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EVIDENCE FOR METABOLIC ACTIVITY OF AIRBORNE BACTERIA

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Abstract—Aerosols of the bacterium *Serratia marcescens* and of uniformly labelled ^{14}C glucose were produced simultaneously by two separate atomizers and mixed within tubing leading to an aerosol chamber where the r.h. was 90–95%. About 15% coagulation occurred. During a subsequent period of about 5 hr. $^{14}CO_2$ was produced metabolically within the chamber and labelled material incorporated within the suspended particles first increased and then decreased. This constitutes the first direct evidence of microbial metabolism of bacteria suspended in the air.

INTRODUCTION

Current NASA planetary exploration projections include near fly-bys and probes of Jupiter, Saturn and possibly one or more of their satellites. Planetary quarantine constraints developed in international agreements are applicable to such probes. Jupiter has a deep gaseous atmosphere which includes methane, ammonia, water (Neil, 1971), and ethane and acetylene (Kazarian, 1974), all in a dynamic convective mass in which there is a zone about 80 km thick where temperature and moisture conditions do not preclude the possibility of microbial growth. Examination of the probability of metabolism and growth (Pg) of spacecraft-borne terrestrial micro-organisms accidentally or deliberately seeded into such clouds and possibly maintained as aerosols, is an important factor in determining the degree of control required to meet international commitments.

There are numerous experimental aerobiology publications citing presumptive evidence that airborne microorganisms support metabolic functions (Benbough and Hambleton, 1973; Cox and Baldwin, 1966; Hambleton, 1971) but direct evidence has been lacking. The question is of importance not only for estimation of Pg in the Jovian atmosphere, but also is implicated in mechanisms of microbial survival in air (Hatch and Wolochow, 1969) and is of fundamental, practical importance with respect to the airborne biota of our own planet (Parker, 1970).

Our laboratories are studying the problems in three phases, listed here generally in order of presumed 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 (Woeller and Ponnamperuma, 1969). 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 was to mix two aerosols in the inlet tubing leading to a closed chamber; one aerosol consisted of semi-starved bacteria and the other of ^{14}C labelled glucose. If a "living" microbe collided with a glucose droplet, the glucose could be transported into the cell and incorporated into metabolic pathways for eventual production of labelled CO_2. Labelled 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. Our effort was directed

only toward a qualitative demonstration of metabolism. No attempt was made to demonstrate specific metabolic pathways.

MATERIALS AND METHODS

Cultures of *Serratia marcescens* (SM) were grown in 0·01 M phosphate buffer with added trace elements and ammonium ions, (Dimmick, 1965) but with 0·5% glucose substituted as sole carbon source, which was just sufficient to support growth of the population to a final density of about 5×10^9 cells/ml. Cultures were inoculated 16 hr prior to a given experiment. Cells were harvested by centrifugation within 10 min of the expected start time and resuspended to a density of about 10^{10} cells/ml in growth medium without glucose.

Uniformly-labelled ^{14}C-glucose (Schwarz/Mann) solutions were adjusted with unlabelled 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 labelled release technique (Levin *et al.*, 1962; Levin, 1972) established that *in vitro* mixing of these glucose solutions with SM resulted in the immediate 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 (DeOme, 1944) having outputs of 0·2 ml min. The atomizer construction and output characteristics are described by Dimmick (1969). The mass median diameter of the emitted particles is approximately 2 μm from a solution containing 2% dissolved solids. 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 1·6 cm dia. and 33 cm long that had a restrictive orifice, one-half the diameter of the tube, in a 1 mm thick disk. This tube was connected to the input tubing (3 cm dia., 44 cm long) of one or the other of a pair of rotating drums (1500 l.) (Goldberg *et al.*, 1958; Goldberg, 1971). In independent experiments (Dimmick *et al.*, 1975), the coagulation efficiency during the 5 sec sojourn of particles in the 33 cm tubing was found to be about 15% with the disk and less than 5% without the disk; interaction of the two particulate species was not solely dependent upon additional coagulation within the drum.

