QUANTITATIVE STUDIES OF THE EFFECT OF ORGANIC SUBSTRATES AND 2,4-DINITROPHENOL ON HETEROTROPHIC CARBON DIOXIDE FIXATION IN *HYDROGENOMONAS FACILIS*

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ABSTRACT

McFADDEN, BRUCE A. (Washington State University, Pullman), AND H. ROBERT HOMANN. Quantitative studies of the effect of organic substrates and 2,4-dinitrophenol on heterotrophic carbon dioxide fixation in *Hydrogenomonas facilis*. J. Bacteriol. **86**:971–977. 1963.—Whole cells of *Hydrogenomonas facilis* under heterotrophic conditions fixed levels of $C^{14}O_2$ which depended upon the nature of the carbon source being oxidized. It was established that oxidative rates varied as a function of p_{CO_2}. Therefore, all studies were conducted in the presence of 1.5 mole % CO_2 in the gas phase. With glucose-grown cells supplied with glucose as substrate, the heterotrophic fixation was curtailed 98% by the addition of 8.3×10^{-4} M 2,4-dinitrophenol (DNP). A coupling between reductive fixation of CO_2 and heterotrophic oxidation of substrate is consistent with the observed effect of DNP. The efficiency of coupling of fixation with oxidation was studied for acetate, D-glucose, L-glutamate, D,L-lactate, D-ribose, and succinate as substrates. Kinetic studies showed that the efficiency of coupling (expressed as disintegrations per minute of C^{14} per microliter of O_2) was initially time-variable for all substrates; however, it approached a constant value after 30 to 45 min for acetate, glutamate, lactate, and succinate. The initial variation of the ratio with time was due primarily to $C^{14}O_2$ uptake, which was nonlinear with time. Control studies in the absence of exogenous substrate indicated that CO_2 fixation may also be linked to oxidation of endogenous stores accumulated during heterotrophic growth. D-Ribose appears to be the most promising substrate for short-term fixation studies owing to the rapid incorporation of C^{14} and the unusually low endogenous fixation rate by cells grown on ribose. Calculations reveal that, after isotopic equilibration has occurred, the amount of CO_2 utilized during glucose oxidation is almost 50% of O_2 uptake during the same interval. Even during succinate oxidation, which was shown to be coupled much less effectively with CO_2 fixation, the CO_2 utilized during the same interval is 8% of O_2 uptake.

The carbon metabolism of the facultative hydrogen autotroph *Hydrogenomonas facilis* is of considerable interest because the organism grows either autotrophically or heterotrophically. Ready reversion to a markedly different mode of growth poses an interesting question of regulatory mechanism(s). It is well established (Bergmann, Towne, and Burris, 1958; McFadden, 1959; Hirsch, Georgiev, and Schlegel, 1963) that a major pathway of assimilation of CO_2 by *H. facilis* under autotrophic conditions (H_2, O_2, CO_2) is identical with the photosynthetic cycle [for a review, see Calvin (1962)].

McFadden (1959), while studying the autotrophic pathway, found that substitution of N_2 for H_2 in control vessels resulted in radioautographic labeling patterns qualitatively similar to those obtained in the presence of hydrogen. This observation led to the hypothesis that energy and electrons gained from the oxidation of organic reserve materials (i.e., heterotrophic oxidations) might also be used for carbon dioxide fixation by a mechanism similar to that for autotrophic fixation. The present communication describes investigations of CO_2 fixation under normal heterotrophic conditions, i.e., in the presence of an exogenous organic carbon source. The effects of various organic substrates on the magnitude and efficiency [expressed as disintegrations per minute (dpm) of C^{14} uptake per microliter of O_2 uptake] of heterotrophic CO_2 fixation are described. The ultimate objective was selection of the most appropriate substrate for in-

vestigations of the pathway(s) of $C^{14}O_2$ fixation under heterotrophic conditions.

