ASSIMILATION OF GLUCOSE AND RELATED COMPOUNDS BY GROWING CULTURES OF PSEUDOMONAS SACCHAROPHILA

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During the last decade there has been increased interest among microbiologists in assimilation by heterotrophic organisms. With the introduction by Barker (1936), Giesberger (1936), and Clifton (1937) of the Warburg manometric technique for the measurement of assimilation by resting suspensions, a new estimation of the process became possible. The method used for such studies consists of providing a "starved" suspension of organisms with a known amount of a single organic carbon source in the Warburg vessel, and measuring the amounts of oxygen uptake and carbon dioxide production during the period of increased metabolic activity following the addition of substrate. If these amounts are found to be less than those required for complete respiration of the organic compound and no products of incomplete oxidation are present in the medium, it is assumed that the remaining fraction of the compound has been synthesized into cell material. It is to be remembered, however, that the organisms used in such studies are not proliferating, and that their activities may be somewhat different from those of growing cultures. We have as yet almost no real knowledge of the steps involved in the synthesis of protoplasmic material.

The investigations reported here were made in an attempt to compare the assimilation from certain compounds by *Pseudomonas saccharophila* (Doudoroff, 1940) during cell multiplication with the assimilation shown by resting suspensions of the same organism. It is hoped that data on this subject will increase our present understanding at least of the over-all mechanism of synthesis.

Pseudomonas saccharophila is a potentially autotrophic hydrogen bacterium, capable of utilizing carbon dioxide as sole carbon source. It grows readily, however, with any of a number of organic compounds as a source of this element, and is strictly aerobic, incapable of fermenting sugars. Except under certain unfavorable conditions to be described, no known metabolic waste products other than CO_2 are formed in heterotrophic metabolism, a fact which makes the organism particularly suitable for the type of studies reported here. Previous investigations (Doudoroff, 1940) using resting suspensions of the organism indicated that its efficiency of assimilation is relatively high. Furthermore, the amount of synthesis which it attains with different carbon sources is remarkably constant and to a certain extent independent of the energy content of the substrate molecule. With glucose, sucrose, maltose, trehalose, lactate, and pyruvate, approximately two-thirds synthesis and one-third oxidation (as measured by oxygen consumption) take place.

METHODS

The general plan of the present work was to provide the bacterium with a known amount of an organic carbon source and, after a given period of growth,

to determine, by measurement of the amount of organic substrate remaining in the medium, how much had been utilized. From this, the weight of carbon used by the organism could be computed. Then, by carrying out carbon analyses on the bacteria, the percentage of utilized carbon actually appearing in the protoplasm could be found. Thus the figures given in this paper for "per cent synthesis" refer to the percentage of substrate carbon which is assimilated.

The basic medium used for growth of the organisms contained a known amount of an organic carbon source in a mineral medium with the following constituents: M/30 KH_2PO_4-Na_2HPO_4 buffer at desired pH (pH 6.64 was usually employed); 0.1 per cent NH_4Cl; 0.05 per cent $MgSO_4 \cdot 7H_2O$; 0.005 per cent $FeCl_3 \cdot 6H_2O$; 0.0005 per cent $CaCl_2$. Organic compounds were sterilized by autoclaving in distilled water, with the exception of sodium pyruvate, which was sterilized by filtration. The phosphate buffer, ferric chloride solution, and a mixture of the remaining salts were autoclaved separately in order to avoid precipitation during the application of heat. For growth experiments, the media were placed in Erlenmeyer flasks in amounts such that a large surface was exposed to air (e.g., 30 ml in a 200-ml Erlenmeyer) and the flasks were placed on a rotary shaker to provide constant agitation and ample aeration of the medium during the development of the organisms. Except where otherwise indicated, the temperature of incubation was 30 C. In most cases, it was attempted to remove the flasks toward the end of the logarithmic period of development. When sugars were used as substrates, the amount remaining in the medium after growth was determined according to the method of Hassid (1937), the disaccharides being subjected to a preliminary acid hydrolysis. Acetic acid was measured by steam distillation and titration with standard base. Of the methods tried for estimation of lactic acid, that of Friedemann and Graeser (1933) was most satisfactory. Pyruvic acid determinations were carried out as suggested by Clift and Cook (1932; see also Elliot, Bency, and Baker, 1935). The bacteria on which carbon analyses were to be made were centrifuged, washed once in distilled water acidified with KH_2PO_4 to remove carbonates, and then dried on a steam bath. Carbon determinations were carried out either by the dry combustion method described by Pregl (1930) using an MnO_2-PbO_2 catalyst or by the wet combustion method of McCready and Hassid (1942), in which the Van Slyke oxidation mixture is used. In both cases, carbon dioxide was determined gravimetrically after absorption. Results with the two methods were found to check fairly well, and both gave 100 per cent recovery when used with known organic compounds.

