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Screening Test for Assessment of Ultimate Biodegradability: Linear Alkylbenzene Sulfonates

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A relatively simple shake-flask system for determining CO_2 evolution was developed to assess the ultimate biodegradability by soil and sewage microorganisms of chemicals which enter the environment. Linear alkylbenzene sulfonates (LAS) were used as model compounds to evaluate the method and were found to undergo substantial biodegradation in this dilute system. At the 30 mg/liter test concentration, higher-molecular-weight LAS compounds were biodegraded at a slower rate and to a lesser extent than lower-molecular-weight LAS, an effect which was eliminated or greatly reduced upon incremental addition of the LAS to the test medium during the first week of incubation. $LA^{35}S$ was used to demonstrate rapid LAS desulfonation, and $^{14}CO_2$ evolution studies with [^{14}C]benzene ring-labeled LAS indicated concomitant biodegradation of the entire LAS molecule as well as the LAS aromatic component. The test can be employed to examine numerous compounds at the same time and is readily adapted to studies of the effect of variation in temperature and oxygen concentration on biodegradation.

The need for assessing the microbial alteration of a molecule in various environments has stimulated development of numerous test methods (4). Many of these test systems, particularly in the area of surfactant biodegradation, focus mainly on primary biodegradation, the microbial alteration of a molecule so that some particular property, such as foaming, colorimetric reaction, or a gas chromatographic peak, is no longer present. Hypothetically, a molecule could undergo primary biodegradation but be quantitatively converted to a recalcitrant product of different physical and chemical properties. Consequently, in an era of increasing environmental awareness there is an increased need to determine ultimate biodegradation, the extent of complete mineralization of a chemical to components such as CO_2, water, and inorganic salts.

In view of the above and our need for assessing the extent of ultimate biodegradation of a large number of diverse chemicals, a more manageable, routine test procedure is required. Since carbon is a major component of almost all of the samples we encounter, measurement of the extent of CO_2 evolution or dissolved organic carbon (DOC) disappearance could be used as an index of the degree of ultimate biodegradation. The former is preferable since DOC methods are not well adapted for assays involving particulate, adsorbed, or insoluble compounds.

Other investigators (2, 5, 7, 13) have developed test methods based on CO_2 evolution as an index of ultimate biodegradation. We have successfully employed the Thompson-Duthie (13) CO_2-evolution test as modified by Sturm (7) for the past several years. The test vessels, however, are large (9 liter), space consuming, cumbersome to manipulate, and time consuming to set up. We required the capability of examining the biodegradability of numerous related classes of compounds at the same time and preferably in duplicate. The present paper describes a system developed and used successfully for the past 2 years in assessing ultimate biodegradability of detergent ingredients, and in particular linear alkylbenzene sulfonates (LAS). LAS represents a class of environmentally important compounds whose primary biodegradation has been studied extensively (3) but whose ultimate biodegradation has not.

(Portions of this work were presented previously [Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, P238, p. 184].)

MATERIALS AND METHODS

Shake-flask CO_2 evolution system. The shake-flask apparatus (Fig. 1) contained 10 ml of 0.2 M $Ba(OH)_2$ in an open container suspended over 500 ml of culture medium in a 2-liter Erlenmeyer flask. The $Ba(OH)_2$ container was made by attaching a 50-ml heavy duty centrifuge tube to a glass tube (10 mm outer diameter by 3 mm inner diameter) by means of three supporting rods. The centrifuge tube

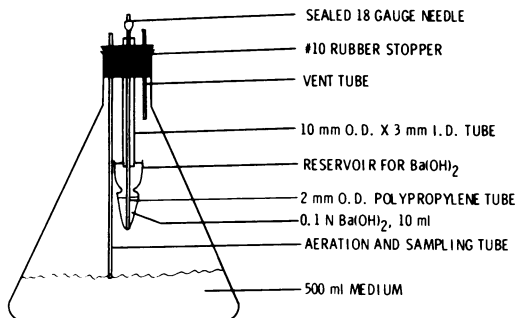

FIG. 1. *Specially equipped shake flask for use in assaying CO_2 evolution.*

opening is large enough to permit air-CO_2 to diffuse into the $Ba(OH)_2$. A constriction was placed just above the 10-ml level in the tube to permit flask placement on and withdrawl from the shaker without spilling $Ba(OH)_2$ into the medium. For periodic removal and addition of base from the center well, a polypropylene tube, sealed externally by a glass-stoppered 18-gauge needle extending from the outside of the flask to the bottom of the centrifuge tube, was incorporated. Glass vent tubes were added for aeration, flask additions, or medium sampling.

