OXIDATIVE ASSIMILATION BY PSEUDOMONAS SACCHAROPHILA WITH C^{14}-LABELED SUBSTRATES[1]

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In 1936, Barker discovered that during the oxidation of many organic compounds by resting cells of colorless alga *Prototheca zopfii*, a large part of the substrate undergoes "oxidative assimilation," and becomes converted to organic material within the cell. Since then, the occurrence of massive assimilation has been confirmed for many other microorganisms and for a wide variety of substrates; and it has been shown that the phenomenon occurs during fermentations as well as oxidations, by both resting and growing cells (Clifton, 1946). Heretofore, the techniques used for the detailed study of the assimilatory process have been largely manometric, in some cases accompanied by gross material balances; despite the considerable volume of work undertaken, the somewhat indirect nature of these experimental approaches has made it impossible to draw any but the most tentative conclusions concerning the mechanism of assimilation. Baddiley *et al.* (1950) have used labeled acetate in the studies of assimilatory processes of growing yeast. In the present studies, several labeled substrates were used in an attempt to determine the metabolic fate of the individual carbon atoms in these compounds during their oxidation by resting cell suspensions of *Pseudomonas saccharophila*. In addition, it was found possible to obtain direct evidence concerning the effect of the oxidation of exogenous substrates on the endogenous respiration.

The results are in general agreement with earlier, tentative conclusions based on the use of less direct methods.

METHODS

The following C^{14}-labeled compounds were used as substrates: acetate labeled in both carbon atoms equally as well as in the C_1 and C_2 positions separately, lactate labeled in the C_1, C_2, and C_3 positions respectively, succinate labeled in the first and second carbon atoms respectively, and glucose with unequal and uncertain content of C^{14} in the various carbon atoms.[3] Their oxidation by cell suspensions of *P. saccharophila* was investigated in Warburg respirometer vessels. The distribution of the labeled carbon atoms in the respiratory CO_2, and in the other products of metabolism, either assimilated by the cells or excreted into the medium, was determined by measuring the radioactivity of the respiratory

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CO_2, the cells and the supernatant after the completion of the oxidation. In all cases, the bacteria were grown in a synthetic medium (Doudoroff, 1940) with the substrate to be studied as sole carbon source. Then they were washed and "starved" for from 2 to 12 hours by incubation in buffer with continuous aeration. Limited amounts of substrate were supplied to the cells in a respirometer vessel, and the oxidation was allowed to proceed until the decrease in the rate of oxygen uptake indicated that the compound had disappeared from the medium. The CO_2 produced during the oxidation was trapped in NaOH in the center well and converted to $BaCO_3$ at the end of the experiment, after addition of a known amount of Na_2CO_3 as "carrier." The $BaCO_3$ was washed then with water and alcohol and spread on aluminum discs for C^{14} determinations (Calvin *et al.*, 1949). The cell suspension in the main compartment was cooled rapidly to 5 C and centrifuged. Aliquots of the supernatant liquid were adjusted to pH 8.3, dried on aluminum discs, and analysed for C^{14} content. The sedimented cells were resuspended in water, and aliquots of this suspension were also dried on aluminum discs for C^{14} determination.

Thus it was possible to determine the distribution of C^{14} among the CO_2, the bacteria, and the products of oxidative metabolism excreted into the medium by analysing the contents of a single vessel. In some experiments two vessels were used, one being sacrificed to determine only the activity of the CO_2 by adding strong acid to the main chamber at the completion of substrate oxidation. Results obtained in this manner did not differ materially from those obtained with a single vessel, provided the vessel was allowed to remain shaking in the bath for ten minutes after the substrate had disappeared and provided the medium was slightly acid throughout the experiment, thus showing that the CO_2 absorption is quantitative under the usual conditions for the manometric determination of O_2 uptake.

RESULTS

CO_2 assimilation. It is well recognized that heterotrophic metabolism leads to the assimilation of CO_2 and its incorporation in both cell substance and certain metabolic waste products of the cell. It was therefore important to determine whether, under the conditions of the experiments, there would be a significant equilibration between C^{14} supplied externally as CO_2 and the products of assimilation of an unlabeled compound. The possibility of such equilibration seemed particularly likely in the organism studied, since *P. saccharophila* is a facultative chemoautotroph, capable of rapid development in an inorganic medium at the expense of the energy obtained from the oxidation of molecular hydrogen (Doudoroff, 1940).

