purification can be accomplished by partitioning between suitable pairs of immiscible solvents and chromatography on silicic acid.

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A Study of Detergent Biodegradability as Shown by Various Analytical Techniques[1]

R. C. ALLRED, E. A. SETZKORN, and R. L. HUDDLESTON, Research and Development Department, Continental Oil Company, Ponca City, Oklahoma

Abstract

The data discussed in this paper indicate the importance of the application of a number of different analytical procedures to a detergent biodegradation study, since no single technique is an adequate means of measuring the various aspects of detergent biodegradability.

This paper describes surface tension, colorimetric, manometric, radioactive, and GLC analytical methods applicable to detergent biodegradation analyses, with an interpretation of the results given by each method.

The biological system used in the studies is also described.

Introduction

DURING THE PAST few years, a number of investigators have studied detergent biodegradation using established chemical and biological methods.

In these studies, workers have generally used one or more of the following analytical techniques: direct colorimetric measurements using a dye capable of forming a detergent-dye salt, Warburg manometry, radioactive tracer techniques, and BOD tests. Although these methods can provide important biodegradation data, each method has distinct limitations. The application of several different analytical methods to a study will frequently provide data to support conclusions which could not be established from a single method of analysis.

The present study reports detergent biodegradability studies in which three of the above analytical methods and two additional methods—surface tension measurements and vapor chromatography—were used to evaluate the results. BOD tests were not used.

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Procedure and Data

Preparation of Samples

The microbial culture employed in our studies was a stable mixed culture obtained from an activated sludge type waste treatment plant (Enid, Okla.). The culture was adapted to growth in the presence of detergent by three successive transfers in a medium containing 30 mg/liter of a sodium alkylbenzene sulfonate.

The composition of the medium used for this purpose, and also for all experimental detergent biodegradation studies, is shown in Table I.

One-liter quantities of the medium plus 30 mg of the detergent under study were added to 2-liter Erlenmeyer flasks and sterilized by autoclaving for 20 min at 20 pounds pressure. After cooling, the flasks were inoculated with 10 ml of a 72-hr bacterial culture and incubated at room temperature (25C) on a gyrorotary shaker. It was found that this apparatus provided adequate agitation and aeration for bacterial growth.

At desired intervals during the incubation period, aliquots were withdrawn from the flasks for analysis; or entire flasks were removed, depending upon the nature of the analysis desired.

Analytical Methods

Colorimetric. The principal colorimetric method used was the methyl green dye technique of Moore and Kolbeson (1). The method depends upon the formation of a dye-anionic detergent salt in aqueous buffered solution. The organic soluble dye-salt is extracted with benzene, and the concentration is determined from the absorbance at 610 mμ, using a suitable spectrophotometer.

Warburg Manometry. The manometric technique

TABLE I

Synthetic medium	
NH_4Cl	3.00 g
K_2HPO_4	1.00 g
$MgSO_4 \cdot 7H_2O$	0.25 g
KCl	0.25 g
$FeSO_4$	Trace
Yeast extract	0.30
Distilled water	1.0 liter
pH after sterilization	7.1
(Detergent	0.030 g)

employed was essentially that of Umbright, Burris, and Stauffer (2). Washed cell suspensions for the Warburg analyses were prepared by centrifuging 24-hr old cells from 1 liter of medium, and washing three times in a buffer solution (3). Prior to use, the washed cell suspensions were placed on a shaker with buffer solution for an additional 3 hr to reduce endogenous respiration.

One hundred μg of substrate (detergent) were added to the Warburg flasks which contained 2.5 ml of liquid. Fluted filter paper and 0.2 ml of 20% KOH were added to the Warburg flask center wells for CO_2 adsorption.

Surface Tension. Surface tension measurements were made with a Du Nuöy interfacial tensiometer on 10-ml aliquots from the flasks under study. Sampling was discontinued when the surface tension reached a definite plateau. A control flask containing medium but no detergent was also inoculated and incubated, and the surface tension was determined at the same time intervals as the sample flasks.

Radioactivity Technique. Biodegradation data using a radioactive technique were obtained from an experiment with a straight chain alkylbenzene sodium sulfonate containing randomly labeled C^{14}. A Tri-Carb spectrometer was used for sample radioactivity counting.

