2. Lineweaver, H., Burk, D., *J. Am. Chem. Soc.*, 1934, v56, 658.
3. Snedecor, G. W., *Statistical Methods*, 5th Ed., Iowa State College Press, 1959, p122.
4. Shapiro, B., *Biochem. J.*, 1947, v41, 151.
5. Lotspeich, W. D., *Metabolic Aspects of Renal Function*, Charles C Thomas, 1959, p169.
6. LeFevre, P. G., Marshall, J. K., *J. Biol. Chem.*, 1959, v234, 3022.
7. Csaky, T. Z., Fernald, G. W., *Am. J. Physiol.*, 1960, v198, 445.

Received Novmeber 14, 1961. P.S.E.B.M., 1962, v109.

Oxidative Assimilation and C^{14} Distribution in *Azotobacter agilis*. (27220)

J. M. SOBEK AND C. E. CLIFTON
Department of Medical Microbiology, Stanford University, Stanford, Calif.

Azotobacter agilis(1) has a high rate (Q_{O_2}) of oxygen consumption in the presence of a readily oxidizable substrate but exhibits low endogenous activity and relatively low efficiency of oxidative assimilation. *A. agilis* oxidatively assimilates approximately 15-20% of the glucose utilized, the remainder being oxidized to carbon dioxide and water. Values around 18% were reported for acetate and succinate and 22% for pyruvate or lactate (1). This study is concerned with both the influence of the growth substrate on assimilation and distribution of assimilated material within the cells.

Materials and methods. Cells were grown for 20 hours in Burk's medium(2) gelled with 2% agar and containing 1% glucose, acetate or succinate as the carbon and energy source. All manometric tests were carried out at 30° C using standard technics. The cell suspensions were prepared in 0.05 molar phosphate buffer of pH 7.2 containing magnesium chloride in a final concentration of 0.005 molar and were adjusted to a Klett-Summerson colorimeter reading (green filter, #42) of 300. The methods for cell fractionations and measurements of radioactivity were those described in a study on *Bacillus cereus*(3), all samples being converted to CO_2 and measured in a Dynacon (Nuclear Chicago Corp.). This procedure yields more accurate and reproducible values than barium carbonate plating and counting methods.

Results. Of the various compounds tested manometrically, acetate, pyruvate, succinate, fumarate, malate, α-ketoglutarate, ethanol and glycerol were oxidatively assimilated by *A. agilis*, generally in the range of 20-25%, by acetate-, succinate-, or glucose-grown cells. Xylose, arabinose, glutamate, glycine, glyoxylate, glycolate, and formate were not oxidized by *A. agilis*. Lag periods of the order of 30-50 minutes were observed for oxidation of most substrates (excluding growth substrate) except no lag was noted for oxidation of acetate, pyruvate and ethanol by all cells and, in the case of succinate-grown cells, fumarate and malate also were oxidized without lag. Glucose, however, was an exception. It was oxidized readily by glucose-grown cells but not by cells grown on acetate or succinate. Long lag periods, 100-200 minutes, were commonly observed and the rate of glucose oxidation in suspensions containing 4 μ-moles of glucose never approached by more than one-sixth the rate observed with glucose-grown cells. On the other hand, the rate of oxidation of succinate by acetate-grown cells approached that of succinate-grown cells after approximately a 50-minute lag period. Increasing the initial concentration of glucose 10-fold or more decreased to some extent the duration of the lag period, particularly with succinate-grown cells. Typical response of acetate- or succinate-grown cells to oxidation of glucose and of acetate-grown organisms to succinate oxidation is illustrated in Fig. 1. The behavior with glucose was confirmed with the use of uniformly labeled C^{14}-glucose. Glucose oxidation went to completion within about 40 minutes with glucose-grown cells while oxygen consumption in the presence of glucose by succinate-grown cells, corrected for endogenous respiration, amounted to 5.2% of the theoretical value for complete oxidation at the end of 1 hour, and 14.1, 41.0, and