To measure the extent of CO_2 fixation during the oxidation of an organic compound, a cell suspension was allowed to oxidize 10 μ moles of unlabeled acetate in a Warburg vessel without the addition of KOH to the central well. Approximately 1.2 μ moles of labeled carbonate were added simultaneously with the acetate. A duplicate vessel with KOH in the central well permitted the computation of the extent of oxidative assimilation of the acetate. After completion of

the oxidation the carbon dioxide was removed by acidification and aeration, and collected in $Ba(OH)_2$. The $BaCO_3$, the bacteria, and the supernatant medium were tested then for radioactivity.

A parallel experiment was conducted in the presence of 0.1 per cent NH_4Cl and 0.2 per cent $MgSO_4$ to determine whether the addition of minerals essential for protein synthesis would influence greatly the assimilation of CO_2 during a brief experiment. The results are summarized in table 1.

From these data, it is evident that even when the CO_2 produced in respiration is allowed to accumulate in the vessel, the degree of equilibration between it and the products of acetate metabolism is negligible. Under the conditions of the experiment, only about 0.5 per cent of the assimilated carbon is derived from CO_2 in the absence of a nitrogen source, and a little over 1.0 per cent in the presence of NH_4Cl.

In the subsequent experiments with labeled substrates, metabolic CO_2 was constantly removed with KOH, permitting even less opportunity for equilibration.

TABLE 1

CO_2 assimilation during substrate oxidation

	BUFFER ONLY	WITH NH_4Cl, $MgSO_4$
Acetate C assimilated	10.1 μ atoms	9.8 μ atoms
CO_2 present at end of experiment	11.1 μ moles	11.4 μ moles
Total radioactivity in CO_2	36400 counts/min	38800 counts/min
Radioactivity in cells	141 counts/min	350 counts/min
Radioactivity in supernatant	66 counts/min	98 counts/min

Autorespiration. To determine whether endogenous respiration continues during substrate oxidation, resting cells were allowed to incorporate C^{14} into their reserve products by oxidizing acetate labeled equally in both carbon atoms with C^{14}. The cells were washed then and allowed to respire both in the presence and in the absence of unlabeled acetate. A parallel experiment was conducted in which M/4,000 2,4-dinitrophenol (DNP) was added just prior to the addition of unlabeled substrate. The total amount of CO_2 produced and the total content of C^{14} in the CO_2 were determined for each set of conditions over a period of 155 minutes, beginning 15 minutes after the addition of unlabeled acetate to the cell suspensions. The results are summarized in table 2.

It is clear from table 2 that the presence of substrate largely inhibits autorespiration, except when assimilation is prevented by dinitrophenol. This is in complete agreement with previous conclusions reached on the basis of indirect evidence (Doudoroff, 1940). Similar experiments in which the cells were exposed to C^{14}-labeled glucose[4] and then allowed to oxidize unlabeled glucose indicated that in the absence of DNP the oxidation of glucose resulted in 96 per cent inhibition of endogenous respiration. The above observations are contrary to

[4] This glucose was labeled in all carbon atoms, but unequally.

those made recently with *Streptomyces coelicolor* by Cochrane and Gibbs (1951), who used a similar experimental approach.

Unfortunately, the nature of the products of "primary assimilation" by *P. saccharophila* has not yet been elucidated. It had previously been shown that the assimilated material is, on the average, considerably more reduced than carbohydrate. The formation of some carbohydrate during oxidative assimilation has since been demonstrated, but this material (which appears to have some properties of glycogen) has not been identified with certainty (unpublished experiments). It had been hoped that the tracer technique would be useful in determining the class of cellular constituents formed by resting cells. Although the experiments to date have not been extensive, the preliminary results have not been encouraging.