Early in these studies, it became apparent that a meaningful, quantitative comparison for various substrates of the efficiency of coupling of fixation with oxidation would be affected by the observation that oxidative rates were a function of p_{CO_2}. Therefore, to ensure comparability of efficiencies, all studies were conducted at the same p_{CO_2}, 1.5 mole %.

MATERIALS AND METHODS

Culture methods. Autotrophically grown cells of *H. facilis* (McFadden, 1959) were adapted to heterotrophic conditions by growth on tap water-agar slants containing 0.3% yeast extract, 0.3% NH_4Cl, 0.05% $MgSO_4$, and 0.05% K_2HPO_4. The pH was adjusted to 7.0. Cells were washed from the slants into 50 ml of liquid medium in a flask, and were shaken at 30 C until growth was heavy. The liquid culture medium (pH 7.0) consisted of 0.3% carbon source and 0.01% yeast extract in a basal minerals medium (hereafter referred to as basal medium) containing the following components: 0.2% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.02% $MgCl_2 \cdot 6H_2O$, and tap water-deionized water (1:1). The flask contents were then used as an inoculum (20%, v/v) for 250 ml of the same medium in a 1-liter flask fitted with a side arm permitting turbidity measurements. After growth at 30 C, the cells were harvested in the log phase [cell densities, 0.15 to 0.36 mg (dry weight)/ml] and washed twice with 0.01 M potassium phosphate buffer (pH 7.0). Cells were then resuspended in the basal medium.

Manometric methods. Work by Schlegel, Lafferty, and Stellmach-Helwig (1961) and the results presented later in this paper indicated that uptake of oxygen in manometric experiments was dependent on the p_{CO_2} in the atmosphere of the flask. Therefore, a constant CO_2 atmosphere of 1.5 mole % was maintained in the flasks during the fixation studies by means of a Pardee (1949) buffer. This buffer permitted the measurement of O_2 uptake in the presence of evolved CO_2.

For a typical experiment, a series of double-arm Warburg vessels were filled as follows: main compartment, 1.5 ml of cell suspension in basal medium [1.75 mg (dry weight)/ml]; side arms, 0.5 ml of 0.10 M carbon source in basal medium (or 0.5 ml of basal medium for controls) and 0.4 ml of $KHC^{14}O_3$ solution at pH 7.0 (0.87×10^7 to 1.61×10^7 dpm/ml); center well, 0.4 ml of Pardee buffer (to maintain 1.5 mole % atmospheric CO_2 at 30 C) on an accordionated wick. Wicks were cut from filter paper to a uniform size (4.5 cm by 2 cm), since removal of CO_2 appears to be a function of flask geometry and exposed absorbing surfaces (Schlegel et al., 1961). All experiments were performed at 30 C.

After thermal equilibration for 5 min, the fluid level in the manometers was adjusted by expiration of breath into the flasks. This procedure enriched the CO_2 content of the flask atmosphere and shortened the interval necessary for attainment of the desired CO_2 level. After equilibration of CO_2 for 1 hr, the substrate was tipped to the cells. All substrates were used at concentrations well into the zero order range with respect to kinetics of O_2 consumption. Thus, quantitative transfer to the main compartment was unnecessary. A period of up to 20 min prior to C^{14} addition was then allowed to compensate partially for the lag in oxidation known to occur with various substrates. The $KHC^{14}O_3$ solution was then tipped to the cells (zero time for $C^{14}O_2$ fixation and oxygen uptake).

At intervals of 15, 30, 45, 60, and 75 min, two flasks were rapidly removed and opened, and 0.3 ml of 5 N H_2SO_4 containing 0.5% Tween 80 (v/v) was added. About 20 sec were required for the killing operation. Control flasks were treated in like manner after a 75-min fixation period.

Plating and counting. Samples of 0.50 or 1.00 ml of cell suspension were removed from the flasks and plated on Millipore membrane filters (Atkinson and McFadden, 1956). The cell mat was washed twice with water and air-dried. The filters were then combusted in oxygen by use of a modified flask of Kelly et al. (1961). Carbon dioxide was absorbed (98% in 25 min) by the addition of 5 ml of 0.5 M Hyamine hydroxide in methanol. A sample of the Hyamine absorbent (0.50 or 1.00 ml) was then added to a toluene scintillator solution and counted in a Packard Tri-Carb spectrometer.