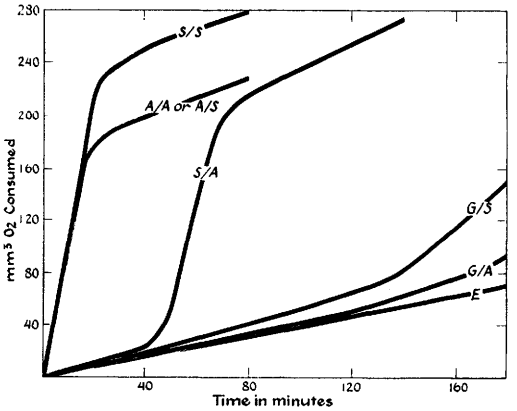

FIG. 1. Time course of oxygen consumption by suspensions of *Azotobacter agilis* utilizing acetate *(A)*, glucose *(G)*, succinate *(S)*, or endogenous reserves *(E)*. Second letter represents growth substrate.

57.0% at the end of 2, 3, and 4 hours, respectively. During these time periods corresponding values for utilization of C^{14}-glucose were 2.5, 16.5, 48.3 and 70.0% of total C^{14}.

Succinate-grown cells were disrupted in a Raytheon 10 kc oscillator and the cell-free extract was tested for oxidative ability. In 100 minutes 2 ml of the extract or extract plus 4 μmoles of glucose consumed about 300 mm^3 of oxygen while 560 mm^3 were consumed in the presence of 4 μmoles of succinate. Hence enzymes for oxidation of glucose appear to be lacking in succinate-grown cells and this suggests that adaptation to glucose involves more than the synthesis of a "permease," a finding similar to that reported(4) by Bruemmer and Wilson for succinate oxidation by *Azotobacter*. The break in rate of oxidation of succinate by the extract occurred at approximately the same percentage of the theoretical value as observed with intact cells.

Results are presented in Table I of C^{14} utilization from glucose, pyruvate and acetate labeled in various positions by glucose-grown *A. agilis*. Essentially the same results were obtained with cells grown on acetate and utilizing acetate or pyruvate. The cell/CO_2-C^{14} ratios give an estimate of the efficiency of assimilation of different carbon atoms in these substrates.

Cells from cultures grown on uniformly labeled glucose or acetate and from unlabeled cells exposed to these labeled substrates were fractionated as previousy described(3), the radioactivity of components soluble in cold 5% trichloroacetic acid (TCA), 75% ethanol of pH 2.5 at 45°C, hot 5% TCA and residue being determined. Another portion of each suspension was digested with hypochlorite(4) and then extracted with chloroform. Values for the radioactivity of the ethanol soluble fraction were subtracted from those for the radioactivity of the chloroform soluble material to give values for poly-β-hydroxybutyrate. This procedure probably gives low values for the polymer. Somewhat more poly-β-hydroxybutyrate was found in other experiments with chloroform extracts of the hot TCA insoluble residue. Little radioactivity was noted in ether extracts of the cells made after extraction with ethanol and hence ether extractions were omitted in the tests reported here. Typical C^{14}-distribution values are recorded in Table II. It is apparent that the label is distributed in various compounds, relatively little going into the storage polymer.

Discussion. Of the various substrates found to be oxidatively assimilated by *A. agilis*, utilization of glucose by cells grown in the absence of this sugar presents a very anomalous pattern. Dependent on the growth substrate, some substrates, including the one

TABLE I. Assimilation of C^{14} from Labeled Substrates by Washed, Glucose-Grown *A. agilis*.

