Anionic Surfactant Biodegradability Studies by Warburg Respirometry

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Abstract

A series of alkylbenzene sulfonates and alkyl sulfates was prepared and examined for biodegradability by measuring the oxygen uptake when exposed to substrate adapted microorganisms in the Warburg respirometer. The Warburg results showed that certain linear alkylate alkylbenzene sulfonates and alcohol sulfates are oxidized completely to carbon dioxide and water. The use of radioactive carbon compounds confirmed the Warburg results.

Introduction

THE SUBJECT OF SURFACTANT biodegradability has received considerable attention over the past few years and several excellent reviews are available (1–4). While the use of Warburg respirometry to study the metabolism of tissue slices is well established (5), its use in studying the biodegradability of synthetic surfactants is less well-known. The apparatus measures quantitatively changes in gas volume or pressure and can be used to follow a reaction in which a gas is evolved or taken up. In practice the Warburg respirometer is most commonly used to study the rate of oxygen utilization and carbon dioxide production by tissues or cells metabolizing specific substrates. The classical procedure is to determine the rate of oxygen uptake, at constant temperature, by the tissues under consideration in the absence of a substrate to be certain it is constant. The substrate is then added and oxygen uptake again determined until it reaches the endogenous rate (the respiration rate in the absence of the substrate). Evolved carbon dioxide is absorbed in potassium or sodium hydroxide solution contained in a center well of the Warburg flask.

For Warburg studies, Bogan and Sawyer (6), in 1954, used an activated sludge fed on synthetic sewage and acclimated these microorganisms to the specific surfactant for 48–60 hr followed by 24 hr of no feedings. In 1956 Sawyer, Bogan and Simpson (7) reported similar work using an acclimation period of 14 days. In 1957 Barden and Isaacs (8) reported the use of Warburg respirometry in their studies on the biological stabilization of sewage. In 1960, Nelson et al. (9) described an interesting investigation which compared degradation results obtained on several ABS surfactants. Their experimental conditions did not prevent the microorganisms from utilizing significant portions of the degraded surfactants for cell growth and these workers made the assumption that complete metabolism of the surfactant would cause 45% of the theoretical oxygen uptake which one would calculate from the empirical formula of the test material. Because of this the quantitative results are open to some question. Qualitatively, however, their results were quite significant and showed, among other things, that ABS with unbranched alkyl groups and with the benzene ring at the end of the chain are degradable, that the presence of one or two methyl branches on the carbon atom adjacent to the ring does not alter significantly the biodegradability and that a quaternary carbon atom situated near the terminal methyl end of the alkyl chain does give a bioresistant structure. Allred, Setzkorn and Huddleston (10) compared the biodegradation of lauryl sulfate, straight-chain dodecylbenzene sulfonate and tetrapropylbenzene sulfonate (TPBS) by Warburg manometry using 24-hr old cell cultures washed with buffer solution. Oxygen uptake results in their studies indicated nearly 100% oxidation of the lauryl sulfate in 6 hr while about 47% of the theoretical oxygen requirement for complete oxidation had been consumed in the oxidation of the linear alkylate sulfonate (LAS) and about 20% of theory in the case of the TPBS. Colorimetric analysis of the Warburg flask contents indicated 100% degradation of the LAS and these workers interpreted this as showing that about one-half of the LAS added remains at the end of the run as metabolic intermediates. They also mentioned the fact that the substrate may itself effect endogenous respiration and lead to erroneous conclusions. They reported that they had observed such effects by adding surfactants to cultures made radioactive with C^{14} and observing the $C^{14}O_2$ produced.

Vath (11), Blankenship and Piccolini (12) and Garrison and Matson (13) have all reported applying Warburg studies to the biodegradation of nonionic surfactants.

In our studies we attempted to overcome some of the limitations of the Warburg method and to determine the extent to which various anionic surfactants would degrade. Nearly all of the studies of others had made use of microbial cultures suspended in buffer, salt solution or medium from which they could derive all of the necessary growth factors, with the exception of carbon in some studies. It was therefore possible for a portion of the substrate to be utilized for cell growth. We hoped to prevent appreciable utilization of the substrate for cell growth by eliminating any readily available nitrogen sources from our microbial suspensions. This was also designed to minimize any errors due to nitrification—the uptake of oxygen for the oxidation of reduced nitrogen—as, for example, the conversion of NH_4^+ to NO_2^-.