EXPERIMENTAL

Composition of the bacteria. Elemental analyses of the bacteria grown in a glucose medium were made by the dry combustion method for carbon and hydrogen and by the Kjeldahl method for nitrogen. The following average values were obtained (on the basis of dry weight): carbon, 54.5 per cent; nitrogen, 11.05 per cent; hydrogen, 7.4 per cent; ash, 3.3 per cent. These figures are fairly comparable with those obtained by Van Niel (1936) for certain purple bacteria. From these figures, the average reduction state of the carbon in the bacterial pro-

toplasm may be computed as approximating the formula ($CH_{2.12}O$). The nitrogen content, which is high as compared with that of many tissues, can be decreased very considerably by the removal of the nitrogen source from the medium, as will be shown later.

Assimilation with different carbon sources. The average amounts of assimilation obtained from various compounds during growth under optimal conditions, together with the minimal division times observed during the exponential phase of growth at 30 C, are presented in table 1. Obviously, the percentages of synthesis from different compounds by proliferating cells are not at all constant. Furthermore, in all cases the amount of synthesis is less than that found with resting cells (table 1). In experiments with resting cells oxidizing lactic acid or

TABLE 1

Assimilation of various substrates by resting suspensions and growing cultures of Pseudomonas saccharophila.

(a) Minimum division time during the phase of exponential (logarithmic) growth at 30 C under optimal conditions (pH, concentration of nutrients) tried.

(b) Percentage of substrate carbon assimilated during growth under optimal conditions.

(c) Percentage of substrate carbon assimilated by resting suspensions on the basis of CO_2 production (computed from Doudoroff, 1940). Figure for maltose based on corrections discussed previously. Value for pyruvate based on highest values obtained previously and by Bernstein (1944). Acetate value from unpublished data.

(d) Ratio of assimilation during growth to that shown by resting suspensions (b:c).

SUBSTRATE	(a) DIVISION TIME	(b) PER CENT ASSIMILATION DURING GROWTH	(c) PER CENT ASSIMILATION BY RESTING SUSPENSIONS	(d) RATIO b:c
	min			
Glucose	178	54	60	0.90
Maltose	170	49	59	0.83
Sucrose	109	49	61	0.80
Trehalose	105	51	61	0.84
Lactate	136	44	61	0.72
Pyruvate	152	36	60	0.60
Acetate	196	28	45	0.62

glucose, Doudoroff (1940) observed that carbon dioxide is produced in slight excess of the oxygen consumed. This has been taken to indicate the formation of storage products which are, on the average, more reduced than carbohydrates. If it is assumed that the product of primary synthesis is a carbohydrate with the empirical formula $(CH_2O)_n$, the extent of its formation may be computed roughly from the oxygen consumption by respiring cells, as has been done in the previous studies. For comparison with actual synthesis during growth, on the other hand, it was necessary to recompute the amount of assimilation by resting cells from the observed evolution of carbon dioxide, so that these results, too, might be expressed on the basis of the percentage of carbon synthesized. Even with comparable methods of expression, however, it will be seen from the table that growing cultures are less efficient in their over-all synthesis than resting suspensions.