Microbial acclimation. Acclimation (induction) of microorganisms to the ability to catabolize the test compound was accomplished by inoculating a minimal salts-vitamin medium (Table 1) supplemented (per liter) with 25 mg each of Difco vitamin-free Casamino Acids and yeast extract, 2.5 g of fresh topsoil (prepared by standard procedures [6]), and 50 ml of freshly settled raw sewage from a local domestic sewage treatment plant. These components were mixed thoroughly for 10 to 15 min, sieved through a 24-mesh (707 μm) screen, and added in 100-ml volumes to 300-ml Bellco baffled shake flasks. The test compound was added incrementally during incubation at a concentration of 10 mg/liter on day 0, 20 mg/liter on day 7, and 10 mg/liter on day 11. At 14 days the inocula from growth on a series of related chemicals were pooled, and 5 volumes of pooled inocula was mixed with 1 volume of freshly settled raw sewage.

CO_2-evolution test. Sixty milliliters (dry weight $\cong$ 25 mg) of the inoculum prepared in the above manner was added to 440 ml of MSV medium (Table 1) in the shake-flask CO_2-evolution units. Test compound was added to the medium to give a final concentration of 20 or 30 mg/liter, and either 10 ml of 0.2 M $Ba(OH)_2$, or 0.5 M KOH for radiotracer studies, was placed in the center reservoir. Flasks were sparged with 70% oxygen in nitrogen, sealed, and placed on a rotary shaker (125 rpm) at room temperature in the dark. Periodically, usually at days 3, 7, 14, 21, and 28, base from the center well was removed for analysis and replaced with fresh base, and the flasks were sparged with 70% oxygen. On the day prior to terminating the test, usually day 27, 3 ml of 20% H_2SO_4 was added to the medium to convert carbonates to CO_2.

For each set of experiments duplicate control flasks receiving inoculated media and no test compound were always included. Results from analysis of these controls (DOC, CO_2 evolution, etc.) were subtracted from the corresponding values obtained from experimental flasks containing test compound. The nature of the test system was such that these corrections were quite small; however, for all results presented in this paper these corrections were made. With compounds of unknown chemical stability in aqueous systems, sterile controls containing inoculated media, test compound, and 50 mg of $HgCl_2$ per liter should be included to correct for chemical degradation. With LAS materials corrections for chemical degradation were found not to be necessary.

Analytical procedures. As the test compounds biodegraded, evolved CO_2, trapped as $BaCO_3$, was quantitated by titration of the entire $Ba(OH)_2$ sample with 0.05 M HCl to the phenolphthalein end point. DOC in centrifuged samples was determined with a Beckman model 915 carbon analyzer with a model 865 infrared detector.

In studies involving ^{14}C-labeled compounds, total CO_2 trapped in the KOH was determined by either titration of a 5-ml sample with 0.25 M HCl after precipitation of dissolved CO_2 by 5 ml of 1.0 M $Ba(OH)_2$ or by injection of a 20-μl sample into the Beckman carbon analyzer. $^{14}CO_2$ evolution was assayed by adding 1 ml of the KOH sample to 15 ml of Instagel (Packard Instrument Co., Downers Grove, Ill.) in a scintillation vial, storing the vials

TABLE 1. *Medium employed for assay of CO_2 evolution during biodegradation of LAS*

Solution	Compound	Stock solution concn (g/liter)[a]
I	NH_4Cl	35
	KNO_3	15
	$K_2HPO_4 \cdot 3H_2O$	75
	$Na_2HPO_4 \cdot H_2O$	25
II	KCl	10
	$MgSO_4$	100
	$FeSO_4 \cdot 7H_2O$	1
III	$CaCl_2$	5
	$ZnSO_4 \cdot 7H_2O$	0.1
	$MnCl_2 \cdot 4H_2O$	0.5
	$CuCl_2$	0.05
	$CoCl_2$	0.001
	H_3BO_3	0.001
	MoO_3	0.0004
IV	One-A-Day multiple vitamin[b] (coat removed)	2
	Biotin	0.005
	Inositol	0.1

[a] Each liter of test medium contained 1 ml of each of these four solutions. Final pH was adjusted to 7.1.
[b] Miles Laboratory, Elkhart, Ind.

for 48 h to minimize chemiluminescence, and counting on a Nuclear Chicago Isocap 300 liquid scintillation counter. The quantity of ^{14}C remaining in the medium after centrifugation was determined by adding 1.0 ml to Instagel. Radioactive counts were corrected for chemiluminescence, background, and quenching. Counting efficiencies in Instagel ranged from 76 to 85%.