Experimental

Activated Sludge

An activated sludge was maintained in the laboratory on a synthetic sewage consisting of nutrient salts, glucose, nutrient broth, sodium benzoate, potassium monohydrogen phosphate and distilled water. The original inoculum was obtained from the Schuylkill River in Philadelphia. The activated sludge stock was maintained on a 24-hr fill and draw cycle. The mixed liquor was settled for 1 hr after which approximately two-thirds of the supernatant liquor was removed and replaced with synthetic sewage. The unit was aerated for 23 hr and the procedure repeated.

Acclimation of Activated Sludge to Surfactant

Fifteen hundred milliliters of the activated sludge were transferred to a plexiglass acclimation cell constructed as described by McKinney and Donovan (14). Medium was prepared as follows: $MgCl_2 \cdot 6H_2O$, 18.6 mg/l; $CaCl_2$, 27.5 mg/l; $FeCl_3 \cdot 6H_2O$, 0.25 mg/l; glucose, 0.3 g/l; nutrient broth, 0.3 g/l; KH_2PO_4, 0.3 g/l; sodium benzoate, 0.3 g/l. The me-

dium was added to the acclimation chamber at the rate 1 liter per 24 hr by using a peristaltic pump. Surfactant, typically, was added to the sterile medium in increasing concentration over a 14-day period starting with 10 mg/l during the first 24-hr period and ending with 75 mg/l during the last 24 hr. During the development of the acclimation procedure a study was made of the microbial ability to attack substrates at various times during the acclimation period. Results are shown in Figure 1 for n-dodecyl sulfate, a C_{11-14} LAS and TPBS.

Warburg Manometry

Warburg flasks were prepared by placing a small pleated piece of filter paper (Whatman No. 42) into the center well of each flask and adding 0.3 ml of 10% KOH to it.

About 40 ml of microbial suspension were withdrawn from the aeration section of the acclimation chamber and centrifuged at 1000 to 2000 g for 10 min. The supernatant was discarded and the settled solids resuspended in 40 ml of a physiological saline solution. The saline solution employed is known as Krebs-Ringer solution and contains NaCl, KCl, $CaCl_2$, KH_2PO_4, and $MgSO_4$. The microbial suspension was recentrifuged and resuspended three times in the saline solution to thoroughly wash the cells. The microbial suspension was then transferred to a cell-suspension apparatus (Scientific Glass Apparatus Co.) designed to dispense 1 ml aliquots of uniformly mixed cell suspensions.

Two milliliters of microbial suspension were then dispensed into the main compartment of each Warburg flask. The dry weight of the microorganisms in the 2 ml was generally 300 to 400 mg. In the studies a minimum of six flasks were used for each substrate tested. Half of the flasks received 1 ml of distilled water and the other half 1 ml of 150 mg/l surfactant solution (to give a final concentration of 50 mg/l). The flasks were then placed on the manometers and incubated in a 30C bath in the usual manner. Normally the manometer readings were made every hour for the first 6 or 7 hr. The manometers were then readjusted to operated overnight without attention. On the following day readings every 2 hr were generally sufficient. Incubation was terminated when the oxygen uptake in the sample flasks returned to the endogenous rate. The oxygen uptake results for all replicates were averaged in calculating the percent oxidation.

