UTILIZATION OF L(-)-GLUCOSE BY NATURALLY OCCURRING MICROORGANISMS

by

ROBERT C.J. FEWKES

B. Ch.E., University of Delaware
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Signature of Author..... Signature redacted .....
Department of Nutrition and Food Science
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Certified by..... Signature redacted .....
Thesis Supervisor

Accepted by..... Signature redacted .....
Chairman, Departmental Committee
on Graduate Students

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ABSTRACT

Carbon recycle by means of physicochemically synthesized carbohydrates has been proposed. These artificial sugars can be used to generate single cell protein. However, it is not known what effects the unnatural components will have on the yield, productivity, and metabolic regulation of the organisms used.

We have obtained from natural populations, a number of organisms which utilize L-glucose as sole carbon source. Of the twelve organisms isolated, five are gram-negative aerobic rods, one is a gram positive coccus, two are thermophilic bacilli, three are yeasts, and one is a mycelial form. Preliminary taxonomy was done on these organisms.

When fully adapted to growth on L-glucose, one pseudomonad grows exponentially with a doubling time of 14 to 16 hours with 5 g/L L-glucose in the medium. Cell yields are about 0.46 g dry cells/g L-glucose, and cell densities as high as 2.8 g/L have been acheived in shake flasks. The apparent maximum growth rate is 0.0506 $hr.^{-1}$ and the apparent overall K_m for growth is 0.14 g/L L-glucose. However, substrate inhibition sets in at about 4.5 g/L L-glucose.

L-glucose transport takes place by facilitated diffusion at V_{max} = 2.63×10^{-3} mg L-glucose/(mg cells-min) and K_m = 0.65 g/L L-glucose.

The organism probably utilizes the entire L-glucose molecule. There is evidence that carbon 1 is eliminated as CO_2 and subsequently reassimilated from the medium. One or more growth factors appear to be necessary for L-glucose utilization. They are made by the organism under good growth conditions and one appears to be excreted into the medium.

A hypothetical mechanism of L-glucose utilization consistent with the growth kinetics is proposed. This mechanism involves a catabolic sequence with at least two limiting reactions. The first is incipient transport limitation and the second is inhibition by an intracellular metabolite derived from L-glucose.

Thesis Supervisor: Dr. Anthony J. Sinskey
Title: Associate Professor of Applied Microbiology

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I. INTRODUCTION

A. Requirements for Waste Recycle and Food Resources

The dense populations and high per capita materials consumption of modern industrial societies are combining to make human wastes a major problem. Mere disposal of these wastes is no longer acceptable since, as the biosphere deteriorates under the increasing load, overall "quality of life" is noticeable lowered. Moreover, people are subjected to harmful concentrations of primary and secondary pollutants. Thus, there are strong pressures for waste recycle beyond that currently being used, and it is likely that these pressures will increase.

Simultaneously, an appreciable portion of the world's population suffers malnutrition because protein is not available in sufficient quantity and/or at reasonable prices. This condition too, is expected to worsen, especially in countries with expanding populations and poor agricultural potential. In addition to the spreading of current protein shortages, a worldwide shortage of edible carbohydrates has been predicted (President's Science Advisory Committee, 1967).

Similar pressures of waste accumulation and limited stores of nutritional food threaten man's health in the microcosm of the space vehicle. Consequently, aerospace technology has a strong interest in the technology of carbon and nitrogen recycle, and nutrient generation similar to those which would benefit mankind in the near future.

The problems encountered with relatively inert wastes such as paper and metal scrap are serious, but ecological deterioration is largely a result of biologically active carbon and nitrogen compounds. It is this class of wastes which has the strongest immediate potential for fruitful

recycle as human nutrients. Various methods of carbon and nitrogen waste recycle have been proposed, ranging in practicality from chemical reduction followed by electrical spark synthesis of amino acids, then thermal polymerization to proteins (Shapira, 1969), to hog or chicken feed formulations and the manufacture of fertilizer from sewage. The latter methods are quite practical, economical, and currently in use, but they generally require an agricultural base. Aquaculture[1] and algal production are somewhat more compact, utilize presently unexploited (perhaps) areas, are presently being piloted, and are undoubtedly practical where applicable. The greatest potential for protein productivity per unit volume, however, lies with the unicellular nonphotosynthetic bacteria and yeasts dubbed single cell protein by Mateles and Tannenbaum (1968). Many of these organisms can double their mass in less than two hours, utilizing a wide variety of simple carbon and nitrogen sources, and yield up to 30 g/L dry solids containing 60 to 80% protein.

B. Formose Sugars

One apparently practical means of carbon recycle via the reduction of carbon dioxide to formaldehyde is the base-catalyzed condensation of formaldehyde to yield "formose" sugars. A.M. von Butlerow first reported the discovery of formose in 1861, and it has received sporadic attention up to the present. Now, under the pressure of aerospace technology's requirement for material recycle (Shapira, 1969[b]), the current world shortage of protein (Bhattachargee, 1970), and the projected world shortage of carbohydrates (President's Science Advisory Committee, 1967), the technical

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[1] Intensive, mechanized culture of oyster, clams, mussels, crabs, lobsters, etc. on sewage effluents (Ryther, 1971; Gordon, 1972).

aspects of producing formose sugars are being studied (Mayer and Jannasch, 1960; Shapira, 1969[b]; Weiss and Shapira, 1970; Mizuno et al, (1970).

Formose results from a purely chemical reaction and the carbohydrates formed lack the configurational specificity of those produced in biological systems. Thus, it is a mixture of isomeric and racemic carbohydrates of two to seven carbon atoms, only a portion of which commonly occur in nature. One analysis[2] of a formose mixture by Akerlof and Mitchell (1964) is shown in Table I. Note that, since each of these samples has racemic forms distinguishable by enymatic mechanisms or by optical rotation, about 50% of the sugars identified by Akerloff and Mitchell would not be utilizable in normal biological systems. The 16.8 wt.% glucose, for example, is actually 8.4 wt.% each of D-glucose (natural) and L-glucose (unnatural). D-glucose is a ubiquitous carbon source since it is the most common hexose produced in nature, and is assimilated by most plants, animals, and microorganisms. The utilization of its mirror image raceme, L-glucose, as energy or carbon source in any biological system has never been reported to our knowledge.

Any of the straight chain and methyl branched[3] carbohydrates of formula $(CH_2O)_{2-7}$ are possible products of the formose reaction. Of the twenty-four straight chain aldo- and ketohexoses, only D-galactose, D-mannose, D-fructose, and D-glucose are generally considered to be edible by man.

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2 Weiss et al (1970) have reviewed formose analyses exhaustively up to 1970, and Mizuno et al (1971) have analyzed more recent samples.

3 Dendroketose was reported in a formose mixture by Katzscmann (1944).

TABLE I

COMPOSITION OF A FORMOSE SUGAR

Percentage by Weight	Identity
0.4	Glycolic aldehyde
1.1	Glyceraldehyde
1.8	Dihydroxyacetone
3.2	Erythrose, Erythrulose
2.5	Xylulose
6.5	Ribose
17.2	Xylose
16.5	Fructose, Mannose
17.5	Sorbose, Arabinose
16.8	Glucose
8.5	Galactose
4.4	unidentified
Less than 2.0	A Heptulose

Reprinted from Akerloff & Mitchell (1964).

C. Proposed Utilization of Formose

Shapira (1970) proposes isolation of the utilizable carbohydrates for human diets by the use of stabilized immobilized enzymes. He does not specify the system. Weiss and Shapira (1970) propose the use of "chromatographic techniques" to separate the sugar species and estimate the cost of formose, 15 to 30% of which is metabolizable, at three to five cents/lb. The non-metabolizable species are to be recycled to the formaldehyde condensation for equilibrium suppression of the non-metabolizable forms. To achieve high capacity in the large scale separation, only homologous groups are to be resolved. The authors cite Abcor's proposed chromatographic separation of xylene isomers (Timmins et al, 1969) in support of this separation scheme.

Production of edible sugars by chromatographic separation and recycle of the nonedibles is not economical or adaptable to light, portable equipment at the present level of technology. The homologous groups (aldohexoses, for instance) consist of several isomeric and racemic forms and there is no reason to expect "edible" and "inedible" fractions. Furthermore, sugars lack the very different intramolecular functional groups which are the basis of the racemic chromatographic separations reported to date (Buss and Vermeulen, 1968). Comparison with the relatively easily separated xylene isomers is not apt. Even if the inedible racemes of the edible sugars can be separated and recycled to the formaldehyde condensation reactor, they will perform exactly like the edible ones in these reactions. The result should be accumulation of inedible sugars in the closed system and a low level of edible hexose production.

Granting the feasibility of enzymic separation it is likely to be

expensive, and recycle of the inedible sugars would lead to the same accumulation problem.

Thus, if microorganisms capable of converting the edible sugars to edible protein, fat, and carbohydrate can be obtained, fermentation would be a practical and more economical means of utilizing the formose sugars. There is good reason to believe that such organisms can be obtained (see Section III, Literature Review of Rare Sugar Utilization).

D. Microbial Utilization of Formose

Some work has already been reported on direct microbial utilization of formose. Finn and Edwards (1967) observed a fifteen hour lag and growth at 0.35 $hr.^{-1}$ for Candida (Torula) utilis on formose. The lag was eliminated by using formose adapted inoculum. Cell yield was about 50% of that on D-glucose on an available carbon basis. Yeast extract (1 g/L) in their medium contributed a background growth of about 6% of the growth on D-glucose. Ewart (1925) reported growth of mixed populations on formose, but found substantial amounts of pentoses unutilized after 10 months (!) incubation. Akerlof and Mitchell (1964) reported only 4.5% of the growth from D-glucose with Saccharomyces cerevisiae. Without further detail, Shapira (1969[b]) reported growth of Saccharomyces carlsbergensis on formose from a continuous reactor at about 20-25% of that on D-glucose. He concluded from this that about 50% of the mixture was D-glucose, D-fructose, and D-mannose (!). Chermside et al (1971) reported on six yeast species, that Rhodotorula pilimanae gave "good utilization", and Saccharomyces cerevisiae did not grow on 1% solutions of a formose made in their laboratory. Recent studies on formose utilization at MIT (Bok, 1972) shows that microorganism growth yield can vary widely with

with different formose mixtures. Bok found several organisms capable of utilizing about 50% of the carbon in a Japanese formose sample and offered good prospects for higher utilization by sequential combinations of these organisms.

A major problem in the evaluation of formose as a carbohydrate source is the variability of its composition. The reaction can be run over a wide spectrum of conditions[4], and analysis of the complex mixtures is difficult and controversial. Some components toxic to mammalian systems have never been identified (Shapira, 1969_b). The sugars which have been identified are often difficult to confirm because of incomplete resolution of physically similar isomers and inconclusive color reactions. Nevertheless, cultivation of microorganisms on formose sugars is potentially very useful and there is no reason to believe that it is not feasible. The technology required for formose-production is being developed with renewed energy and research is in progress on its microbial utilization. The latter work, however, is hampered by the complexity of formose, and much could be learned about the dynamics of mixed substrate utilization and rare carbon source metabolism in simpler systems. Therefore, a study of the utilization of a single rare sugar in pure culture was conducted with a view to predicting the implications of such utilization to the organism's performance on more complex substrates.

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[4] An exhaustive review of literature on the catalysts, conditions, and systems used prior to 1970 is in Shapira's (1970) paper. Refer also to articles by Mizuno et al (1970, 1971) and by Kagiya and Narita (1969).

II OBJECTIVES AND APPROACH

A. The Direction and Utility of this Research

The ultimate objective of this line of research is microbial utilization of formose sugars. The specific approach was to study the catabolism of a rare sugar as sole carbon source by one or more organisms in pure culture. Knowledge of this catabolism will help predict its effects on other metabolic pathways; i.e. whether loss or replacement of other functions can be expected, and it will help to predict the modes of utilization of other unnatural substrates. Such information can be applied to the evaluation of chemically generated microbial feed streams, heterogeneous waste recycle streams, and other systems containing rare and unnatural carbon sources. Catabolism of novel carbon sources is of academic interest also, since it affords opportunities for direct observation of the evolution of enzyme structure, inducer affinity, and control systems resulting in the acquisition of new metabolic functions.

Microbial metabolism of the rare sugars occuring in nature is regulated by the well-known mechanisms of enzyme induction (Monod, 1966; Umbarger, 1964) and catabolite repression (Magasanik, 1961; Perlman et al, 1969). Most of the inducible catabolic enzymes are simple ones; i.e. they are not highly specific or multifunctional allosteric proteins. Several are known to have some activity on substrates other than the primary one. Some organisms posessing a versatile repertoire of inducible enzymes for carbon source utilization are capable of metabolizing substrates rare in nature. A review of such organisms and enzymes is presented in Section III of this thesis.

B. Selection of Rare Sugar Utilizing Microorganisms

The initial objective of this research was to obtain one or more

microorganisms utilizing a rare sugar. More than one organism is desirable because some generalization of the results might be possible, and because different organism types have unique advantages. Bacteria are generally adaptable to different carbon sources; some even utilizing olefinic and aromatic hydrocarbons. Thus, bacteria are most likely to utilize really unusual compounds. Yeasts and molds are larger organisms and cell recovery in a commercial process, often up to 50% of the product cost, would be easier and more economical. Yeast is already commonly used in human and animal food and consumer acceptance would be good relative to "bacterial" or "fungal" protein. Thermophiles would have an important advantage in operating temperature since heat removal from high cell density, high growth rate cultures can be a serious problem in areas without an adequate coolant supply.

Suitable microorganisms can probably be derived from three sources; enrichment and isolation from wild populations[5], adaptive cultivation of organisms known to be nutritionally versatile[6], and by forced mutation of likely organisms with selection on the desired sugar[7]. These approaches were to be used in the above order, and the search was to be terminated when a suitable organism(s) was found.

Wild microbial populations were chosen as the first source of organisms because a great variety of heterotrophs can be found in common aerobic environments (Alexander, 1961; Gilman, 1945). Indigenous organisms in non-eutrophied environments are typically very death-resistent under starvation

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[5]Examples were reported by Shaw (1956), Simpson et al (1958), Beverin (1971), and Guirard (1971).

[6]An example was reported by Mortlock (1971).

[7]An example was reported by Lerner et al (1964).

conditions because they have very low energy requirements in the resting state, and they preserve the integrity of the cytoplasmic membrane (Jannasch, 1971). Indeed the best characterized rare sugar utilizer to date, Aerobacter aerogenes, PRL-R1 was described by Mortlock (1971) as an "atypical, death-resistant strain". These characteristics, which aid survival in natural non-eutrophied environments could be useful if adaptation to a foreign carbon source is very slow.

Chances of finding a suitable organism would probably be enhanced by sampling wild populations at sites likely to contain sugars with a variety of configurations. Such sites are deposits of polysaccharides and complex sugar-like compounds undergoing decomposition, e.g. rotting leaves, organic-rich muds grass mats etc. (Alexander, 1961). Several thermophilic molds and at least one thermophilic yeast are known to occur in self-heated hay decomposition (Cooney and Emerson, 1964). An initial screening experiment by this author in November, 1970, showed that there are airborne microorganisms in MIT 16-241 that metabolize D-arabinose and possibly L-glucose.

Identified organisms reported as being versatile carbon source utilizers are listed in Table II (Stanier et al, 1970; Doelle, 1969; Gottlieb, 1963; Steinberg, 1950; Ainsworth and Sussman, 1965; Alexander, 1961; Gunsalus and Stanier, 1961; Ornston, 1971; Rose and Harrison, 1969).

C. Choice of Rare Sugar

L-glucose was chosen as the first rare sugar for investigation because:

1. It is commercially available in high purity.
2. It has never been reported to be metabolized in biological systems.
3. It is the mirror image of the most ubiquitous natural hexose, D-glucose.

TABLE II

MICROORGANISMS WITH VERSATILE CARBON SOURCE REQUIRMENTS

Yeasts	Bacteria	Molds
Nematospora coryli	*Pseudomonas multivorans*	*Aspergillus niger*
Candida utilis	*P. aeruginosa*	*A. oryzae*
Candida intermedia	*P. pseudomallei*	*Penicillium luteum*
Candida tropicalis	*Azotobacter vinelandii*	
Debaryomyces hansenii	*A. chroococcum*	
Torulopsis candida	*Zooglea ramigera*	
	Bacillus circulans	
	B. macerans	
	B. megaterium	
	B. stearothermophilus	
	B. subtilis	
	Actinoplanes philipinensis	
	Corynebacterium laevaniformans	
	C. poinsettiae	
	Selenomonas sp.	
	Streptococcus mutans	
	Spirillospora sp.	
	Thermoactinomyces sp.	

4. Any physico-chemical process optimized for D-glucose production will be equivalently optimized for L-glucose.

Succinctly put, L-glucose is available, its metabolism is novel research, it is the archetypical unnatural sugar, and its utilization will be necessary for efficient, economical disposition of chemically generated sugars.

If one or more L-glucose utilizers had not been isolated, this research would have been transferred to another rare sugar, preferably one commercially available in sufficient quantities (50 to 100 g), but quite rare in nature.[8] L-ribose is a sugar of this description. D- and L-lyxose are commercially available. Failing these, L-xylose and D-arabinose are readily available rare sugars already known to be metabolized by a variety of microorganisms.

D. Organism Enrichment Medium and Conditions

One important feature of the medium is fixed by the immediate objectives. It must be defined. Within this constraint, a variety of trace elements and vitamins will be added to aid any reactions requiring them when the cell is forced to operate with low energy reserves. The total vitamin concentration will be low enough to preclude significant growth if it were utilized as carbon source.

If the wild population samples are already at low metabolic levels, it might not be a good starting point for adaptation to a new carbon source. On the other hand, there is little advantage in growing a healthy population on D-glucose, since catabolite repression could be expected to inhibit adaptation to a novel carbon source. If such a culture were grown to a high cell density under favorable conditions, the last traces of D-glucose would be

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[8] A literature survey of rare sugar occurence and utilization is in Section III of this thesis.

used so quickly that the population would be carbon-starved while still under catabolite repression. For L-glucose adaptation, it was decided to start the wild samples at relatively high concentrations of L-glucose (2.5 g/L) in combination with enough D-glucose (0.0125 g/L) to permit energy generation and (relatively) healthy growth, then to subculture sequentially to 0.00125 and zero g/L D-glucose while maintaining the L-glucose concentration at 2.5 g/L. Since the inception of our study, a gradual adaptation of this type has been reported for Pseudomonas sp. MA-1 (Guirard and Snell, 1971) from pyridoxamine to pyridoxine. Their case was somewhat different, however, in that first, the two substrates are metabolically related, and second, they started with high concentrations, gradually decreasing, of pyridoxamine, and zero concentration, gradually increasing, of pyridoxine.