For technical reasons, it was not possible to use identical $KHC^{14}O_3$ solutions in trials with different substrates, although the specific activities were similar. For this reason, samples were taken of the $KHC^{14}O_3$ at the time of filling of the Warburg flasks and assayed in the scintillation solution of Bray (1960). Comparative fixation results presented later have been normalized by

multiplication by a factor which equalizes the amount of activity used in each experiment. Experimentally determined efficiencies of the various counting systems have enabled expression of observed counts per minute as dpm.

Effect of 2,4-dinitrophenol (DNP). Cells were prepared as described for the efficiency runs. Experimental flasks contained: 2.0 ml (3.5 mg, dry weight) of cells in basal medium, 0.4 ml of $KHC^{14}O_3$ (5×10^6 dpm) solution (pH 7.0) in a side arm, and Pardee buffer as described. The remaining arm contained 0.5 ml of: (i) glucose in basal medium, (ii) glucose and DNP in basal medium, or (iii) basal medium alone (controls). All other manipulations were as described earlier, except that a single fixation period of 60 min was studied.

Studies of isotopic distribution. Interpretation of the data from efficiency studies required some knowledge of the time necessary for mixture of added $HC^{14}O_3^-$ with related unlabeled species, e.g., CO_2, HCO_3^-, and $CO_3^=$, in each liquid phase. Therefore, an experiment was conducted with lactate-grown cells in a manner similar to that described above under *Manometric methods;* however, the cells were not killed. Rather, at each designated time, the wick was rapidly removed from the center well and, after removal of excess Pardee buffer by manipulation with forceps, was transferred to a vial containing Bray solution and counted.

Results

Effect of p_{CO_2} on O_2 uptake. Figure 1 illustrates the necessity of maintaining a constant p_{CO_2} in manometric experiments from which data are used to compare O_2 uptake with different substrates. The rate of oxidation of glucose was found to vary inversely with the percentage of CO_2. Moreover, the lag period prior to a constant oxidative rate was extended as the percentage of CO_2 increased.

Efficiency of coupling of CO_2 fixation with oxidation of various substrates. For representation of the data, the ratio of dpm of C^{14} fixed to microliters of O_2 absorbed has been employed. To a first approximation, this ratio might be expected to remain constant over a time interval if fixation and oxidation are, in fact, coupled by a time-invariant mechanism. Preliminary studies with L-glutamate indicated that the ratio became constant only after a prolonged time interval

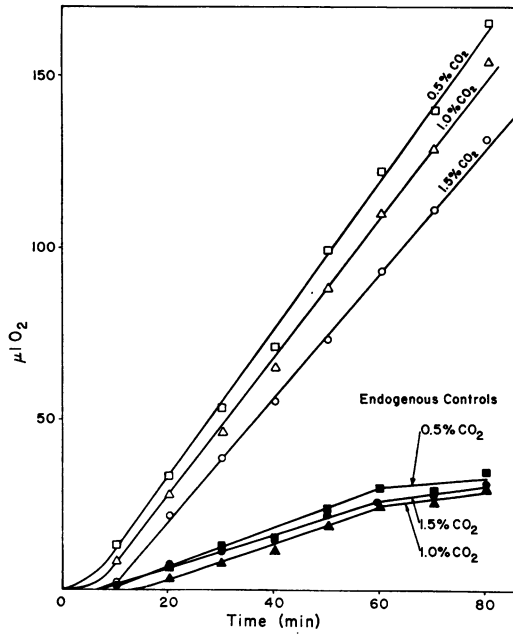

FIG. 1. *Effect of mole % CO_2 on oxygen uptake by glucose-grown Hydrogenomonas facilis oxidizing glucose. All points represent averages from duplicate trials. Filled symbols represent endogenous controls. The p_{CO_2} defined was maintained in each case by the appropriate Pardee buffer.*

