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EVIDENCE FOR METABOLIC ACTIVITY OF AIRBORNE BACTERIA

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There are numerous experimental aerobiology publications citing presumptive evidence that airborne microorganisms support metabolic functions (2), but direct evidence has been lacking. The question is of importance not only in the approach to estimation of Pg in the Jovian atmosphere, but also is implicated in mechanisms of microbial survival in air (3), and is of fundamental, practical importance with respect to the airborne biota of our own planet (4).

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ABSTRACT

Aerosols of the bacterium Serratia marcescens, and of uniformly labeled ^{14}C glucose were produced simultaneously and mixed in tubing leading to an aerosol chamber. During a subsequent period of about 5 hrs, $^{14}CO_2$ was produced metabolically within the chamber, and labeled material incorporated within the suspended particles first increased, then decreased. This constitutes the first direct evidence of microbial metabolism of bacteria suspended in the air.

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There are numerous experimental aerobiology publications citing presumptive evidence that airborne microorganisms support metabolic functions (2), but direct evidence has been lacking. The question is of importance not only in the approach to estimation of Pg in the Jovian atmosphere, but also is implicated in mechanisms of microbial survival in air (3), and is of fundamental, practical importance with respect to the airborne biota of our own planet (4).

Groups in our laboratory are studying the problems in three phases, listed here generally in order of ease of proof. They are: (a) demonstration of the presence of some metabolic functions; (b) demonstration of transcription or of gene replication and (c) demonstration of growth (Propagation) of selected microorganisms in atmospheres providing organic matter potentially present or found in the Jovian atmosphere (5). The work described is the demonstration of the uptake of glucose and subsequent CO_2 production by airborne Serratia marcescens (SM) in fulfillment of objective (a).

The strategy of the experiment was to mix two aerosols in a closed chamber - one aerosol of semi-starved bacteria and the other of ^{14}C labeled glucose. If a "living" microbe collided with a glucose droplet, the glucose could be transported into the cell and incorporated into metabolic pathways for production of labeled CO_2. Labeled materials within microbes would increase by incorporation, then decrease through metabolism, and CO_2 within the chamber would increase to some maximal level; this sequence is essentially what was observed.

Cultures of SM were grown in 0.01 M phosphate buffer with added trace elements and ammonium ions, and with 0.5% glucose as sole carbon source which was just sufficient to support growth of the population to about 5×10^9 cells/ml. Cultures were inoculated 16 hrs prior to a given experiment. Cells were harvested by centrifugation within

10 minutes of the expected start time, and resuspended (ca 10^{10} cells/ml) in growth medium without glucose.

Uniformly-labeled ^{14}C-glucose (Schwarz/Mann) solutions were adjusted with unlabeled glucose to concentrations of 0.5% and a specific activity of 100 μCi/ml. Purity was established by thin-layer chromatography on silica gel using n-butanol: acetone: water (30:50:20) as the solvent system. Acidification of the glucose solutions released less than 0.1% of the radioactivity as $^{14}CO_2$. Independent tests using the labeled release technique (6) established that in vitro mixing of these glucose solutions with SM resulted in the rapid evolution of $^{14}CO_2$ of biological origin.

Measurements of radioactivity were performed on a Beckman liquid scintillation counter equipped with a cesium external standard. The counting "cocktail" consisted of 0.25 g of 1,4-bis(2-(5-phenyloxazolyl)) benzene (POPOP) and 2 g of 2,5-diphenyloxazole (PPO) added to 500 ml of toluene and 500 ml of methanol. Counting efficiency was established with ^{14}C benzoic acid (New England Nuclear) as prime standard.

A cell suspension and a glucose solution were atomized separately and simultaneously from two refluxing Wells atomizers having outputs of 0.2 ml/min; efficiency of particulate emission was about 20% of total mass loss from the atomizer (7). The two atomizers were positioned in a tee-joint, opposed, and separated by about 4 cm. The side outlet of the tee led to a copper tube 0.9 cm in diameter and 33 cm long which

was connected to the input tubing (0.3 cm in diameter, 44 cm long) of one or the other of a pair of rotating drums (1500 liters)(8). In independent experiments the coagulation efficiency during the 5-second sojourn of particles in the tubing was found to be about 15 percent; interaction of the two particulate species was not dependent upon additional coagulation within the drum.