Briefly, the coagulation tests employed two fluids atomized separately but simultaneously. The aerosols were allowed to mix, as above and be drawn through an illuminated glass tube. The two fluids were 2% aqueous solutions of $AgNO_3$ and KBr. This concentration had the same dissolved solids content as the biological fluids and thus should have produced an equivalent average particle size and distribution (Dimmick, 1969), although the actual size was not measured in either case. When particles from the two different fluids collided, AgBr was formed, which, after exposure to light in the glass tube and collection in samplers protected from light, could be developed, measured photometrically and compared to samples collected in light.

Drums were located in a temperature-controlled chamber (21°C) and, prior to each run, drums were washed with clean air at 90–95% r.h. until the humidity of the exit air was identical to the input air. Atomization then proceeded for 5 min, after which each drum contained about 22 μCi (30 000 dpm/l.) glucose with (or without, as appropriate) approximately 10^{10} (5×10^6 cells/l.) viable bacterial cells suspended in the aerosol state. At various times after aerosolization, AGI-30 (Brachman *et al.*, 1964) impinger samples of 12·5 l. were collected into 20 ml of phosphate buffer during 1 min. These

samples were analyzed for radioactive content and for viable bacterial count by serial, ten-fold dilution in gelatin–phosphate medium and then by cultivation on trypticase soy agar.

Gas samples were also collected for 30 min intervals at various times throughout a run. "Standard" methods of gas collection were inadequate because of either large fluid volume requirements or difficulties of extraction of the small sample from solids. The gas sampler system consisted of a 5 ml immunological pipette with the tip immersed

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

A Glucose & SM					B Glucose				
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*	—	—	130	—	BA*	—	—	0	—
1 min	$3{\cdot}8 \times 10^4$	$3{\cdot}7 \times 10^6$	—	460	1 min	$2{\cdot}7 \times 10^4$	—	—	0
10 min	—	—	92	—	32 min	—	—	91	—
3·5 hr	$3{\cdot}0 \times 10^4$	$3{\cdot}0 \times 10^6$	—	1400	3·3 hr	$2{\cdot}6 \times 10^4$	—	—	150
3·6 hr	—	—	555	—	4·0 hr	—	—	34	—
5·5 hr	$2{\cdot}8 \times 10^4$	$2{\cdot}4 \times 10^6$	—	1700	5·3 hr	$2{\cdot}3 \times 10^4$	—	—	150
5·6 hr	—	—	798	—	5·6 hr	—	—	137	—
22·5 hr	—	—	2,630	—	21·5 hr	—	—	0	—
23·0 hr	$1{\cdot}6 \times 10^4$	$2{\cdot}1 \times 10^5$	—	360	23·0 hr	$2{\cdot}1 \times 10^4$	—	—	0
27·5 hr	$6{\cdot}8 \times 10^3$	$4{\cdot}2 \times 10^4$	—	—	27·5 hr	$1{\cdot}8 \times 10^4$	—	—	—
27·6 hr	—	—	2,270	100	28·0 hr	—	—	307	0
46·5 hr	—	—	566	—	47·0 hr	—	—	0	—

^{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 = Corrected for fallout and background; see text.

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 during sampling. In independent experiments, we found essentially 100% collection of CO_2 at this flow rate, but efficiency decreased at higher flow rates. It was noted in these tests that some evaporation of the fluid occurred and corrections were made accordingly. During collection of gas, samples were first passed through a 0·10 μm membrane filter to remove most radioactive particles and the actual volume collected during the sampling period was measured by water displacement. Duplicate 0·2 ml samples were then assayed for total radioactivity. Acidification of a Hyamine hydroxide aliquot, with entrapment of subsequently evolved gases on a filter pad moistened with barium hydroxide, established that the radioactivity present in Hyamine hydroxide used to collect gas was CO_2 (or possibly traces of weak volatile acids) and was devoid of glucose.