Only with glucose, and to a lesser extent with the disaccharides, does the "growth synthesis" even approach the synthesis by resting suspensions.

The utilization of disaccharides by *P. saccharophila* both in growing cultures and with resting suspensions is somewhat unusual in that sucrose, maltose, and trehalose are all used more rapidly than their constituent hexoses. The rapid utilization of sucrose is particularly striking since fructose is attacked only with very great difficulty. Recent work with enzyme preparations from this organism (Doudoroff, Kaplan, and Hassid, 1943; Doudoroff, 1943) has shown the occurrence of a reversible phosphorolysis of sucrose with the production of glucose-1-phosphate and fructose. As these authors point out, however, this does not explain entirely the behavior of the bacteria with regard to this sugar or throw any light on their action on other disaccharides. With resting suspensions of the bacteria, sucrose and trehalose, though oxidized more rapidly than glucose, are assimilated to about the same extent as this monosaccharide.

The postulation of a two-thirds primary synthesis from pyruvic acid was based on selected experiments using the Warburg technique and depended on the application of certain corrections for autorespiration which were not made with other substrates. In practice, it has been found difficult to obtain closely reproducible results with this substrate, since the previous history of the bacteria, as well as the experimental conditions, appear to affect the efficiency of assimilation from pyruvic acid to a much greater extent than that from sugars. In general, the fraction assimilated was found to be less than two thirds, but, from a large number of experiments, Bernstein (1944) concludes that this figure represents the maximal attainable value for the isolated process of "primary synthesis." It should also be stated that complications are encountered in manometric studies with acetic acid as substrate. Not only does the per cent of assimilation appear to decrease when increasing amounts of this compound are given to the bacteria, but the pH of the medium has a striking effect on the efficiency of synthesis. At high pH values (pH 7.5 to 8.5) the rate of respiration was found to increase and the efficiency of synthesis to decrease progressively with increasing alkalinity. The figures for acetic acid utilization by resting suspensions given in table 1 are based on the average values obtained in experiments conducted in neutral or slightly acid environment. With growing organisms, the amount of assimilation is lower with lactate than with any of the sugars studied, still lower when pyruvate is used as carbon source, and lowest of all with acetate.

Effect of iron concentration. The concentration of ferric chloride used in the medium had a pronounced effect on the rate of development of the cultures with all the substrates used. A maximum growth rate was obtained in all cases with about 0.0025 per cent $FeCl_3 \cdot 6H_2O$. With lower concentrations, not only was the rate of growth, but also the amount of assimilation, decreased. The effect of iron on both growth rate and assimilation was more pronounced with those carbon sources which gave rapid development (e.g., sucrose) than with substrates utilized more slowly (e.g., glucose). See table 2. With sucrose and trehalose, the iron exerted a striking effect on the course of metabolism during growth. If

insufficient iron was added, pyruvic acid[1] was found to accumulate in the medium, sometimes to such an extent as to result in the death of the organisms. Thus, up to about one third of the sugar was converted into this compound when 0.0001 per cent $FeCl_3 \cdot 6H_2O$ or less was present. No pyruvic acid, or only traces, could be detected when the concentration was increased to 0.005 per cent. Except in those experiments where the iron concentration was so low that the bacteria appeared to be incapable of further activity after the pyruvic acid accumulation had reached a maximum, this compound was found to disappear more or less rapidly after the depletion of the sugar from the medium, the rate of disappearance depending on the amount of iron available to the organisms. No pyruvic acid was found under any condition in cultures using glucose, maltose, or lactic acid as substrates, nor could its production be shown with suspensions of resting cells grown in iron-deficient media and allowed to oxidize sucrose. That the effect ascribed to iron concentration was not due to impurities present in the

TABLE 2

Effect of iron concentration on assimilation

Cultures grown at pH 6.64, 30 C, and harvested just before the exhaustion of substrate

SUBSTRATE	AMOUNT OF $FeCl_3 \cdot 6H_2O$ INITIALLY ADDED (IN MG PER L)	PERCENTAGE OF SUBSTRATE CARBON ASSIMILATED
M/75 Glucose	5	46
	17	53
	50	53
M/150 Sucrose	5	35
	17	43
	50	48

ferric chloride was shown by the fact that the salt could be replaced by a highly purified solution of ferrous tartrate (tartrate is not oxidized by the bacterium), whereas a mixture of trace elements failed to have the same effect.[2] On the other hand, the amount of iron added to the medium does not truly represent the amount available to the organisms, since during growth of the cultures the colloidally dispersed iron compounds tend to be precipitated out.

