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OXIDATION OF RADIOACTIVE GLUCOSE BY AERATED SLUDGE

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Treatment of organic waste by aerated sludge depends on the metabolic activities of many microorganisms present in the mixed liquor. Although aerated sludge is a melange of many types of microorganisms (1) in which all possible pathways of carbohydrate metabolism could occur to complicate the picture, studies on the biochemical oxidation of dairy wastes (2) show that complete oxidation of milk sugar involves such distinct activities as (a) removal of soluble material from solution, (b) intracellular storage, (c) oxidative conversion of stored material, and (d) endogenous respiration of cellular components.

Intensive studies in recent years show that carbohydrate dissimilation by bacteria is a complex procedure and may involve more than one metabolic pathway, yet this information has not been applied to the degradation of organic wastes by sludge organisms. Investigations were started to follow gross distribution of a substrate utilized by sludge organisms. Radioactive glucose, labeled at the carbon-1 (C-1) or carbon-6 (C-6) atoms, was used to follow the fate of the glucose molecule with respect to CO_2 evolution and sludge formation.

These studies appear to be the first of this nature in the fields of sanitation and industrial wastes. Radioactive tracers have been used in measuring sewage flow times and in procuring other operational data. Rorschach and Reid (3) designed a laboratory-scale pilot plant to study effects of radiophosphorus on biological sewage oxidation. In 1954 Grune and Eliassen (4) investigated effects of P^{32} on microbial life to establish tolerance levels for amounts of radioactivity to be allowed in sewers and sewage treatment plants. Skrinde and Sawyer (5) applied Warburg techniques to the safe and economical determination of radiophosphorus effects on the BOD of domestic sewage. One C^{14} study applicable to the investigation has been mentioned in a recent note. Dawes and Holmes (6) noticed that *Sarcina lutea* build up a non-polysaccharide reserve within the cells which, when later metabolized, seemed to suppress oxidation of exogenous substances.

The results obtained in these preliminary experiments, although incomplete, indicate that the glucose molecule is degraded, and the component carbon atoms are used at various rates and enter into various reactions.

Methods

Sludge

Propagation of an active aerated sludge commenced two weeks before studies were initiated. A sludge culture containing 5,000 ppm cells was built up in the Humfeld fermentor by daily feedings of 20 g dried skim solids to 20 l of culture. Temperature was maintained at 30° C, aeration was continuous and in excess. The sludge was settled, washed twice with distilled

water, then adjusted to a concentration of 2,500 ppm COD, which was considered equal to 2,000 ppm sludge solids (7). This served as seed for the Warburg studies. Seed for the later studies in sintered glass aerators was prepared in a like manner.

Warburg Respirometer

Manometric studies were carried out by the "direct" method of determining O_2 and CO_2 (8). Double side-arm flasks were used, a set of two for each time interval sample. In one side-arm was placed 0.5 ml of sludge containing 1 mg cell solids. The second side-arm contained 0.2 ml 2N H_2SO_4. The reaction chamber of each flask contained 0.5 ml of 0.05M phosphate buffer (pH 6.7), 0.1 ml $(NH_4)_2SO_4$ containing 10μg N, 0.1 ml glucose-1-C^{14} (12,000 cpm), and 0.8 ml water. One flask of each pair received 0.2 ml 20 per cent CO_2-free NaOH in the center well immediately prior to adjusting the manometer.

After 10-min equilibration in the bath at 30° C, the sludge cells were tipped from the side-arm into the main chamber of each flask and measurements in gas changes were begun. The acid in the second side-arm of each pair of flasks was tipped into the main chamber at 0, 0.5, 1, 4, and 5.5 hr to stop the cell activity and to liberate bound CO_2.

The flask containing NaOH in the center well of each pair was used for the determination of radioactivity. The NaOH was carefully removed and the center well was rinsed several times with CO_2-free NaOH. The rinsings were combined with the NaOH and treated with 5 ml 2M NH_4Cl and 10 ml 0.5M $BaCl_2$. The precipitated $BaCO_3$ was collected on filter paper with the aid of a precipitation apparatus.