For studies with ^{35}S-labeled compounds, the test media was supplemented with 100 mg of Na_2SO_4 per liter. $^{35}SO_4^{2-}$ release was monitored by acidifying a 5-ml sample of the shake-flask culture with 10 M HCl, adding 200 mg of Na_2SO_4, and extracting three times with 5-ml volumes of ethyl ether. Ether extracts, containing intact $LA^{35}S$ and possibly intermediates of the degradation of $LA^{35}S$, were combined in a scintillation vial, evaporated to dryness at 50 C, and quantitatively assayed for radioactivity by scintillation counting after addition of 15 ml of Instagel. Residual ether in the aqueous layer was removed by aeration. Bromine water (0.2 ml) was added, and the sample was heated at 80 C for 15 min to convert all inorganic sulfur compounds to sulfate. Sulfate was precipitated with 95% ethanol and ethyl ether and dried. Approximately 10 mg of precipitate was added to a scintillation vial, dissolved in 1 ml of water, and assayed after addition of Instagel. Values from studies that involved ^{35}S were corrected for radioactive decay, chemical quenching, and background interference. ^{35}S-counting efficiencies ranged between 82 and 86% in Instagel.

Methylene blue-active substances were measured to determine primary biodegradation of LAS by a modification of the Hellige procedure (9).

Test materials. Glucose served as a readily biodegradable control in each test set, and analyses typically indicated evolution of 80 to 90% of the carbon as CO_2 and removal of 95 to 100% of the DOC in the 28-day test period. Typical high and low 2-phenyl isomer commercial LAS products with average chain lengths ranging from 10.8 to 13.3 carbon atoms ($C_{10.8\,avg}$ to $C_{13.3\,avg}$) were obtained from our chemical stock (Table 2). Individual LAS homologues with chain lengths of C_{10} through C_{15} (Table 2) were synthesized in our laboratory using the HF-catalyzed alkylation of benzene with the individual C_{10} through C_{15} olefins to form the respective alkylbenzenes. LAS was subsequently prepared by sulfonation of each pure alkylbenzene homologue. [^{14}C]benzene ring-labeled C_{12} LAS (specific activity, 33.0 μCi/mmol) and C_{13} LAS (specific activity, 33.3 μCi/mmol) pure homologues were prepared using a smaller scale of the above process and [U-^{14}C]benzene. $C_{12\,avg}$ $LA^{35}S$ (specific activity, 5.38 mCi/mmol) was also employed. Radiopurity of the three radioactive LAS compounds was >95% as determined by desulfonation-gas chromatography (8). Radioactive LAS compounds simulating commercially mixed homologue LAS products were prepared by blending appropriate amounts of each C_{10} to C_{15} pure LAS homologue with the desired ^{14}C- or ^{35}S-labeled LAS material to yield $C_{12\,avg}$ or $C_{13\,avg}$ LAS (Table 3).

RESULTS AND DISCUSSION

Nonionic surfactants. Nonionics were biodegraded in a manner similar to that reported by

TABLE 2. *Composition of LAS samples employed*

Avg alkyl chain length	Chain length distribution (%)							2-Phenyl isomer (%)
	C_9	C_{10}	C_{11}	C_{12}	C_{13}	C_{14}	C_{15}	
Commercial LAS products								
Low 2-phenyl								
10.8		37	42	20				18
11.2		15	54	30				14
11.7		5	27	54	13	1		22
13.1			3	14	57	26		14
13.3			1	9	52	38		17
High 2-phenyl								
11.3		19	43	33	6			33
11.7		12	31	32	25	1		34
12.8		4	8	18	47	23		28
LAS pure homologues								
10	1	99						24
11		1	99					18
12			2	98				14
13				1	99			19
14					1	99		17
15							100	15