(at least 45 min). For this reason, all subsequent studies were conducted over a 75-min period. Before proceeding with a study of the other substrates, it was necessary to obtain evidence that the C^{14} fixed was related to oxidation and not merely the result of an exchange process. This evidence is presented in Table 1. It can be seen that glucose-grown cells oxidizing glucose fixed large amounts of $C^{14}O_2$. Omission of the substrate led to greatly decreased O_2 uptake and an approximately parallel decrease in $C^{14}O_2$ fixation. The curtailment of heterotrophic fixation by DNP is striking. Even at a concentration of 4.1×10^{-5} M which stimulated O_2 consumption, the $C^{14}O_2$ uptake was sharply diminished.

The other substrates were subsequently tested. As may be seen from Fig. 2, the efficiency ratios were found to be initially time-variable. For four of the six substrates, however, a leveling of the curve occurred after 30 to 45 min. Leaching of C^{14} from killed cells did not occur; heat-killed cells were stable to leaching for 3 hr when stored under the usual conditions, i.e., in the presence of acid. Acid-killing resulted in about 90% of the level of

TABLE 1. *Heterotrophic CO_2 fixation and its sensitivity to 2,4-dinitrophenol (DNP)*

Flask contents[a]	O_2 uptake[b]	$C^{14}O_2$ uptake[b]
Complete[c]	(100)[d]	(100)[e]
Complete − glucose	18	12
Complete + 4.1×10^{-5} M DNP	117	16
Complete + 4.1×10^{-4} M DNP	58	3
Complete + 8.3×10^{-4} M DNP	52	2

[a] See Materials and Methods for experimental details.

[b] All percentages were calculated from averages of duplicate flasks.

[c] This medium contained the minerals (see text) plus 50 μmoles of D-glucose.

[d] The actual value was 139 μliters per flask.

[e] The actual value was 2.02×10^4 dpm per flask.

expect its variation with time to be attributable to nonlinearity with time of: (i) the numerator (dpm of C^{14}), (ii) the denominator (microliters of O_2), or (iii) both numerator and denominator. A plot of oxygen uptake during the fixation (Fig. 3) reveals that the uptake was linear after 15 min, glucose being an exception. The numerator, then, appears to vary nonlinearly with time. Examination of the C^{14}-fixation data reveals that this is indeed the case. If equilibration of C^{14} throughout the system were the sole explanation for the change in ratio with time, the initial negative slopes would be very similar, if not identical, for all substrates. This is not the case. However, that such equilibration does contribute to the negative slopes observed was established by the results shown in Fig. 4. The scatter of

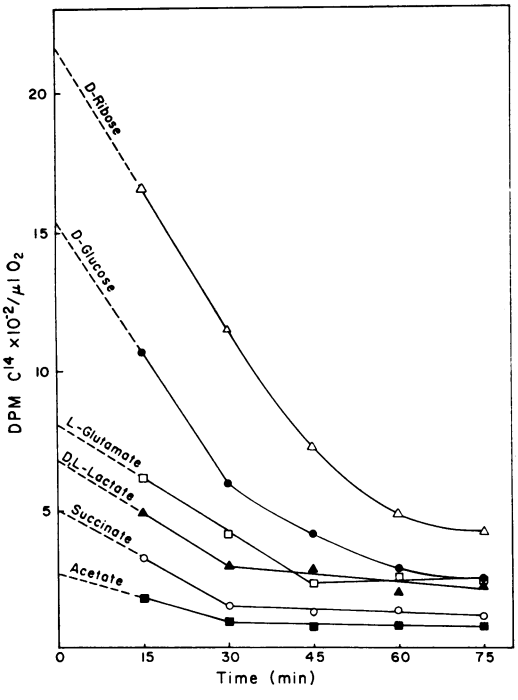

FIG. 2. *Efficiency (dpm of C^{14} per microliter of O_2) of coupling of CO_2 fixation with oxidation of various carbon sources as a function of time. Oxidative data are uncorrected for endogenous O_2 uptake. See Materials and Methods for experimental details.*