Effect of pH and temperature. Although assimilation from different substrates by growing cultures shows rather wide variations, the amount from a single substrate is fairly independent of certain changes in environmental conditions. For example, the amount of synthesis during growth with glucose is almost constant between pH 6.2 and 7.4, even though the rate of development is considerably lower in the alkaline range (see table 3). Furthermore, no appreciable difference

[1] Pyruvic acid was precipitated from the medium and identified as the 2,4 dinitrophenyl hydrazone. M.P. (uncorrected): 2,4 dinitrophenyl hydrazone of pyruvic acid, 212.9 C; unknown, 213.0 C; mixed, 213.0 C.

[2] The trace element mixture and ferrous tartrate solution were obtained through the kindness of Dr. D. I. Arnon.

could be detected between glucose cultures grown at room temperature, which showed a synthesis of 52 per cent of the carbon, and those incubated at 30 C.

Effect of the composition of atmosphere. Certain alterations of the atmosphere during growth with glucose affected the amount of synthesis rather markedly. As will be seen from table 3, neither the provision of extra carbon dioxide, nor a variation of the oxygen content of the atmosphere between 5 and 20 per cent affected the amount of assimilation. However, removal of all carbon dioxide by its absorption in a cup of alkali within the culture flask resulted in a decreased synthesis. In view of the now well-known role of carbon dioxide in heterotrophic assimilatory processes, this is hardly surprising. The most pronounced effect of

TABLE 3

Effect of pH, temperature and composition of the atmosphere on assimilation during growth with glucose

Initial concentration of glucose, M/75 in experiment 1; M/700 in experiment 2. Free access of air allowed in experiment 1; while experiment 2 was carried out in closed flasks. Bacteria were harvested in each case before the exhaustion of either glucose or oxygen.

EXPERIMENT NUMBER	INITIAL COMPOSITION OF ATMOSPHERE	pH	TEMPERATURE	PER CENT OF SUBSTRATE CARBON ASSIMILATED
			C	
1	Air	6.64	20–22	52
	Air	6.2	30	54
	Air	6.64	30	53
	Air	7.4	30	51
2	5% O_2, 95% N_2	6.64	30	54
	5% O_2, 5% CO_2, 90% N_2	6.64	30	54
	20% O_2, 80% N_2	6.64	30	54
	20% O_2, 80% N_2, CO_2 removed*	6.64	30	48
	20% O_2, 5% CO_2, 75% N_2	6.64	30	54
	50% O_2, 50% N_2	6.64	30	40

* CO_2 removed from atmosphere by absorption in KOH placed in a cup within the culture flask. In all other cases, CO_2 was allowed to accumulate in the atmosphere.

a change in atmosphere was observed with high partial pressures of oxygen. Not only did the assimilation decrease as the amount of this gas present was increased up to 50 per cent; it was also observed that growth was entirely absent if as much as 80 per cent oxygen was used. In manometric experiments, resting suspensions were found to assimilate the same percentage of glucose in an atmosphere composed entirely of oxygen as in air. It would therefore appear that the "primary synthetic" mechanism is not affected by an excess of this gas. Furthermore, the autorespiration of resting cells remained the same in an oxygen atmosphere. Apparently, then, oxygen affects some system in the organism connected with growth but not with "primary synthesis."