Drums were located in a temperature-controlled chamber (21° C) and, prior to each run, were washed with clean air at 90-95% relative humidity until the humidity of the exit air was identical to the input air. Atomization then proceeded over a 5-minute spray time, after which each drum contained about 22 μCi (30,000 dpm/liter) glucose with (or without, as appropriate) approximately 10^{10} (5×10^6 cells/liter) viable bacterial cells suspended in the aerosol state. At various times after aerosolization, AGI-30 (9) impinger samples of 12.5 liters were collected into 20 ml of phosphate buffer during one minute. These samples were analyzed for radioactive content and for viable bacterial count by serial, ten-fold dilution in gelatin-phosphate medium and plating on trypticase soy agar.

Gas samples were also collected for 30-minute periods at various times throughout a run. The gas sampler system consisted of a 5 ml immunological pipette with the tip immersed into 2 ml of Hyamine hydroxide held in a small cup. The pipette and cup were incorporated into a closed container such that air entering the container was removed via the pipette at a rate of about 60 ml/min., and almost the entire 2 ml was suspended as a bubbling liquid within the pipette.

In independent experiments, we found essentially 100% collection of CO_2 at this flow rate. During collection of gas, samples were first passed through a 100 μm membrane filter to remove radioactive particles, and the volume collected during the sampling period was measured by water displacement. Samples were then assayed for total radioactivity. Acidification of a Hyamine hydroxide aliquot with entrapment of subsequently evolved volatiles, e.g. CO_2, on a filter pad moistened with barium hydroxide, established that the radioactivity present in the Hyamine hydroxide samples from the drums was CO_2 (or possibly a weak volatile acid) and was devoid of glucose.

Results of a typical experiment in which separate preparations of ^{14}C-glucose and SM were mixed in the aerosol state are shown in Table 1. These results are compared to a parallel experiment in which glucose alone was aerosolized into a second drum in the absence of SM. As shown, both glucose and the bacterial particles fell onto the walls of the drum with time, decreasing the concentration in the aerosol state. This fallout rate is a direct function of particle size and, for glucose, the disappearance of radioactive-labeled particles from the drum represents the actual fallout rate. For viable bacterial cells, however, the decrease in concentration within the drum represents both fallout and the loss of cell viability. It should be noticed that the fallout rate of radioactive glucose is considerably larger in the presence of SM than in the absence of bacterial cells and that the humidity was the

same in both drums. This indicates that coagulation had occurred between glucose and SM forming larger particles than in the absence of SM. As shown, in the drum containing both glucose and SM, the amount of $^{14}CO_2$ present gradually increased until a plateau was reached after about 24 hours. At the plateau, the total amount of $^{14}CO_2$ represents approximately 7% of the original amount of radioactivity added to the drum. We do not understand the unusual, delayed disappearance of $^{14}CO_2$ in this one test (compare Table 3). Omission of SM from the drum resulted in production of less than 15% of the carbon dioxide accumulated in the presence of SM during the entire run. This indicates that most of the CO_2 observed in the presence of both SM and glucose is metabolic in origin and not a result of some nonbiological interaction of glucose with residual material possibly present on the walls of the drums. As for $^{14}CO_2$ released in the absence of SM, separate experiments have shown that, despite scrupulous cleaning procedures between sequential experiments, some residual radioactivity remained on the walls of the drum at the start of the next experiment. Trace amounts were observed to be released very slowly as CO_2 under the experimental conditions. Sometimes, after extended intervals, CO_2 was comparable in magnitude to that seen in Table 1 in the absence of SM.

The results shown in Table 1 do not allow a clear determination of whether the source of CO_2 production is metabolism occurring from particles in the aerosol form, or is a result of particulate fallout and subsequent metabolism on the wall of the chamber.

Indeed, the possibility of metabolism on the walls is suggested in Table 1 because CO_2 increased as the concentration of airborne bacterial cells and glucose decreased by fallout; although 99% of the bacterial cells fell to the walls within 24 hours, the amount of CO_2 in the drum was still increasing. The pair of experiments in Table 2 show that, although cellular metabolism on the wall did, indeed, occur, it did not account for all the metabolic $^{14}CO_2$ evolved when SM and glucose were simultaneously aerosolized. In this experiment, one drum was first preconditioned by aerosolizing with labeled glucose alone and allowing all the glucose to fall out to the walls of the drum during a 72-hr period. The drum was subsequently air-washed and then filled with airborne SM. Any resulting $^{14}CO_2$ in the drum would then represent metabolism on the walls (plus any bleeding effects). These results are compared to a parallel drum experiment in which both SM and glucose were aerosolized simultaneously. The results of this experiment (Table 2) show that metabolism on the walls is not a significant contributor of CO_2 during the first 5 hours of the reaction. The CO_2 formed soon after aerosolization of both SM and glucose must, then, be attributable to metabolism in the aerosol state.