The 2.2-ml acid-treated cell suspension was transferred from the main chamber of the flask to a centrifuge tube and the cells were separated. The Warburg flask was washed with 1.8 ml CO_2-free water and the cells were resuspended in this wash and centrifuged again. The supernatant and the wash water were combined and an aliquot taken for combustion by the persulfate wet combustion method of Calvin *et al.* (9). The total quantity of the separated cells was decomposed by the same method. The liberated CO_2 was trapped in N NaOH, precipitated as $BaCO_3$, and collected. The radioactive material was counted and the values corrected to infinite thinness.

Sintered Glass Aerator.

The second series of experiments was conducted in an apparatus previously described for measuring CO_2 evolution (10). Each aerator held 200 ml of mixed liquor consisting of 25 ml 0.5M K_2HPO_4, 25 ml 0.5M KH_2PO_4, 5 ml 0.1 per cent $(NH_4)_2SO_4$, 100 mg sludge in 100 ml, and 45 ml containing 45 mg glucose-6-C^{14} (1,200,000 cpm). The sludge was added after purging the mixed liquor with CO_2-free air. Aeration was continued by passing CO_2-free air through the sintered glass sparger at the rate of 200 ml per minute.

The spent air was passed through measured amounts of 0.03 N NaOH to capture the evolved CO_2. The use of NaOH avoided clogging of the glass dispersion tubes, as often occurred with $Ba(OH)_2$. Also, uniform dispersion of the CO_2 was required for accurate sampling and assaying of radioactivity. The two receiving tubes of each set contained sufficient amounts of the standard NaOH as predetermined by a trial run using plain glucose. These tubes were prepared under nitrogen gas.

At the indicated sampling times, the NaOH tubes were removed and replaced with another prepared set. The NaOH, now containing CO_2, was transferred quantitatively to 100-ml flasks under nitrogen gas and made up to volume with CO_2-free water. A 10-ml aliquot was transferred to a 50-ml flask for the volumetric determination of CO_2 by treating with neutral $BaCl_2$·-

$2H_2O$. After standing for 4 hr or longer (11), an aliquot of the supernatant was titrated with 0.01 N HCl, and the carbon content of the original samples was determined.

Four aerators containing glucose-6-C^{14} were prepared, permitting the removal of an aerator at 0, 5, 24, and 48 hr. Samplings at 0.5, 1, and 3 hr were made from the one aerator removed at 5 hr. Two ml of the aerated mixture were added to 0.2 ml 2N H_2SO_4 in a centrifuge tube and treated as previously described in the Warburg respirometer studies. A 25-ml sample was also removed, placed in a tube containing 0.04 ml conc H_2SO_4, and an aliquot taken for the determination of total COD (12). Supernatant COD and reducing sugar (13) were determined after filtering 10 ml through asbestos. (Reducing sugar was also run in the Warburg experiment.) At 0, 5, 24, and 48 hr the sludge was inactivated with 0.3 ml conc H_2SO_4; aeration, except for the primary sample, was continued for 10 min. CO_2-free water was added to replace water losses prior to assaying. In addition, one aerator was run 48 hr without glucose in order to obtain endogenous evolution of CO_2. A water blank was also prepared. This experiment was conducted at room temperature.

The manometric experiment with glucose-1-C^{14} was completed in 5.5 hr and gave information on the distribution of the radioactive C^{14}-1 between the cells, CO_2, and supernatant solution. The second experiment conducted in sintered glass aerators gave like information concerning C^{14}-6. Aeration was continued over a period of 48 hr in the latter experiment, giving an opportunity to follow sludge oxidation and other changes, since there was sufficient material for analyses.

Distribution changes in radioactivity are reported on the basis of the total count at the initiation of each experiment (12,200 for the 0.45 mg glucose-1-C^{14} used in the Warburg flasks, and 1.2 million for the 45 mg glucose-6-C^{14} in the aerators).

Results and Discussion

Marked differences were observed in the rate of utilization and distribution of the two labeled carbon atoms of the glucose substrates. Direct comparisons were made of the results obtained from the manometric and aerator experiments because prior studies on dilute substrates showed results to be comparable.