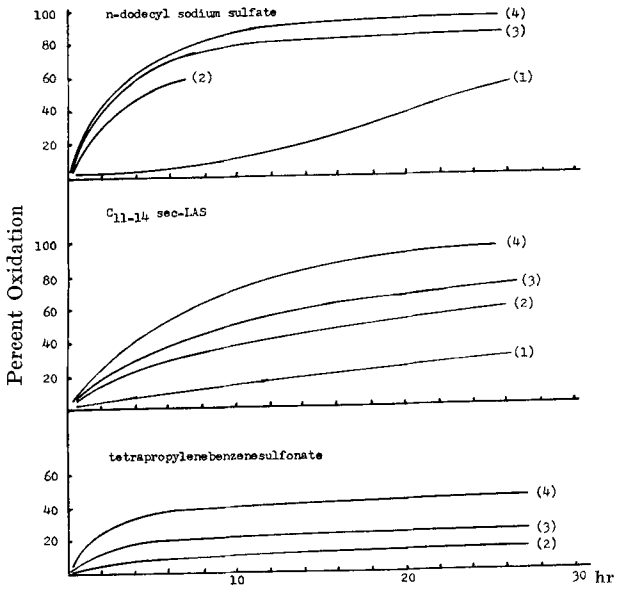

FIG. 1. Surfactant biodegradation in Warburg respirometry using microorganisms acclimated to the substrate as follows: (1) no acclimation, (2) acclimation for 1 day to a surfactant concentration of about 10 mg./l, (3) acclimation for 6 days to a concentration of about 22 mg/l, and (4) acclimation for 14 days to a concentration of about 60 mg/l. Initial surfactant concentration within the Warburg flasks was 50 mg/l in all runs.

Radioactive Carbon Studies

Activated sludge was adapted to surfactant in the usual fashion except that for the last 72 hr of the adaptation period the sludge feed was supplemented by uniformly labeled C^{14}-glucose. The labeled sludge was washed by centrifugation and used in a Warburg run where the evolved CO_2 was trapped in 10% sodium hydroxide placed in the side arm of the flask. The center well was left empty. At the conclusion of the run, the sodium hydroxide solutions from the blank and sample flasks respectively (six replicates of each) were pooled. The carbonate was precipitated with $BaCl_2$ and the $BaCO_3$ collected, dried and weighed. The $BaCO_3$ was then crushed to a fine powder and dispersed in a scintillator-gel for radioactivity analysis in a Packard Tri-Carb liquid scintillator spectrometer. An internal standard was subsequently added to allow the calculation of the activity as distintegrations per minute (dpm).

Results and Discussion

If one considers the empirical formula of the substrate it is possible to express oxygen uptake, at any given time, as a percent of total oxidation. A number of surfactant structures were synthesized in our laboratory and we studied the effects on biodegradability of variations in alkyl chain length, the extent of branching and the position of the phenyl ring along the chain. Some of our results are shown in Table I. The relative biodegradabilities of these structures as determined by this method agree generally with results reported by others and with our own observations using other methods such as the colorimetric procedure employing methylene blue.

To evaluate the effect of carbon dioxide removal the method of Pardee (15) was employed. The results showed no appreciable "CO_2 effect."

Oxygen uptake vs. incubation time was examined at varying concentrations of surfactant. It was found that oxygen uptake is directly proportional to surfactant concentration up to the maximum concentration investigated—50 mg/l.

We were interested in the fact that this Warburg method gave results approximating 100% oxidation for some of the substrates. This indicated that those structures were being completely converted to carbon dioxide and water under the experimental conditions employed but the possibility that the surfactants had altered the endogenous respiration of the microbial cultures could not be eliminated without undertaking further studies. The most rigorous proof could have been obtained by synthesizing C^{14}-labeled substrates, feeding these to the cultures and following the fate of all the radioactive carbon atoms. Since we were primarily interested in establishing whether those structures indicating 100% oxidation on the basis of oxygen uptake were really being completely oxidized, we decided to follow a less rigorous but quite satisfactory alternate procedure. By labeling the microorganisms with C^{14} and trapping the evolved $C^{14}O_2$ it was possible to determine whether or not the endogenous respiration had been altered by the presence of the substrate. The results using random secondary phenyl C_{11-14} LAS and n-dodecyl sodium sulfate, as

TABLE I

Anionic Surfactant Biodegradability

Substrate	Percent oxidation after			Maximum oxid'n observed [b]	
	3	6	24 hr	Percent	Time (hr)
Alkyl sulfates					
n-Dodecyl	52	64	96	103	28
n-Hexadecyl	21	44	97	103	30
Straight chain alkylbenzene sulfonates					
Phenyl-C_{5-6} alkyl, random secondary[a]	3	3	8	11	30
Phenyl C_{7-8} alkyl, random secondary	0	0	0	0	24
1-Phenyldecane	17	39	66	66	24
1-Phenylundecane	19	25	94	94	24
2-Phenyldodecane	30	54	87	89	30
3-Phenyldodecane	14	27	77	77	24
3-Phenyltetradecane	18	32	40	40	24
Phenyl C_{11-14}, random secondary	26	44	97	103	30
Phenyl C_{11-20} random secondary	28	42	98	98	24
Branched chain alkylbenzene sulfonates					
2-phenyl-2-methylundecane	35	51	81	83	26
1-Phenyl-5,7,7-trimethyloctane	0	0	0	0	24
Tetrapropylbenzene	26	33	44	44	24
Dialkylbenzene sulfonates					
Dialkylate from 1-heptene and benzene	11	25	52	53	26