Both the "glycogen" and the lipid fractions of cells which had oxidized either labeled acetate or glucose had relatively low specific activities as compared with the activity of other cellular constituents. There is considerable evidence, which

TABLE 2

Effect of substrate oxidation on endogenous respiration

	WITHOUT DNP	WITH DNP
(*1*) Total CO_2 produced without substrate	62 μl	81 μl
(*2*) Total CO_2 produced with acetate	450 μl	605 μl
(*3*) Total radioactivity of CO_2 produced without substrate (counts/min)	409	630
(*4*) Total radioactivity of CO_2 produced with acetate (counts/min)	73	591
(*5*) Inhibition of autorespiration by acetate [100 − ((*4*)/(*3*))(100)]	82 per cent	6 per cent

will not be discussed here, that during the oxidation of a substrate, the assimilated carbon is rapidly distributed among the various components of the cell, including the proteins.

Assimilation with various substrates. In previous comparative studies on the oxidative assimilation of various compounds by *P. saccharophila* certain patterns of metabolism have been detected. For example, the probability of the oxidation of the carboxyl groups of pyruvic and lactic acids to CO_2 has been supported by the demonstration of the almost identical degree of assimilation with the two compounds. The role of pyruvate as a product of intermediate metabolism in the oxidation of both carbohydrates and dicarboxylic acids has been postulated on similar grounds, as well as on the basis of the observation that this compound accumulates under special conditions where carbohydrates or dicarboxylic acids are undergoing oxidation (Doudoroff, 1940; Whelton and Doudoroff, 1945; and Bernstein, 1944).

It has never been possible, however, to distinguish directly between two mechanisms, either of which could lead to the assimilation of a given fraction of a substrate. The first is the complete oxidation of some molecules of the substrate accompanied by the complete assimilation of others; the second is the

oxidation of a part of each molecule to CO_2, concomitant with the assimilation of the rest of the molecule.

Thus, the oxidation of lactate has been shown to be in approximate accord with the following empirical equation in which assimilated material is very roughly, and somewhat inaccurately, represented by the formula (CH_2O):

$$C_3H_6O_3 + O_2 \rightarrow CO_2 + 2(CH_2O) + H_2O$$

This could be interpreted either as an oxidative decarboxylation leading to the assimilation of a C_2 fragment or as the complete oxidation of one molecule of lactate to CO_2, accompanied by the incorporation of two molecules into cell material, as shown in equations (a) and (b):

TABLE 3

Assimilation of various substrates by P. saccharophila

SUBSTRATE	PER CENT OF INITIALLY SUPPLIED C^{14} APPEARING IN			
	CO_2	Cells	Supernatant	Total recovery
$C^{14}H_3COOH$	48	49	4	101
$CH_3—C^{14}OOH$	61	36	4	101
$C^{14}H_3COOH$*	44	52	2	98
$CH_3—C^{14}OOH$*	73	24	2	99
$C^{14}H_3—CHOH—COOH$	21	77	7	105
$CH_3—C^{14}HOH—COOH$	29	63	3	95
$CH_3—CHOH—C^{14}OOH$	91	3	12	106
$COOH—C^{14}H_2—C^{14}H_2—COOH$	32	71	3	106
$C^{14}OOH—CH_2—CH_2—C^{14}OOH$	90	1	9	100

* In the presence of 0.1 per cent NH_4Cl, 0.02 per cent $MgSO_4$.

$$\text{(a)}\quad C_3H_6O_3 + O_2 \rightarrow CO_2 + (C_2H_4O_2) + H_2O$$

$$\text{(b)}\quad \begin{cases} C_3H_6O_3 + 3O_2 \rightarrow 3CO_2 + 3H_2O \\ 2C_3H_6O_3 \rightarrow (C_3H_6O_3)_2 \end{cases}$$

The use of isotope-labeled compounds has made it possible to determine which carbon atoms of various substrates (acetate, lactate, and succinate) are actually assimilated and which are oxidized to CO_2.

The results of a series of experiments, using substrates labeled with C^{14} in different C atoms, are presented in table 3. In studies on acetate utilization, the cell suspensions were allowed to metabolize the acetate both in the presence and in the absence of NH_4Cl and $MgSO_4$. These salts were added to permit the synthesis of proteins during the respiration. The oxidation of lactate and of succinate was studied only with "resting cells" in the absence of an exogenous nitrogen source.

It is clear from the table that both carbon atoms of acetate undergo assimilation, slightly more of the methyl than of the carboxyl carbon being assimilated

by resting cells. Under conditions more favorable for growth, which decreases the efficiency of assimilation (Whelton and Doudoroff, 1945), preferential assimilation of the methyl carbon is even more marked. Ehrensvärd *et al.* (1951) have shown that growing yeast produces twice as much CO_2 from the carboxyl as from the methyl carbon of acetate.

