BBA 12137

STUDIES WITH A CONSTITUTIVE ALDOSE DEHYDROGENASE IN *PSEUDOMONAS FRAGI*

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(Received June 25th, 1962)

SUMMARY

Pseudomonas fragi contains a constitutive, particulate aldose dehydrogenase. The enzyme oxidizes its sugar substrates to the γ-lactone of the corresponding aldonic acids. An investigation of properties of the enzyme showed that all substrates are oxidized by a single enzyme.

Though the enzyme is constitutive, growth did not seem to be correlated with oxidation of the sugar but rather with metabolism of the product of oxidation, *i.e.* the sugar acid. The enzymes oxidizing the sugar acids and D-arabinose have different properties from those of the aldose dehydrogenase. These enzymes are soluble, are formed by induction and are highly specific for their individual substrates.

INTRODUCTION

Pseudomonas fragi is an organism capable of growing on a variety of carbohydrates including the four common pentoses[1]. In a study of the mechanism by which this organism degrades the pentoses, it was found that the sugars were all oxidized via similar pathways which converged in forming α-ketoglutarate. However, some differences were found in the enzymic response of the organism to the various pentoses. The entire enzyme system for oxidizing D-arabinose was in the soluble fraction of sonic extracts of cells grown on D-arabinose. On the other hand, the first step for oxidizing L-arabinose, D-xylose and D-ribose was catalyzed by the particulate fraction of extracts while the enzymes for the remaining steps were present in the soluble fraction.

This study is concerned with the physiological conditions under which the various enzyme systems are formed, and with some of the properties of the particulate pentose dehydrogenases.

Abbreviation: PMS, phenazine methosulfate.

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EXPERIMENTAL

P. fragi NRRL B-25 was grown, harvested and suspended as resting cells or extracted into cell-free preparations as previously described[1].

Sugars were determined by the method of SCHALES AND SCHALES[2], lactone as hydroxamic acid[3] and aldonic acid as lactone after heating in acid solution[1]. Oxygen consumption was measured by standard Warburg manometry. Pyridine nucleotide reduction was assayed on a Beckman* DU spectrophotometer at 340 mμ, dichlorophenol-indophenol reduction was followed at 600 mμ, cytochrome *c* at 550 mμ and potassium ferricyanide at 440 mμ.

L-galactose was a gift of Dr. W. Z. HASSID. Phenazine methosulfate was supplied by Dr. J. W. NEWTON. All other chemicals were prepared or obtained as reported previously[1].

RESULTS

Relationship of sugar utilization to growth

Cells of *P. fragi* grown on a medium of 0.1% tryptone, 0.1% yeast extract and 0.5% glucose, were inoculated into a synthetic medium containing one of the pentoses as the sole carbon source. For reasons that will be discussed later, the same medium with either glucose or galactose as sole carbon source was also inoculated. The results (Fig. 1) show that only in the case of D-arabinose was there any appreciable lag in growth and in sugar consumption. With the other sugars, oxidation of the substrate seemed to begin immediately upon inoculation. Almost all the sugar was consumed before much growth took place. Growth seemed to be better correlated with lactone disappearance. The results with galactose are especially interesting in that, even though the organism could oxidize the substrate, no growth of the organism occurred on this substrate.

In culture media with L-arabinose, xylose or ribose as substrates, it is the corresponding lactone rather than the free acid that accumulates. The reverse is true when the cells are growing on D-arabinose. Undoubtedly this accumulation of lactone is due to the absence of any lactonases in cells grown on the first three substrates, whereas the cells growing on D-arabinose possess an enzyme that will hydrolyze D-arabono-γ-lactone[1]. Cells to which galactose has been supplied as substrate accumulate galactono-γ-lactone in the culture fluid. Since the organism uses neither the lactone nor galactonic acid for growth, the acid accumulates in the medium as the lactone slowly hydrolyzes spontaneously. With glucose as substrate, little lactone or aldonic acid accumulates and then for only a short time. Cells grown on glucose contain a lactonase[4], but it is specific for glucono-δ-lactone. The results with growth on glucose are not strictly comparable to those obtained with the other substrates since the inoculum had been grown on a medium containing glucose.

