example, Cartwright and Cain (*4*) experienced great difficulty in isolating organisms metabolizing *m*-nitrobenzoate, although strains active on *o*- and *p*-nitrobenzoate were found with ease. Investigations of 34 soil pseudomonads revealed that some were capable of utilizing *o*- and *p*-methoxybenzoate, *o*- and *p*-aminobenzoate and *p*-nitrobenzoate, but not one of the 34 was active on *m*-methoxy-, *m*-amino-, or *m*-nitrobenzoates (*9*). Likewise, aniline but not nitrobenzene is readily oxidized by a sewage microflora (*7*), and a phenol-adapted culture was found by Tabak, Chambers, and Kabler (*15*) to be capable of oxidizing dihydric phenols and cresols, but mono- or disubstituted benzenes containing nitro, chloro, or sulfonate groups were degraded slowly if at all by the bacteria.

The results have considerable bearing on the persistence of synthetic chemicals in natural environments. Pesticides, household and industrial wastes, and other materials are entering terrestrial and aquatic environments at ever increasing rates, and many are slowly degraded biologically in the environments into which they are introduced or through which they pass. The data presented herein demonstrate the significance of chemical structure of a variety of simple aromatic compounds on the susceptibility of these molecules to attack by a diverse microbial flora.

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STRONTIUM-90

Accumulation of Strontium in Bovine Bones

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The strontium, strontium-90, and calcium content of bovine bone ash from Nevada range cattle at three locations was studied from 1958 to 1962, inclusive. The level of strontium-90 was influenced more by world nuclear testing than by location of the animals within the state. Peak concentrations occurred in 1959 (1 year after initiation of the moratorium) and in 1962 (1 year after testing was resumed). Total strontium was characteristic of the location of the herd. When a herd was moved from an area of high concentration to a correspondingly low area, the total strontium in the bovine bone ash gradually became similar to that in the new environment. The strontium-90 content of bovine bone ash was unrelated to either total strontium or calcium. Calcium level was related to total strontium, but the correlation was not high ($r = 0.5$).

PHYSIOLOGICALLY and chemically strontium is similar to calcium, and, thus, both are concentrated in the osseous tissues of animals. The turnover rate of mineral elements from the skeleton is usually low; hence, the content of somewhat unusual elements in the bones of animals represents a long-time effect.

Since the advent of nuclear devices, much interest has been expressed in the accumulation of fission products in the tissues of both men and animals. Of the major fallout products of biological importance, only strontium-90 is retained in animal tissues for a long time (7.4 years) (*7*). Iodine-131 has a short physical and biological half life. While the physical half life of cesium-137 is long, its biological half life is short (17 days) (*7, 9*). Many other fission products are not readily absorbed and, thus, disappear from the animal in a short time (*12*).

The purpose of this study was to measure the stable strontium and strontium-90 in the bones of range cattle grazing on different range areas in Nevada and to determine the relation between the stable and radioactive elements under these conditions.

Methods

During the 5 years from 1958 to 1962, grade and purebred Hereford cattle from three locations in Nevada were slaughtered twice each year: the Nevada Test Site of the Atomic Energy Commission (NTS) and Delamar Valley (DV), located 80 km. (50 miles) east of the NTS herd in southern Nevada, and the Knoll Creek Field Laboratory (KC) of the University of Nevada, located 90 km. (70 miles) south of Twin Falls, Idaho, and 480 km. (300 miles) north of the DV herd. The experimental areas and sampling procedures have been described in detail (*1, 2*).

At each sampling, one third of the shaft and entire femur head and the distal third of the eighth rib, including

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