RESULTS

Data from a typical experiment in which separate preparations of ^{14}C-glucose and SM were mixed in the aerosol state are shown in Table 1A. These results are compared to an experiment run at the same time in which glucose alone was aerosolized into a second drum in the absence of bacteria (Table 1B). As shown, both glucose and the bacterial particles drifted onto the walls of the drum with time, decreasing the concentration of particles in the aerosol. This fallout rate is a direct function of particle size and for glucose alone the disappearance of radioactive-labelled particles from the

* Reg Trade Mark. Rohm and Hass Co. An organic hydroxide compatible with the cocktail. Packard Instruments, La Grange, Ill., U.S.A.

drum represents the actual particulate fallout rate (Hatch and Wolochow, 1969). For viable bacterial cells, however, the apparent 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 larger in the presence of SM than in the absence of bacterial cells, although the humidity was the same in both drums. This indicates that an interaction had occurred between glucose and the bacteria, forming larger particles than in the absence of SM. As shown, in the drum containing both glucose and bacteria, the amount of $^{14}CO_2$ present gradually increased until a plateau was reached after about 24 hr. At the plateau, the total amount of $^{14}CO_2$ represents approximately 7%

Table 2. Test of metabolism; glucose followed by bacteria against glucose plus bacteria

A Glucose then SM A				B Glucose & SM B				
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	—	150
22 min	—	—	0	7 min	—	—	487	—
3·7 hr	—	—	119	3·4 hr	1.9×10^4	1.2×10^6	—	—
5·3 hr	—	2.7×10^6	—	3·6 hr	—	—	910	300
6·0 hr	—	—	425	5·5 hr	1.3×10^4	7×10^5	—	—
21·0 hr	—	—	1,230	5·6 hr	—	—	1.030	300
23·5 hr	—	6.2×10^5	—	22 hr	—	—	1.640	—
28·5 hr	—	—	1,210	23·7 hr	5.2×10^3	1.4×10^5	—	0
				28·4 hr	—	—	2.680	—

^{14}C-glucose was aerosolized into one drum and allowed to fallout 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 = Corrected for fallout and background; see text.

of the original amount of radioactivity added to the drum. We do not understand the unusual, delayed disappearance of $^{14}CO_2$ observed in this one test (compare Table 3), although in about seven prior tests (not run as comparative sets) the phenomenon was observed in one other instance. Omission of bacteria from the drum resulted in production of less than 15% of the CO_2 accumulated in the presence of bacteria 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. Separate experiments have shown that, despite scrupulous cleaning procedures between sequential experiments, some residual radioactivity remained on the walls of the drum and was released as $^{14}CO_2$ slowly. After 72 hr, the amount was comparable in magnitude to that reported in Table 1 in the absence of SM at 28 hr.

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 hr, the amount of CO_2 in the drum was still increasing. Data in Table 2 show that, although cellular metabolism on the wall did 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 labelled glucose alone and all the glucose was allowed to fall 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 from residual CO_2 on the walls). 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 hr of the reaction. The CO_2 formed almost immediately after aerosolization of both SM and glucose must, then, be attributable to metabolism in the aerosol state. Note also that the number of airborne cells was nearly 3 times as much in the test where glucose and bacteria were added sequentially (2A) as when they were added simultaneously, thus increasing the potential for metabolism on surfaces.

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

	A Glucose & SM ($-Na_2CO_3$)					B 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	$3{\cdot}6 \times 10^4$	$4{\cdot}5 \times 10^6$	—	—	1 min	$3{\cdot}6 \times 10^4$	$5{\cdot}9 \times 10^6$	—	—
4 min	—	—	—	642	3 min	—	—	—	254
20 min	—	—	349	—	8 min	—	—	289	—
3 hr	$2{\cdot}2 \times 10^4$	$2{\cdot}2 \times 10^6$	—	578	3·2 hr	$2{\cdot}1 \times 10^4$	$1{\cdot}9 \times 10^6$	—	340
3·5 hr	—	—	729	—	3·3 hr	—	—	462	—
4·7 hr	$1{\cdot}5 \times 10^4$	$1{\cdot}4 \times 10^6$	—	119	5·2 hr	$1{\cdot}6 \times 10^4$	$1{\cdot}2 \times 10^6$	—	362
5·6 hr	—	—	511	—	5·5 hr	—	—	332	—
23·5 hr	—	—	1,448	—	23·4 hr	—	—	318	—
24·6 hr	$5{\cdot}4 \times 10^3$	$2{\cdot}2 \times 10^5$	—	0	24·7 hr	$5{\cdot}0 \times 10^3$	$2{\cdot}4 \times 10^5$	—	0
28·0 hr	4×10^3	$1{\cdot}4 \times 10^5$	—	0	28·5 hr	$3{\cdot}7 \times 10^3$	$1{\cdot}2 \times 10^5$	—	—
28·6 hr	—	—	1,218	—	28·6 hr	—	—	374	0
48·7 hr	$1{\cdot}8 \times 10^3$	3×10^4	—	—	49·2 hr	$2{\cdot}0 \times 10^2$	—	—	—
49·5 hr	—	—	1·348	—	51·5 hr	—	—	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 = Corrected for fallout and background, see text.