An experiment (Table 3) was conducted in an effort to eliminate the effects of metabolism on the walls. SM and glucose were simultaneously aerosolized into each of two drums, one of which was coated with a 0.2N Na_2CO_3 solution to trap any $^{14}CO_2$ formed by metabolism on the walls. Initial values of CO_2 were similar in both drums.

However, as shown, CO_2 gradually increased in the absence of carbonate, whereas, in the presence of carbonate, CO_2 remained constant or increased only slightly after the initial evolution. These results suggest that metabolism in air occurs soon after contact of the cell with the glucose particle and that further increases in CO_2 contain a partial contribution of metabolism from cells on the walls of the drum. The magnitude of the wall effect seen in Table 3 is in general agreement with the results shown in Table 2. The apparent magnitude of the CO_2 increase (Table 3) is undoubtedly lower than the actual increase because of the known solubility of CO_2 in bases.

As an additional indication of microbial metabolic activity in the air, we collected samples for 1 minute on 0.45 μm membrane filters at flow rates of 5 liters of air/min. Filters were then rinsed 5 times with 2.0 ml quantities of unlabeled 0.5% glucose and the filters counted for ^{14}C retention. Corrections were made for small amounts of glucose which tend to be retained on the filter even without cells. The remaining counts are referred to as insoluble particulate label (IPL), and presumably represent an estimate of the amount of incorporated glucose not yet metabolized to CO_2. Although the dynamics of the IPL are complex, as is CO_2 evolution, in all tests the IPL first increased, then decreased to background levels during the first 2 to 5 hr period of aerosol life. In one experiment (Table 2), the IPL obtained with glucose plus SM appears to be the same as glucose alone (Table 1). However, there was a gross difference in physical fallout between the two experiments. When corrections are made accordingly, the IPL with glucose plus SM is significantly greater than that with glucose alone.

Together, these experiments demonstrate that metabolism can and does occur in the aerosol state. Under experimental conditions, metabolism starts soon after initial interaction between glucose and bacterial cells. After the initial aerosolization, which optimizes coagulation, little additional coagulation occurs between SM and glucose in the airborne state within the drum. The fact that CO_2 evolution from the airborne cells did not continue for an extended time after interaction with substrate may reflect total utilization of all glucose available to the bacterial cells, as the decline of IPL indicates, or it may reflect inhibitory pH changes and/or accumulation of waste products in the micro-environment of the droplets.

Alternatively, the outcome is similar to results of experiments conducted at Biospherics (10) in which ^{14}C-labeled substrates were added to coliform organisms. An initial burst of $^{14}CO_2$ evolution was followed by a slower but continual rate of $^{14}CO_2$ release. Since coliform organisms were in continual contact with ^{14}C-labeled substrates, this kinetic phenomenon could not reflect lack of substrate availability. The rate of $^{14}CO_2$ evolution in these studies (10) increased again as growth and reproduction of the microorganisms began to occur.

At present, we have no evidence that growth and/or reproduction are occurring within the drum. It is possible that the kinetics observed for airborne metabolism may, therefore, reflect the "early burst" phenomenon and that, if the aerosol were maintained over longer periods, until growth and reproduction could occur, the metabolic rate would be resumed.

Thus, whether or not the kinetics of airborne metabolism in these experiments represent a substrate-limited situation or reflect lack of growth is an issue to be resolved by further experimentation.

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Table 1. Test of metabolism; glucose against glucose plus bacteria.

Glucose					Glucose & SM				
Aerosol time	dpm/l (Impinger)	cells/l (Impinger)	dpm/l $^{14}CO_2$	IPL[F]	Aerosol time	dpm/l (Impinger)	cells/l (Impinger)	dpm/l $^{14}CO_2$	IPL[F]
BA*	-	-	0	-	BA*	-	-	130	-
1 min.	2.7×10^4	-	-	70	1 min.	3.8×10^4	3.7×10^6	-	560
32 min.	-	-	91	-	10 min.	-	-	92	-
3.3 hrs	2.6×10^4	-	-	250	3.5 hrs	3.0×10^4	3.0×10^6	-	1400
4.0 hrs	-	-	34	-	3.6 hrs	-	-	555	-
5.3 hrs	2.3×10^4	-	-	250	5.5 hrs	2.8×10^4	2.4×10^6	-	1700
5.6 hrs	-	-	137	-	5.6 hrs	-	-	798	-
21.5 hrs	-	-	0	-	22.5 hrs	-	-	2,630	-
23.0 hrs	2.1×10^4	-	-	60	23.0 hrs	1.6×10^4	2.1×10^5	-	280
27.5 hrs	1.8×10^4	-	-	-	27.5 hrs	6.8×10^3	4.2×10^4	-	
28.0 hrs	-	-	307	100	27.6 hrs	-	-	2,270	150
47.0 hrs	-	-	0	-	46.5 hrs	-	-	566	-

^{14}C-glucose was aerosolized into each of two drums with and without simultaneous aerosolization of Serratia marcescens, as indicated. At various times after aerosolization, impinger samples were withdrawn to determine the remaining suspended ^{14}C-glucose and bacterial cell count. Gas samples were also withdrawn to determine the amount of $^{14}CO_2$ in the drums.