The oxidation of both lactate and succinate results in the recovery of almost all of the carboxyl carbon as respiratory CO_2. This is in agreement with the previously postulated schemes, according to which carbon atoms 2 and 3 of pyruvate, of lactate, and of the dicarboxylic acids were proposed as the building blocks for anabolism (Bernstein, 1944).

Certain discrepancies may be noted between the fractions of substrate carbon actually appearing in the cells in the foregoing experiments, and the values expected on the basis of previously published equations. In experiments with labeled substrates, only 48 per cent of lactate carbon and 36 per cent of the succinate carbon appear in the bacteria. Previous computations of the assimilation at the expense of these compounds gave values of 66 per cent and 50 per cent, respectively. This reflects in part the fact that in all previous experiments all the organic products of metabolism were considered as products of assimilation, whether they appeared in the cells or in the medium. Two other factors must also be taken into consideration in explaining this discrepancy. First, the previous estimations of the efficiency of assimilation were based largely on the idealized interpretation of curves showing oxygen uptake and CO_2 production. In the present experiments, a certain amount of endogenous respiration had to be allowed after the completion of substrate oxidation, in order to ensure the complete disappearance of substrate and absorption of carbon dioxide. Second, there appear to have been some slight but definite changes in the metabolism of *P. saccharophila* in the course of several years of cultivation under laboratory conditions. A tendency towards lower efficiencies of assimilation of lactate, pyruvate, succinate, malate, and fumarate has been noted, as well as a striking difference in the course of oxidation of maltose.

DISCUSSION

The present work supports the view that two-carbon fragments of the nature of "active acetate" are the fundamental building blocks in the oxidative assimilation of *P. saccharophila*. It is such C_2 units, rather than entire molecules of C_3 or C_4 compounds derived directly from lactate and succinate which are involved not only in the synthesis of assimilated materials, but also in exchange reactions with other protoplasmic constituents. It would appear that neither pyruvate nor the C_4 dicarboxylic acids can be themselves "activated" in such a way as to enter the anabolic pathways directly. This may be due to the inability of the organism to phosphorylate these compounds directly or to form phosphopyruvate in the oxidation of either lactate or succinate except through a cycle involving C_2 condensation. This conclusion is supported by the previous observations that oxidative assimilation with pyruvate is almost as efficient as with lactate and that under certain conditions there is a transient accumulation

of pyruvic acid in the metabolism of succinate, malate, and fumarate (Bernstein, 1944).

The findings do not shed any light on the mechanism of the oxidation of the substrates beyond the C_2 stage. The observed distribution of the substrate atoms in the metabolic products could be explained by invoking the operation of either a di- or a tricarboxylic acid cycle.

It must be pointed out, however, that with the exception of acetate, about half of which is completely oxidized to CO_2, the substrates tested give rise to such great amounts of assimilation from the C_2 (active acetate) portion that a cycle, if operative, can be receiving only a relatively small amount of this fragment. Thus, of the second and third carbon atoms of lactate, only 29 and 21 per cent, respectively, appeared as CO_2. This would indicate that only a fifth of the "active acetate" had been completely oxidized, while an additional small fraction had been partially degraded.

It is interesting to note that Fraser and Tolbert (1951), using C^{14}-labeled lactate, have found an almost identical pattern of assimilation of the carbon atoms of lactate in growing cultures of *Escherichia coli* and in the formation of bacteriophage.

SUMMARY

By the use of C^{14}-labeled substrates it has been shown that (1) During the oxidative assimilation of organic compounds by the facultatively autotrophic *Pseudomonas saccharophila* the reduction of CO_2 to cell material is negligible. (2) Endogenous respiration of the cells is inhibited during the oxidation of substrates, except in the presence of dinitrophenol. (3) Both carbon atoms of acetic acid are assimilated, the methyl carbon being favored, especially in the presence of nitrogen source. (4) The carboxyl carbon of lactate is almost completely oxidized, the remaining C_2 fragment being largely used for synthetic reactions. (5) Both carboxyl carbons of succinate are oxidized to CO_2, while the methylene carbons are largely assimilated.

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