An experiment was conducted in an effort to eliminate the effects of metabolism on the walls (Table 3). SM and glucose were simultaneously aerosolized into each of two drums, one of which was coated with a 0·2 N 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 glucose particle and that further increase in CO_2 does 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 observed magnitude of the CO_2 production by airborne cells (Table 3) is undoubtedly lower than the actual production because of the known solubility of CO_2 gas in basic solutions which will remove CO_2 from the air. In fact, we found that when $^{14}CO_2$ gas was held in a drum coated with Na_2CO_3, 60% disappeared from the air in 5 hr and 90% disappeared in 24 hr. We have not applied corrections for this in Table 3.

As an additional indication of microbial metabolic activity in the air, we collected samples for 1 min intervals at various times on 0·45 μm membrane filters at flow rates of 5 l. air/min. Filters were then rinsed 5 times with 2·0 ml quantities of unlabelled 0·5% glucose and the filters counted for ^{14}C retention. A correction was made for amounts of glucose which tend to be retained on the filter even without cells. Usually the amount was less than 100 counts, which was the correction applied.

The remaining counts are referred to as insoluble particulate label (IPL), and presumably represent an estimate (±20%) 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 with mixed aerosols the IPL first increased, then decreased to background levels during the first 1–5 hr period of aerosol life.

DISCUSSION

A successful outcome of these experiments was, of course, dependent upon a number of variable factors that could only be partially controlled, or even measured. For example the bacterial culture is a collection of individual cells. It is well known that the "age" of the culture can affect the ability of the cells to survive stress as well as influence the rate of *in vitro* metabolism. Day-to-day production of truly uniform populations is virtually impossible (Dimmick, 1973). Also, coagulation efficiency was not precisely 15%, but was shown in other tests to vary from 10 to 22% and undoubtedly varied more in the drum runs. Further, during analysis of the IPL fraction, some CO_2 must have been evolved and lost from the filter pad during the washing. Theoretically, the integrated, total IPL amounts should have been the same as the total CO_2 formed: It varied from about 30 to 50%. In some instances, the IPL was maximal in the first sample, indicating that some cells must have metabolized glucose in a short period of time, thus decreasing the measured, total IPL.

The total amount of radioactivity ($^{14}CO_2$ + IPL) recovered, varied from 5 to 10% of the initial input, or 30–50% of the amount that could have coagulated with the cells.

Low recovery can be attributed to many factors. Carbons 1 and 2 on the glucose molecule are converted to CO_2 more readily than the remaining four. Hence, with uniformly labelled glucose, the rate of CO_2 evolution is not constant. When this effect is combined with the uncertainty of the rate of loss of $^{14}CO_2$ in the collected IPL fraction, some $^{14}CO_2$ evolution from the walls, limited additional coagulation and the fact that the observed fallout rate is that of all labelled particles, not just the bacteria–glucose particles, then it is obvious that to attempt to account for all possible factors would be prohibitive.

Despite problems hindering quantification these experiments demonstrate that metabolism can and does occur in the aerosol state at moderate temperature and high humidity. Under experimental conditions, metabolism starts soon after initial interaction between glucose and bacterial cells. After the initial aerosolization, which optimizes coagulation, little additional coagulation appears to occur 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 microenvironment of the droplets.

Alternatively, the outcome is similar to results of experiments conducted at Biospherics (Levin *et al.*, 1957) in which ^{14}C-labelled substrates were added to coliform organisms. An initial burst of $^{14}CO_2$ evolution was followed by a slower rate of $^{14}CO_2$ release. Since coliform organisms were in continual contact with ^{14}C-labelled substrates, this kinetic phenomenon could not reflect lack of substrate availability. The rate of $^{14}CO_2$ evolution in these studies (Levin, 1963) 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 in the airborne state, but these data establish the basis for further experiments investigating those possibilities. Perhaps the kinetics observed for airborne metabolism are indicative of 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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