Enzymic patterns of cell-free extracts of P. fragi grown on various substrates

L-Arabinose, xylose, ribose and glucose: The soluble fractions from cells grown on these substrates were devoid of dehydrogenase activity on any of the compounds.

* Beckman Instruments, Inc., Palo Alto, California. Products mentioned are not endorsed by the U. S. Department of Agriculture over other products of the same quality.

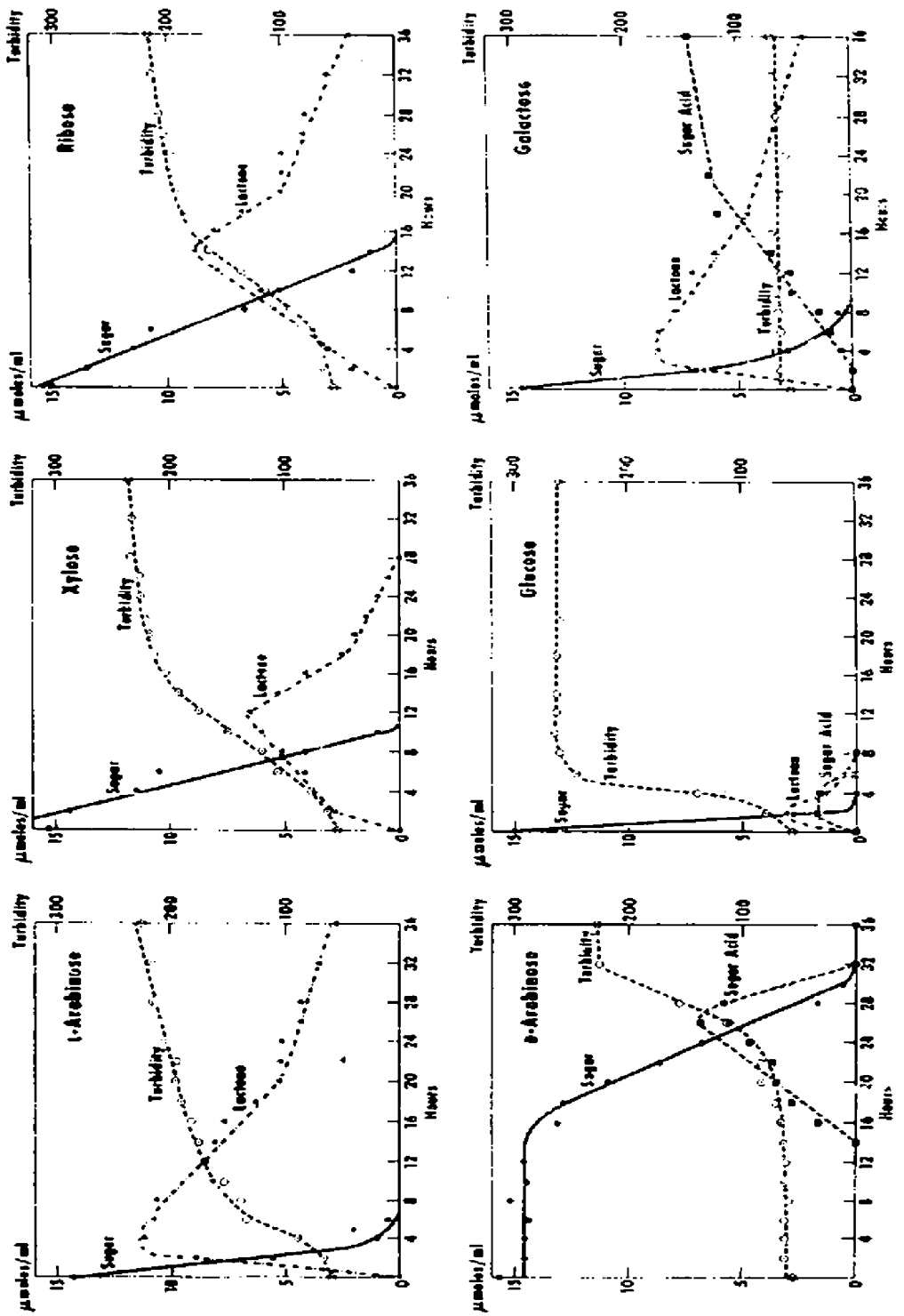

Fig. 1. Growth of *P. fragi* on a series of substrates. Media (100 ml) were inoculated with 20 ml of a rich culture of the organism grown on 0.1% tryptone, 0.1% yeast extract and 0.5% glucose. The cultures were incubated aerobically at 28°. At intervals, 5 ml samples were removed for turbidity measurements and chemical analyses.

Oxidative ability was found only in the particulate fractions. These particles showed a low degree of specificity in that they were capable of catalyzing the oxidation of a number of sugars. As an example, the results with the particulate fraction from cells grown on L-arabinose are presented in Table I. This same enzymic pattern was observed with all particulate fractions of *P. fragi* regardless of the substrate upon

TABLE I

RELATIVE RATES OF OXIDATION OF VARIOUS SUGARS BY WASHED PARTICLES OF *Pseudomonas fragi* GROWN ON L-ARABINOSE

In the main compartment of a Warburg flask were placed 0.3 ml crude particles (0.8 mg N/ml), 0.1 ml 0.05 M $MgSO_4$ and 1.6 ml 0.1 M phosphate (pH 6). The sugars, at a concentration of 1.0 M, were added to the enzyme from the vessel's side arms in a volume of 0.1 ml. The center well contained 0.2 ml 20% KOH.

Sugar	$\mu l\ O_2/h$	Sugar	$\mu l\ O_2/h$