Another useful carbon source refinement might be to add an inducer for enzymes having some activity on the new carbon source. Tanaka et al (1967) were successful in growing a D-mannitol negative Escherichia coli on D-mannitol by induction of the enzymes of D-arabitol utilization with catalytic amounts of D-arabitol. Unfortunately, we don't know which enzymes have activity on L-glucose. D-arabinose has the same configuration at carbons I, II, and III as L-glucose, and the enzymes of L-fucose metabolism are known to have activity on D-arabinose in Aerobacter aerogenes PRL-R3 (Mortlock, 1965). Therefore, it was decided to include in the medium a stagewise decreasing concentration of L-fucose, similar to that of D-glucose, from 0.0125 to zero.

Incubation temperatures of 30°, 37°, and 55° C were used because temperatures of 30° C or above have commercial process advantages, most mesophiles can grow at this temperature (Nickerson, 1970), 37° C favors organ-

isms associated with mammalian physiology, and 55° C favors thermophiles. Facilities at these temperatures are readily available in the microbiology laboratories of the Department of Nutrition and Food Science at MIT.

A hydrogen ion concentration of pH 6.8 was used in the initial stage of enrichment because most organisms grow at this pH and the filter-sterilized medium did not readily precipitate. Second stage enrichment and subsequent cultivation of yeast-like and mycelial forms was done at pH 3.0 - 3.5 to favor their growth over most bacterial forms. The bacterial forms were cultivated at pH 6.4 to 6.8 throughout these studies.

Samples were to be incubated for up to ninety days at all stages before being considered negative. Mortlock's (1971) L-xylose utilizing Aerobacter aerogenes PRL-R3 required 40 to 60 days in resting culture before adaptation.

The enrichment technique to be used for this study has four important features:

1. L-glucose at 2.5 g/L is the major carbon source.
2. Low concentrations of natural sugars are included in the initial stages.
3. The medium is completely defined, but comprehensive with respect to vitamins.
4. Long incubation times will be used if necessary.

We think that this is the first time D-glucose has been purposely incorporated into an organism enrichment medium in the manner stated.

E. Characterization of Rare Sugar Utilizers

The second specific objective of this research is to characterize the growth of the rare sugar utilizers obtained. The acquisition of and use of the metabolic pathway involved should have some implications on the

use of the organism as a protein producer on mixed carbohydrate substrates. It is our intention to predict some of these implications.

An obvious step in characterization is enough taxononomy to determine the biological Division, Class, Order, Family, and, if possible, Genus of the organism(s). The known metabolism of the organism in conjunction with observable characteristics of its growth on the rare sugar might be informative.

Knowledge of the actual steps of the metabolic pathway is desirable. Some clues to these may be obtained by looking at the lag times and growth rates of the adapted organism on substrates normally requiring induction.

Definitive determination of a metabolic pathway may require identification and proof of the intermediates and/or identification and proof of the enzymes involved. The former approach is feasible if the intermediate pools are large enough to recover and identify. The first step for any *in vivo* analysis of metabolite pools or enzyme activities is determination of the uptake kinetics for the substrate in question. In particular, if substrate transport is not limiting growth rate, a substantial intracellular pool of the substrate or an early metabolite(s) may be present.

In consideration of the above requirements, the steps proposed for attacking the second objective are:

1. Identification of the organism(s) to the genus level, if possible.
2. Determination of the growth kinetics and carbon yield on the rare sugar.
3. Characterization of the growth of the rare sugar-adapted organism on a variety of sugars.
4. Determination of the transport kinetics of the rare sugar.

III. LITERATURE REVIEW OF RARE SUGAR UTILIZATION

A. Definitions

Sorbitol. This is the common name for the hexitol resulting from hydrogenation of D-glucose, L-glucose, D-fructose, or L-sorbose. In the literature it is sometimes called L-Sorbitol and sometimes D-sorbitol. Note that D-sorbose would be the C_5 ketose of the L-glucose configuration. According to the conventions of carbohydrate nomenclature (Stanek, 1963) the short name of the naturally occurring sorbitol is properly either D-glucitol or L-gulitol. The name "D-glucitol" will be used in this thesis.

L-Glucitol. Hexitol of the L-glucose configuration resulting from the hydrogenation of L-glucose, D-gulose, L-fructose, or D-sorbose.

Psicose. The C_2 ketose of the D-allose or D-altrose configurations. It can properly be called D-allulose. For consistency with the major references on D-allose metabolism, the name "D-allulose" will be used in this thesis.

Tagatose. The C_2 ketose of the D-galactose and D-talose configurations. In deference to the references on tagatose utilization, all of which use this term, "D-tagatose" will be used in this thesis.

B. Five and Six Carbon Sugars

The normal pentoses and hexoses of formula $(CH_2O)_5$ and $(CH_2O)_6$ have thirty-six isomers; eight aldopentoses, four ketopentoses, sixteen aldohexoses and eight ketohexoses are shown in Figure 1. Reduction of the keto-oxygen of each of these compounds to a hydroxyl leads to four pentitols and eight hexitols. These polyols are shown in figure 2. In addition to the normal keto sugars, structures of four of the 6-deoxy hexoses, two branched-chain carbohydrates, abequose (2, 6 dideoxy D-glucose) and the heptulose (sedoheptulose), mentioned in this thesis are

FIGURE 1

LINE PROJECTION FORMULAE OF SOME CARBOHYDRATES

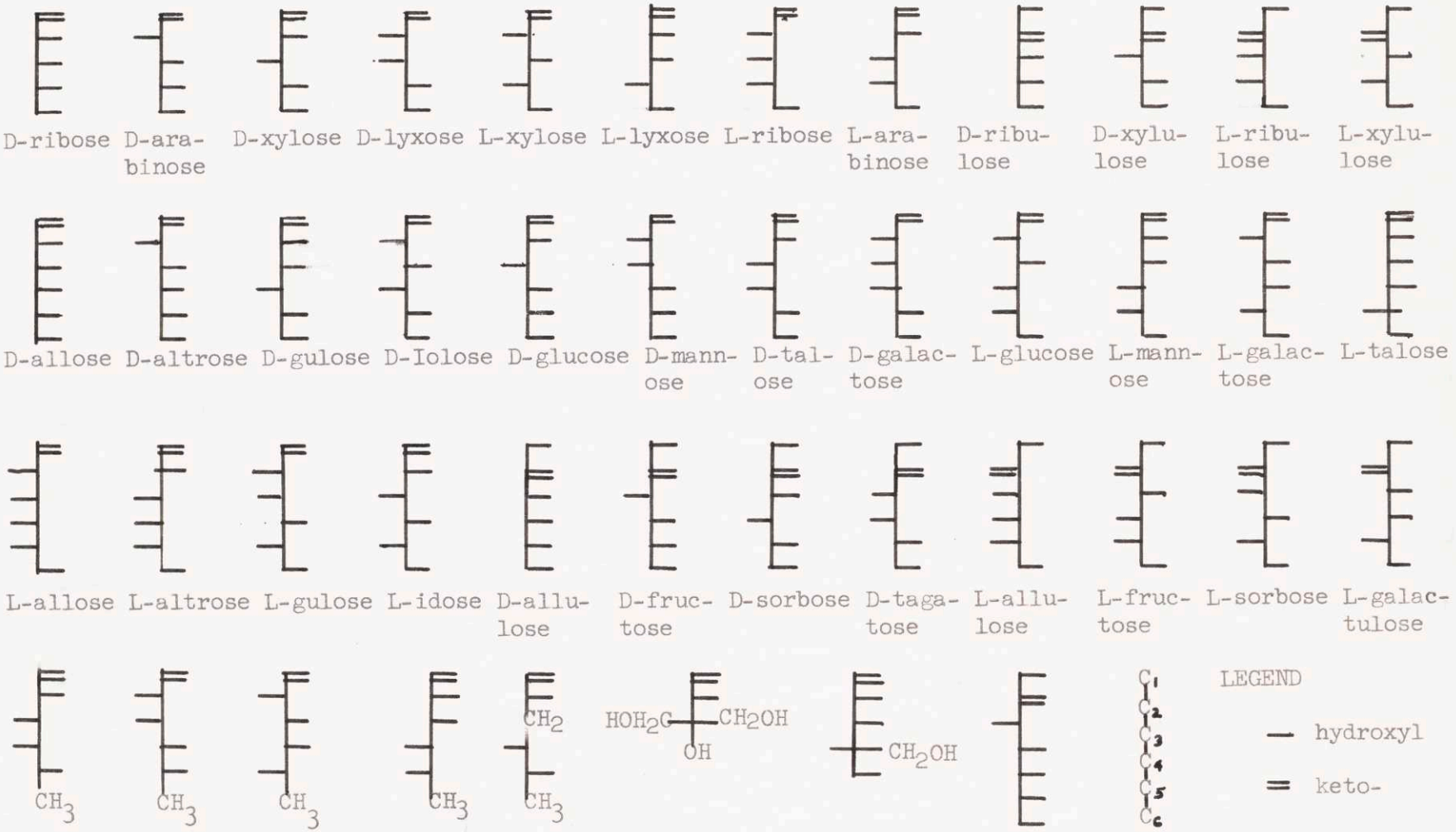
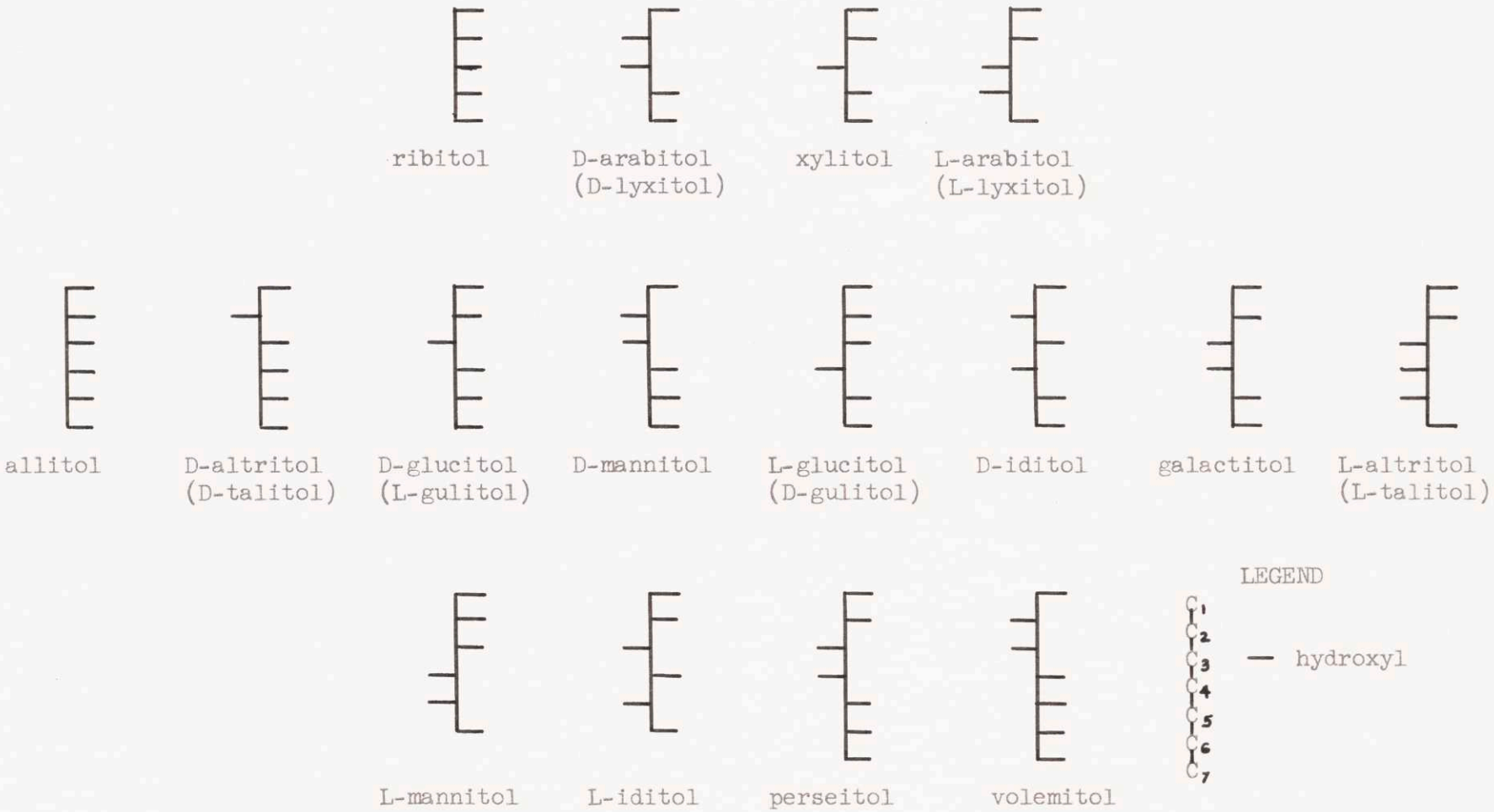


FIGURE 2

LINE PROJECTION FORMULAE OF SOME POLYOLS



included in figure 1.

Most of the D-series sugars are considered to be natural and the L-series to be unnatural. Exceptions to this are the rare D-sugars; D-arabinose, D-lyxose, D-sorbose, D-fucose, and D-rhamnose and the (relatively) common L-sugars; L-arabinose, L-sorbose, L-fucose, and L-rhamnose. Of these L-sugars, L-arabinose is a homomorph of D-galactose and L-sorbose is 5-keto-D-glucose. L-fucose and L-rhamnose are produced from D-sugars by nucleotide phosphate-coupled epimerization reactions to be mentioned later. For reference later in this section, note that D-arabitol is a common polyol and L-arabitol is rare...contra their aldose derivatives.

C. Occurence of Rare Sugars in Nature

Rarity does not make a sugar unnatural. Table III lists the sources of many of these sugars isolated from nature. Most are hydrolysis products of polysaccharides, oligosaccharides or glycoproteins. Aside from Saha and Choudhury's work (1922), the only other report of L-glucose incidence is that of Power and Tutin (1905). However, Stanek (1963) considers the 1905 data not "too convincing".

Blumson (1961) found one of the three sugar moieties of streptomycin from Streptomyces griseus to be N-methyl-L-glucosamine. Akamatsu and Arai (1967) did a radioactive tracer study by using D(1-C^{14})-glucosamine and concluded that the L-glucose molecule was assembled from D- and L-glyceraldehyde-3-P. Bruton et al (1967) did a similar study using D-glucosamine labelled at carbon 1 and D-glucose labelled at carbons 2, 3, and 4. They concluded that the L-glucose structure arose by multiple inversions of the assymetric carbons. Demain and Inamine (1970) suggested a similarity to the glucosyl thymidine diphosphate-rhamnosyl thymidine diphosphate pathway.

TABLE III

OCCURENCE OF RARE SUGARS IN NATURE

Sugar	Source	Reference
D-arabinose	tobacco smoke	Stanek (1963)
L-lyxose	curamycin	Galmarini (1961)
L-glucose	Corchorus capsularis	Saha & Choudhury (1922)
L-galactose	agar	Stanek (1963)
		Duckworth & Yaphe (1971)
	red algae	Stanek (1963)
	corn roots	Roberts (1971)
	snails	Bell & Baldwin (1941)
	sea urchin eggs	Hunt (1970)
	flax	Smith & Montgomery (1959)
	corn cobs	Aspinall (1959)
D-talose	hygromycin	Stanek (1963)
D-gulose	dextran sulphate	Stanek (1963)
L-xylulose	urine	Stanek (1963)
D-allulose	psicofuranin	Stanek (1963)
D-fucose	chartreusin, many heterosides	Stanek (1963)
D-rhamnose	Gram negative capsules	Markowitz (1962)
D-tagatose	heated milk	Stanek (1963)
	Sterculia setigera	Stanek (1963)
D-arabitol	lichens	Stanek (1963)
	Fistulina hepatica	Stanek (1963)
L-iditol	Sorbus aucuparia	Stanek (1963)
Perseitol	Persea gratissima	Stanek (1963)
Volemitol	Lactarius volemus	Stanek (1963)
D-apiose	Apiin	Hudson (1949)
	Posidonia australis	Bell et al (1954)
	Hevea brasiliensis	Patrick (1956)
abequose	Salmonella typhimurium	Stanier et al (1970)

D. Microbial Utilization of Rare Sugars

1. Organisms Utilizing Rare Sugars

Microbial utilization of rare sugars has been of interest to investigators for characterization and purification of sugars in early carbohydrate chemistry and for scientific curiosity. Since the development of defined media, biological activity on a few rare sugars has become a useful tool for classification of a wide variety of organisms. Emil Fischer (1890) incubated "bierhefen" with his newly synthesized L-glucose and L-mannose as part of his elucidation of the structure of these sugars. Schweisfurth et al (1969) investigated the growth of twenty bacteria and four yeasts on D-ribulose, D-tagatose, D-sedoheptulose, and three disaccharides. They found that while most of these organisms utilized D-ribulose, only Salmonella typhimurium LT_2, Streptococcus faecalis, Bacillus subtilis, Candida albicans, and Candida pseudotropicalis utilized D-tagatose. S. typhimurium LT_2, four species of Proteus, S. faecalis, B. subtilis, C. albicans, and C. pseudotropicalis utilized sedoheptulose. Sternfeld and Saunders (1967) reported growth of 5 enterics and 3 staphylococci on D-arabinose and L-ribose, but it should be noted that their medium contained 8 gm/L peptone and beef extract; both excellent carbon sources for most of these organisms.

The literature on microbial growth utilizing L-glucose is all negative. Fischer's yeast (1890) did not grow in the twenty-four hour incubation period employed. Rudney (1940) observed no growth with Bacterium (Aerobacter) aerogenes, B. (Escherichia) coli, and mammalian cells. Streptomyces griseus was found to transport L-glucose into resting mycelia, but did not metabolize it (Bruton, 1967). Dr. R.P. Mortlock (1971) informed

this author that Dr. W.A. Anderson had an L-glucose utilizing strain of Aerobacter aerogenes, but it was deadapted during storage on agar. There is no record of any investigator purposely enriching a microbial culture for L-glucose utilizers.

D-arabinose and inositol are carbohydrates almost invariably included in the carbon assimilation profiles used in taxonomic studies with yeasts (Lodder, 1970). In Pseudomonas taxonomy, utilization of D-fucose is diagnostic for differentiation of the Pseudomallei group from the rest of the genus (Stanier et al, 1966).