Supernatant

Glucose, measured by the routine reducing value determination, apparently disappears from the supernatant solution very rapidly (Figure 1). In 1 hr approximately 75 per cent is utilized, and by 4 hr less than 5 per cent remains. However, measurement

TABLE I.—Distribution of Radioactivity in Mixed Sludge Liquor Containing 500 PPM Sludge and 225 PPM Glucose (Glucose-1-C^{14} Used in Respirometers and Glucose-6-C^{14} in Aerators)

Aeration Period (hr)	Radioactivity (%)		
	Sludge	CO_2	Supernatant
(*a*) Warburg Study: 1 Mg Sludge Plus 0.45 Mg Glucose-1-C^{14}, 12,200 cpm			
0	1.1	0	98.9
0.5	4.0	2.5	97.1
1	11.2	6.5	87.2
4	27.6*	42.0	16.4
5.5	21.3*	37.3	22.9
(*b*) Aerator Study: 100 Mg Sludge Plus 45 Mg Glucose-6-C^{14}, 1.2 Million cpm			
0	0.7	0	99.3
0.5	26.0	0.9	70.2
1	11.8	1.7	57.5
3	23.7	3.0	62.7
5	38.8	4.5	40.3
24	26.0	26.6	37.4
48	24.0	45.7	29.0

* Low, should probably be about 40.

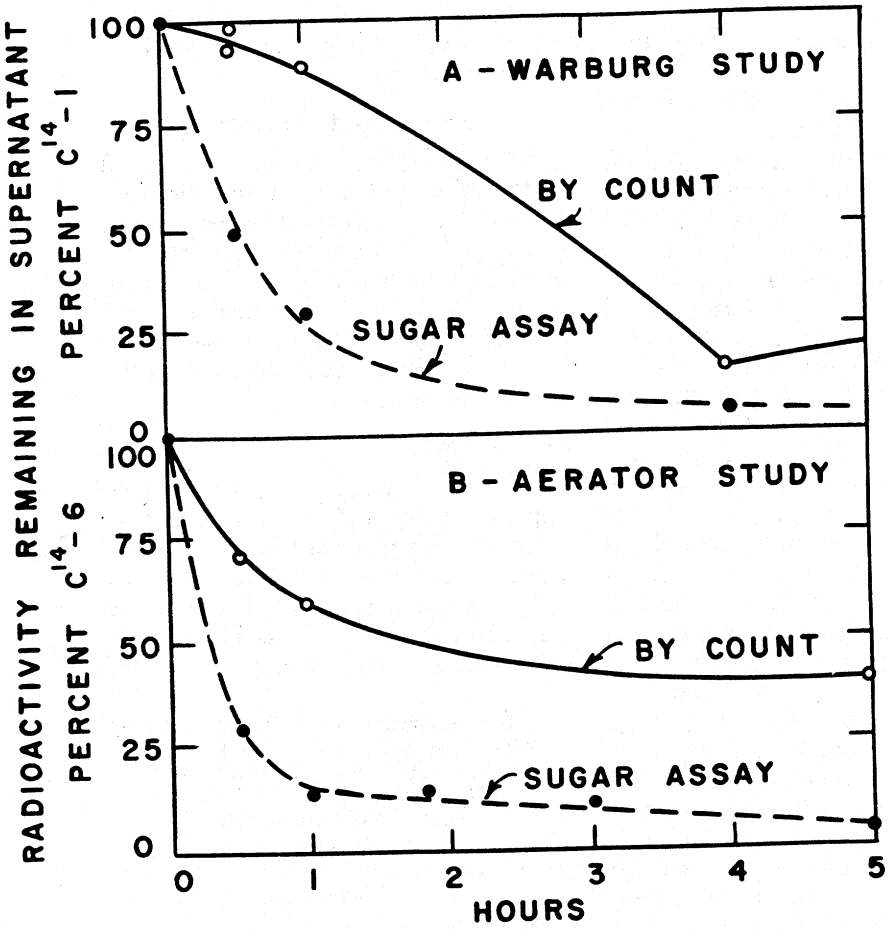

FIGURE 1.—Glucose reduction from supernatant solution in (a) Warburg study and (b) aerator study.