Glucose	264	Trehalose	0
Galactose	264	D-Arabinose	0
L-Arabinose	264	D-Lyxose	0
Xylose	178	L-Rhamnose	0
Mannose	165	Maltose	0
Glucosamine	108	Sucrose	0
Potassium gluconate	102	Potassium L-arabonate	0
Ribose	35	Potassium D-arabonate	0
Lactose	29	Potassium galactonate	0
Cellobiose	18	Ammoniumxylonate	0
L-Fucose	0	Potassium ribonate	0

which the organism was grown. Also, the relative rates at which the sugars were oxidized were the same in all cases. This same pattern occurred in particles from cells grown on D-arabinose (which is not a substrate for the enzyme) and even cells grown in a medium composed only of 0.5% yeast extract plus 0.5% tryptone. Thus, the enzyme system catalyzing the oxidation of L-arabinose, xylose, ribose and glucose can be considered to be an aldose dehydrogenase that is constitutive in *P. fragi*.

On the other hand, the soluble fraction from these cells contained the enzyme systems for oxidizing the pentonic acids to α-ketoglutarate[1]. Here there is some relationship between the growth substrate and enzymic activity. Cells grown on L-arabinose and xylose contained a specific oxidizing system for the corresponding sugar acid (Fig. 2A, B). As reported[1], no system for oxidizing ribonate can be found in cell-free extracts from cells grown on ribose; however, the extracts from these cells can oxidize xylonate (Fig. 2C). The relationship of xylonate oxidation to ribose metabolism in ribose-grown cells has not been investigated. Cells grown either on glucose or in a medium lacking any carbohydrate source contain none of these sugar acid-oxidizing enzyme systems in the soluble portion of cell-free extracts (Fig. 2E, F).