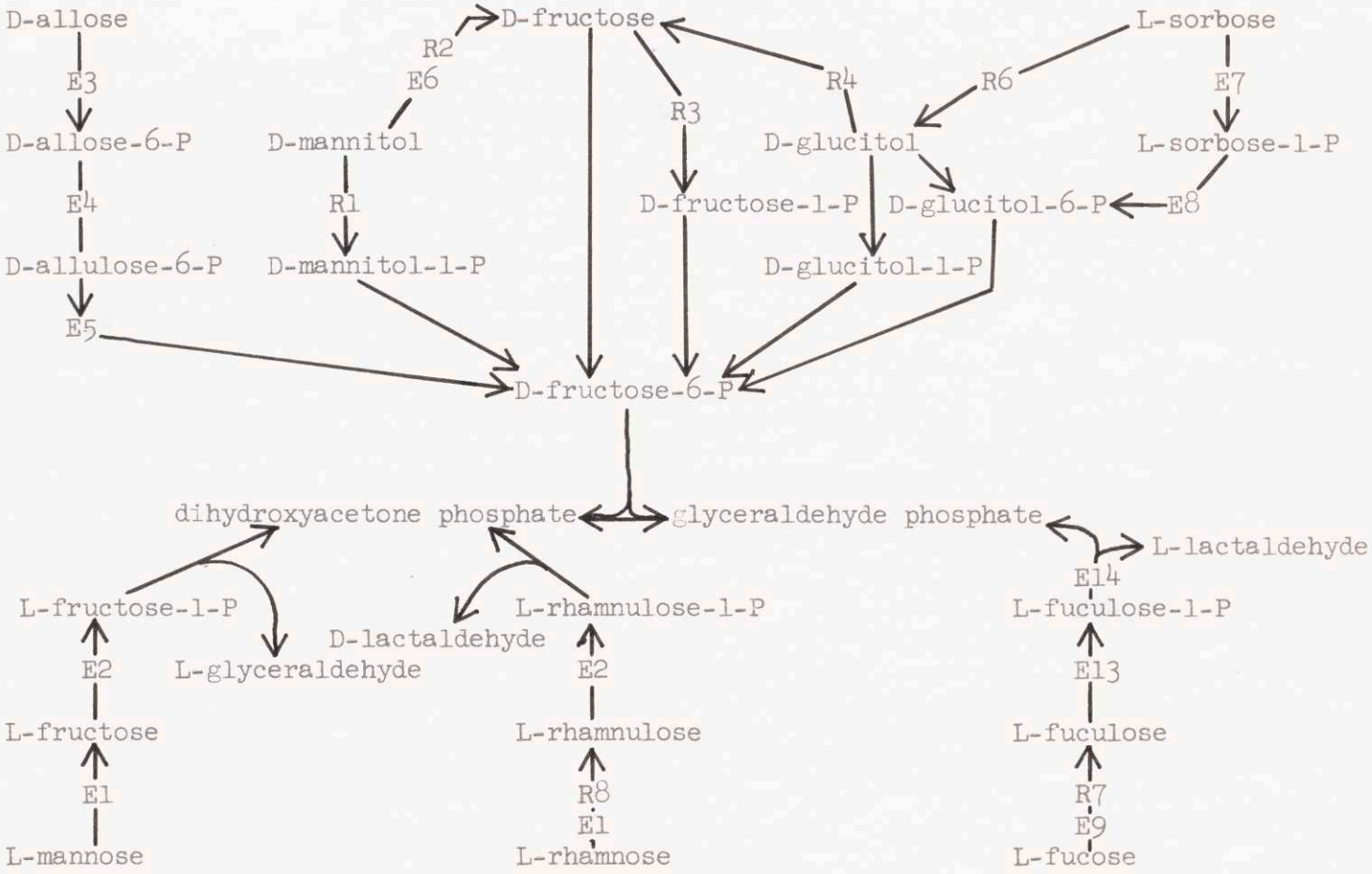
2. Metabolic Pathways of Rare Sugar Utilization

Microbial utilization of several rare sugars other than L-glucose is well documented. The metabolic pathways are known, and in some cases, the enzymes involved have been analyzed in detail. Figures 3 and 4 show several of these pathways for hexoses and pentoses respectively. Note that many of the reactions depicted are reversible, perhaps even unfavored under normal metabolic conditions in vivo. The familiar Emden-Meyerhoff-Parnas pathway and the initial D-glucose-associated steps of the Hexose Monophosphate and Entner Doudoroff pathways are omitted since they have no apparent bearing on rare sugar utilization. The glucuronate and galactonate pathways have not yet been implicated either, but the D- and L-arabonate pathways (via the gamma-lactones) are shown on figure 4 for comparison. Nucleoside diphospho-sugars will be briefly discussed later.

Of special interest are those pathways in which the method of acquiring a pathway for catabolism of a novel carbon source is known. This is the case for the utilization of L-fructose, L-mannose, D-ribitol, L-arabitol, D-arabinose, and L-xylose by Aerobacter aerogenes PRL-R3 originally isolated by Neish and Simpson (1954). The enzymes used in these

FIGURE 3

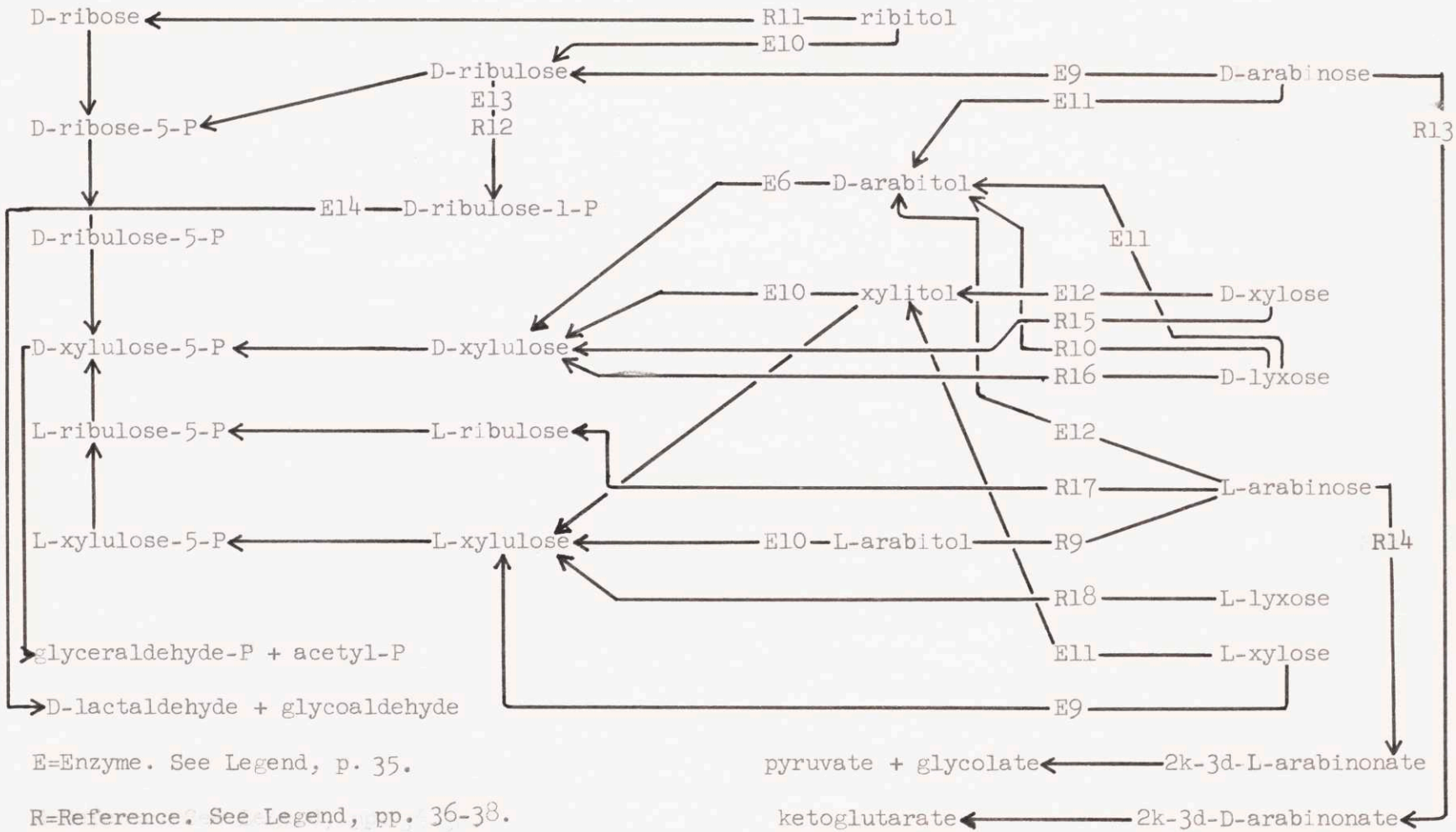
CATABOLIC PATHWAYS FOR SOME 6-CARBON CARBOHYDRATES



E=Enzyme. See Legend, p. 35.
R=Reference. See Legend, pp. 36-38.

FIGURE 4

CATABOLIC PATHWAYS FOR SOME 5-CARBON CARBOHYDRATES



E=Enzyme. See Legend, p. 35.

R=Reference. See Legend, pp. 36-38.

LEGEND, FIGURES 3 AND 4

Enzyme(s)	Natural Substrate	Organism	Reference
E1, E2	L-rhamnose	Aerobacter aerogenes	Mayo & Anderson (1968a, 1968b, 1969)
E3, E4, E5	D-allose	Aerobacter aerogenes	Gibbins & Simpson (1963, 1964)
E6	D-arabitol	Aerobacter aerogenes	Tanaka et al (1967)
	D-arabitol, D-mannitol	Rhizobium meliloti Rhizobium leguminosarum Rhizobium trifolii	Martinez-DeDrets & Arias (1970)
		Cellvibrio polytrophicus	Scolnick & Lin (1962)
E7, E8	L-sorbose	Aerobacter aerogenes	Kelker et al (1972)
E9	L-fucose	Aerobacter aerogenes	Mortlock et al (1964, 1968) Mortlock (1965) Oliver & Mortlock (1970)
E10	ribitol	Aerobacter aerogenes	Camyre & Mortlock (1965) Mortlock et al (1968) Wu et al (1968) Hulley et al (1963)
E11	unknown	Torulopsis candida	Karasevich (1971)
	unknown	Candida pulcherrima	Karasevich (1970)
E12	D-xylose	Torulopsis candida	Karasevich (1971)

continued next page

Reference	Organism	Reference
R1	Aerobacter aerogenes	Liss et al (1962)
	Salmonella typhimurium	Berkowitz (1971)
	Bacillus subtilis	Horwitz & Kaplan (1964)
	Diplococcus pneumoniae	Marmur & Hotchkiss (1955)
	Escherichia coli	Wolff & Kaplan (1956a, b)
	Lactobacillus plantarum	Chakravorty (1964, 1965)
	Salmonella gallinarum	Zancan & Bacila (1964)
	Staphylococcus aureus	Murphey & Rosenblum (1964)
	Clostridium thermocellum	Patni & Alexander (1971)
	Piricularia oryzae	Yamada et al (1961)
	Diplodia viticola Desm.	Strobel & Kosuga (1965)
R2	Lactobacillus brevis	Martinez et al (1963)
	Acetobacter suboxydans	Arcus & Edson (1956)
		Peterson et al (1956)
	Azotobacter agilis	Marcus & Marr (1961)
	Cellvibrio polytrophicus	Scolnick & Lin (1962)
	Pseudomonas fluorescens	Sebek & Randles (1952)
	Diplodia viticola Desm.	Strobel & Kosuga (1965)
	Agaricus campestris	Edmundowicz & Wriston (1963)
	Acetobacter melanogenum	Sasajima & Isono (1968)
	Acetobacter aceti	DeLey & Schell (1959)
	Gluconobacter oxydans	Kersters et al (1964)
	Leuconostoc mesenteroides	Sakai & Yamanaka (1968)
	Lactobacillus bifidus	DeVries & Stouthamer (1968)
R3	Aerobacter aerogenes	Hanson & Anderson (1966)
	Bacteroides symbiosus	Reeves et al (1966)
	Escherichia coli	Fraenkel (1968)

continued next page

LEGEND, FIGURES 3 AND 4 (continued)

Reference	Organism	Reference
R4	Aspergillus niger	Desai et al (1967)
	Rhizobium meliloti and two other Rhizobia	Martinez DeDrets & Arias (1970)
	Cellvibrio polytrophicus	Scolnick & Lin (1962)
	Acetobacter melanogenum	Sasajima & Isono (1968)
	Gluconobacter oxydans	Kersters et al (1965)
	Azotobacter agilis	Marcus & Marr (1961)
	Acetobacter suboxydans	Arcus & Edson (1956)
	Candida utilis	Arcus & Edson (1956)
R5	Aerobacter aerogenes	Kelker & Anderson (1971)
R6[a]	Gluconobacter oxydans	Kersters et al (1965)
	Acetobacter suboxydans	Arcus & Edson (1956)
	Candida utilis	Arcus & Edson (1956)
R7	Escherichia freundii	Heath (1958)
	Aerobacter aerogenes	Mayo & Anderson (1968)
		Heath & Ghalambor (1962)
	Escherichia coli	Green & Cohen (1956)
		Huang & Miller (1958)
R8	Escherichia coli	Wilson & Ajl (1955, 1957)
		Frontali & Tecce (1959)
	Pasteurella pestis	Englesberg (1957)
R9	Penicillium chrysogenum	Chiang & Knight (1960)

continued next page
a.presumptive pathway.

LEGEND, FIGURES 3AND 4 (continued)

Reference	Organism	Reference
R10	*Penicilium chrysogenum*	Chiang & Knight (1960)
	Candida utilis	Scher & Horecker (1966)
	Candida albicans	Veiga et al (1960)
R11	*Candida pulcherrima*	Karasevich (1970)
R12	*Escherichia.coli*	LeBlanc & Mortlock (1971)
R13	*Pseudomonas saccharophila*	Palleroni & Doudoroff (1957)
R14	*Pseudomonas saccharophila*	Weimberg (1959)
R15	*Pseudomonas hydrophila*	Hochster (1955)
	Pasteurella pestis	Slein (1955)
	Lactobacillus pentosus	Mitsuhashi & Lampen (1953)
R16	*Aerobacter aerogenes*	Anderson & Allison (1965)
	Lactobacillus pentosus	Lampen (1956)
	Lactobacillus pentoaceticus	Rappoport et al (1951)
	Propionobacterium pentosaceum	Volk (1956)
R17	*Aerobacter aerogenes*	Simpson et al (1958)
R18	*Aerobacter aerogenes*	Anderson & Wood (1962)

pathways and their derivation has been well characterized through the efforts of Drs. Anderson, Mortlock, Wood, and their coworkers. Mayo and Anderson (1968) found that a single mutation to constitutivity of the L-rhamnose operon permitted A. aerogenes to grow, albeit slowly, on L-mannose or L-fructose using the same enzymes. Mayo and Anderson (1969) then observed a structural mutation which conferred resistance to the toxicity of L-glyceraldehyde (Cutinelli, 1967; Lardy, 1950), permitting higher growth rates on L-mannose or L-fructose. Anderson and Wood (1969) point out that it is the first reported instance in which the first step in catabolism of a non-derived aldohexose is an aldose→ketose isomerization.

Tanaka, et al (1967) reported an instance in which a D-mannitol negative revertant acquired a new pathway. They isolated mutants defective in the mannitol phosphotransferase portion of the phosphoenolpyruvate-phosphotransferase system normally used by enterics for D-mannitol catabolism. This defective mutant thereby lost its ability to utilize D-mannitol alone, but it was able to grow on D-mannitol in the presence of catalytic amounts of D-arabitol. The investigators determined that D-arabitol was inducing the D-arabitol dehydrogenase, permitting metabolism of D-mannitol via D-fructose. Of ten uv-induced revertants to growth on D-mannitol as sole carbon source, nine were found to produce D-arabitol dehydrogenase constitutively. One revertant reacquired D-mannitol phosphorylation activity. It is logical that mutagenesis is more likely to harm the D-arabitol repressor system than to restore function in the D-mannitol phosphotransferase. Any changes resulting in loss of DNA binding capacity for the repressor or in an unrecognizable sequence for the

operator would result in constitutivity, whereas only a frameshift in a limited section of the genome or very specific translational mutations (depending on the original lesion) would result in recovery of the phosphotransferase activity (Mortlock, 1971).

Other instances of a single constitutive mutation permitting utilization of a new sugar(s) are D-ribitol dehydrogenase permitting use of xylitol and L-arabitol (Camyre and Mortlock, 1965), and L-fucose isomerase and the coordinately induced permease permitting use of D-arabinose, L-lyxose, and L-xylose (Mortlock, 1965b). A similar constitutive mutation of the L-fucose operon was observed in Escherichia coli K-12, permitting utilization of D-arabinose. In E. coli, the operon contains three enzymes; L-fucose isomerase, L-fuculose kinase and L-fuculose-1-phosphate aldolase. These convert D-arabinose to D-ribulose, to D-ribulose-1-phosphate and finally to D-lactaldehyde plus glycoaldehyde. D-ribulose-1-phosphate is a unique compound in intermediary metabolism.

The isomerase which permits D-lyxose utilization in A. aerogenes also resulted from a single mutation, but it appears to be a new enzyme and it is induced only by D-lyxose (Mortlock and Wood, 1964).

Hegeman and Rosenberg (1970) credit Lerner et al (1964) with the first systematic study of the evolution of a metabolic pathway. A. aerogenes was adapted stepwise to faster growth on xylitol by:

1. A constitutive mutant for ribitol dehydrogenase production to oxidize xylitol to D-xylulose; selected by growth on xylitol.

2. a structural modification of the constitutive xylitol dehydrogenase resulting in 2.5x specific activity on xylitol; induced by nitrosoguanidine treatment.

3. a constitutive mutant for the D-arabitol active transport system which accepts xylitol; induced by nitrosoguanidine (Wu et al, 1968).

Most organisms which acquire the ability to catabolize a new carbon source can do so by a single mutation permitting them to:

1. transport the carbon source across the membrane if it is normally excluded.

2. constitutively produce enzymes from a different pathway which have some activity on the new substrate.

3. produce a structurally altered constitutive enzyme with activity on the new substrate.

4. produce a structurally altered repressor with affinity for the new substrate.

5. produce a structurally altered enzyme with lower sensitivity to an inhibitory product.

Case 2. has been the most common means of rare sugar adaptation in A. aerogenes. The lag times for rare sugar utilization in Mortlock's (1971) studies were quite long because control mutations were required to permit constitutive enzyme production without inducer being present. Table IV lists the lag times of the wild type and the generation times of each initial mutant on five natural sugars and five rare ones. This organism required five to eight weeks to adapt to L-xylose and Mortlock (1971) feels that cell permeability was involved.

The L-sorbose pathway in Aerobacter aerogenes via L-sorbose-1-phosphate was recently elucidated by Kelker and Anderson (1972). L-sorbose production via D-glucose and D-glucitol in Acetobacter suboxydans (Arcus and Edson, 1956) and other pseudomonads has been known for some time. These reactions equilibrate freely and possibly regulate (or are regulated by) NAD vs. NADP levels in the cell (Hollman, 1964). It is assumed that the major catabolic pathway for L-sorbose in these organisms proceeds by

TABLE IV

LAG TIMES AND GENERATION TIMES FOR RARE SUGAR UTILIZATION IN AEROBACTER AEROGENES PRL-R_3

Sugar	Lag Time (days)	Generation Time (hours)
D-Glucose	0.06	0.9
D-Arabitol	0.1	1.2
Ribitol	0.2	1.2
D-Xylose	0.2	1.5
L-Arabinose	0.1	1.2
D-Arabinose	1 - 1.5	2.0
Xylitol	4 - 5	4 - 5
L-Arabitol	1	4 - 5
L-Xylose	35 - 56	6 - 8

this oxidoreduction. It should be noted, however, that D-glucitol and L-sorbose are oxidized to 2-keto-L-gulonic acid by more than two-hundred strains of the genera Acetobacter, Gluconobacter, and Pseudomonas (Isono et al, 1968). It is not known if this ketoacid is an intermediate or a byproduct.