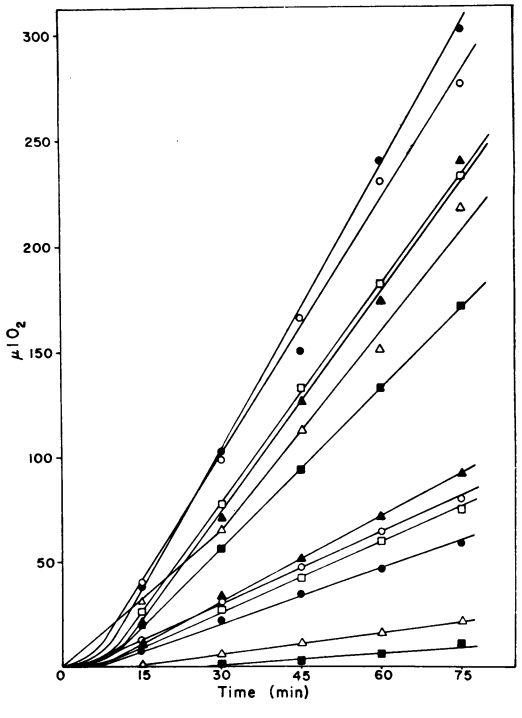

FIG. 3. *Oxygen uptake during $C^{14}O_2$ fixation by Hydrogenomonas facilis. The oxidative data obtained in the experiment described by Fig. 2 are plotted. The upper six lines show uptakes (averages of duplicates) observed during oxidation of: acetate, ○; D-glucose, △; L-glutamate, ▲; D,L-lactate, ●; D-ribose, ■; and succinate, □, by cells which had been grown on the same substrate provided during the oxidative studies. The lower six curves represent the corresponding endogenous uptakes (averages of duplicates).*

incorporation observed with heat-killing, demonstrating that a small fraction of labeled compounds are acid labile.

Since the ordinate (Fig. 2) is a ratio, one might

points describing this curve, which shows the time course of C^{14} uptake into the center well during lactate oxidation, is not too surprising considering the experimental procedure employed. It is obvious that leveling off occurs about 30 min after $KHC^{14}O_3$ tipping and thus about 2 hr after closing of the flask. This is most probably a result of isotopic equilibration, in the sense that related species such as CO_2, HCO_3^-, and $CO_3^=$ have reached a constant and identical specific activity. That such equilibration would be expected in the 30-min time period observed is supported by the data of Mills and Urey (1940).

In the period after addition of $KHC^{14}O_3$ and prior to isotopic equilibration, the wick increased in C^{14} content. Therefore, the cell suspension, in contact with the wick via the gas phase, may be initially enriched in C^{14}, the C^{14} then being slowly lost to the wick via $C^{14}O_2$. This is consistent with the initially high values of C^{14} uptake compared with O_2 uptake observed in all cases.

Comparison of efficiencies of coupling during oxidation of exogenous and endogenous compounds. As noted, controls lacking substrate were run concurrently to the 75-min interval during the experiments depicted in Fig. 2. In Fig. 5, the $C^{14}O_2$ fixed, O_2 consumed, and efficiency of coupling are compared in the presence and absence of exogenous substrate.

It is interesting that, with acetate and succinate, fixation of $C^{14}O_2$ was not only considerably less but substantial fixation by cells grown on each of these substrates occurred in the absence of exogenous substrate. This latter fixation was ostensibly due to the oxidation of endogenous stores formed during growth on these compounds.