D-Arabinose. The soluble fraction from cells grown on this substrate contains enzymes for oxidizing D-arabinose and D-arabonate. As mentioned above, the particulate fraction is inactive on D-arabinose, although it can catalyze the oxidation of the other pentoses. D-Arabinose dehydrogenase is an inducible enzyme because it

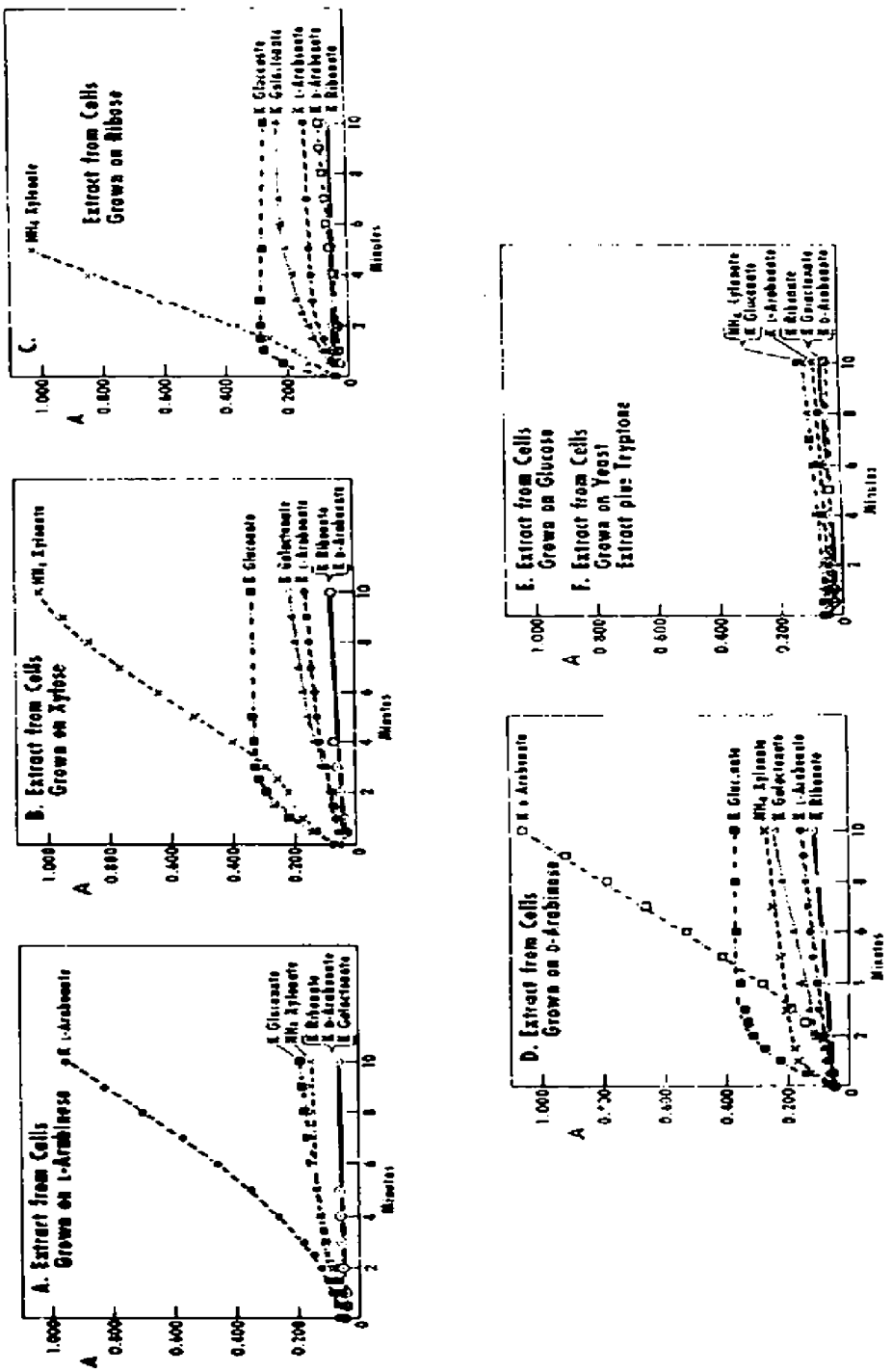

Fig. 2. Oxidation of aldonic acids by extracts of *P. fragi* grown on various substrates. To a reaction mixture composed of 0.1 ml of enzyme, 0.05 ml of 0.05 M $MgSO_4$, 0.1 ml $5 \cdot 10^{-3}$ M of TPN and 0.8 ml of 0.1 M Tris (pH 8.0) in a cuvette was added 0.1 ml 0.1 M of substrate. Change in absorbancy was followed at 340 mμ.

is absent in cells grown on other substrates. Like D-arabinose, D-arabonate is oxidized only by cells and extracts of cells grown on D-arabinose, and the enzyme system is specific for this aldonic acid (Fig. 2D). The only other compounds, besides D-arabinose and D-arabonate, that can be oxidized by crude extracts under the same conditions are L-galactose and glucose 6-phosphate.

Properties of the particulate aldose dehydrogenase

Hydrogen acceptors: Crude particles are capable of coupling the oxidation of the sugars to oxygen. Other reagents that can act as hydrogen acceptors with these particles are 2,6-dichlorophenol-indophenol, cytochrome *c* and PMS. Potassium ferricyanide was only slowly reduced under the same conditions. Triphenyl tetrazolium chloride, methylene blue, FAD, FMN, DPN and TPN were inactive either as hydrogen acceptors or as stimulators of the rate of oxygen uptake.

Inhibitors: EDTA inhibited the reaction, demonstrating a metal requirement. Mg^{2+} stimulated the reaction, but not if added after the particles had been treated with EDTA. Other cations, such as Mn^{2+}, Ca^{2+} or Co^{2+}, had no stimulatory action.

Cyanide at a concentration of $1 \cdot 10^{-3}$ M was another effective inhibitor. This inhibition by cyanide suggested that the oxidation is cytochrome linked. Under anaerobic conditions or in the presence of cyanide, it can be shown that cytochromes are present in the particles and reduced upon addition of substrate.