In general, enteric bacteria metabolize hexitols via phosphorylation, sorbose via phosphorylation, and aldopentoses via isomerization to ketopentoses. Yeasts and fungi metabolize aldopentoses via reduction to pentitols, then oxidation to ketopentoses. Many of the perfect fungi utilize hexitols by oxidation to ketohexoses. Pseudomonads and acetic acid bacteria utilize hexitols by reduction, sorbose via hydrogenation to D-glucitol and possibly by oxidation to 2-keto-L-gulonic acid. D- and L-arabinose are utilized via the gamma-arabino-lactones and arabonic acids in the latter organisms.

3. Polyol Dehydrogenation

The oxidoreductases which equilibrate the aldose→alditol→ketose systems have three features which make them interesting when considering novel carbon sources. They are relatively nonspecific, they are often induced simultaneously (that is, derepressed by compounds other than their substrates), and they are freely reversible, with relatively high concentrations of all species, depending on the oxidoreduction potentials in the cell.

Reports of multisubstrate activities for polyol dehydrogenases from several organisms are listed in Table V.

Marcus and Marr (1961) reported D-mannitol dehydrogenase induction in Azotobacter agilis by D-mannitol and by all of the pentitols except

TABLE V

REPORTS OF MULTISUBSTRATE ACTIVITY IN POLYOL DEHYDROGENASES

Normal Substrate	Organism	Reference
ribitol	Aerobacter aerogenes	Mortlock et al (1965[a])
ribitol	A. aerogenes	Mortlock et al (1964a), (1964b)
D-arabitol	A. aerogenes	Mortlock et al (1964a), (1964b)
xylitol	A. aerogenes	Mortlock et al (1964a), (1964b)
D-mannitol	Acetobacter suboxydans	Arcus & Edson (1956)
D-mannitol	Candida utilis	Arcus & Edson (1956)
Galactitol	Pseudomonas sp.	Shaw (1956)
D-iditol	Pseudomonas sp.	Shaw (1956)
xylitol	Candida utilis	Chakravorty et al (1962)
xylitol	Lactobacillus plantarum	Chakravorty et al (1962)
D-mannitol	Pseudomonas fluorescens	Sebek & Randles (1952)
D-mannitol	Diplodia viticola Desm.	Strobel & Kosuge (1965)
D-mannitol	20 Acetobacter species	Sasajima & Isono (1968)
D-mannitol	Acetobacter melanogenum	Sasajima & Isono (1968)
xylitol	Candida utilis	Scher & Horecker (1960)
xylitol	Gluconobacter oxydans	Kersters et al (1965)
ribitol	Gluconobacter oxydans	Kersters et al (1965)
D-glucitol	Gluconobacter oxydans	Kersters et al (1965)
D-mannitol	Gluconobacter oxydans	Kersters et al (1965)
xylitol	Penicillium chrysogenum	Chiang & Knight (1960)
D-mannitol	Azotobacter Agilis	Marcus & Marr (1961)
L-iditol	Azotobacter Agilis	Marcus & Marr (1961)

L-arabitol. Ribitol is an excellent inducer but it is not metabolized. In the same organism, L-iditol dehydrogenase is induced by D-glucitol and by xylitol. Kersters et al (1965) used only D-glucitol (and yeast extract) to induce xylitol dehydrogenase, D-glucitol dehydrogenase, ribitol dehydrogenase, and D-mannitol dehydrogenase. In Rhizobium meliloti, Rhizobium trifolii, and Rhizobium leguminosarum, growth on either D-mannitol, D-arabitol, or D-glucitol induced all three dehydrogenases simultaneously (Martinez-De Drets and Arias, 1970). Growth of the same organism on galactitol yielded 10-fold lower activity for the other three dehydrogenases, and growth on other carbon sources yielded none. The Pseudomonas sp. isolated by Shaw (1956) was induced for galactitol dehydrogenase by either galactitol or D-glucitol. The same conditions induce the enzyme for D-glucitol oxidation, but not for D-mannitol. D-mannitol induced D-mannitol and galactitol oxidation, but not D-glucitol oxidation. All three of these polyols induced xylitol oxidation at a "slow" rate.

Shaw (1956) found that cell extracts from his pseudomonad had very broad dehydrogenase specificity, as shown in Table VI. Of special interest is the very high oxidation rate of L-glucitol. Shaw did not say where he got his L-glucitol, but he did identify the oxidation product as L-fructose. The crude extract used for these studies apparently contained galactitol, D-iditol, and D-mannitol dehydrogenases. By ammonium sulphate fractionation and storage stability, Shaw determined that the L-glucitol dehydrogenation activity was associated with an unstable D-iditol dehydrogenase with activity on the D-threo-configuration.

The xylitol dehydrogenase of Candida utilis studied by Chakravorty et al (1962) was reported to have 5% relative activity on L-glucitol. The ribitol dehydrogenase from Gluconobacter oxydans was measured by Kersters

TABLE VI

POLYOL OXIDATION RATES OF CELL EXTRACTS
FROM A PSEUDOMONAS GROWN ON GALACTITOL

Substrate	Rate
Nil	0.14
Erythritol	0.18
D-Arabitol	0.41
L-Arabitol	0.27
Ribitol	0.31
Xylitol	1.52
Allitol	0.18
D-Mannitol	0.00
L-Gulitol (sorbitol)	1.29
D-Gulitol	3.35
D-Talitol	3.04
Galactitol (dulcitol)	2.19
6-Deoxy-L-gulitol	5.13
D-manno-D-talo-Heptitol (volemitol)	0.18
D-manno-D-gala-Heptitol (perseitol)	0.54
L-gulo-D-gala-Heptitol	0.36
L-gulo-D-talo-Heptitol (B-sedoheptitol)	0.31

Reprinted From Shaw (1956).

et al (1965) as having 2% relative activity on L-glucitol. Kersters et al synthesized their L-glucitol by reduction of "L-gulono-γ-lactone" which should have yielded D-glucitol. However, their L-glucitol was not reduced at all by D-glucitol dehydrogenase, so it seems likely that their starting material was D-gulono-γ-lactone. They proved that D-sorbose is the oxidation product.

It is potentially important to rare carbon source utilization, that an oxidoreduction "network" resulting in epimerization of the β-carbons can be established. Such an equilibrium system (Figure 5) was reported by Chiang and Knight (1960) in Penicillium chrysogenum. It could result in isomerization of L-arabinose to D-xylose if a reaction were removing D-xylose from the system.

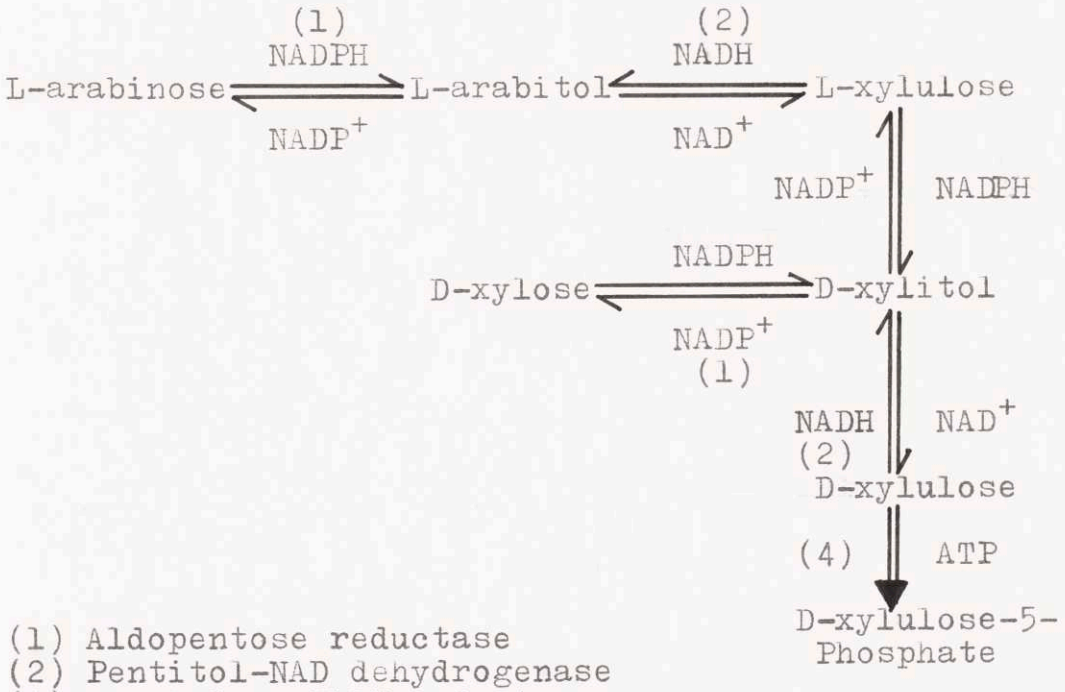
4. Sugar-Diphosphonucleotide Reactions

A number of isomerization reactions occur with sugar nucleotides. Glucosyl-uridine-diphosphate (UDP-glucose) for instance gives rise to UDP-galactose, UDP-xylose, UDP-arabinose, UDP-mannose, and UDP-N-acetyl-glucosamine (Hollman,1964; Stanier et al, 1970). Similarly, glucosyl cytidine diphosphate (CDP-glucose) gives rise to CDP-abequose, glucosyl thymidine diphosphate (TDP-glucose) to TDP-rhamnose, and glucosyl guanosine diphosphate (GDP-glucose) to GDP-fucose. These compounds are intermediates for all oligo- and polysaccharide synthesis, probably glycoprotein synthesis, and are known to participate in the synthesis of some antibiotics as sugar moiety donors. However, UDP-mannose and UDP-galactose are used in pathways of mannose and galactose assimilation. In fact, this is the major pathway of galactose utilization in enterics and in many yeasts. If a carbohydrate can be phosphorylated and transferred to the nucleotide,

FIGURE 5

OXIDOREDUCTION SYSTEM OF PENTOSE UTILIZATION

IN PENICILLIUM CHRYSOGENUM



(1) Aldopentose reductase
(2) Pentitol-NAD dehydrogenase
(3) L-xylulose-NADP reductase
(4) D-xylulokinase

Adapted from Chiang & Knight (1960).

the nucleotide phosphate derivative could be a powerful means of isomerization. As already discussed, Bruton (1967) and Akatmatsu (1966) have given evidence of an L-gluco-structure arising from D-glucose, possibly via the UDP derivative.

5. Structural Basis for Enzyme Activity on Sugars

The affinity of an existing enzyme for a new substrate is logically considered in terms of structural similarity between the new substrate and the enzyme's natural substrate. A familiar example is the use of gratuitous inducers in regulatory studies. Gratuitous inducers work for various reasons, but in some specific cases, like thiomethylgalactoside induction of β-galactosidase and 2-deoxy-D-glucose induction of hexose kinase, they are known to work by imitating the enzyme affinity of the primary substrate.

Aldoses in aqueous solution exist as pyranoses, that is, a C_5-C_1 hydrogen exchange balances the C_1 aldehydic group and the molecule assumes a pyran ring structure. This structure in turn has four possible conformations with the hydrogens and hydroxyls assuming axial and equatorial attitudes to the planes of the carbon-carbon bonds. By analogy with cyclohexane, these can be termed "boat" and "chair" conformations. Because of the bulk of the hydroxyl groups, however, the two chair conformations are sterically disadvantageous and need not be considered. Steric and energetic considerations also dictate which boat conformation will predominate, and Reeves (1951), Kelly (1957) and Edward (1955) have established them for the aldoses. All of these except D-lyxose, D-altrose, and D-idose have a single predominant conformation. The three exceptions are constrained by their carbon configurations to an equilibrium between the so-called C1 and 1C conformations. These are defined as conventional clockwise (C1) and

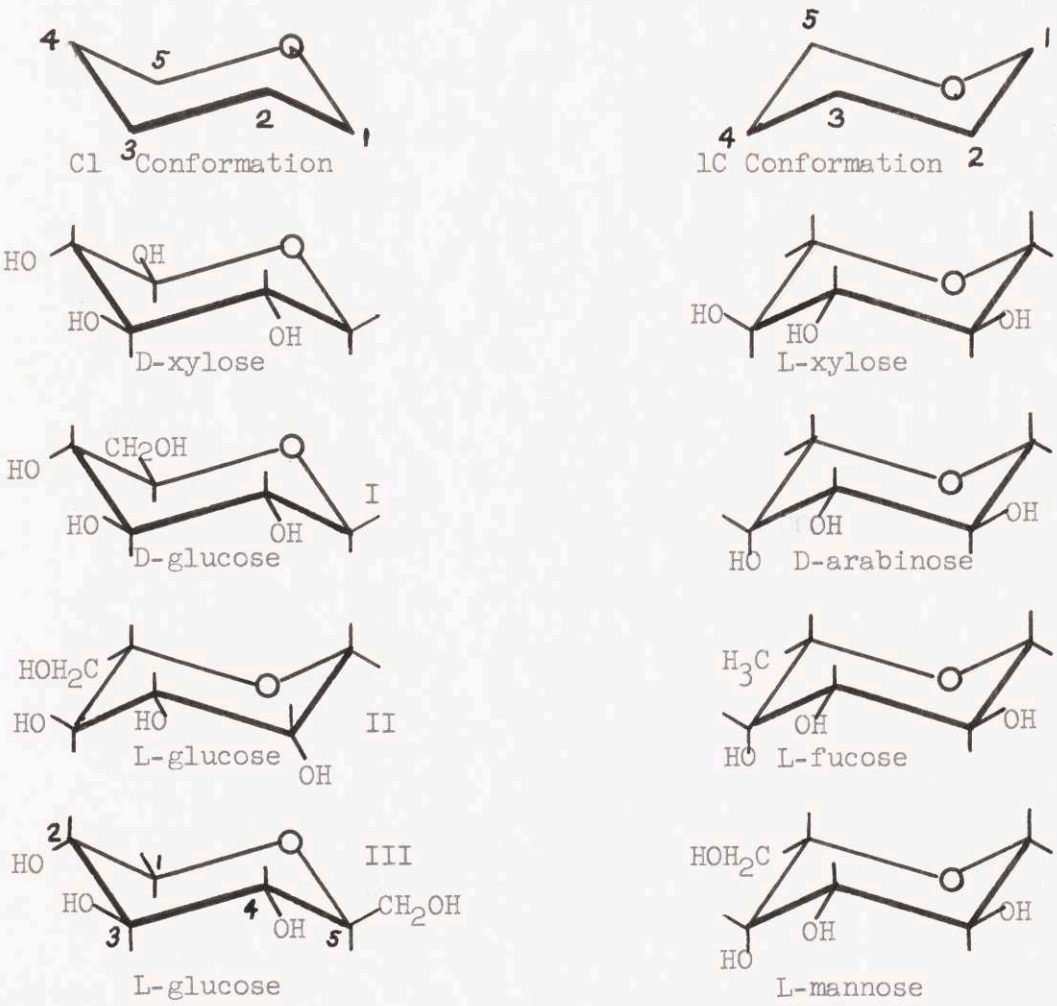
counterclockwise (1C) numbering of the carbon atoms, or as carbon 1 pointing downward (C1) or upward (1C).

Consider the case of D-arabinose, L-xylose, and L-mannose utilization by enzymes whose natural substrate is L-fucose. Referring to Figure 6, it can be seen that the D-arabinose molecule in the 1C conformation is exactly like 1C L-fucose with the exception of the equatorial methyl group on carbon 5. Thus, an enzyme utilizing hydroxyl group orientation should find D-arabinose a perfectly acceptable substitute for L-fucose. Oliver and Mortlock (1971) found Aerobacter aerogenes L-fucose isomerase to have 60 to 70% of its L-fucose activity on D-arabinose. The Michaelis constants (K_m) were 0.05M for D-fucose and 0.15M for D-arabinose and the maximum velocities (V_{max}) were identical. L-xylose has an equatorial hydroxyl at carbon 4 rather than the axial hydroxyl as in D-arabinose and L-fucose. The same A. aerogenes L-fucose isomerase on L-xylose has a K_m of 0.4M and V_{max} only 0.83% of that for L-fucose or D-arabinose. When A. aerogenes derived from the same strain (PRL-R3) is adapted to growth on L-mannose by relief of a metabolite inhibition (Mayo and Anderson, 1969), it has a generation time of two to three hours compared to about one and a half hours on L-fucose. Mortlock's (1971) L-xylose utilizer from the same wild type had a generation time of six to eight hours. Thus it appears that the carbohydroxy group on carbon 5 of L-mannose might improve enzyme activity over that on L-xylose. The influence of the equatorial hydroxyl on carbon 4 of L-mannose and L-xylose is not clear.

The correspondence of L-fucose and D-arabinose activities for the L-fucose enzymes is also noteworthy in E. coli K12 (LeBlanc and Mortlock, 1971). The L-fucose isomerase had 35-50% of its L-fucose activity on

FIGURE 6

CHAIR STRUCTURES OF PYRANOSES



All vertical substituents are axial and angled substituents are equatorial. The anomeric hydroxyls are omitted.

D-arabinose, the L-fuculose kinase had 150% (!) of its L-fuculose activity on D-ribulose, and the L-fuculose-1-P aldolase had 23% of its L-fuculose-1-P activity on D-ribulose-1-P. The induction of the operon was not as efficient, D-arabinose having 3-10% of the effectiveness of L-fucose as an inducer. It is not known whether this resulted from transport or from repressor affinity. In either case, structural influences vary with individual enzymes.

In the case of L-arabinose and D-fucose catabolism in Escherichia coli B/r, the repressor protein is less specific than the catabolic ones since D-fucose will hyperinduce the L-ara operon without being metabolized[9] (Beverin et al, 1971; Englesberg et al, 1966). An important structural difference in this case is that L-arabinose is the natural substrate of the enzymes and the new carbon source, D-fucose is one methyl group bigger...the reverse of the L-fucose, D-arabinose case.

Cirillo (1968) and Kotyk (1967) have evaluated the transport by Saccharomyces cerevisiae of a number of pentoses and hexoses in terms of their structure. They showed that each of the substituents on the glucopyranosering with the exception of the carbon 2 hydroxyl contributes to transport specificity. Their data are very consistent except for three important cases. The hexose transport system responsible for D-glucose uptake in S. cerevisia apparently also transports D-fructofuranose, L-sorbofuranose (Cirillo, 1968; Kotyk, 1967) and the linear D-mannitol molecule (Maxwell and Spoerl, 1970) at high rates.