Sample Isolation-Desulfonation-GLC Analysis. The samples were prepared for isolation from the flasks by acidification to 1 *N* with hydrochloric acid and clarification by boiling on a hot plate for 15 to 20 min. After cooling, pH was adjusted to ca. 7.0 with sodium hydroxide. Isolation of the sample was accomplished by passing through a 1.3 × 8 in. column containing ca. 15 g of charcoal. The active matter was subsequently eluted with 500 ml of a 1:1 methanol benzene solution containing 1% ammonium hydroxide (4). The charcoal was then removed from the column and extracted with an additional 500 ml

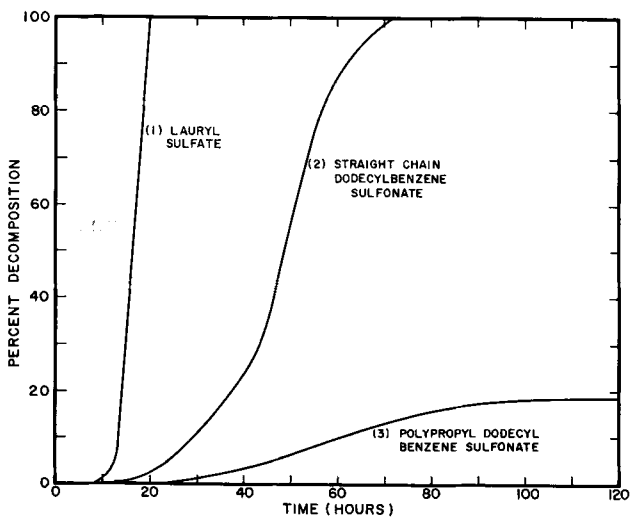

FIG. 1. Biodegradation of lauryl sulfate, straight chain dodecylbenzene sulfonate and polypropyl dodecylbenzene sulfonate as determined by colorimetric analysis.

TABLE II

Comparison of Methylene Blue, Methyl Green, and the Longwell and Maniece Spectrophotometric Methods

Compound	Methylene blue		Methyl Green		Longwell & Maniece	
	ppm added	Found	ppm added	Found	ppm added	Found
Dodecylbenzene sodium sulfonate	30	30	30	30	30	30
p(ω-sodium carboxydecyl) sodium benzene sulfonate	33	30–33	33	12	33	0

of the elution solvent by warming on a steam bath for 20–25 min. After filtration to remove charcoal, all solvents were combined and evaporated. The residue was then transferred to a small flask for desulfonation.

The desulfonation technique, using phosphoric acid hydrolysis, was an extension of the method of Knight and House (5), to allow processing samples of 5–30 mg. In this procedure, using micro equipment (6), the desulfonated alkylbenzenes were distilled from phosphoric acid boiling at ca. 215C, and collected on the surface of a water trap. The oils were then removed by petroleum ether extraction, and the extracts were transferred to a test tube for careful removal of solvent by vacuum.

GLC analyses were made using either a 6 ft Apiezon L column on Celite, or a 15-ft column containing 30 wt percent Resoflex 728 on 42–60 mesh Chromosorb-W. The column aperating temperature was ca. 200C.

Results and Discussion

The extent of biodegradation vs. time of three structurally different anionic detergents is shown in Figure 1. The results were obtained by methyl green colorimetric analysis.

A number of different procedures are available using various dyes for analysis of anionic detergents (7). The methyl green technique was used for these particular studies rather than the better known standard methylene blue method, because methyl green seemed to have fewer positive interferences, and was also less reactive to certain postulated metabolic intermediate compounds which may be formed during detergent biodegradation.

Some time after completion of these studies, however, information was obtained indicating that the Longwell and Maniece (8) modification of the methylene blue method was unreactive to p(ω sodium carboxy decyl) sodium benzene sulfonate. Since this may be one of the early compounds which would occur in the pathway of detergent biodegradation, the compound was prepared in our laboratories and its response to the standard methylene blue, methyl green, and the Longwell and Maniece procedures were determined. Results are shown in Table II.