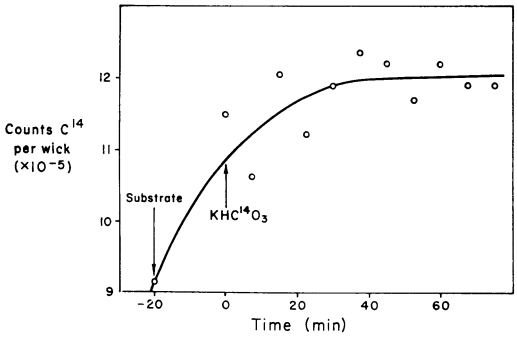

FIG. 4. *C^{14} content of the wick as a function of time during oxidation of D,L-lactate. See Materials and Methods for experimental details.*

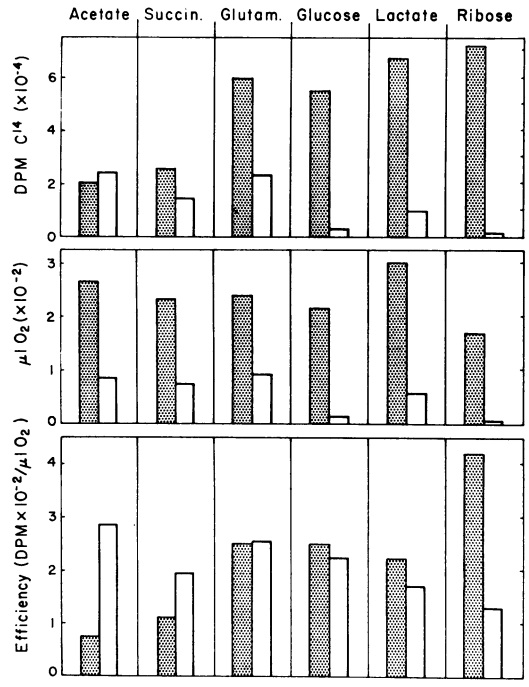

FIG. 5. *C^{14} uptake, O_2 uptake, and efficiency of coupling at 75 min by cells in the presence and absence of substrate on which they were grown. Darkened areas are for data in the presence of substrate; open areas are for data with substrate absent. Values represented are averages obtained from data for two flasks after 75 min of fixation.*

Ribose gave the greatest amount of fixation for the least O_2 uptake. Therefore, its calculated efficiency at 75 min was far greater than that of any other substrate tested.

Data from endogenous controls revealed a wide range of values for fixation and oxidation. Generally, the C^{14} uptake paralleled the extent of endogenous oxidation. Ribose-grown cells exhibited an unusually low and constant endogenous rate of 0.15 μliters of O_2 per min. Comparison of efficiencies for fixation coupled with oxidation of exogenous and endogenous compounds again revealed a wide range of values. With acetate and succinate-grown cells, C^{14} fixation was more efficiently coupled to oxidation of storage material than to oxidation of the substrate itself. In the presence of ribose, oxidation of the substrate was coupled much more efficiently with C^{14} fixation than it was with oxidation of cell storage material. Finally, for cells grown on glutamate, glucose, and lactate, the coupling efficiency was about equal and not

greatly affected by the presence of exogenous substrate.

Discussion

The data presented should be regarded as semiquantitative. Precision of the dpm of C^{14} per microliter of O_2 values was often rather poor. Part of the problem resulted from clumping of cells, affecting each stage requiring transfer of cell suspensions. More subtle factors may also have resulted in some imprecision. These include the geometries of flasks and of folded wicks, both of which would alter the rate of isotopic equilibration. Additionally, mention should be made of the fact that undoubtedly some $C^{14}O_2$ incorporation occurred prior to provision of $KHC^{14}O_3$ to cells. This was unavoidable because of the time necessary for equilibration of Pardee buffer with the gas and liquid phase. It was desirable to use $KHC^{14}O_3$ at pH 7 to maximize the rate of equilibration after it was added to cell suspensions at pH 7. Finally, some C^{14} incorporation occurred in the brief period between substrate addition and $KHC^{14}O_3$ addition. In summary, the experiments were necessarily complicated. However, they were carefully standardized, and reasonably meaningful comparisons of the characteristics and efficiency of heterotrophic fixation for different substrates are possible.