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9 A mutation to D-fucose insensitivity is required. D-fucose is also a D-galactose analog, and it irreversibly traps UDP-galactose empimerase.

Cirillo has pointed out that the 1C conformation of L-glucose (structure II in Figure 6) can be turned upside down and end for end (III) to look very much like the C1 D-glucose (I). However, the carbohydroxy group and the anomeric carbon are on opposites sides of the cyclized oxygen atom. Thus, they present completely different structures to the active site of an enzyme. Cirillo measured the K_m of L-glucose uptake at $2x10^3$mM by its inhibition of L-sorbose uptake. This represents a vanishingly small affinity. Kotyk (1967) used a different strain of S. cerevisiae to measure the K_m at 10^{-2}mM and the V_{max} at 1 μg/(mg cells-min). At a yield of 0.5 g dry cells per g L-glucose, this rate would support a doubling time of 23 hours.

IV METHODS

A. Materials

Calbiochem L-glucose Lot No. 901873 was used for the organism enrichment and isolation. Sigma Chemical Co. L-glucose lots No. 120C-0450 and 32C-2330 were used for the remaining studies. The radioactive L(1-^{14}C)-glucose used for the transport experiments was obtained from Nuclear-Chicago (Amersham/Searle) and had a specific activity of 16.7 μCi/mg. The uniformly labelled D(^{14}C)-glucose from the same source had a specific activity of 16.1 μCi/mg.

One quart of Dupont "Ludox" silica gel suspension was kindly given to us by Dr. R.P. Mortlock of the University of Massachusetts at Amherst. This material was intended for development of a carbon-free plating medium for organism storage.

B. Culture Media

The basal medium used for organism enrichment, isolation, and characterization has the composition listed in Table VII. Besides having the normal nutrient salts, note that it is supplemented with trace elements and vitamins (0.2mg/L). The major carbon sources were L-glucose or D-arabinose at 2.5 g/L. In addition, in the first stages of isolation and then present in diminishing quantities on subculturing, 1:1 D-glucose and L-fucose were added as shown in Table VIII. The broth medium used for initial enrichment was pH 6.4 and, if a lower pH was desired, it was adjusted to pH 3.5 with HCl. For the later growth rate and yield studies, pH 6.8 (6-CFM) and pH 3.5 (3-CFM) carbon-free media were made directly by proportioning 0.05 molar KH_2PO_4 and K_2HPO_4 buffers. The CFM media were used directly for controls and blanks and 50 or 100 g/L sugar solutions were added for carbon sources.

TABLE VII

BASAL MEDIA COMPOSITION FOR L-GLUCOSE ORGANISMS

Component	Final Concentration in 6-CFM[a]	Final Concentration in 3-CFM[b]
Nutrient Salts	g/L	g/L
$(NH)_2SO_4$	1.2	1.2
K_2HPO_4	7.0	
KH_2PO_4	3.0	3.0
$MgSO_4 \cdot 7H_2O$	0.1	0.1
$CaCl_2$	0.04	0.04
NaCl	0.1	0.1
Trace Elements	Mg/L	Mg/L
$FeCl_3.6H_2O$	7.5	7.5
$ZnSO_4.7H_2O$	7.0	7.0
$MnSO_4.4H_2O$	5.5	5.5
$CuSO_4.5H_2O$	1.0	1.0
$CoSO_4.7H_2O$	1.0	1.0
H_3BO_3	1.0	1.0
$Na_2MoO_4.2H_2O$	2.5	2.5
KI	2.0	2.0

(Table VII continued on next page)

[a] pH 6.4 to 6.8 Carbon-Free Medium

[b] pH 3.2 to 3.5 Carbon-Free Medium

TABLE VII (continued)

Component	Final Concentration in 6-CFM[a]	Final Concentration in 3-CFM[b]
Vitamins	ug/L	ug/L
Riboflavin	25.0	25.0
Folic Acid	15.5	15.5
Biotin	2.0	2.0
Thiamine	40.0	40.0
Pyridoxine	44.5	44.5
Pyridoxamine	34.5	34.5
Calcium Pantothenate	39.5	39.5
Nicotinic Acid	27.0	27.0
Inositol	25.5	25.5
p-Aminobenzoic Acid	8.0	8.0
Other[c]	Ml/L	Ml/L
HCl, 37 wt.% aqueous	0.3	0.3
NaOH, 0.2 N	0.625	0.625

[c] HCl for trace salt and riboflavin dissolution, NaOH for folic acid and biotin dissolution.

TABLE VIII

CARBON SOURCES IN L-GLUCOSE UTILIZER ENRICHMENT MEDIA

Sugar	Stage 1 g/L	Stage 2 g/L	Stage 3 g/L
D-glucose	0.025	0.0025	0.00
L-fucose	0.025	0.0025	0.00
L-glucose or D-arabinose	2.50	2.50	2.50

The agar form of this medium was made by adding 15 g/L Difco Bacto-Agar to the nutrient and trace salt solution, autoclaving at 15 psig steam for fifteen minutes and cooling to 45°C. Immediately upon cooling, stock vitamin and carbon source solutions were added aseptically, and the plates poured. Control (blank) agar plates are made in identical fashion without the addition of carbon source.

Because of the expense of L-glucose, these plates were poured very thin and incubated in hydraters to keep them from dessicating.

C. Wild Microbial Samples

Twelve wild microbial populations were collected as soil, hay, and aquatic mud samples of about 250 ml bulk volume from the sites listed in Table IX on the following page. The dry samples were humidified overnight by addition of five weight per cent sterile distilled water. All samples containing solids were suspended in sterile broth medium 6-CFM (See Table VIII) without carbon source and coarsely filtered through cheese-cloth and glass wool. One ml of this filtrate was inoculated into 50 ml of medium in 250 ml shake flasks for the first culture stage.

D. Cultivation Conditions

Samples were incubated in shake flasks at 55°C, 37°C and 30°C. At each temperature, initial incubations were at pH 6.8, cultures developing yeasts or molds were enriched at pH 6.8 and at pH 3.5.

E. Organism Enrichment

Stage 1 cultures were monitored for growth by visible turbidity and inspected microscopically for organism type. When turbidity developed, the culture was transferred to stage 2 but maintained in stage 1 for further inspection. If an obvious shift in population type was later observed, the culture was inoculated into another stage 2 flask. If mycelia

TABLE IX

NATURAL POPULATION SAMPLING SITES

Sample Type	Sample Number	Site
Soil	1.	Soil under rotting leaves from woods by Quanapowitt Yacht Club, Wakefield, Mass.
	2.	Soil under rotting leaves from bushes on median lawn, Memorial Drive, Cambridge, Mass.
	3.	Soil under rotting leaves along North Ave. at Rte. 128 intersection, Reading, Mass.
	4.	Soil under peat moss mulch, yews on NE side of building 6, MIT.
Hay	1.	Hay from field beside municipal disposal area, Reading, Mass.
	2.	Dried grass from median lawn, Memorial Drive, Cambridge, Mass.
Mud	1.	Bottom mud and water from Lake Quanapowitt, Wakefield, Mass.
	2.	Bottom mud and water from Charles River, MIT sailing pavillion.
Sputum	1.	Sputum and oral epithelial tissue scrapings from experimenter.
Air	1.	Airborne organisms collected 48 hrs. in open 100 cc beaker on lab bench 16-141.
	2.	Airborne organisms similarly collected on a magnetic stirrer which kept medium at 40°C.

or yeastlike cells were observed, the culture was inoculated into another stage 2 flask at pH 3.5. Stage 3 cultures were similarly developed from stage 2. Stage 3 cultures were considered negative and discarded if visible turbidity did not appear in ninety days.

F. Organism Isolation

Samples of appropriately diluted broth culture were spread on plates of the defined L-glucose medium with 1.5% Difco Bacto-Agar. Duplicate samples were spread on plates of Trypticase-Soy-Yeast Extract agar supplemented with 1 g/L dextrose (TSY) for bacteria or Sabouraud Dextrose agar (SDA) for yeasts and molds. Single colonies were transferred from the L-glucose plate to fresh, sterile L-glucose broth as soon as the colonies were big enough to assure isolation. A culture was considered pure after a minimum of three such transfers.

G. Organism Identification

The bacterial isolates analyzed were keyed to the taxonomic system of Bergey(Breed et al, 1957), (Skerman, 1967) using the media and reagents detailed in Table X. The yeast-like isolates analyzed were keyed to the taxonomic system of Lodder (1971) by the methods shown in Table XI.

H. Carbohydrate Analyses

L-glucose was analyzed for purity by optical rotation, by liquid chromatography on a three foot Bio-Rad AG-50-W140 cation exchange resin with ethanol-water solvent, and by paper chromatography with 5:1:2 n-butanol: acetic acid: water solvent. L- and D-glucose concentrations in broths were measured by the anthrone-sulfuric acid colorimetric method detailed by Wood (1966). D-glucitol concentrations were measured by an adaptation of this method (Graham, 1963) utilizing a longer color development and absorption at a different wavelength.

TABLE X

TAXONOMIC TESTS METHODS FOR BACTERIAL ISOLATES

Method	Reference	Organism Tested	Control
Gram Stain	a	RF-1,2,3,4,6,9,10,11	Mouth scrapings
Motility, microscopic inspection		all	no control
SIM agar	b	RF-1,2,3,4,6,11	Ps. aeruginosa
Flagellation, Leifson's flagella stain	c	RF-1,2,3,6	Ps. aeruginosa
Endospores, microscopic inspection		all	
Acid fastness	c	RF-1,2,3,4,6,11	no control
Aerobiosis, SIM agar	b	RF-1,2,3,4,6,11	E. coli
TSI agar	b	RF-1,2,3,4,6	E. coli
Hugh and Leifson	c	RF-1,2,3,4,6	E. coli
Anaerobic jars		RF-1,2,3,4,6	E. coli
Nitrogen fixation, Skerman	c	RF-1,2,3,4,6	E. coli
Agar hydrolysis[d]			
Catalase, Topley-Wilson	b	RF-1,2,3,4,6,9,10,11	no control
Oxidase, Kovacs	c	RF-1,2,3,4,6	E. coli
Ewing	c	RF-1,2,3,4,6	E. coli
H_2S evolution, TSI agar	b	RF-1,2,3,4,6	none

(Table X continued on next page)

TABLE X (continued)

Method	Reference	Organism Tested	Control
Acid and gas on glucose,			
Hugh and Leifson	c	RF-1,2,3,4,6	E. coli
TSI agar	b	RF-1,2,3,4,6	E. coli
Brom-cresol Purple agar	b	RF-1,2,3,4,6	E. coli
Gas evolution on lactose	b	RF-1,2,3,4,6	E. coli
Indole, SIM agar	b	RF-1,2,3,4,6,9,10	no control
Urease, Brilliant Green Bile agar	b	RF-1,2,3,4,6	no control
Urea agar base	b	RF-1,2,3,4,6	no control
Methyl Red and Voges-Proskaues			
MRVP agar	b	RF-1,2,3,4,6,11	no control
10% alcohol medium	c	RF-1,2,3,4,6	no control
40% Bile Salts medium	c	RF-4	no control
Blood agar	b,c	RF-4	Staph. aureus

a.
Harrigan and M^{c}Cance, 1966.

b.
Difco Manual, 1953.

c.
Skerman, 1967.

d.
Observation of liquefaction and deep pitting of agar surface. Some organisms grew slightly on agar blanks but this was not considered positive.

TABLE XI

TAXONOMIC TEST METHODS FOR YEAST ISOLATES

Test	Reference	Organisms Tested	Control
Growth and Presporulation morphology			
YM broth	a	RF-7, 13, 14	Sacc. cerevisiae Candida utilis
Colony morphology			
Wort agar	b	RF-7, 13, 14	S. cerevisiae Rhodotorula rubra
Carbohydrate utilization			
Yeast Nitrogen Base	b	RF-7, 13, 14	microscopic inspection, plating[e]
Nitrate Assimilation			
Yeast Carbon Base	b	RF-7, 13, 14	C. utilis
Vitamin-Free Growth	b	RF-7	vitamin + medium
	c	RF-13, 14	vitamin + medium
Amyloid Formation			
Lugol's Iodine	a	RF-7, 13, 14	no control
Urease			
Urea Agar Base	b	RF-13, 14	no control
Temperature Range			
Yeast Nitrogen Base with 0.5% D-glucose		RF-7, 13, 14	
Acid-fast Structures			
Ziehl-Neelsen Stain	d	RF-13, 14	no control

a. Lodder, 1970
b. Difco Manual, 1953
c. Pfaff et al, 1966
d. Skerman, 1967
e. Contamination check after positive utilization.

I. Growth Rate and Lag Phase Measurements

250 ml culture flasks with Klett tube sidearms containing the sterile defined basal medium 6-CFM supplemented with the appropriate carbon source were preincubated at 30°C. Lag phase measurements were made by aseptic introduction of enough exponentially growing inoculum to give a Klett reading of 50 - 60. The absorption was monitored at appropriate time intervals with a Klett-Summerson absorption meter with the red filter (640-700 mμ).

Lag phase 1 was measured from the time of inoculation to that of the intersection of the initial growth line with the horizontal line representing the initial absorption of the culture. Lag phase 2, where it differs appreciably from lag phase 1, was measured from the time of inoculation to the onset of exponential growth. Measurements of lag phases 1 and 2 are shown on Figure 12 in section IV, RESULTS.

Growth rates in the carbohydrate utilization phases of this study were obtained on the same or similar cultures. The growth rates used for the kinetic plots were obtained similarly, but with inocula of 10^2 to 10^3 cells/ml. Cell number was then monitored at appropriate intervals by TSY plate counts up to Klett absorption of about 10 and by Klett-Summerson absorption meter thereafter.

J. Cell Concentration

Cell dry weights (CDW) were obtained by centrifuging the cells from the broth at 1,000 x G, 15 minutes, decanting the supernate and washing the cells once with distilled water in a similar fashion. The cells were then washed from the centrifuge tube with distilled water, evaporated to dryness without browning in an aluminum weighing dish at 100°C and dried to constant weight in a dessicator (usually about 24 hours).

Cell numbers were obtained by plate count on TSY agar and by visual microscopic count in a Petroff-Hauser counting chamber. In the latter case, the cells were diluted one to ten with 10% formaldehyde to arrest motility and placed on the chamber with an inoculating loop. The cover slip was settled into place and the slide was left for fifteen minutes to permit the cells to settle to the bottom of the chamber. A minimum of 600 cells was counted for each of three chamber preparations. Cell weight and number were calibrated to Klett absorption meter readings separately for fast growing (<10 hr. doubling) and for slow growing (>15 hr. doubling) cultures.

K. Cell Yield

Yields were measured on exponentially growing cells over three to four doublings of cell mass. Assuming 0.5 gCDW/g L-glucose, makeup L-glucose was added to the broth to keep the concentration $\pm$ 20% of the stated value. Yield was calculated from the dry cell weights (CDW) and L-glucose concentrations as:

$$\text{Yield } \frac{\text{g cells}}{\text{g L-glucose}} = \frac{(\text{CDW harvested}) - (\text{CDW inoculated})}{(\text{L-glucose fed}) - (\text{residual L-glucose})}$$

L. Carbon Starvation Experiments

Cells were grown at 5 g/L L-glucose in pH 6.8 medium (6-LGM). When CDW reached about 1 g/L by Klett measurement, the cells were harvested by centrifugation at 1000xG, fifteen minutes, 30°C. They were washed once by resuspension in 6-CFM, then similarly recentrifuged. This pellet was resuspended in sufficient 6-CFM to give 1.7 to 2 g/L CDW, placed in a 250 ml baffled sidearm shake flask, and incubated on a shaker table at 30°C. The Klett absorption of this flask was monitored throughout the experiment. At appropriate time intervals, portions of the carbon starved suspension were withdrawn and inoculated into fresh 6-LGM in 250 ml baffled sidearm

flasks. Lag time and growth rate were measured on these flasks by Klett absorption.

M. Transport Rate Measurements

Cells were grown exponentially in 5 g/L 6-LGM. When the culture reached about 0.75 g/L cells, additional L-glucose was added to return the concentration to 5 g/L. A yield of 0.5 g CDW g L-glucose was assumed for this makeup. When the culture reached 1 to 1.2 g/L (about four to six hours later), the cells were harvested by centrifugation at 1000xg, fifteen minutes at 30°C. The centrifugate was sampled for residual L-glucose analysis. The cells were resuspended in 6-CFM, then recentrifuged similarly. The cells were resuspended in enough 6-CFM to yield a suspension of about 2 g/L cells. This suspension was aseptically dispensed in 15 ml portions into 250 ml baffled sidearm Klett flasks which were incubated on a rotary shaker at 30°C. The remaining suspension (75 to 100 ml) was used for CDW determination. This large CDW sample was used to minimize inaccuracy in cell concentration since specific transport rates were to be obtained. When two hours had elapsed since decanting the growth medium from the first centrifuge pellet, the series of transport measurements was started. The experiments in each group were confounded by random number tables. A typical group and its confounded order is shown in Table XII. Alternate flasks were sampled for cell number by plate count.

Measurement was initiated by pipetting radioactive carbohydrate solution into the flask. At suitable time intervals, typically 0.5, 1, 1.5, 2.0, 2.5, 3.0, 4.0, and 10.0 minutes, 0.5 ml samples were withdrawn and diluted into 5 ml of cold (nonradioactive), 0°C 6-LGM containing the same carbohydrate concentration as the uptake vessel. This 5.5 ml was immediately poured onto a 25 mm 0.22μ Millipore filter under suction, and, as

TABLE XII

TYPICAL GROUP OF TRANSPORT MEASUREMENTS

Radioactive Species	Concentration G/L	Remarks	Order of Performance
L-glucose	5.0		6
L-glucose	10.0		4
L-glucose	1.0		3
L-glucose	0.5		1
L-glucose	5.0	30 mM NaN_3	7
D-glucose	5.0		2
D-glucose	5.0	30 mM NaN_3	5

TABLE XIII

LIQUID SCINTILLANT FOR DRY SAMPLES

Component	Amount	Wt/Vol %
PPO[a]	8 gm	0.4
POPOP[b]	1 gm	0.05
Toluene	to 2 liters	

a 2,5 - Diphenyloxazole
b P-Bis(2-5- Phenyloxazolyl) - Benzene

soon as the liquid pulled through, the filter was washed with 1 ml cold, 0°C 6-LGM. Each filter was washed with 1 more ml cold, 0°C 6-LGM, removed, dried thirty minutes under a 250 watt infrared bulb at 14 inches, and inserted into a scintillation vial. Each scintillation vial was given 10 ml of the liquid fluor solution listed in Table XIII. The activity of each vial was then read for ^{14}C activity in a Beckman CPM-100 liquid scintillation counter.

Zero time samples of each uptake flask, taken prior to carbon source addition, were washed, filtered, dried, and read in idential fashion.