Since neither the detergent biodegradation mechanism, nor the half life and chemical composition of transient metabolic intermediates, is yet clearly defined, it is difficult to evaluate the colorimetric methods on a completely unequivocal basis. Based on data from the single compound in Table II, however, the Longwell and Maniece procedure appears to be the best method to apply to studies where it is desired to measure only unaltered detergent.

In the methyl green colorimetric procedure, the sample aliquots, usually 10 ml, were collected in 125 ml Erlenmeyer flasks and immediately acidified to stop further degradation by addition of ca. 2 ml

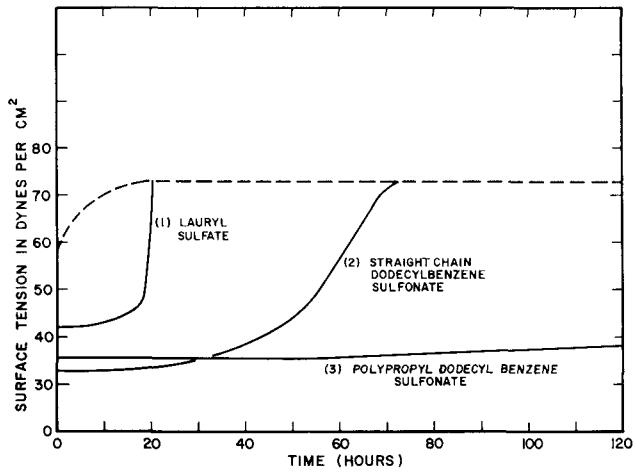

FIG. 2. Biodegradation of lauryl sulfate, straight chain dodecylbenzene sulfonate and polypropyl dodecylbenzene sulfonate as indicated by surface tension change.

of 12 *N* hydrochloric acid. Prior to analysis, the samples were hydrolyzed by careful evaporation to near dryness on a hot plate. It was found that acid hydrolysis is frequently beneficial in reducing emulsion problems in the extraction step of the procedure. It is also believed that the hydrolysis is necessary to free detergent adsorbed within or on the surface of cell walls.

In all colorimetric studies, several identical aliquots were also removed from each flask at zero time to serve as control samples to establish the initial detergent concentration. These samples were also immediately acidified and were later hydrolyzed and analyzed with each group of samples under study.

Since alkyl sulfate samples are unstable in acid solution, both controls and regular samples were frozen as quickly as possible after sampling to stop bacterial action. These samples were later thawed, and the analysis was started without delay.

Figure 2 shows the curves obtained from surface tension measurements of the same biodegradation study discussed under colorimetric analysis. The dashed line is the surface tension curve obtained from a flask containing only the biological medium (no detergent) as shown in Table I. The principal advantages of the surface tension method are the speed of analysis and the minimum manpower requirements. The over-all results obtained from surface tension measurements are similar to colorimetric results; however, the surface tension method is not sensitive to small changes in detergent concentration. This fact is usually most apparent in the early stages of a study when the detergent is in the 10–20% oxidized range. Surface tension results would, of course, be adversely affected if surface active metabolic products were formed during the biodegradation.

Even though neither the surface tension method, nor the methyl green colorimetric procedure are completely quantitative for unaltered detergent only, the principal positive contribution to the results of both analyses is from unaltered detergent. This is illustrated in Table II where the methyl green analysis shows only about 35% response to the postulated early metabolic intermediate compound. The effect of this compound on surface tension would also be expected to be small, since the hydrophobic chain has been altered by the addition of a hydrophilic group.

The results of a Warburg manometric study of the three detergents are shown in Figure 3. Bio-