TABLE 3. *Composition of radioactive LAS products employed*

Sample	Avg alkyl chain length	Sp act (μCi/mmol)	Chain length distribution (%)						2-Phenyl isomer (%)
			C_{10}	C_{11}	C_{12}	C_{13}	C_{14}	C_{15}	
Stock radioactive compounds									
A[a]	12.0	33.0			100				17
B[a]	13.0	33.3			6	94			17
C[b]	12.0	5,378	8	25	30	30	7		22
Formulated radioactive products[c]									
D	12.0	16.6	5	20	50	20	5		16
E	12.0	6.4	5	20	51	19	5		16
F	12.0	35.5	5	20	50	20	5		16
G	12.0	7.5			100				15
H	13.0	5.2		5	15	61	18	1	18
I	13.0	20.2		5	15	61	18	1	18
J	13.0	35.5		5	15	60	18	2	18
K	13.0	20.0			3	97			19

[a] Uniformly labeled with ^{14}C in the benzene ring.
[b] Uniformly labeled with ^{35}S in the sulfonate group.
[c] Formulated by mixing (per 100 mg of product) stock radioactive compounds (A, B, or C, above) with pure LAS homologues (Table 2): (D) 50 mg (4.74 μCi) of sample A; (E) 20 mg (1.84) μCi) of sample B; (F) 0.66 mg (10.2 μCi) of sample C; (G) 22.5 mg (2.13 μCi) of sample A; (H) 15.1 mg (1.43 μCi) of sample A; (I) 60.6 mg (5.57 μCi) of sample B; (J) 0.66 mg (10.2 μCi) of sample C; (K) 60.0 mg (5.52 μCi) of sample B.

Sturm (7) in his studies with 9-liter bottles, indicating the comparability of our method with his. Briefly, our work and that of Sturm indicated substantial CO_2 evolution (>65% of theoretical) as a result of microbial degradation of linear primary and secondary alcohol ethoxylates. Changing the alkyl chain length in the range of C_8 to C_{20} and the ethoxylate chain length in the range of C_3 to C_{11} ethoxylate groups had little effect on the rate and extent of CO_2 evolution. Branched C_8 alkyl phenol ethoxylates were poorly biodegraded (<20% CO_2 evolution) in our shake-flask system and the bottle test used by Sturm (7).

Anionic surfactants. In contrast to rates of degradation of the nonionic surfactants, the rates of ultimate biodegradation of commercial LAS products (Fig. 2) and pure LAS homologues (Fig. 3) were significantly affected by alkyl chain length. Rates and extents of CO_2 formation from higher-molecular-weight commercial ($C_{12.8\ avg}$ to $C_{13.3\ avg}$) and pure homo-

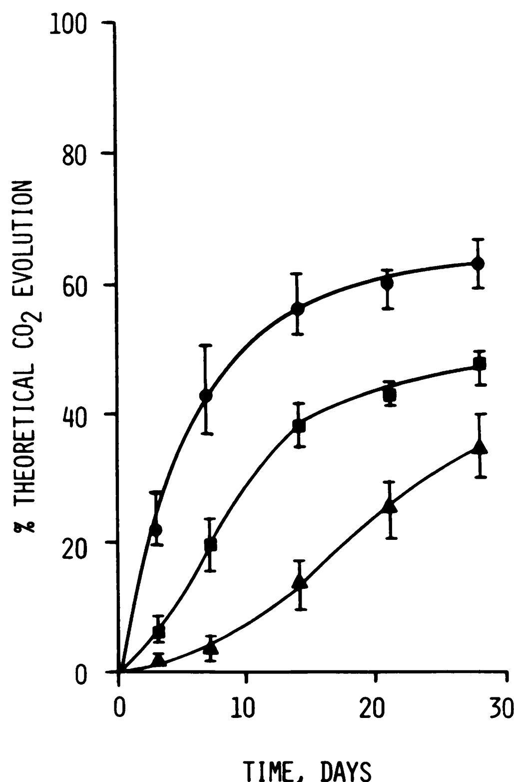

FIG. 2. *CO_2 evolution during biodegradation of commercial LAS products. Average number of carbon atoms per alkyl chain: ●, 10.8 to 11.7; ■, 12.8; and ▲, 13.1 to 13.3. Vertical bars represent the range of values obtained in testing two to four replicates of each sample.*