of the radioactive carbon moieties contributed by the glucose indicates that considerable carbonaceous material remains in solution and that the various carbon atoms of glucose are utilized at different rates and to different extents. Table I and Figure 1 show that C-6 is initially removed from solution more rapidly than C-1; in 1 hr 43 per cent of the C-6 activity disappeared, as compared to only 13 per cent of C-1. After 5 hr, however, the sludge removed 80 per cent of C-1 and as much as 60 per cent of C-6 (at a time when tests of reducing values indicated 95 per cent of the glucose had disappeared). Further aeration of the sludge for 48 hr resulted in the slow removal of an additional 11 per cent of the C-6 from the supernatant.

Carbon Dioxide

Column 3 in Table I shows the proportion of the total radioactivity evolved as CO_2. The breakdown of C-1 occurred more rapidly than that of C-6. In 4 hr 42 per cent of C-1 appeared as CO_2, whereas in 5 hr only 4.5 per cent of C-6 was released in the gas. Only after 48 hr of incubation did the sludge evolve 45.7 per cent of the C-6 as CO_2.

The total CO_2 evolution, 42 per cent of the weight of C in glucose, approximates the previously reported values for the breakdown of lactose by sludge

TABLE II.—CO_2-C and Radioactive C Evolved from Aerating Sludge-Glucose Mixture Containing Glucose-1-C^{14} and Glucose-6-C^{14}

Aeration Period (hr)	CO_2-C (ppm)	Labeled C (ppm)	Labeled C (%)
(*a*) WARBURG STUDY: 1 MG SLUDGE PLUS 0.45 MG GLUCOSE-1-C^{14}			
0.5	3.2	0.4	12.5
1	6.7	1.0	14.9
4	35.6	6.3	17.7
5.5	38.6	5.6	14.5
(*b*) AERATOR STUDY: 100 MG SLUDGE PLUS 45 MG GLUCOSE-6-C^{14}			
0.5	8.7	0.2	2.3
1	14.2	0.3	2.1
3	20.4	0.5	2.5
5	28.3	0.7	2.5
24	50.6	4.0	7.9
48	71.7	6.8	9.5

(14). Because each C represents 16.7 per cent of the total glucose-C, and because only one C was labeled, the ratio of labeled to total CO_2 might be of value in assessing the breakdown of the C atoms. Table II shows the percentage of the total CO_2 present as radioactive C, based on the conversion of the data to ppm. If each C atom of glucose was equally converted to CO_2, $C^{14}O_2$ would be equal to 16.7 per cent of the total CO_2 formed. This value was approached, as determined by the labeled activity of C-1. However, that fraction of C-6 present in the CO_2 at the end of 5 hr was only 2.5 per cent of C^{14} present originally in glucose.

It would appear, therefore, that because C-6 was not dissimilated to CO_2, the C atoms were not equally utilized and the other carbons, -2, -3, -4, or -5 must contribute more than C-1 to the total CO_2 evolved and, therefore, were oxidized more rapidly and to a greater extent than C-1 or C-6. Similarly, such differences in the utilization of C was obtained with *Serratia marcescens* by Wasserman and Hopkins in work as yet unpublished.

Sludge

The C^{14} content of the sludge increased as the experiment progressed (Table I). However, the greatest uptake was observed with C-6; 26 per cent of the total activity was transformed to sludge material in 0.5 hr. The assimilation of C-1 occurred more slowly in the earlier stages of sugar utilization, but by the conclusion of the experiment the maximum incorporation had occurred. (It is believed that the 4- and 5.5-hr radioactivity determinations for the sludge utilizing glucose-1-C^{14} are too low, and by calculation of the total radioactivity should be approximately 40 per cent. Because of the nature of the sludge and the manner in which it grew, it was difficult to obtain a proper sampling, particularly from the manometric experiment.)