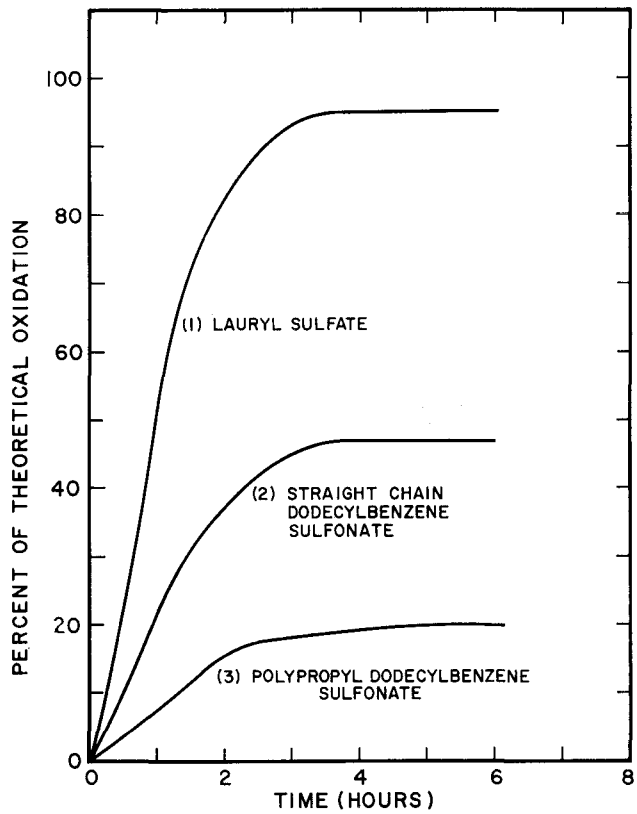

FIG. 3. Biodegradation of lauryl sulfate, straight chain dodecylbenzene sulfonate and polypropyl dodecylbenzene sulfonate as determined by Warburg manometry.

oxidation data obtained by this method are considered to be the maximum oxidation that can be expected for a given detergent by the microorganisms employed. While we have obtained satisfactorily consistent Warburg data from alkyl sulfates, some difficulty has been encountered in the reproducibility of Warburg data for the alkylbenzene sulfonates in repeat analyses over a period of time. Data shown in Figure 3 are the average values of several analyses.

Warburg data are usually interpreted by plotting μl of theoretical oxygen uptake against time; however, unless 100% of the theoretical oxygen uptake is realized (complete conversion to CO_2 and H_2O), valid interpretation of the results is not possible without additional analysis of the Warburg flask contents. As shown in Figure 3, an average of 47% of the theoretical oxygen requirement for complete oxidation had been consumed in the oxidation of the straight chain alkylbenzene sulfonate. In most instances with this detergent, when Warburg data indicated oxygen uptake in the 40–50% of theoretical range, a colorimetric analysis of the Warburg flask contents indicated 100% degradation of the detergent. The colorimetric result indicates that all of the detergent molecules were sufficiently altered to destroy the ability of the detergent to form an organic extractable dye-detergent salt. With this additional knowledge, we can interpret the Warburg result as showing that about half of the straight chain detergent originally added remains at the end of the Warburg run, probably in the form of detergent metabolic intermediates.

Conventional Warburg technique requires endogenous respiration to be measured in flasks containing no substrate. The endogenous values are then subtracted from the substrate flask reading to give the amount of oxygen consumed by the substrate. This

TABLE III
Charcoal Isolation of Organic Matter after Biodegradation of C^{14} Labeled Straight Chain Alkylbenzene Sulfonate

	Sample treatment			
	Acid hydrolyzed		Non-hydrolyzed	
	Activity cpm	%	Activity cpm	%
Initial	880,000	100.0	880,000	100.0
Charcoal column water effluent	3,300	0.4	119,550	13.6
Solids desorbed from charcoal	686,000	78.0	554,400	63.0
Charcoal after desorption	1,000	.....	1,000	.....

correction does not consider the possible effect of substrate on endogenous respiration. Several Warburg studies were made in which radioactive CO_2 produced from C^{14} labeled bacterial cells was counted to establish the level of endogenous respiration. Using detergents as substrate, a considerable change in endogenous respiration was noted; therefore valid interpretation of Warburg data can be made only when the effect of the substrate on endogenous respiration of the bacterial cells is known.

In a study made to correlate colorimetric analysis with the extent of oxidation to CO_2 and H_2O, and also to check the efficiency of the charcoal isolation method, a portion of the straight chain alkylbenzene sulfonate used in these studies was randomly labeled with C^{14}, and the material biodegraded in the usual manner. The degradation was stopped when colorimetric analysis indicated only 10% of the detergent originally added remained undegraded. One group of flasks was acid hydrolyzed, and the organic matter isolated as described earlier. A second group was autoclaved to stop further bacterial action, and the organic matter was isolated in the usual manner except for the omission of the acid hydrolysis. Table III shows the results based on the radioactive count of the various fractions.