The specific activities of the labelled glucose solutions were determined in quadruplicate by drying 1 ml of the solution onto two 24 mm glass fiber filters (Reeve Angel 934AH) and reading the activity in the same manner as the samples. It was determined that one to three of these filters did not quench significantly.

Initial velocities were determined by plotting the reciprocal average velocities ($t/(cpm/gDCW)$) versus time and determining the zero time intercept by linear regression analysis. This method eliminates the innaccurate determination of initial velocity at the first data point and places equal weight on later data points for which the higher radioactivity can be more accurately counted (deRibichon-Szulmaster, 1961). Use of the straight line regression requires the assumption of saturation (Monod-type) kinetics.

N. Disposition of Carbon 1 of L (1-^{14}C)- Glucose

Cells charged with radioactive labelled L-glucose were washed with unlabelled 5 g/L 6-LGM medium the same as for the carbon starvation and transport runs. After two washes, they were resuspended in unlabelled 5 g/L 6-LGM. This suspension was dispensed 0.5 ml per flask into 10 ml unlabelled 5 g/L 6-LGM in baffled 250 ml flasks, two of which were equipped

with sidearms for Klett readings. Growth was monitored in the sidearm flasks. The entire flask was harvested for each sample, reserving the sidearm flasks for the last two samples. Each flask's contents was filtered on a 25 mm or 50 mm 0.22 um Millipore filter, depending on the cell concentration, shifted to a different filter flask, and washed with two 5 ml portions of 6-LGM. The filters were dried 1/2 hour under a 250w infrared bulb at 14 inches. One ml of the filtrate was absorbed onto two 24 mm (Reeve Angel 934 AH) glass fiber filter discs in 0.1 ml doses and the discs were similarly dried. The dried filters were placed in scintillation vials, irrigated with 10 ml of the liquid scintillation fluid in Table VIII, and the ^{14}C activity was counted on a Beckman CPM-100 liquid scintillation counter. Filtrate counts were multiplied by an elevenfold sampling factor.

V RESULTS

A. Natural Incidence of L-Glucose Utilizers

The three stage enrichment of the natural population samples on L-glucose yielded the results shown in Table XIV. The site of each sample is detailed under Methods (Table IX). Growth in stage 1 appeared in about five days and the samples were subcultured to stage 2 after ten to twenty-five days. The stage 2 cultures showed visible growth in about ten days and were subcultured to stage 3 at between two and six weeks. The stage 3 cultures showed visible growth in two to six weeks, with initial populations of small motile rods, a few spores, cocci, yeastlikes, and mycelia. The L-glucose agar plates spread at this time typically took five to ten days for the appearance of 0.5-1 mm colonies. Simultaneous plating of duplicate samples on control plates (no carbon source) typically showed no growth or pinpoint colonies 0.1 mm in diameter. In a group of 22 control plates, one of them showed growth equivalent to that on the corresponding L-glucose plate.

During this portion of the study, it was found that agars autoclaved at low pH (3.0 to 4.5) or stored overnight at 45° C, pH 6.8, supported growth as well or better than L-glucose plates. The purified Difco Bacto-agar is a polysaccharide containing D- and L- galactose residues, some of which are esterified with sulfate, and (1-4) linked 3, 6-anhydro-α-L-galactose (Lehninger, 1970; Duckworth & Yaphe, 1971). Identical pH 6.8 agar plates poured immediately after autoclaving grew these organisms poorly, indicating that most of the growth was due to agarolysis products. To forestall this problem, all L-glucose and control plates made thereafter were formulated at pH 6.5 and poured immediately after autoclaving.

Of the four soil samples, three contained organisms capable of

TABLE XIV

NATURAL INCIDENCE OF L-GLUCOSE UTILIZERS

Culture Source		Incubation Temp.°C.	Stage 1[a]	Stage 2[b]	Stage 3[c]	Control Broth[d]	L-glucose Plate[e]	Control Plate[f]
Soil	1	30	+	+	+	-	+	(-)[g]
	2	30	+	+	+	-	+	-
	3	30	+	+	+	-	+	(-)[g]
	4	30	+	+	-	-	0	0
	1	55	-	-	0	0	0	0
	2	55	+	+	-	0	0	0
	3	55	+	+	+	-	+	(-)[g]
	4	55	+	+	+	-	+	-
Hay	1	30	+	+	+	-	+	(-)[g]
	2	30	+	+	-	-	0	0
	1	55	+	+	+	-	+	(-)[g]
	2	55	+	-	0	0	0	0
Mud	1	30	+	+	-	0	0	0
	2	30	+	+	-	0	0	0
Air	1	30	0	0	+	-	+	(-)[g]
	2	42, 55	0	0	+	-	+	(-)[g]
Sputum								
	1	37	+	+	+	-	+	+[h]

\+ Significant growth
\- No growth
0 Not cultured

a. 0.025 g/L natural sugars.
b. 0.025 g/L natural sugars.
c. No natural sugars.
d. Complete medium without sugar.
e. Agar medium with L-glucose.
f. Agar medium with no sugar.
g. Slight growth.
h. Slow growth.

utilizing L-glucose at 30° C, and two contained thermophiles. L-glucose utilizers were also in some of the hay, air, and sputum samples. Neither of the mud samples yielded L-glucose utilizers when enriched by our method.

Two soil samples taken one year later at sites #2 and #4 were treated identically except for the ommission of the natural sugars, D-glucose and L-fucose, from the stage 1 and 2 media. These samples showed slight turbidity in the first flask at fourteen days, but did not grow in subcultures.

Since L-glucose utilizers were obtained and D-arabinose utilizers are not difficult to find, the D-arabinose utilizers were discarded.

At this stage of the project, sixty-eight mixed cultures were being maintained at 30°, 37°, and 55° C, pH 3.5 and 6.8.

B. L-glucose Utilizing Organisms

Eleven microorganisms were isolated from the enrichment cultures. After a minimum of 3 single colony isolations on L-glucose agar alternated with L-glucose broth cultivations as soon as the colonies were visible, these isolates were assigned strain numbers as listed in Table XV. Classification tests consistent with the Bergey system (Breed et al, 1957; Skerman, 1967) were run on seven of the bacteria as reported in Table XVI.

All of the bacteria examined were catalase postive, methyl red negative, Voges Proskauer negative, and indole negative. They are all quite versatile in carbon source utilization. RF-9 does not grow at 37° C and grows up to 65° C whereas RF-10 prefers temperatures of 37° and 45° C and will not grow above about 60° C. The gram positive coccus (RF-4) prefers 37° C over 30° C and does not grow anaerobically on TSY or Nutrient agar.

About 0.5 g RF-2 colonies from a TSY plate was suspended in petroleum ether, extracted for 2 hours, and centrifuged. The absorption of the supernate was then read at 418, 437, and 463 mu. No absorption was found.

TABLE XV

L-GLUCOSE UTILIZING MICROORGANISMS

Strain Number	Form	Temperature °C	Gram Reaction	Source
RF-1	Bacterial rod	30	-	air
RF-2	Bacterial rod	30	-	soil
RF-3	Bacterial rod	30	-	soil
RF-4	Coccus	37	+	sputum
RF-6	Bacterial rod	30	-	hay
RF-7	Yeast	30	nd	soil
RF-8	Mycelium	30	nd	air
RF-9	Bacterial rod	55	nd	soil
RF-10	Bacterial rod	55	+	soil
RF-11	Bacterial rod	30	-	soil
RF-13	Yeast	30	nd	soil
RF-14	Yeast	30	nd	soil

nd. Not determined.

TABLE XVI

CHARACTERISTICS OF L-GLUCOSE UTILIZING BACTERIA

STRAIN NUMBER	RF-1	2	3	4	6	9	10	11
Source	Air	Soil	Soil	Sputum	Hay	Soil	Soil	Soil[9]
Form	rod	rod	rod	coccus	rod	rod	rod	rod
Gram reaction	-	-	-	+	-	+	+	-
Endospores	-	-	-	-	-	+	+	-
Motility	+	+	+	-	+	+	+	+
Flagellation[h]	polar	per.	ND	none	polar	ND	ND	ND
Acid Fastness	-	-	-	-	-	-	-	-
Colony Color--								
Rich agar	yellow	yellow	tan	white	tan	tan	tan	yellow to pink
L-glucose agar	hyaline	yellow[f]	hyaline	white	tan	tan	tan	pink
Temperature °C	30	30	30	37[a]	30	55	55[b]	30
Aerobiosis	obligate	obl.	obl.	obl.	obl.	facult.	facult.	obl.
N_2Fixation	-	-	f	-	-	ND	ND	ND
Agar hydolysis	-	-	-	+	-	+	-	ND
Catalase	+	+	+	+	+	+	+	+
Oxidase	+	+	+	-	+	+	+	+
H_2 evolution	-	-	-	-	+	-	-	ND
Acid (on D-glucose)	+	+	-d	+	+	+	+	ND
Gas (on D-glucose)	-	-	-	-	-	-	-	ND
Indole	-	-	-	-	-	-	-	ND
Urease	+	-	-	-	-	-	-	ND
Methyl Red	-	-	-	-	-	ND	ND	ND
Voges-Proskauer	-	-	-	-	-	ND	ND	ND

(Table XVI continued on next page)

TABLE XVI (Cont.)

STRAIN NUMBER	RF-1	2	3	4	6	9	10	11
Carbon Assimilation								
D-glucose	+	+	+	+	+	+	+	+
D-fructose	+							
L-sorbose	+	-	+	+	+	ND	ND	ND
L-fucose	+	+	+	+	+	ND	ND	ND
D-fucose	-	ND	ND	ND	ND	ND	ND	+
L-rhamnose	+	+	+	+	+	ND	ND	ND
D-arabinose	+	+	+	+	+	ND	ND	ND
L-arabinose	+	+	+	+	+	ND	ND	ND
D-ribose	+	+	+	+	+	ND	ND	ND
D-xylose	+	+	+	+	+	ND	ND	ND
L-xylose	+	ND	ND	ND	ND	ND	ND	ND
D-glucitol	+	+	+	+	+	+	ND	+
D-mannitol	+	ND	+	ND	ND	ND	ND	ND
D-arabitol	+	ND	+	ND	ND	ND	ND	ND
L-arabitol	+	ND	+	ND	ND	ND	ND	ND
D-ribitol	+	ND	ND	ND	ND	ND	ND	ND
xylitol	+	ND	+	ND	ND	ND	ND	ND
lactose	+	+	+	+	+	+	+	+
Saccharose	+	+	+	+	+	ND	ND	ND
Hexadecane	+							
10% ethanol	-	-	-	+	-	ND	ND	ND
Growth in Bile Salts	ND	ND	ND	-	ND	ND	ND	ND
Blood Agar greening	ND	ND	ND	-	ND	ND	ND	ND

a. Isolated at 30° C.
b. Lower temp. preferred
d. Alkaline reaction on Brilliant Green agar.
f. Slight
g. Derivative of RF-1.
h. By Leifson's stain; not definitive.
nd. Not determined.

Taxonomic tests on the three yeasts are shown in Table XVII. RF-14 is unusual in that its colonies are black as soon as they become visible.

When growing on L-glucose, RF-14 appears to have stalks between the buds and mother cells as depicted in Figure 7. Most separate cells are ovoid, 2-6 μ in diameter, and a few are appiculate. It is much slower to emerge on agar and broth media than RF-13, typically not visible in 48 hours on rich presporulation medium. After 5 days, however, turbidity and colony size are about the same for RF-13 and 14. On rich broth media, reproduction is exclusively by multilateral budding. Two week old colonies on yeast extract - malt extract agar develop green-grey-white aerial mycelia with some elongated, branching cells and arthrospores (?). Even in these mycelia, however, single cells with multilateral buds predominate. Growth on Kleyn's, Fowell's, potato dextrose, and Cornmeal sporulation agars was essentially the same, but not as heavy.

RF-13 grows quickly on broth or agar media. After 24 hrs. at 30° C in presporulation medium, cells are almost spherical to ovoid. Size is 4-5 μ in diameter. Growth is dense at the surface, with some turbidity, and a white deposit at the bottom. Surface growth is not a pellicle; it settles at a slight jar. Reproduction is by multilateral budding, no mycelia or pseudomycelia were observed. After 3 days at 25° C, cells have a similar appearance, but more variability in size (3-7 μ dia.) A few chains of three to six cells might be considered very primitive pseudomycelia, but there is no other indication of a different vegetative morphology. No sporulation was observed in colonies up to 90 days old on Yeast-Malt extracts, Wort, Fowell's, Kleyn's or Cornmeal agar. Old colonies were tan in color.

RF-7 grows well on Wort agar, producing 0.5-0.8mm creamy white colonies in twenty-four hours at 30° C. It also grows well at 37° C. After seven

TABLE XVII

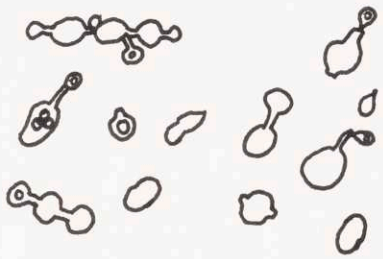
CHARACTERISTICS OF L-GLUCOSE UTILIZING YEASTS

	RF-7		13		14	
Source	Soil		Soil		Soil	
Vegetative Form	Bud-fission, Mycelia		Multilateral buds		Sterigma?, Multilat. buds, Mycelia	
Pseudomycelia	+		v. primitive		v. primitive	
Ascospores	+		-		?	
Arthospores	+		-		?	
Ballistospores	-		-		-	
Color (on Wort Agar)[a]	cream		white		black	
Carbohydrate Utilization	Growth	Gas	Growth	Gas	Growth	Gas
D-galactose	+	-	+	-	+	-
D-glucose	+	weak	+	-	+	-
L-glucose	+	-	+	-	+	-
D-xylose	nd	nd	+	-	+	-
i-inositol	nd	nd	+	-	slow	-
D-glucitol	+	nd	+	-	+	-
Cellobiose	nd	nd	+	-	nd	nd
Lactose	+	-	+	-	v.slow	-
Maltose	+	-	+	-	+	-
Melibiose	+	-	+	-	slow	-
Raffinose	+	-	+	-	+	-
Sucrose	+	weak	+	-	+	-
Trehalose	nd	nd	+	-	+	-
Salicin	nd	nd	+	-	+	-
Nitrate Assimilation	-		-		+	
Growth without vitamins	+		+		-	
Starch formation	-		-		-	
Urease	nd		-		+	
Temperature 4° C	nd		-		-	
20° C	nd		slow		+	
25° C	+		++		+	
30° C	++		++		++	
37° C	+		++		-	
44° C	nd		trace		-	
55° C	nd		-		-	
Acid-fast Structures	nd		-		-	

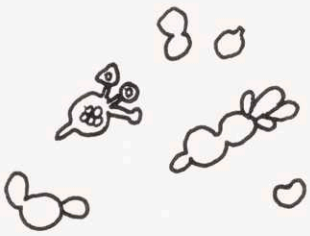
a. Young colonies
nd. Not determined

FIGURE 7

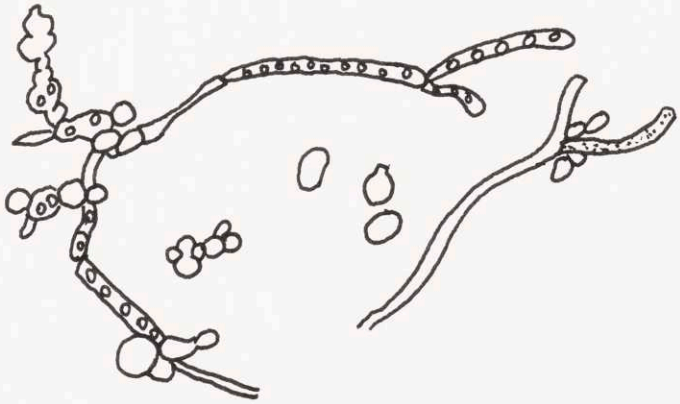
SKETCHES OF THE L-GLUCOSE-UTILIZING YEAST, RF-14



Growth on L-glucose



48 hours on malt-yeast extract



14 days on wort agar

5μ

days at room temperature, the dull white colonies are 3 to 8 mm, have raised centers and a hard folded brainlike surface with powder in the folds. The interior is creamy and soft. Mycelia, pseudomycelia, ascospores, and arthrospores are present as sketched in Figure 8. The asci contain two to four sperical spores per ascus, but occasionally one to seven. Vegetative growth in young cultures is almost exclusively by bud fission, but when broth becomes obviously turbid, many true mycelia are present. On rich broth media, a pellicle forms only in old cultures, but growth on L-glucose is characterized by pellicle formation above about Klett=40. RF-7 does not grow anaerobically on Wort, Yeast-Malt extract, or SDA agars.

Tentative identification of ten of the L-glucose utilizers based on the tests and observations, is in Table XVIII.

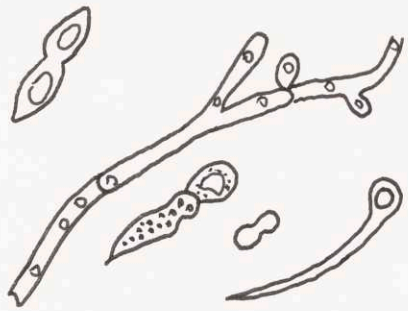
To this point, no organism had been successfully transferred from TSY or SDA agar plates to L-glucose broth even at quite high inoculum densities (Klett=50). Similar lack of outgrowth was observed when transferring colonies from L-glucose agar to L-glucose broth if the colonies were left on the agar more than about ten days. The most dependable means of colony isolation and recultivation was found to be to transfer the colony from L-glucose agar as soon as it reached recognisable size (0.3 to 1 mm), about 3 to 8 days.

During the isolation work with L-glucose and blank agar plates, the incidence of agar utilizers, as evidenced by good growth on the blank agar increased from 1/22 to 7/18.