Effect of availability of nitrogen source. Over a limited period of time, the lack of an adequate nitrogen source does not greatly affect the efficiency of assimilation,

as might be expected on the basis of experiments with resting cells, which are provided with no nitrogen source. It will be seen from table 4 that organisms supplied with additional sugar in the absence of ammonium chloride assimilate approximately the same percentage of substrate carbon as when a nitrogen source is present. The composition of the bacteria, however, undergoes a striking change in the absence of nitrogen. A decrease in the nitrogen content of the cells is, of course, to be expected. By analysis of the figures, it can be shown that the total nitrogen in this crop of organisms did not change materially, although there was a small loss, possibly due to experimental error. It will be noted that the amount of sugar used in the same period of time was nearly twice as great in the medium with NH_4Cl as in the nitrogen-free medium. This, of course, was due to the multiplication of the bacteria and the resulting increase in the total

TABLE 4

Effect of the availability of nitrogen source an assimilation and bacterial composition

Two aliquots of a culture were centrifuged, washed, and resuspended in medium containing M/150 glucose, buffer and all usual minerals,* but in one case without any nitrogen source provided, while in the other with 0.1% NH_4Cl. Original total carbon content of bacteria in each aliquot: 46.6 mg. Incubation at 29 C for 4 hours with constant agitation. Per cent C and N content of bacteria expressed on dry weight basis.

	WITH NH_4Cl	WITHOUT N SOURCE
mg sugar used	222.25	126.2
mg C in sugar used	88.9	50.5
mg increase in bacterial C	44	22.5
% synthesis	49.5	44.7
	%	%
N content of bacteria	11.0	7.2
C content of bacteria	56	56

* Note: Only 5 mg of $FeCl_3 \cdot 6H_2O$ per L was added to the suspension, since a large amount of iron was already present in the precipitate which was harvested with the bacteria. The somewhat low amount of assimilation observed in N-containing medium may have been due to an insufficiency of this element.

metabolism of the culture. The efficiency of the assimilation was actually somewhat lower in the nitrogen-free medium. This might seem contrary to expectations, since resting cell suspensions have been found to assimilate even greater quantities of substrate than growing cultures. It must be remembered, however, that the manometric experiments are of very short duration and involve very small amounts of substrate. If the growth experiments had been continued for a longer period of time, the efficiency of assimilation would undoubtedly have shown an eventual decline and would have approached zero, for presumably the synthesis of only nonnitrogenous compounds cannot continue indefinitely.

Autotrophic development. As has been mentioned, *P. saccharophila* is a potentially autotrophic bacterium, capable of utilizing carbon dioxide as the sole carbon source in its nutrition. It obtains the energy necessary for the reduction

of carbon dioxide to cell material by the oxidation of gaseous hydrogen to water. In order to study the autotrophic assimilation, a culture of the organism was allowed to develop in the basic mineral medium without any added organic substrate, but in an atmosphere composed initially of 74.5 per cent H_2, 18 per cent O_2, and 7.5 per cent CO_2. Growth was stopped before the partial pressure of any one of the component gases had decreased to half its original value. From the total uptake of gases, and from the amount and reduction state of cell material formed during growth under such conditions, the following over-all formulation for the autotrophic metabolism could be made:

$$CO_2 + 11.68\ H_2 + 4.81\ O_2 \rightarrow (CH_{2.12}O) + 10.62\ H_2O.$$

It will be seen that the ratio of oxygen to CO_2 used in the process is approximately 4.81 to 1. This may be considered to be a very inefficient or wasteful use of hydrogen gas, since much lower O_2:CO_2 ratios have been observed with other hydrogen bacteria (Ruhland, 1924). In unpublished studies Doudoroff found that different strains of hydrogen bacteria showed O_2:CO_2 ratios ranging from 2:1 to 5:1 during growth. The ratio appeared to be fairly characteristic of the species or strain and in selected cases was found to be relatively independent of wide variations in the O_2:CO_2 ratio in the atmosphere.