Iodoacetate, floride, arsenate, arsenite, 2,4-dinitrophenol, Na_2S, cysteine and glutathione are without effect on the oxidation. However, BAL at concentrations of $1 \cdot 10^{-3}$ M will inhibit the reaction. The inhibition by BAL, more than likely, is due to its metal chelating properties.

Product of oxidation: The crude particles from all sources oxidized L-arabinose, xylose, ribose and galactose at pH 6.0 with the consumption of 0.5 μmole O_2/μmole substrate. When the oxidation was inhibited with cyanide, PMS could be used to couple the reaction to oxygen, and the amount of oxygen consumed was doubled. A stable lactone accumulated in the reaction mixtures in quantitative yields. The products were identified by chromatography as the γ-lactones of the corresponding aldonic acids.

With glucose as substrate, an unstable lactone was detected in the reaction mixture along with gluconic acid. The yields were never stoichiometric, probably because the particles also contained a gluconate dehydrogenase. By following the rate of hydrolysis of the gluconolactone[4], it, too, was characterized as the γ-isomer. Apparently all the sugars are oxidized in the furanose form.

Extraction of particles: Several solvents and enzymes "solubilize" enzymes in particles from other organisms. Application of these reagents to *P. fragi* particles in an attempt to solubilize part or all the oxidative ability in the particles has been consistently unsuccessful. Either the reagent left all activity associated with the insoluble residue, or else it destroyed activity toward all substrates. Reagents which did not "dissolve" the enzyme upon extraction were: Various lipases, ribonuclease, deoxyribonuclease, sodium perfluorooctanoate, sodium deoxycholate at pH 6.0 and butanol-1. Reagents which destroyed activity were: Trypsin, pepsin, lysozyme plus EDTA, acetone and sodium deoxycholate at pH 7.5.