The 1.5% CO_2 atmosphere used in the present studies represents an environment that may obtain in soil (Baver, 1956), which is one, if not the only, natural habitat of *H. facilis* (Schatz and Bovell, 1952). This percentage was employed in the present studies to minimize the change in specific activity of $C^{14}O_2$ as a result of liberated respiratory CO_2. Exchange reactions, which would probably depend on p_{CO_2}, appear to play a minor role in the assimilation of $C^{14}O_2$ during glucose oxidation and presumably during oxidation of other substrates. Confirming this view is the inhibition observed with DNP and the very low C^{14} content of cells (ribose- and glucose-grown) which did not have large endogenous O_2 consumptions.

The relatively high endogenous O_2 consumption by acetate-, glutamate-, lactate-, and succinate-grown cells is presumably due in part to oxidation of storage polymer, poly-β-hydroxybutyrate. These cells were suspended in the basal medium containing NH_4^+, conditions known to favor oxidation of the polymer by *H. facilis* (Schlegel et al., 1961). Furthermore, C^{14} incorporation into the polymer from labeled acetate, lactate, or succinate was reported by Schlegel and Gottschalk (1962). These authors also reported that polymer synthesis does not occur from sugars in *H. facilis*. This is in accord with the very low endogenous O_2 consumption observed in the present work with ribose- and glucose-grown cells.

Assimilation of $C^{14}O_2$ during the oxidation of ribose results in large incorporation of label. It is inviting to speculate that growth on ribose may lead to relatively high levels of enzymes of the autotrophic carbon pathway. Hirsch et al. (1963) proposed that this pathway functions detectably during succinate oxidation by succinate-grown *H. facilis* in the presence of $C^{14}O_2$. Extrapolation to zero time of the efficiency curves (Fig. 2) has indicated the feasibility and desirability of using ribose as an exogenous substrate in studies of the pathway of heterotrophic CO_2 assimilation. To be sure, the mechanisms of such fixation probably vary strikingly as a function of substrate provided.

The most significant finding of the present work is the large magnitude of heterotrophic CO_2 fixation by *H. facilis*. This can be readily estimated by considering the observation (Fig. 4) that distribution of C^{14} of provided $KHC^{14}O_3$ into CO_2, HCO_3^-, $CO_3^=$, and related species apparently occurs in the course of the experiments described, resulting in equivalent specific activities for each of these species. Thus, the microliters of CO_2 utilized can be calculated for an interval after isotopic equilibrium has been established. For example, the interval in the experiment with glucose can be chosen where C^{14} uptake in comparison with O_2 uptake was essentially constant (60 to 75 min) and the number of microliters of CO_2 utilized per microliter of O_2 utilized can be calculated. This ratio is 0.49. The ratio for the same interval with succinate is 0.08. Hence, with dense and presumably nongrowing suspensions of *H. facilis*, the adenosine triphosphate (ATP) afforded by electron transfer to O_2 may be available for extensive CO_2 fixation. Several pathways of CO_2 fixation are known to require ATP. For example, the Calvin pathway requires ATP, as does CO_2 fixation during fatty acid synthesis.

Studies of several obligate heterotrophs in which the uptakes of O_2 and CO_2 were measured

in parallel experiments have yielded data in general agreement with those reported here (McLean, Robinson, and Purdie, 1951; Schlegel et al., 1961; Lafferty, 1963). The magnitudes of CO_2 fixation observed were smaller, but definitely depended upon the organism type and substrate furnished. With *Escherichia coli* growing on glucose, it has been found (Abelson, Bolton, and Aldous, 1952) that the ratio of millimoles of CO_2 fixed to millimoles of glucose consumed is 0.21. Assuredly, it is well documented that utilization of intermediates derived from heterotrophic metabolism is the major assimilatory process for diverse living forms. However, the significance to assimilatory processes of complete combustion coupled with cellular incorporation of some of the end product, CO_2, may have been widely overlooked.

Acknowledgments

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