[a] "Random secondary" indicates distribution obtained on alkylating either straight-chain olefins or random monochloroparaffins using aluminum chloride catalysis under standard alkylating conditions.
[b] Oxygen uptake in sample flasks approximately parallel to uptake in blank flasks at this time.

shown in Table II, show clearly that endogenous respiration was essentially unaltered.

According to the data of Table I the individual LAS compounds with phenyl ring attached to the 2nd or 3rd carbon on the alkyl chain are less degradable than a mixture of C_{11-14} or C_{11-20} random secondary material. The random secondary mixtures contain not only the 2nd and 3rd position isomers but also more internal position isomers generally known to be even more difficult to completely oxidize when tested individually. Swisher (16), using gas chromatography, demonstrated that in a random secondary mixture the 2- and 3-phenyl isomers disappear more rapidly than those with the phenyl group nearer the chain center. Similar findings were reported by Huddleston and Allred (17). Swisher also stated that a mixture of secondary isomers was degraded more rapidly than any of the individual components. He speculated that this might occur because each component in the mixture started at an initial concentration lower than when used alone. The present work suggests that the observed differences between the individual components of random secondary LAS mixtures are differences in rate of biodegradability and not completeness of biodegradability. When the individual phenyl isomers are tested separately a difference in completeness of biodegradability is observed—based on oxygen uptake data alone. It is difficult to imagine why these isomers should degrade partially when tested individually and completely in mixtures. Perhaps the individual isomers at relatively high concentration have a greater effect on the endogenous respiration of the microorganisms as the phenyl ring is moved nearer to the central carbon atom of the alkyl chain. This would give a distorted value for percent oxidation based on oxygen uptake. Further studies would be required to resolve these questions.

TABLE II

C^{14} Studies with Surfactants and Labeled Microorganisms

Material	Radioactivity per flask
A. C_{11-14} sec LAS	
$BaCO_3$ from blank flasks	6621 dpm
$BaCO_3$ from sample flasks	6512 dpm
$\left(\frac{6512}{6621}\right) 100 = 98.3\%$	
B. n-dodecyl sulfate	
$BaCO_3$ from blank flasks	2752 dpm
$BaCO_3$ from sample flasks	2604 dpm
$\left(\frac{2604}{2752}\right) 100 = 94.6\%$	

In summary, the Warburg respirometer has been employed to study the biodegradation of several anionic surfactants. Under the conditions employed some straight chain alkylbenzene sulfonates and alcohol sulfates, on the basis of oxygen uptake, were completely oxidized to carbon dioxide and water. Experiments employing microbial cultures labeled with C^{14} have confirmed this complete oxidation for n-dodecyl sulfate and C_{11-14} sec.-LAS.

The Warburg apparatus is not recommended as a standard tool for the routine determination of biodegradability. The procedure is quite time-consuming for the number of samples which can be accommodated at any one time and for every different type of surfactant or detergent formulation one would need to perform some sort of radioisotope study to demonstrate that the endogenous respiration had not been altered and/or that part of the substrate was not utilized for new cell growth. It should be pointed out here that labeling of the microorganisms rather than the substrate is a valid procedure only if the oxygen uptake values indicate approximately 100% oxidation of the substrate.

These experiments do not mean that alcohol sulfates and LAS are always degraded to carbon dioxide and water. In the field-sewage treatment plants, rivers, etc.—the natural environment is much too complex to allow us to make that conclusion and there are undoubtedly many instances when such substrates are broken down into 2- or 3-carbon fragments which are subsequently used to produce new cellular material.

These experiments do prove that LAS and alcohol sulfates in the appropriate weight range are not refractory to common soil and water microorganisms in aerobic biodegradation. Both of these types of surfactants are readily and rapidly degraded to provide either energy or cellular material for the microorganisms and no organic residue remains to add to the pollution problems in our rivers and lakes.

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