The effect of extraction of crude particles with butanol-1 was investigated in

detail. A 0.1-volume of cold butanol was added to a suspension of particles in 3 mM phosphate (pH 6.8). The mixture was stirred in the cold for 1 h. At the end of this time, the particles were removed by centrifuging 20 min at 25 000 × *g*, washed twice in 3 mM phosphate (pH 6.8) and finally suspended in the original volume in this buffer.

The extraction removed approx. 33% of the nitrogen. When specific activity was measured by various assay procedures, the particles could no longer utilize either O_2 or cytochrome *c* as hydrogen acceptors, and the rate of reduction of 2,6-dichlorophenol-indophenol was reduced 50–80%; however, the specific activity as measured by O_2 uptake, with PMS as the electron carrier, was only slightly affected (Fig. 3).

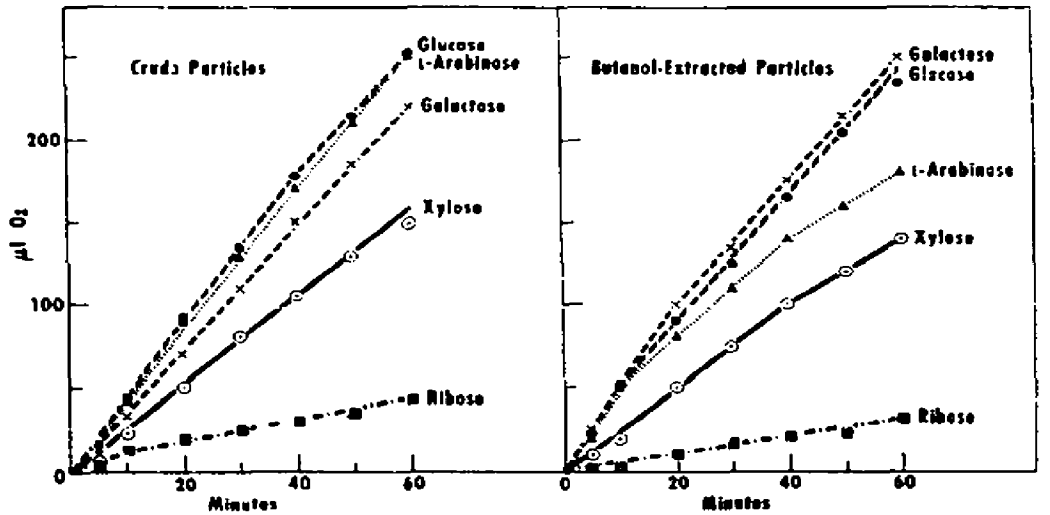

Fig. 3. Oxidation of sugars by crude and butanol-extracted particles from *P. fragi* grown on xylose. Either 0.2 ml of crude particles (0.9 mg N/ml) or 0.2 ml butanol-extracted particles (0.6 mg N/ml) were mixed in the main compartment of a Warburg flask with 0.1 ml 0.05 M $MgSO_4$, 0.1 ml 1% PMS, and 2.0 ml 0.1 M phosphate (pH 6.0). To those flasks containing the crude particles was added 0.1 ml 0.1 M KCN. In the side arms was placed 0.1 ml 1.0 M sugar. The center well contained 0.2 ml 20% KOH.

The relative ability of the particles to oxidize the various sugars remained the same as that of the crude particles, except that the extracted particles were now unable to oxidize potassium gluconate.

pH Optimum: The pH optimum was measured with particles poisoned with cyanide to which PMS was added to couple the reaction to oxygen. With all substrates, the maximum rate of oxygen uptake occurred at approximately pH 6.

Partial and total heat inactivation: Heating butanol-extracted particles to 50° for 5 min had no effect on the rate of oxidation of the various substrates (Table II). Increasing the temperature to 60° resulted in a 70% loss of activity. Temperatures of 70° for 5 min or 80° for 1 min completely destroyed enzymatic activity in the particles toward all substrates.