The data clearly indicate that without acid hydrolysis a substantial amount of detergent-compound apparently remains adsorbed on or within cell walls and is not removed by charcoal adsorption.

Data in Table III also indicate that ca. 75% of the initial radioactivity count was present after the bio-oxidation. The loss in radioactivity was undoubtedly due to sample oxidation to CO_2. Since colorimetric analysis indicated the sample to be 90% degraded, the remaining radioactivity (ca. 65% of the initial count) must be due to detergent intermediate compounds.

The development of the isolation-desulfonation-GLC method described earlier allows a biodegradation study to be made simultaneously in one flask with as many compounds and isomeric mixtures as can be resolved by GLC analysis. Of the various methods discussed, this technique is perhaps the most valuable analytical tool to a fundamental study of the biodegradation of alkylbenzene sulfonates. A biodegradation study was made of a blend containing four straight chain alkylbenzene sulfonates of different molecular weight. Since the compounds had been synthesized by alkylation of benzene with an α-olefin, each compound also contained all possible phenyl positional isomers except the 1-phenyl alkane which is not formed in this alkylation. The compounds studied were the isomeric mixtures of hexyl, octyl, decyl, and dodecylbenzene sodium sulfonate.

Aliquots of the blend containing equal parts by weight of the four compounds were added to a series of flasks at a 40 mg/liter concentration. Two flasks were removed at zero time to serve as controls. Other flasks were removed at selected time intervals during the biodegradation.

The residue obtained from each flask following the isolation procedure described earlier was desulfonated, and the recovered oils were analyzed by GLC. Results are shown in Figure 4.

From an examination of these data, we can draw two major conclusions: 1) in the straight chain alkylbenzene sulfonate series from C_6 alkyl through C_{12}, the 12 carbon alkylbenzene is the most rapidly metabolized; 2) the number of carbon atoms between the terminal methyl group of the alkyl chain and the benzene sulfonate group greatly affects the suscepti-

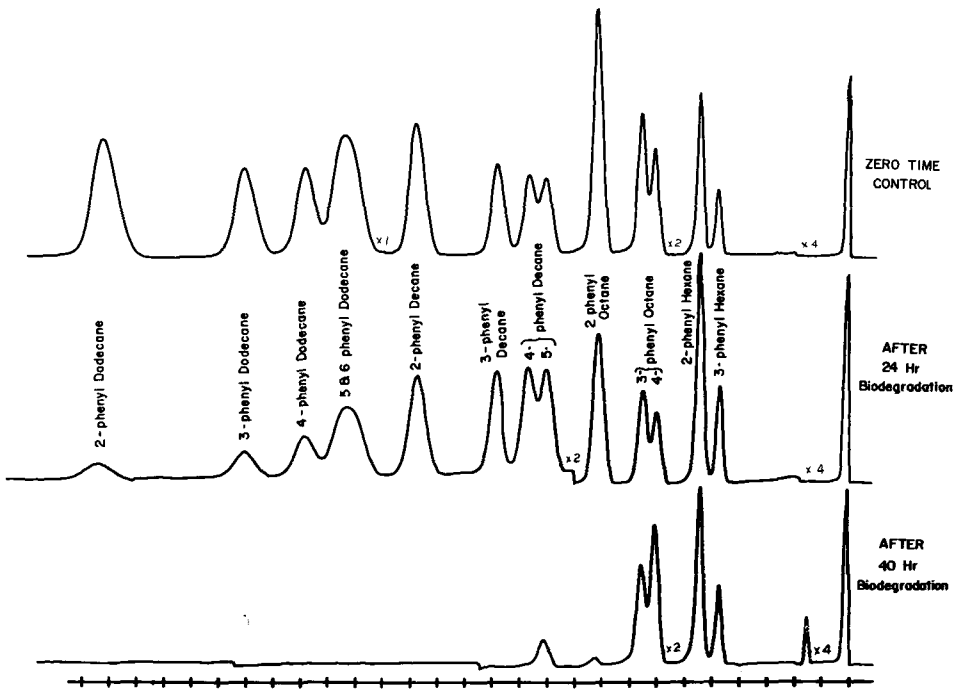

FIG. 4. Chromatogram of alkyl benzenes from desulfonation of an isomeric mixture of hexyl, octyl, decyl and dodecylbenzene sodium sulfonates.