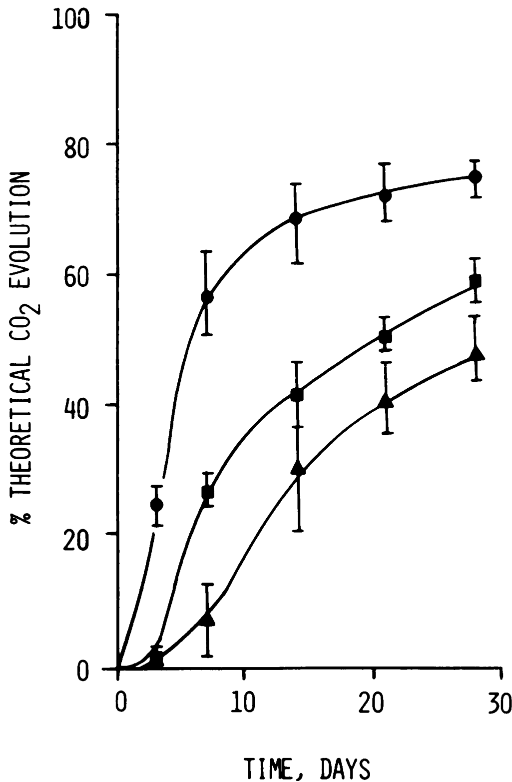

FIG. 3. *CO_2 evolution during biodegradation of LAS pure homologues. Average number of carbon atoms per alkyl chain: ●, 9 to 12; ■, 13; and ▲, 14 to 15. Vertical bars represent the range of values obtained in testing two to three replicates of each sample.*

logue (C_{13} to C_{15}) LAS products were less than those for the lower-molecular-weight LAS products at the 30 mg/liter test concentration. However, the reduced susceptibility of longer-chain-length species to microbial degradation may represent a concentration effect, as has been reported in earlier studies of LAS primary biodegradation (10), and can be eliminated or greatly reduced by incremental addition of the surfactant to the test medium during the first week of study (Fig. 4). Commercial LAS products (Fig. 2) were also mineralized at slower rates, and lesser quantities of CO_2 were evolved from these compounds than from corresponding pure LAS homologues (Fig. 3) of similar alkyl chain length. The presence of higher-molecular-weight LAS homologues in commercial products (Table 2) may explain this effect.

From these results it is evident that within the time period of the test, CO_2 evolution values alone cannot yield a precise assessment of the degree of ultimate biodegradation. To achieve 100% CO_2 evolution a compound must first be metabolized to CO_2 and microbial cells and, secondly, the cells must undergo complete degradation to CO_2. Thus, complete CO_2 evolution did not occur within the time period of the test. The rates and extent of CO_2 production are dependent on such factors as numbers and types of microorganisms capable of metabolizing the test compound, stability of the cells after growth, endogenous respiration, molecular structure and toxicity, and others. Determining the exact extent of ultimate biodegradation would necessitate a detailed material balance of initial and final concentrations of microbial cells, test material, metabolic intermediates, and CO_2. In large-scale screening studies of the biodegradability of a wide variety of chemical classes these measurements would be impractical. CO_2 evolution data, however, are useful in that values approaching the the-

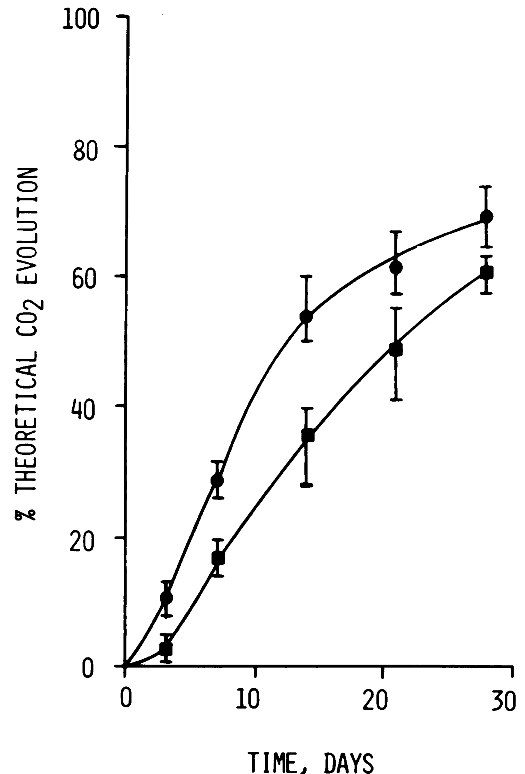

FIG. 4. *Effect of incremental addition to the test medium of higher-molecular-weight LAS pure homologues on rates and extent of biodegradation. Average number of carbon atoms in the alkyl chain: ●, 13, 14; and ■, 15. Addition schedule: days 0 and 1, 5 mg/liter; days 3 and 7, 10 mg/liter. Vertical bars represent the range of values obtained in testing three to four replicates of each sample.*