According to the equation of synthesis,

$$8(CH_2O) + 3O_2 + NH_3 \rightarrow C_5H_7NO_2 + 3CO_2 + 6H_2O,$$

about 60 per cent of the soluble C added to sludge mixture should be assimilated into new cell material or stored as readily utilizable reserve material (14). Determinations made at the end of 5 hr indicated that only about 40 per cent of the labeled carbon was assimilated. With further incubation, appreciably more C-1 could not be stored because so little still remained in the supernatant. However, with C-6 assimilation may have continued beyond 5 hr with degradation occurring in the latter portion of the 24-hr period.

The uneven distribution of the labeled C atoms of glucose in the CO_2 evolved, the sludge, and the residue remaining in solution (aside from irregularities in determination), point up the fact that glucose, or any other substrate, is not used as an entity by

the microorganisms in the sludge. The various metabolic patterns of the sludge population result in the degradation of the sugar into smaller components, which are then used for energy or synthesis at varying rates. Further studies with glucose labeled in the remaining C atoms, and identification of the metabolic products, should allow a complete picture of the degradation of glucose by sludge.

Changes in COD

The aeration experiment gave the opportunity to follow the changes in COD over a period of time. The total amount of starting COD was 625 ppm for the sludge and 240 ppm for the added sugar, making a total of 865 ppm. Table III shows that the actual analysis of the total COD at the start was 851 ppm, of which 274 ppm were soluble COD; apparently, solubles were present with the sludge. In 0.5 hr 167 ppm of glucose had disappeared, yet the total soluble COD decreased only by 27 ppm, whereas the solids gained only 37 ppm. In this experiment the maximum sludge solids were reached in 5 hr, with almost complete removal of glucose and considerable removal of total soluble solids.

If 766 ppm are considered as the maximum sludge solids COD reached in 5 hr, there was a loss of 15 per cent by the 24th hour and 39 per cent by the 48th hour. This may be compared to the 23 per cent loss of endogenous respiration at 48 hr, when 577 ppm sludge solids COD was reduced to 444 ppm. The greater change in the fed sludge must be attributed to assimilated material more easily oxidized and utilized by the multiple population. The significant amount of oxygen-demanding soluble material was apparent. Although the results are somewhat erratic, due doubtless to sampling difficulties, the general trends are apparent. The radioactivity of the sludge solids followed the sludge solids COD, reaching the maximum in 5 hr and then declining.

TABLE III.—Changes in COD of Components in a Buffered Sludge-Glucose Mixture Aerated 48 Hours

Aeration Period (hr)	Chemical Oxygen Demand (ppm)			
	Total Solids	Soluble Solids	Sludge Solids	Glucose
0	851	274	577	240
0.5	851	247	604	73
1	818	235	583	35
3	803	155	648	31
5	862	96	766	14
24	767	114	653	—
48	599	127	472	—
Endo	508	64	444	—

Observations

These experiments were purely exploratory. It is hoped to repeat this study and include glucose containing labeled carbon in each position and the universally labeled carbons. In that way, more information would be obtainable as to whether or not the mixture of sludge microorganisms utilizes glucose in the same manner as selected pure cultures. Studies of this type may give some insight as to the source of residual soluble oxygen-demanding substances that are always present in aeration processes.

It was observed that C-1 and C-6 of the glucose were incorporated into cellular material at different rates and to varying degrees. A substantial amount of the radioactivity remained in the supernatant solution and was used slowly, if at all, and was associated with the residual COD.

Sugar removal, as measured by reducing power, was practically complete within 4 hr, although the actual rate of removal was different in each experiment. Radioactivity of the solution was high, even after such glucose removal. The C^{14}-1 remaining was about equal to the amount that should have been removed during assimila-

tion. The C^{14}-6 remaining was much greater and at the expense of this carbon in the CO_2

It appears that C-1 was more readily available for energy purposes than C-6, because in 5 hr about 37 per cent of the C-1 was found in the CO_2, as compared to 4.5 per cent of the C-6. The latter carbon does become slowly available over long periods of aeration. It required 48 hr to remove as much C-6 as was removed in 5.5 hr of C-1.

Because neither C-1 nor C-6 is completely removed from solution, they are probably integral parts of conversion products of the glucose, which may have been acted upon outside of the cell, or waste products excreted from the sludge cell. These probably contribute to the residual COD found in the aeration process. Additional studies, using both radioactive and chemical techniques, are indicated.

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