*BA = Before Aerosolization

F = Uncorrected for fallout or background; see text.

Table 2. Test of metabolism; Glucose follwed by bacteria against Glucose plus bacteria

Glucose then SM				Glucose & SM				
Aerosol time	dpm/l (Impinger)	cells/l (Impinger)	dpm/l $^{14}CO_2$	Aerosol time	dpm/l (Impinger)	cells/l (Impinger)	dpm/l $^{14}CO_2$	IPL[F]
BA*	-	-	180	BA*	-	-	137	
2 min	-	1.3×10^7	-	1 min.	3.0×10^4	3.7×10^6	-	250
22 min	-	-	0	7 min.	-	-	487	-
3.7 hrs	-	-	119	3.4 hrs	1.9×10^4	1.2×10^6	-	-
5.3 hrs	-	2.7×10^6	-	3.6 hrs	-	-	910	230
6.0 hrs	-	-	425	5.5 hrs	1.3×10^4	7×10^5	-	-
21.0 hrs	-	-	1,230	5.6 hrs	-	-	1,030	200
23.5 hrs	-	6.2×10^5	-	22 hrs	-	-	1,640	-
28.5 hrs	-	-	1,210	23.7 hrs	5.2×10^3	1.4×10^5	-	70
				28.4 hrs	-	-	2,680	-

^{14}C-glucose was aerosolized into one drum and allowed to fall out completely before aerosolizing Serratia into the same drum. For comparison, both ^{14}C-glucose and Serratia were simultaneously aerosolized into the parallel drum. At various times after aerosolization, impinger samples were withdrawn to determine the remaining suspended ^{14}C-glucose and bacterial cell count. Gas samples were also withdrawn to determine the amount of $^{14}CO_2$ in the drums.

*BA = Before Aerosolization

F = uncorrected for fallout and background; see text.

Table 3. Test of metabolism; with and without CO_2 adsorbant.

Glucose & SM (- Na_2CO_3)					Glucose & SM (+ Na_2CO_3)				
Aerosol time	dpm/l (Impinger)	cells/l (Impinger)	dpm/l $^{14}CO_2$	IPL[F]	Aerosol time	dpm/l (Impinger)	cells/l (Impinger)	dpm/l $^{14}CO_2$	IPL[F]
BA*	-	-	0	-	BA*	-	-	0	-
1 min	3.6×10^4	4.5×10^6	-	-	1 min	3.6×10^4	5.9×10^6	-	-
4 min	-	-	-	742	3 min	-	-	-	354
20 min	-	-	349	-	8 min	-	-	289	-
3 hrs	2.2×10^4	2.2×10^6	-	578	3.2 hrs	2.1×10^4	1.9×10^6	-	402
3.5 hrs	-	-	729		3.3 hrs	-	-	462	-
4.7 hrs	1.5×10^4	1.4×10^6	-	119	5.2 hrs	1.6×10^4	1.2×10^6	-	362
5.6 hrs	-	-	511	-	5.5 hrs	-	-	332	-
23.5 hrs	-	-	1,448	-	23.4 hrs	-	-	318	-
24.6 hrs	5.4×10^3	2.2×10^5	-	78	24.7 hrs	5.0×10^3	2.4×10^5	-	82
28.0 hrs	4×10^3	1.4×10^5	-	44	28.5 hrs	3.7×10^3	1.2×10^5	-	-
28.6 hrs	-	-	1,218	-	28.6 hrs	-	-	374	28
48.7 hrs	1.8×10^3	3×10^4	-	-	49.2 hrs	2.0×10^2	-	-	-
49.5 hrs	-	-	1,348	-	51.5 hrs	-	-	419	-

^{14}C-glucose and Serratia marcescens (SM) were simultaneously aerosolized into each of two drums, one of which had previously been lined with Na_2CO_3, as indicated. At various times after aerosolization, impinger samples were withdrawn to determine the remaining suspended ^{14}C-glucose and bacterial cell count. Gas samples were also withdrawn to determine the amount of $^{14}CO_2$ in the drums.

*BA = Before Aerosolization

F = uncorrected for fallout and background, see text.