oretical indicate substantial progress toward ultimate biodegradation, whereas little or no CO_2 evolution may indicate recalcitrance.

Measurement of the extent of DOC removal is also practical in a large-scale screening program, and such information can supplement knowledge gained from CO_2 evolution studies in assessing the extent of ultimate biodegradation of chemicals that do not have a tendency to adsorb to particulate materials in the test system. Table 4 indicates that the extent of DOC removal from LAS products was also influenced by alkyl chain length as indicated by the greater quantity of residual carbon present in media receiving longer chain-length LAS products. The assay system was extremely "dilute" with regard to both nutrients and inoculum, being purposely designed to show these rate differences. Residual DOC may not indicate recalcitrant material. Swisher noted 94 to 97% removal of the DOC of both high- and low-molecular-weight LAS compounds in acclimated sewage conditions (12) and 85 to 100% DOC removal in river waters (unpublished observation). Nevertheless, a study is planned to establish what chemical species are present at the end of the test and what modifications to the CO_2 system are necessary to more closely simulate behavior of LAS in the natural systems.

Monitoring the biodegradation of appropriately radioactively labeled compounds provides a sensitive means for detecting rates of biodegradation of specific portions of a complex organic molecule. Although a previous study by Swisher (11) measuring changes in ultraviolet light adsorption indicated extensive LAS benzene ring biodegradation occurred in other test systems, the use of specifically radiolabeled LAS would more definitively establish the rate and extent of LAS ring desulfonation and ultimate biodegradation in the present test system. The ^{14}C- and ^{35}S-labeled LAS products described in Table 3 were added to the test system at a concentration of 20 mg/liter.

Values derived from studies of the degradation of ^{14}C-labeled, formulated radioactive products of equal average alkyl chain length (Table 3, C_{12} LAS products D, E, and G; C_{13} LAS products H, I, K) were not significantly different and were, therefore, averaged for presentation in the following figures. Comparatively rapid primary biodegradation of $C_{12\ avg}$ LAS was indicated by the slope of the curves from both the methylene blue-active substances and ether-extraction determinations (Fig. 5). Medium DOC and ^{14}C also decreased throughout the study. The chemical nature of residual DOC and ^{14}C was not identified but may represent normal cellular metabolic products, ^{14}C-labeled cells not removed by centrifugation, or more slowly degrading LAS components. Total ^{35}S in the medium did not

TABLE 4. *Ultimate biodegradation of LAS*[a]

Type	Alkyl chain length	% Theoretical CO_2 evolution	Dissolved organic carbon removal (%)
Commercial LAS products[b]	$C_{10.8\ avg}$ to		
	$C_{11.7\ avg}$	64	75
	$C_{12.8\ avg}$	47	68
	$C_{13.3\ avg}$	37	59
Pure LAS homologues[b]	C_9 to C_{12}	77	85
	C_{13}	61	63
	$C_{14,15}$	49	65
	$C_{13,14}$[c]	69	84
	C_{15}[c]	58	76

[a] Summary of 28-day test results from Fig. 2 to 4.
[b] See Table 2 for product description.
[c] Substrates were added to the test medium incrementally—on days 0 and 1, 5 mg/liter; on days 3 and 7, 10 mg/liter.