DISCUSSION

In agreement with Clifton and Logan's observations (1938) it has been found that, under optimal conditions, at least some substrates (e.g., glucose) are assimilated almost to the same extent on the basis of carbon by growing cultures as by resting cell suspensions. Yet the anabolic end products are very different in the two cases, as is shown by the effect of nitrogen deficiency on cell composition. This suggests that the main losses of substrate carbon and the most wasteful dissipation of energy occurs in the initial stages of substrate breakdown, leading to what is commonly referred to as the "primary synthesis." It would seem that the further transformations of the reserve materials into protoplasm need not involve any further great losses of either the anabolized carbon or of energy. The discrepancies in assimilation between resting and growing cells with pyruvate and particularly with acetate as substrates would suggest, on the other hand, that the formation of protoplasm does not proceed entirely from the products of primary synthesis but requires also the participation of the substrate.

The effect of iron concentration on the amount of synthesis is by no means clear. Only with sucrose and trehalose as substrates was a deficiency of this element observed to have a marked influence on the course of metabolism. In iron-deficient media with these sugars, pyruvic acid appeared in large quantities during growth, but not in experiments with resting cells. Bernstein (1944) showed that the iron concentration in the medium, together with the developmental state of the culture, had a marked effect on the course of oxidation of dicarboxylic acids by the same organism. It is interesting that in his experiments pyruvic acid was found to accumulate only in resting cell suspensions and not in the growing cultures, and only when sufficient iron was present.

Pyruvic acid has also been shown to accumulate in the medium when *P. saccharophila* oxidizes glucose in the presence of dinitrophenol (Doudoroff, 1940). In suitable concentrations, this poison appears to inhibit assimilation without affecting materially the rate of oxygen uptake. It seems likely that the iron deficiency has a similar effect on the oxidation of sucrose and trehalose in that the later stages of oxidation, involving pyruvic acid, cannot keep pace with the production of this compound. Whether this is brought about primarily by a suppression of assimilation or by the overloading of the hemin system can only be a matter of speculation, since the two possibilities cannot at present be investigated separately. Bernstein's results can also be explained by assuming that the hemin system is affected by the availability of iron during growth, but that under the conditions of his experiment the rate of oxidation of fumarate to pyruvate and CO_2 is more dependent on iron concentration than are the further oxidations involving pyruvate. It is conceivable that, although the mechanism of "primary synthesis" is the same with disaccharides as with hexoses, some later process necessary to growth cannot quite keep up with the rapid assimilation of disaccharides. This would result in a loss of efficiency and account for the somewhat lower values for assimilation obtained with cultures growing with the disaccharides. Furthermore, with lactic and pyruvic acids which support rapid development, synthesis during growth under optimal conditions is even lower, although assimilation by resting cells is almost as great as with glucose (see table 1). The effect of iron deficiency may then be to accentuate the discrepancy between catabolic and anabolic processes. This would explain the higher iron requirements for maximum efficiency of assimilation with the disaccharides than with glucose.

SUMMARY

In a study of the assimilation with different substrates by growing cultures of *Pseudomonas saccharophila*, it has been shown that:

1. Whereas synthesis during growth with some substrates (glucose) is not greatly different from the "primary synthesis" shown by resting cell suspensions, with other substrates (lactate, pyruvate, and acetate) a rather great discrepancy appears between the results obtained in the two types of studies.

2. With glucose as substrate, moderate variation in the temperature of incubation, the composition of the atmosphere, and the pH of the medium have no appreciable effect on the efficiency of assimilation.

3. Complete removal of CO_2 decreases synthesis, as do high concentrations of oxygen. With very high partial pressures of oxygen no growth occurs, although no effect of such atmospheres can be observed on the behavior of resting cell suspensions.

4. An insufficiency of nitrogen does not materially affect the percentage of synthesis over a limited period of time, although it brings about a striking alteration in the composition of the organisms.

5. An insufficiency of iron in the medium results in a decreased synthesis, particularly striking with those substrates utilized more rapidly than glucose.

Pyruvic acid accumulates in iron-deficient media with sucrose and trehalose, but not with the other substrates studied as carbon sources.

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