Simultaneous addition of two substrates: Butanol-extracted particles were used to minimize the number of steps in the electron-transport system. PMS was used to

TABLE II

HEAT INACTIVATION OF PARTICULATE ALDOSE DEHYDROGENASE FROM *Pseudomonas fragi* GROWN ON L-ARABINOSE

Butanol-extracted particles were heated for 5 min at the temperatures indicated. Samples were removed and assayed for activity at 30°. With glucose, galactose and L-arabinose as substrates, 0.2-ml samples of enzyme were used; with xylose, 0.3 ml; with ribose, 0.6 ml. The enzyme was mixed in a Warburg flask with 0.1 ml 0.05 M $MgSO_4$, 0.1 ml 1% PMS and enough 0.1 M phosphate (pH 6.0) to bring the volume to 2.5 ml. A 0.1-ml volume of 1.0 M substrate was added from the sidearm and 0.2 ml 20% KOH was placed in the center well.

Substrate	Temperature			
	30° $\mu l\ O_2/h$	50° $\mu l\ O_2/h$	60° $\mu l\ O_2/h$	70° $\mu l\ O_2/h$
Glucose	287	305	93	5
Galactose	300	292	89	0
L-Arabinose	298	325	98	0
Xylose	195	195	52	0
Ribose	103	98	5	0

couple the reaction to oxygen. Under conditions where the oxidative enzyme would be saturated with glucose, if it were added as the only substrate, and PMS was present in excess, the rate of oxygen uptake was not increased when either L-arabinose or galactose was added to the particles simultaneously with the glucose (Fig. 4). That

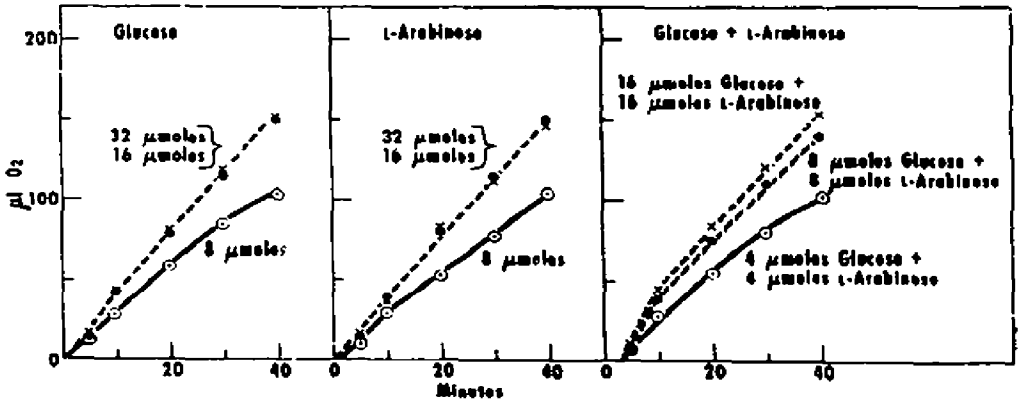

Fig. 4. Simultaneous oxidation of glucose and L-arabinose by butanol-extracted particles from *P. fragi* grown on glucose. A volume of 0.1 ml of particles (0.72 mg N/ml) was added to the main compartment of a Warburg vessel along with 0.1 ml 0.05 M $MgSO_4$, 0.2 ml 1% PMS and 2.0 ml 0.1 M phosphate (pH 6.0). Substrate was placed in the sidearm in amounts listed below. The center well contained 0.2 ml 20% KOH.

this is not due to competitive inhibition could be shown by adding less than saturating amounts of the two substrates. Under these conditions, the rate of O_2 uptake was strictly additive.

These results strongly suggest that a single aldose dehydrogenase with a wide specificity range is present in these particles.