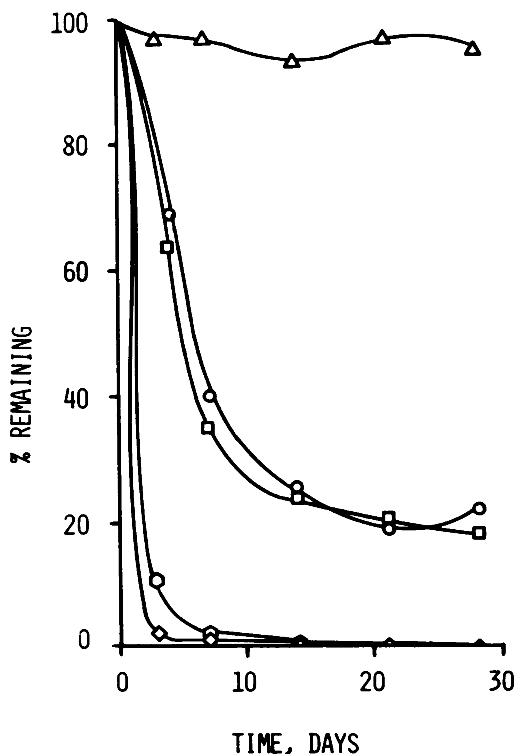

FIG. 5. *Biodegradation of $C_{12\ avg}$ LAS. Symbols:* △, *total ^{35}S in medium;* ○, *DOC;* □, *total ^{14}C in medium;* ⬡, *MBAS;* ◇, *ether-extractable ^{35}S.*

vary significantly throughout the test, although $LA^{35}S$ ring desulfonation released inorganic ^{35}S at a rapid rate (Fig. 6). Desulfonation occurred mostly after primary biodegradation (methylene blue-active substances, ether extractables) and to a greater extent than CO_2 evolution. The non-ether-extractable ^{35}S-labeled material remaining in the medium was not characterized but will be examined in future studies.

The ultimate biodegradation of the total LAS molecule (total CO_2) was concomitant with LAS benzene ring ($^{14}CO_2$) biodegradation. This observation is consistent with that of Swisher (11) and indicates that the aromatic portion of LAS is as susceptible to biodegradation as the rest of the molecule. Corresponding results of assays for the biodegradability of ^{14}C- and ^{35}S-labeled $C_{13\ avg}$ LAS-formulated products were similar to those obtained in experiments with $C_{12\ avg}$ LAS, but with sequences occurring at slightly decreased rates (as might be expected from the results shown in Fig. 2). After incremental supplementation of the medium, the $C_{13\ avg}$ LAS products were biodegraded at rates similar to the $C_{12\ avg}$ products.

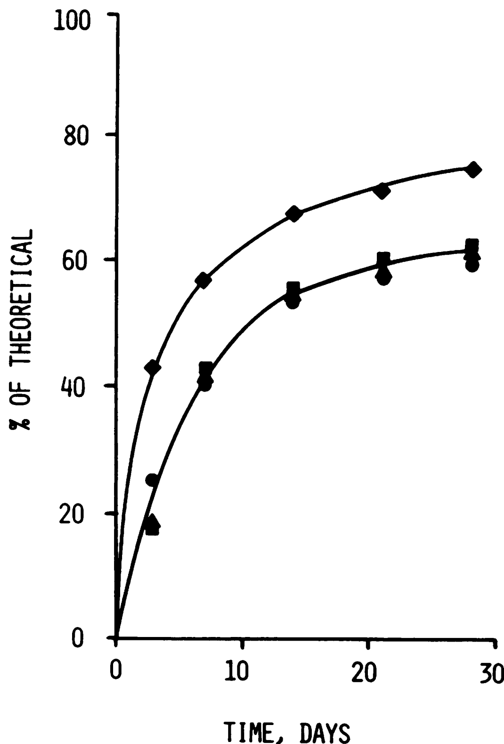

FIG. 6. *Ring desulfonation and ultimate biodegradiation of $C_{12\ avg}$ LAS. Symbols:* ◆, *inorganic ^{35}S formation;* ■, *$^{14}CO_2$ evolution;* ●, *CO_2 evolution via titration;* ▲, *CO_2 evolution via carbon analysis.*

The shake-flask CO_2 evolution system is a relatively simple, reasonably accurate method of assessing the extent of ultimate biodegradability of various classes of molecules. Knowledge of the ultimate biodegradability of chemicals which enter the environment will become increasingly important in the near future. The system described in this paper can be easily used to examine a wide variety of compounds concurrently and is readily adaptable to the study of the effect on biodegradation of varying temperature and oxygen concentrations. The test is a useful supplement to, but cannot replace, studies of biodegradation in sewage, soil, and natural water systems in both the laboratory and the natural environment. These collectively are needed for an overall judgment as to the biodegradability of a particular chemical.

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