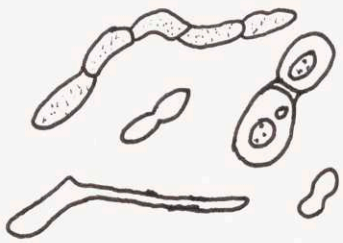
Some organisms isolated from the L-glucose plates spread from the third stage enrichment were lost during further cultivation. For example, a gram positive non-acid-fast rod never observed to sporulate in D-glucose-limited cultures 24 to 96 hours old disappeared during the L-glucose broth

FIGURE 8

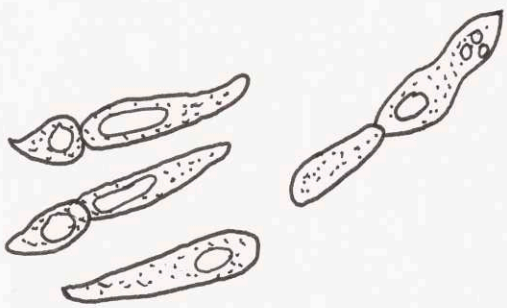
SKETCHES OF THE L-GLUCOSE-UTILIZING YEAST, ENDOMYCOPSIS SP., RF-7



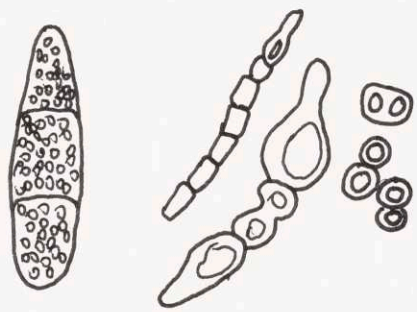
Growth on L-glucose



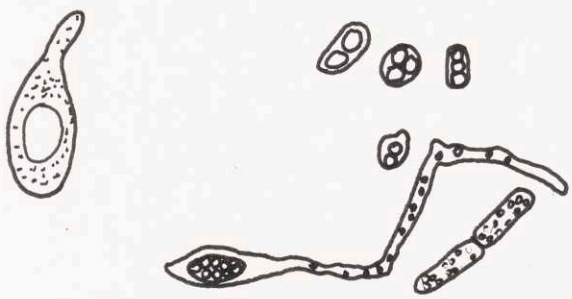
Growth in malt-yeast extract



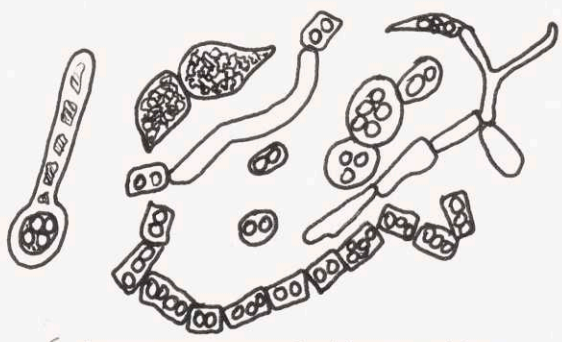
48 hours on malt-yeast extract



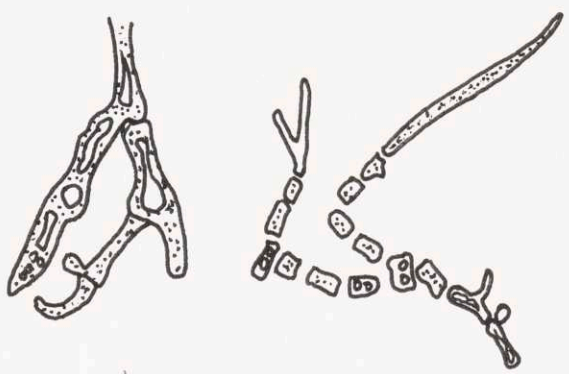
6 days on malt-yeast extract



48 hours on sporulation medium



6 days on sporulation medium



14 days on wort agar

TABLE XVIII

IDENTITY OF L-GLUCOSE UTILIZING MICROORGANISMS

STRAIN NUMBER	FORM	TENTATIVE IDENTIFICATION
RF-1	ROD	PSEUDOMONAS SP.
RF-2	ROD	FLAVOBACTERIUM SP.
RF-3	ROD	PSEUDOMONAS SP.
RF-4	COCCUS	MICROCOCCUS SP.
RF-6	ROD	PSEUDOMONAS SP.
RF-7	YEAST	ENDOMYCOPSIS
RF-8	MYCELIA	
RF-9	ROD	BACILLUS STEAROTHERMOPHILUS
RF-10	ROD	BACILLUS SUBTILIS
RF-11	ROD	PSEUDOMONAS SP.
RF-13	YEAST	TORULOPSIS CANDIDA
RF-14	YEAST	

enrichment from L-glucose agar colonies. A white mycelial thermophile was repeatedly plated from mixed cultures on L-glucose broth and only an organism with the properties of Bacillus stearothermophilus grew up. Similarly, a recurring small yeast-like organism in two of the 55° C broth cultures never grew on L-glucose or TSY plates and thus was never isolated.

At this stage of the project, 87 mixed cultures were being maintained.

Ninety day incubations of Aerobacter aerogenes isolated from an aquatic mud provided by Dr. C.L. Cooney[10], Hansenula holstii isolated on methanol as sole carbon source by Mr. D.L. Levine[10], and Bacillus Stearothermophilus provided by Dr. Randolph Greasham[10] all failed to show growth. Ceratocystis moniliformis was incubated with L-glucose for two weeks by Miss Elaine Y. York[10] and it did not grow in this time.

C. L-glucose Analyses

Purity of L-glucose has been established by:

1. Analysis reported by the supplier.
2. Liquid chromatography on cation exchange resin paper chromatography in our own laboratory.
3. Optical rotation.

Calbicchem, the supplier of L-glucose lot #901873 used in the enrichment and early isolations reports that the material is chromatographically homogeneous, contains 0.00% D-glucose, and has a specific optical rotation $(\alpha)_D^{20^\circ} = -51.3$. The 6.25 wt. % stock solution was chromatographed at MIT on a three foot column packed with Bio-Rad AG-50-W140 cation exchange resin with ethanol-water elution solvent. Under the same conditions found to give baseline resolution of ribose, glucose and fructose, at better than 5 mg/L

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[10] MIT personnel.

sensitivity, the L-glucose had retention time identical to that of D-glucose. There were no extraneous peaks or shoulders. The specific optical rotation was measured at 12.50 g/L to be $(\alpha)_D^{20^\circ} = -51.2^\circ$, ± 0.4. $(\alpha)_D^{20^\circ}$ is reported to be -51.4° for L-glucose (CRC Handbook, 1956).

Sigma Lot 120C-0450 was found to be chromatographically homogeneous with retention characteristics identical to D-glucose by paper chromatography using n-butanol/acetic acid/water (5:1:2) solvent. The specific optical rotation was measured at 15 g/L to be $(\alpha)_D^{20^\circ} = -51.1^\circ$, ± 0.5.

The anthrone-sulfuric acid method was found to analyze for L-glucose down to about 0.5 μg/ml in the presence of up to 5 g/L D-glucitol without interference. An adaptation of the anthrone method developed for polyol analysis by Graham (1963) was found to analyze D-glucitol alone down to about 0.05 mg/ml, but L-glucose at 25 to 50% of the D-glucitol concentration interferes.

D. Growth on L-Glucose

Initial growth rate and yield data for the yeast, RF-7, and the pseudomonad, RF-1, showed doubling times of 43 to 48 hours and yields of 0.16 to 0.22 g CDW/g L-glucose. The thermophilic bacillus, RF-9 had doubling times ranging from 12 to 40 hours in different cultures under similar conditions, with yields ranging from 0.10 to 0.28 g CDW/g L-glucose. RF-9 repeatedly started sporulating at Klett values of 90 to 125 (0.3 to 0.5 g/L), even with 0.5 to 1.0 g/L L-glucose remaining in the medium. One case, where growth and L-glucose concentration were monitored is shown in Figure 9. Substantial numbers of spores were present at all times. This bacillus was judged too erractic in growth and sporulation patterns, and the yeasts grew only in pellets above Klett about 100 (0.4 g/L), so further work was based on the 30° C pseudomonad, RF-1.

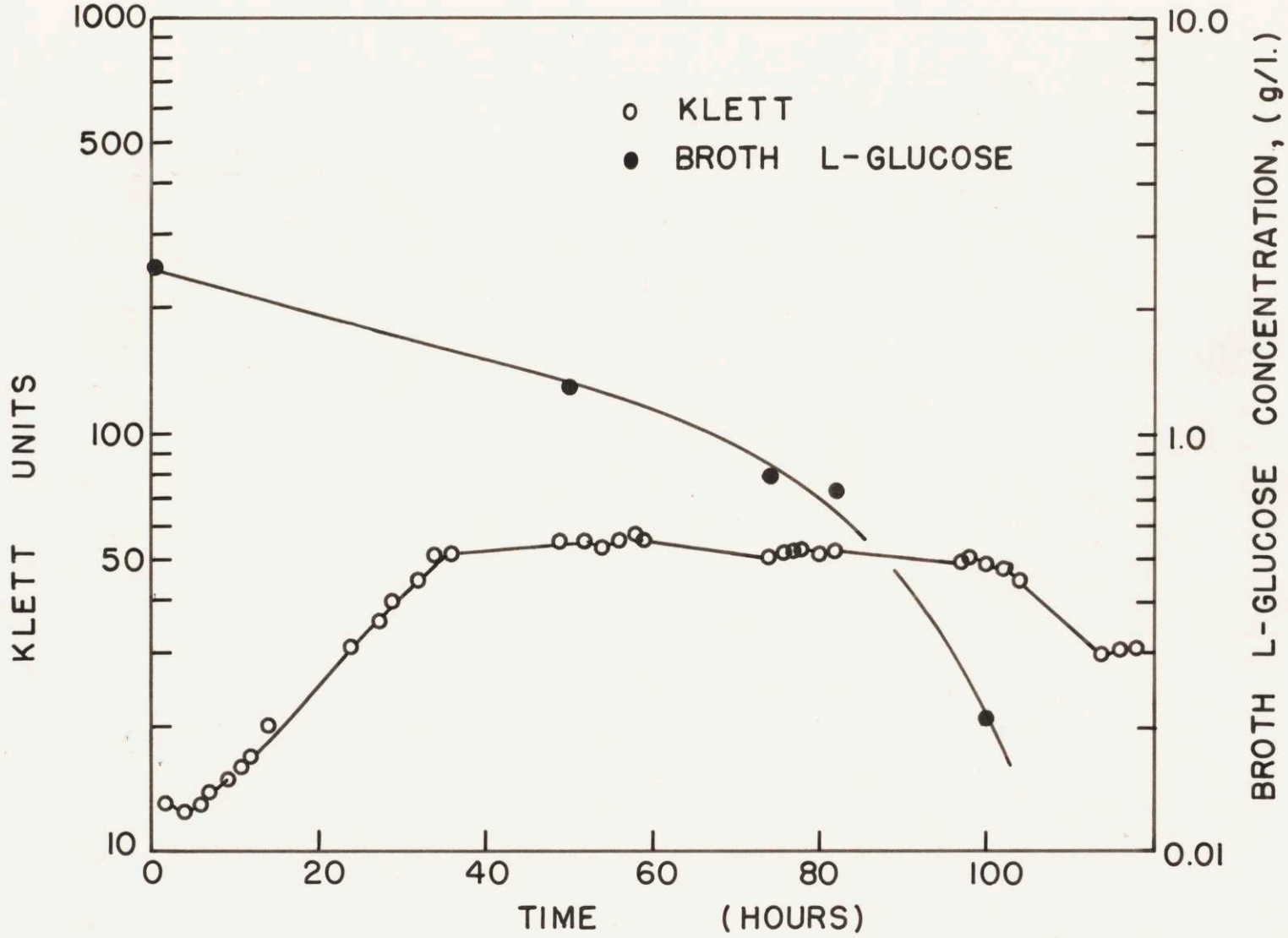


FIGURE 9.- CELL GROWTH AND RESIDUAL L-GLUCOSE CONCENTRATION OF <u>BACILLUS STEAROTHERMOPHILUS</u>. RF-9.

Initial L-glucose broth cultures of RF-1 gave colony counts on L-glucose agar about 50% less than on Trypticase Soy Yeast Extract (TSY) agar, indicating a significant number of non-reproducing cells among the L-glucose utilizers. Repeated inoculation of fresh sterile medium from growing cultures reduced this difference in plate counts, and after the third transfer, doubling times of 25 to 29 hours with a yield of 0.3 grams of dry cells per gram of L-glucose consumed were measured. Three more similar transfers yielded cultures with doubling times of 17 hours and Klett absorption values above 200 (measured at 0.8 g/L dry cells), with yields up to 0.43. Identical inoculated control flasks lacking only the L-glucose showed no growth. Table XIX shows successive doubling times for RF-1 when the organisms were inoculated from resting cultures and sequentially subcultured during growth as described above. Observations on plating efficiency, (the ratio between TSY and L-glucose agar colony counts) and on cell mass yield as functions of apparent doubling time with RF-1 are graphed in Figure 10. Similar data on RF-3 are consistent with these observations and are included. If the organisms are held in resting culture for a few weeks, the low growth rate and yield phenomena are re-established.

The medium formulations all include 0.2 mg/L vitamins. Control flasks of inoculated carbon-free medium throughout these studies have shown that this is not enough to support observable growth. Attempts to start RF-1, RF-2, RF-3, and RF-11 from resting cultures without the vitamins have all failed. Furthermore, small inocula (0.03 ml 0.4 g/L CDW into 10 ml fresh medium) of actively growing cells will not grow without the vitamins. An experiment with 10 parallel flasks inoculated with actively growing RF-1 cells into medium containing 0, 1/2x, 1x, and 2x the standard vitamin

TABLE XIX

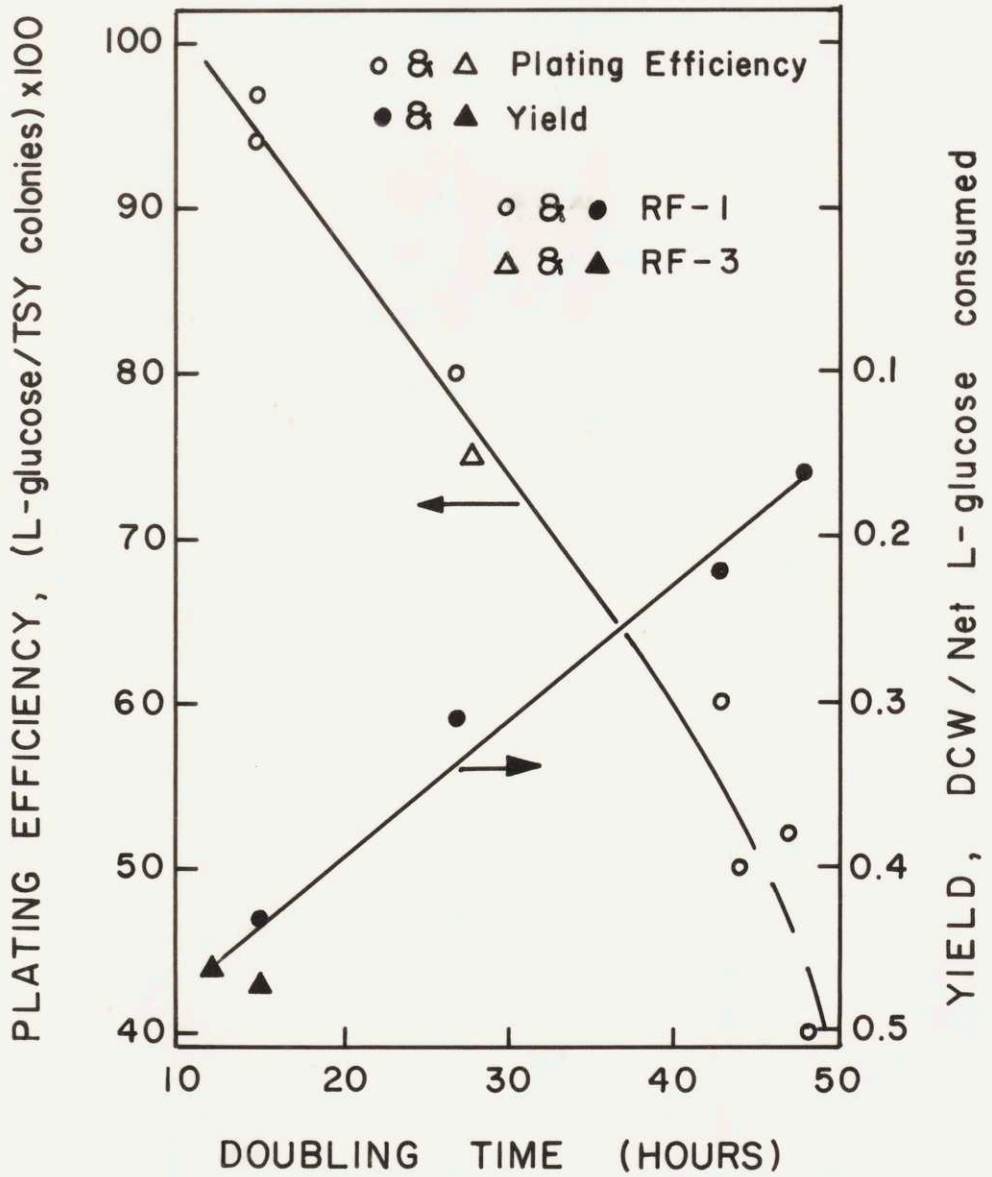
ADAPTATION OF RESTING PSEUDOMONAD (RF-1) CULTURES TO ACTIVE GROWTH ON L-GLUCOSE

Initial Culture Age[a] (Days)	Total Generations from Rest	Doubling Time (Hr.)
19	0	48
	2.75	43
	5.75	29
	9.20	25
	12.70	28
	16.30	15
25	0	55
	4.00	44
	9.00	34
	11.25	31
	14.00	22
	16.00	21
	20.25	16
8	0	35
	6.1	27
	10.4	17

a. Stored at 30° C in 18 mm culture tubes containing 3 ml 6-LGM. Culture age is counted from visible cell outgrowth.

FIGURE 10

PLATING EFFICIENCY AND YIELD OF L-GLUCOSE UTILIZERS



concentration show that measured growth rates will not be sensitive to small errors in vitamin measurement. See Table XX. Resting cultures of RF-11 will not grow on the standard medium, but inocula from growing cultures grow normally. Resting cultures of this organism do start well in sterilized used medium from RF-1, RF-2, or RF-11.

E. Carbohydrate Metabolism of L-glucose Utilizing Pseudomonads

Various carbohydrates were used as sole carbon sources to determine the lag times and growth rates of L-glucose-adapted cells. Typical growth curves for these experiments are shown in Figure 11. These five growth curves for RF-1 on L-glucose, D-glucose, D-glucitol, L-rhammose, and an inoculated control flask with all ingredients except carbon source, are quite straightforward with lag times followed by abrupt transition to exponential growth. Figure 12 shows the response of RF-1 to a shift from L-glucose to L-arabitol. In this case, a 58 hr. lag was followed by a period of slow growth for at least six hours before exponential growth at the steady state doubling time of 9 hours. RF-2 was very similar to RF-1 in general, except that shifts to several sugars elicited the response shown in Figure 13 for L- and D- arabitol in which a short growth period was followed by a resting stage, then exponential growth. The results of these runs are shown in Table XXI (lag times) and Table XXII (growth rates and carbon yields). The lag times for L-glucose-adapted RF-1 and RF-2 when shifted to D-glucitol were very short, especially when the 1-2 hour lag on D-glucose is considered. The growth rates on D-glucitol were similarly at least as good as those on D-glucose. D- mannitol and D- arabitol appear to be as good substrates for RF-2 as D-glucitol, but D-arabitol has an appreciable lag time in both organisms. Except for L-arabitol and L-sorbose, the growth rates are divided neatly into natural and unnatural sugars. D-arabinose is utilized at about

TABLE XX

VITAMIN REQUIREMENTS OF RF-1

Vitamin Concentration mg/L[a]	Doubling Time Hr.
0.0	no growth
0.1	25± 2[b]
0.2	20± 3[b]
0.4	20± 2[b]

[a]Standard amount is 0.2 mg/L. See Table IV in METHODS.