DISCUSSION

The oxidation of glucose by particles in extracts of several bacterial organisms has been described[5-11]. In studying the specificity of the oxidation, it was frequently reported that these particles could oxidize other sugars also. However, there has never been any evidence to show that the activity on these various substrates was due to one or several enzymes. In *P. fragi* the evidence is fairly good that a single enzyme is responsible for the oxidation of these various sugars. The essential structure of the sugar in order to be a substrate for the enzyme is that the hydroxyl group on carbon 2 be in the same configuration as it exists in glucose. Also, because the product of the oxidation is the γ-lactone of the sugar acid, likely, the furanose form of the sugar is oxidized.

There are many enzymes in bacteria associated with the particulate fractions of cell-free extracts. Varying degrees of success have been obtained in attempts to dissolve these enzymes. A recent interesting example is the solubilization by EDTA of D-allohydroxyproline dehydrogenase induced in *Pseudomonas striata*[16]. In an extensive study of glucose and gluconate dehydrogenases in *P. fluorescens*, HERTLEIN[17] succeeded in solubilizing the gluconate dehydrogenase system but was unable to dissociate glucose oxidation from the particles. HAUGE[18], on the other hand, found a soluble glucose dehydrogenase in extracts of *Bacillus anitratum* with properties similar to those reported for particulate enzymes. These cells also contain a typical particle-bound glucose dehydrogenase. The apparent inability to solubilize the particulate enzyme system in *P. fragi* may be due to the fact that it is a multistep reaction. Not only are there several steps following the oxidation which are involved in electron transport, but possibly there are one or more enzymic steps preceding oxidation. For instance, since sugars exist in the pyranose form in aqueous solution[12,13] but the oxidative enzyme only oxidizes those in the furanose form, there may be an enzyme catalyzing a pyranose furanose interconversion, just as there is a mutarotase that interconverts α- and β-pyranose forms of glucose[14,15]. If only part of this multi-enzyme complex were dissolved during an extraction procedure, the assay system would not function and it would appear as if all activity had been lost.

The enzyme system oxidizing L-arabinose, xylose, ribose, glucose and galactose is a constitutive one. The enzyme system is present regardless of the substrate upon which the organism is grown. It is present in the particles even when the organism is grown in a medium lacking any of the enzyme's substrates. Further evidence for the constitutive property of this enzyme is the absence of any lag in oxidation when the organism is introduced into a medium containing the sugar. D-Arabinose, on the other hand, is metabolized by an inducible dehydrogenase system.

Although the oxidation of L-arabinose, xylose, ribose and glucose is catalyzed by a constitutive system, there is a 2- to 4-h lag before growth begins when these compounds are supplied to the cell as substrates. Since cellular cytochromes are reduced, presumably the energy from the sugar oxidation is available to the cell, but the cell is unable to use it. Growth is delayed, perhaps, because no carbon material is available as yet for conversion to cell material. The lag in growth undoubtedly represents the period required for induction of the enzymes responsible for the subsequent steps in the oxidation. Until this happens, the energy obtained from sugar oxidation cannot accrue to the cell. The metabolism of galactose is an extreme

example of this. *P. fragi* is unable to produce enzymes capable of oxidizing galactonic acid. This acid therefore accumulates quantitatively in the culture medium from galactose and none of the energy from the oxidation is used for assimilation and growth.

This phenomon of substrate oxidation without concomitant growth has been observed in other obligately aerobic microorganisms[7,19]. The question arises as to what is the function of these enzymes. The particulate and constitutive nature of these dehydrogenases suggests that they are an essential component of the cell membrane. In this locus, they serve the purpose of oxidizing the sugars to another compound, the aldonic acid, toward which the organism, at least in *P. fragi*, may be induced and, consequently, may use as a substrate for growth. If, as in the extreme case, the organism is unable to use the aldonic acid in this way, then the enzyme is apparently functionless from a metabolic viewpoint.

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