TABLE IV
Biodegradation of an Isomeric Mixture of Straight Chain Sulfonates Calculated from GLC Data

Sulfonate	% Degradation in 24 hr					
	Isomer					
	Total	2-Phenyl	3-Phenyl	4-Phenyl	5-Phenyl	5- and 6-Phenyl
Dodecylbenzene	45	80	55	30		15
Decylbenzene	20	45	15	0	0	....
Octylbenzene	5	10	0	0	....	....
Hexylbenzene	0	0	0	....	....	....

bility of an alkylbenzene sulfonate to bacterial degradation. When this number becomes less than ca. 5 carbon atoms as in 4-phenyl octane and 2 and 3 phenyl hexane sodium sulfonates, biodegradation becomes slower.

Data shown in Table IV summarize the chromatographic data in Figure 4, and indicate the relationship of molecular weight and side chain structure to ease of biodegradation. The governing factor in this relationship seems to be the length of the alkyl carbon chain from the benzene sulfonate group to the terminal methyl group.

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Triglyceride Gas Chromatography as a Means of Detecting Butterfat Adulteration[1]

A. KUKSIS and M. J. McCARTHY, Department of Biochemistry, Queen's University, Kingston, Ontario

Abstract

Gas chromatography was used for the separation and quantitative estimation of triglycerides by carbon number from native and adulterated butterfats. When the triglyceride type composition of the native butter was known, adulteration with vegetable fat could be detected at the 1% level, and with lard at the 3% level, as these adulterants added significantly to only a few of the butterfat triglyceride peaks resulting in a noticeable distortion of the chromatographic elution pattern of this fat. Due to considerable variations observed in the elution patterns with butterfats of different origin, the lower limit for the detection of adulteration of an unknown butter sample with lard or vegetable fat was of the order of 5–10%. The ease of detection and identification of the adulterant varied with the type of fat added. It was demonstrated that mixtures of coconut oil and lard could be made which matched the gas chromatograms of butterfat closely and remained undetected even when added in relatively large amounts. Despite this, the simplicity of the technique and its rapid and relatively reliable applicability to the widest variety of problems of natural fat characterization recommends it as one of the methods in any fat test or control laboratory.

Introduction

DURING QUANTITATIVE studies (1,2) on gas chromatographic fractionation of natural triglyceride mixtures it became necessary to determine the extent to which the individual components were recovered. Although under the chosen experimental conditions the estimated proportional contributions of the recovered triglyceride peaks remained constant, it was possible that constant losses might have occurred. In order to guard against such a possibility, known weights of selected synthetic triglycerides were added to weighed quantities of various natural fats and the proportional recoveries of the components measured. The results obtained were sufficiently encouraging to extend these studies to mixtures of natural fats, and during the course of this work it occurred to us that triglyceride gas chromatography offered great promise as a technique for the detection of adulteration of butterfat. This possibility was explored by examining the quantitative variation among the various triglyceride types observed in butterfats of different origin and the levels at which adulteration with lard and vegetable fats could be reliably detected by means of triglyceride gas chromatography.

Experimental

The samples of butter, lard, vegetable fat, and their mixtures used in these studies were obtained through the courtesy of Madhu Sahasrabudhe, Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Canada. The butterfats were authentic samples and their physical and chemical properties represented the range commonly found in this food. The code and certain chemical constants for these four butter samples are given in Table I. The vegetable fat was partially hydrogenated cottonseed oil. The fatty acid composition of the butterfats and the lard and vegetable fat samples are given in Table II. The fatty acid composition was determined by gas chromatography as previously described (2). The values give approximate weight composition. The triglyceride gas chromatography was performed as described by Kuksis and McCarthy (3) except

TABLE I
Selected Chemical Characteristics of Some Commercial Butterfats[a]

Code	Iodine no.	Reichert-Meissl no.	Polenske no.
Butterfat K	36.9	26.2	1.75
Butterfat L	39.1	26.8	1.95
Butterfat M	38.3	29.7	2.49
Butterfat N	34.8	30.1	2.70

[a] Constants supplied by Madhu Sahasrabudhe, Department of National health and Welfare, Ottawa, Canada.

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