	Cells	CO_2	Supernatant	Total	Original	Cell/CO_2-C^{14}
	μcuries × 10^{-3}					
Glucose-1-C	3.9	96.6	5.4	105.9	100.2	.04
" -2-C	19.9	74.9	15.6	110.4	108.4	.26
" -6-C	11.5	31.2	7.5	50.2	50.2	.36
" -U-C	14.0	71.4	4.5	89.9	88.1	.20
Pyruvate 1-C	1.5	26.9	2.6	31.0	31.4	.06
" 2-C	10.9	56.6	7.9	75.4	79.5	.19
" 3-C	14.8	52.4	9.2	76.4	77.3	.28
Acetate 1-C	12.9	78.1	8.0	99.0	97.1	.17
" 2-C	15.1	67.4	10.3	92.8	92.8	.22

TABLE II. Percentage Distribution of C^{14} in Fractions of *A. agilis* Grown on C^{14}-Labeled Glucose or Acetate and by Washed Suspensions Utilizing These Substances.

	Cold TCA soluble	Alcohol soluble	Hot TCA soluble	Residue	Chloroform soluble*
Glucose culture	5	8	8	79	6
G† *vs* glucose	20	7	11	62	4
G† *vs* acetate	11	6	6	77	12
Acetate culture	4	4	7	85	20
A† *vs* acetate	19	9	15	57	3

* Chloroform soluble material is included in values for the residue fraction.
† Washed suspensions of glucose (G)- or acetate (A)-grown cells.

on which the cells were grown, are oxidized without lag while others are oxidized only after a normal lag period. When the cells are grown on acetate or succinate and glucose is added to washed suspensions there is a much longer lag period, confirmed both by manometric and radioactivity measurements, than with the other substrates and maximum rate of oxidation never approaches that observed with glucose-grown cells. This behavior would not be predicted since glucose is regarded commonly as a universal fuel. Increasing the concentration of glucose many-fold may reduce the lag period and increase the rate of oxygen uptake to a limited extent. No satisfactory explanation has been obtained for this observed behavior but observations with sonicates indicate that glucose adaptation is not dependent only upon formation of a permease. The same lag behavior is also noted with acetate-grown cells to which acetate plus glucose are added. Acetate is utilized in the normal manner and then a very long lag period is noted before glucose oxidation is apparent.

The more accurate results (Table I) obtained on utilization of variously labeled glucoses confirm the earlier report(1) on the preferential assimilation of the glucose-2-C over the 1-C and are extended to include the assimilation of the glucose-6-C which is assimilated to a greater extent than the 2-C atom, as indicated by cell/CO_2-C ratios. It appears that both the Embden-Meyerhof and pentose pathways of oxidation are operative in *A. agilis*. Most of the carboxyl-C of pyruvate is converted to CO_2, assimilation of the 3-C of pyruvate occurring to a somewhat greater extent than of the 2-C. Likewise the methyl-C of acetate is assimilated to a somewhat greater extent than is the carboxyl-C.

The pattern of incorporation of C^{14} from glucose or acetate in growing cells or in washed suspensions is essentially the same, somewhat higher values being noted for the cold TCA soluble fraction of suspension cells as contrasted with culture cells. Probably this represents a more active metabolic pool(s) in the resting cell suspension. Relatively low percentages of the assimilated carbon are found in the poly-β-hydroxybutyrate reserves of the cell. The bulk of the assimilated carbon in either case is found in the hot TCA residue. This consists for the most part of proteinaceous matter and some cell wall material, and since washed cell suspensions do not fix nitrogen it may represent in part the result of turn-over reactions. The general pattern of assimilation is similar to that noted with other organisms.

Summary. *Azotobacter agilis* oxidatively assimilates various substrates, generally to an extent of 20-25%. Most of the C-1 carbon of both glucose and pyruvate is given off as CO_2, assimilation to slightly different extents occurring from the other carbons and from acetate carbons. A lag period, probably involving formation of inducible enzymes, is noted with some substrates and is dependent upon the nature of the growth substrate. This lag is very marked for utilization of glucose by acetate- or succinate-grown cells, the adapted cells are not highly active, and adaptation is dependent upon more than the induced formation of a permease. The assimilated carbon, in resting or growing cells, is found to the greatest extent in materials insoluble in hot TCA, smaller amounts being present in cellular components soluble in cold and hot TCA and ethanol.

1. Johnson, E. J., Sobek, J. M., Clifton, C. E., *J. Bact.*, 1958, v76, 658.

2. Wilson, P. W., Knight, S. C., *Experiments in Bacterial Physiology*, 1952, Burgess Publishing Co.

3. Clifton, C. E., Sobek, J. M., *J. Bact.*, 1961, v82, 252.

4. Bruemmer, J. H., Wilson, P. W., *Bact. Proc.*, 1961, p93, 189.

Received September 13, 1961. P.S.E.B.M., 1962, v109.

Separation of Leucylaminopeptidase Components in Normal Human Sera by DEAE Cellulose Column Chromatography. (27221)

FRANCIS J. BEHAL, CHESTER B. KANAVAGE AND RUTH D. HAMILTON
(Introduced by W. K. Hall)
Department of Biochemistry, Medical College of Georgia, Augusta

As a part of a general program to study the significance of differences of various enzyme activities in serum, a systematic study of leucylaminopeptidase (LAP) in human tissues has been initiated. In the literature there are conflicting opinions about the meaning of altered serum leucylaminopeptidase levels (SLAP). Rutenburg, Pineda, *et al.*(1,2) have proposed that elevated SLAP levels have considerable significance in the diagnosis of carcinoma of the head of the pancreas, whereas Shay *et al.* and Winsten(3,4) maintain that elevated SLAP levels are of little value in this connection. Kowlessar *et al.*(5) and Rutenburg *et al.*(6) have reported the appearance of second and third SLAP components in addition to the "normal component" in patients with hepatobiliary disease. This report will deal specifically with our studies of these SLAP components.

Materials and methods. LAP activity was determined by a modification of the method of Goldbarg(7). The unit of LAP activity is defined as that amount of enzyme that will liberate one microgram of beta naphthylamine from the substrate, L-leucyl-beta-naphthamide, in one hour at 37°C.

Serum samples were fractionated by means of DEAE Cellulose (DEAE Cel) chromatography. Prior to application to the column the samples, 2.0 ml of serum, were dialyzed at 4°C against 100 volumes of 0.005 M phosphate buffer at pH 8.6. DEAE Cel, Type 40, was obtained from Carl Schleicher & Schuell Co. This adsorbant was equilibrated with the above buffer before use. The columns used were 11 mm in diameter and 40 cm long. The column was then eluted at 4°C by means of a gradient elution device that provided a decrease in pH from 8.6 to 4.0 of a 0.005 M phosphate buffer and an increase in sodium chloride concentration from zero to 0.18 M in the course of the delivery of 400 ml of phosphate-sodium chloride solution to the column. Fraction volume was 6.0 ml.

Results. In our laboratories, we have subjected the sera of 22 normal adult persons to DEAE Cel chromatography. It has been found that in the serum of all such individuals, there were 4 distinct SLAP components. In Fig. 1, the elution patterns for a representative normal adult subject and a person with severe obstructive jaundice are presented. The components have been arbitrarily identified in the order of elution, and are defined as follows: α_1 LAP, activity in fractions 25-39; β_1 LAP, activity in fractions 40-50; β_2 LAP, activity in fractions 51-58; and β_3 LAP, activity in fractions 59-74.

There is some variation in amount of this enzyme activity in each of the SLAP components in sera of normal subjects. The most constant component is the first one to be eluted, namely, α_1-LAP, which has always been found to account for a large proportion of total SLAP activity. In Table I, mean and extreme concentrations for each of the SLAP components in normal sera are listed.

The elution pattern (Fig. 1) of a person with obstructive jaundice is typical of 7 cases of elevated SLAP screened so far. These include carcinoma of the pancreas, cholelithia-