[b]95% confidence limits.

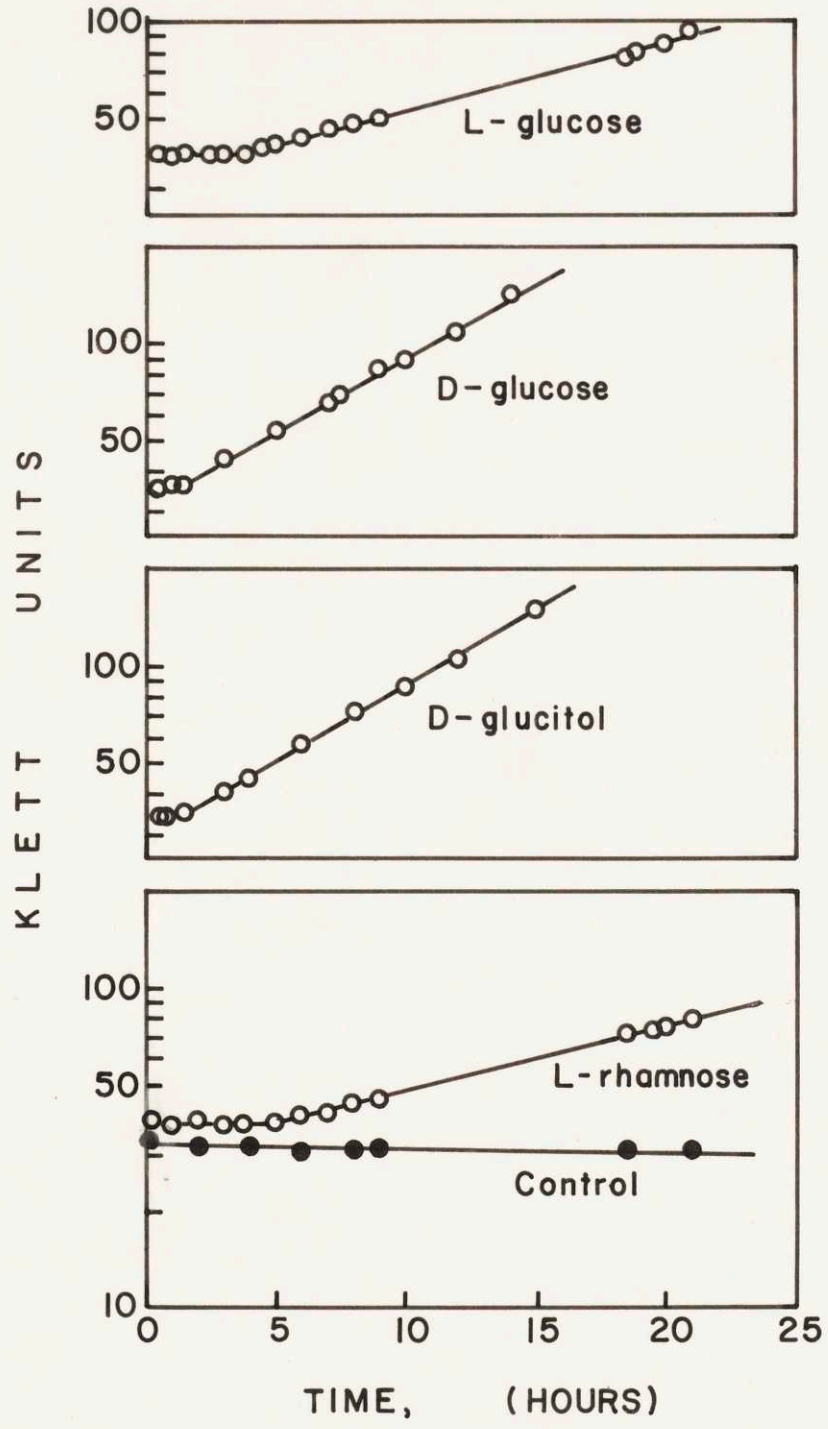


FIGURE II.- GROWTH OF L-GLUCOSE ADAPTED PSEUDOMONAD, RF-I, ON VARIOUS SUGARS.

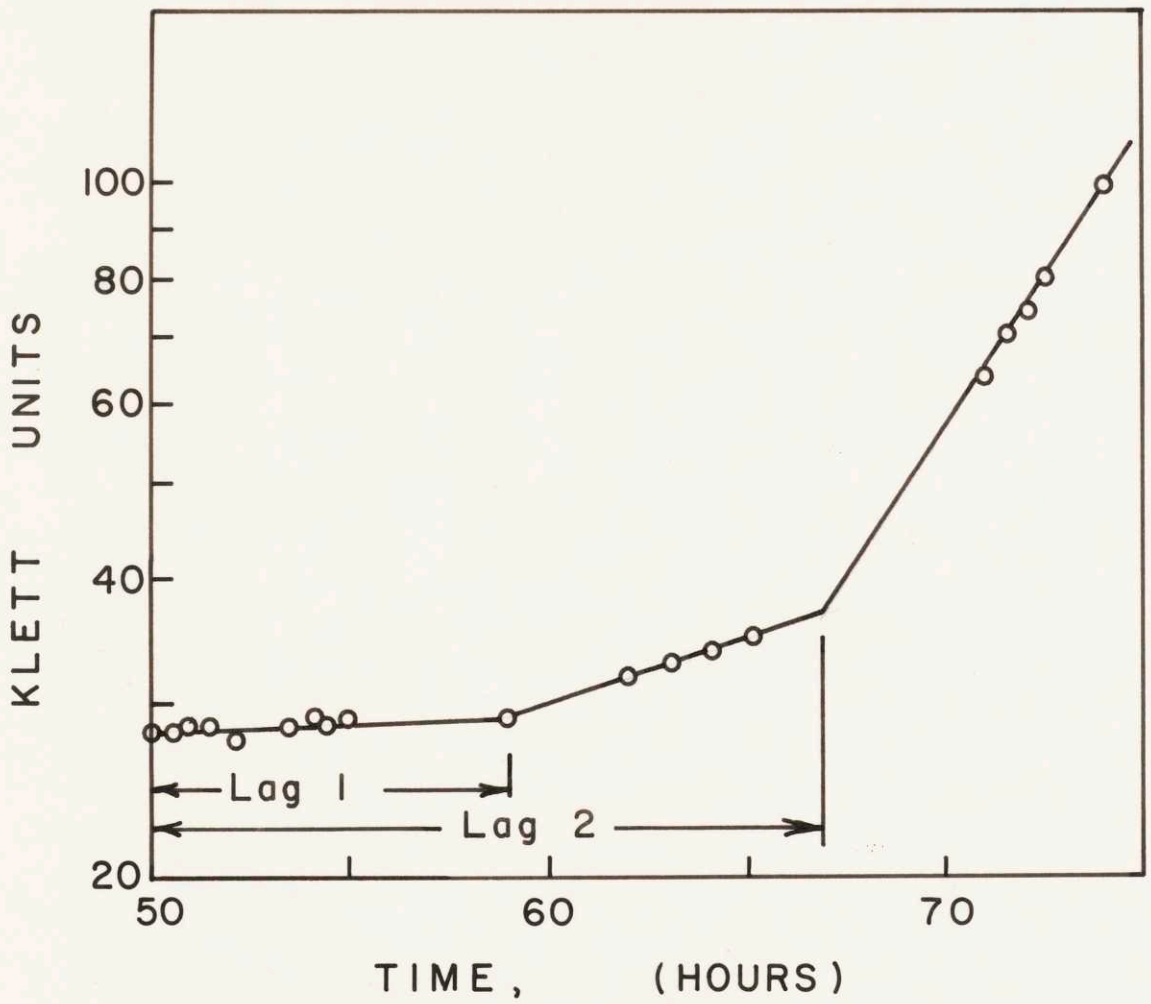


FIGURE 12.— GROWTH OF L-GLUCOSE ADAPTED PSEUDOMONAD RF-1, ON L-ARABITOL.

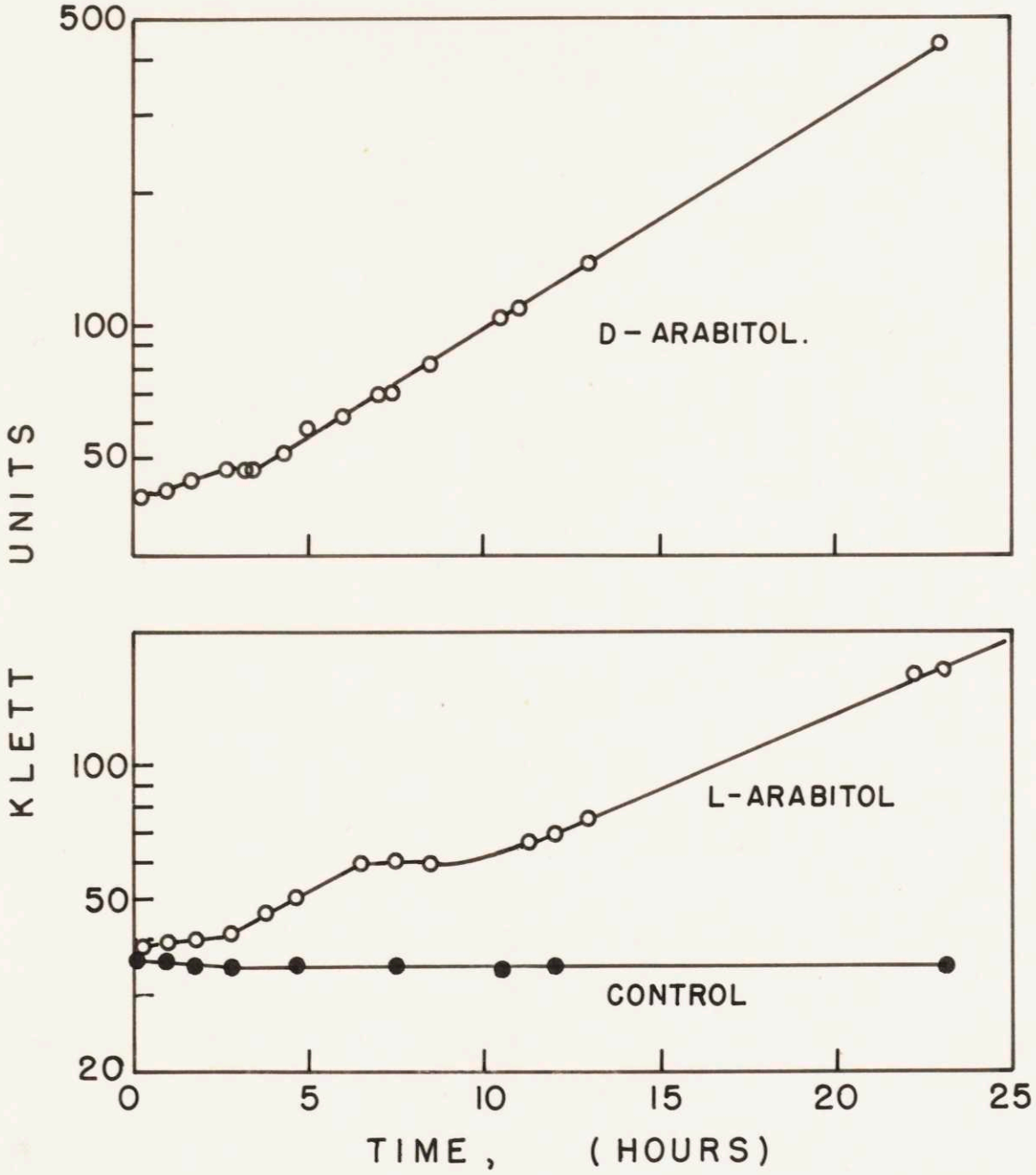


FIGURE 13.- GROWTH OF L-GLUCOSE ADAPTED PSEUDOMONAD RF-2 ON D-ARABITOL AND L-ARABITOL.

TABLE XXI

LAG TIME OF L-GLUCOSE-ADAPTED PSEUDOMONADS ON VARIOUS SUGARS

Sugar[b]	Organism RF-1		Organism RF-2	
	Lag 1,Hr.	Lag 2,Hr.	Lag 1,Hr.	Lag 2,Hr.
D-glucitol	1		0.5	
D-glucose	1-2		0.5	
D-mannitol			0.5	
D-arabitol	2.5		0.5	3.5
D-galactitol	2.75			
L-glucose	3-6		2.5	
L-fucose	3			
L-xylose	6			
L-rhamnose	12			
D-arabinose[a]	16			
L-arabinose	17			
L-sorbose	18			
L-arabitol[a]	58	67	0.5	10
D-ribitol[a]	67	88	2.0	
xylitol[a]	72	90	3.0	
Glycerol	0			
Citrate	0			
Succinate	0			
Acetate	28			
Hexadecane	140			

a. Rare in nature.

b. Each sugar at 2.5 g/L in 6-CFM minimal medium.

TABLE XXII

GROWTH RATE OF L-GLUCOSE ADAPTED PSEUDOMONADS ON VARIOUS SUGARS

Sugar[c]	Organism RF-1		Organism RF-2	
	Doubling Time, Hr.	Yield gCDW/g	Doubling Time, Hr.	Yield gCDW/g
D-glucose	6	0.48	6.8	0.47
D-glucitol	6-6.5	0.53	6.3	0.49
D-arabitol	6.2-6.5	0.50[b]	6.5	0.50[b]
D-mannitol			6.5	0.51[b]
L-rhamnose	7	0.38[b]		
L-arabinose	7	0.36[b]		
L-arabitol[a]	9	0.39[b]	8.3	0.46[b]
D-galactitol	12.5	0.41[b]		
L-fucose	13	0.30[b]		
L-glucose[a]	15-23	0.43-0.47		
xylitol[a]	15	0.42[b]	10.8	0.42[b]
D-ribitol[a]	16.5	0.32[b]	15.8	0.40[b]
L-xylose[a]	20	0.35		
L-sorbose	29	0.29		
D-arabinose[a]	47	0.24		
Glycerol	7.7	0.45[b]		
Citrate	9.3	0.5[b]		
Succinate	9.5	0.5[b]		
Acetate	130	0.1[b]		
Hexadecane	25	0.4[b]		

a. Rare in nature.

b. Calculated on the basis of Klett absorption uncorrected for residual sugar.

c. Each sugar at 2.5 g/L in 6-CFM minimal medium.

the same rate as L-glucose would be by a deadapted resting culture. L-xylose (5- decarbohydroxy-L-glucose) and xylitol are utilized at about the same rate as L-glucose. The slowest growth substrate on the list is acetate at a 130 hr. doubling time. The lag times for L-arabitol, D-ribitol, and xylitol are all above 50 hours for RF-1. L-xylose has a six hour lag.

The reverse experiment, shifting back to L-glucose, was performed with D-glucitol, D-arabitol, and D-glucose with the results shown in Table XXIII. These were performed without washing the cells, but residual sugar in the inoculum was low (0.2 g/L) and the inoculum was diluted 10:1 into new medium. The cells were raised 4 1/2 generations on the respective carbon source. The D-glucitol and D-arabitol-raised cells shifted to a 15-16 hour doubling time on L-glucose without pause. The D-glucose-raised cells did not stop growing, but they had a lag 2 of about 1 hour, after which they grew on the L-glucose with a 16 hr. doubling time. Similarly, addition of 0.1 g/L D-glucose to a growing L-glucose culture containing 0.1 g/L cells resulted in abrupt transition to the higher growth rate until the culture contained about 0.15 g/L cells. The growth rate then returned smoothly to that on L-glucose.

One culture of RF-1 grown on D-glucose for 18 generations did not readapt to L-glucose utilization over a 30 day incubation.

During this portion of the studies, plating efficiencies higher than 100% were first observed. It was found that cultures from long steady-state growth (12-15 generations) on L-glucose yielded three to four times higher colony counts on L-glucose agar than on TSY agar. It was later found that this discrepancy was caused by a very late-blooming population on the TSY plates and too-early counting. The first RF-1 colonies reach 1-2 mm in 24-30 hours at 30° C on TSY agar. After about 3-4 days incubation,

TABLE XXIII

LAGS AND GROWTH RATES OF RF-1 RETURNED TO L-GLUCOSE AFTER 4½ GENERATIONS

Inoculum Substrate	Growth Substrate[a]	Lag 1, Hr.	Lag 2, Hr.	Doubling Time, Hr.	Yield g CDW / g Substrate
D-glucose	D-glucose	0	0	7½	0.48
	L-glucose	0	1	16	0.47
D-glucitol	D-glucitol	0	0	6½	0.48
	D-glucose	½	0	6	0.46[b]
	L-glucose	0	0	15	0.44
D-arabitol	L-glucose	0	0	18	0.42[b]
	D-arabitol	0	0	6.2	0.51[b]

[a]Cells not washed. Carryover estimated to be about 0.02 g/L inoculum substrate. Growth substrate at 2.5 g/L.

[b]Yields estimated by Klett absorption and original sugar concentration. Not corrected for residual sugar.

a second population with otherwise identical color, appearance, and growth rate on D-glucose appear. RF-2 and RF-11 emerge in 2-4 days under similar conditions. All of these organisms emerge in approximately the same time (6-8 days for 0.5 mm colonies) on L-glucose agar.

Shiftup and shiftdown experiments on L-glucose concentration yielded the results shown in Figure 14. When the L-glucose concentration was shifted from 0.5 to 5.0 g/L L-glucose by addition of L-glucose to a growing culture of RF-1, the doubling time shifted from 29 to 18.5 hours within 2 hours. The drop in Klett absorption at shiftup is an artifact of dilution with L-glucose solution. When the L-glucose concentration was shifted from 5.0 to 0.5 g/L by a 10:1 dilution into fresh carbon free medium, the doubling time shifted from 18 hours to 24 hours after a lag of approximately 3 hours.

F. Carbon Starvation in L-Gluose Utilizing Pseudomonads

The initial carbon starvation studies were done with a mixed culture of RF-2 and RF-11. The results were sufficiently interesting to merit consideration in terms of the L-glucose metabolism of these organisms. Cells from a steady state growing L-glucose culture were washed and resuspended in carbon-free medium. Portions of this suspension were then inoculated into fresh L-glucose medium at different times and the lag times and growth rates of the starved cells were measured. Cell number and mass (Klett absorption) was monitored in the starving suspension. The early results showed that starvation up to about ten hours made little difference to the lag time or the initial doubling time of the cultures. After 44 hours, however, a distinct shortening of lag time occurred and the initial growth rate was higher than the steady state growth rate. Figure 15 shows the growth curve of this culture in 0.5 and 5.0 g/L L-glucose after 116.5 hours of carbon starvation. Four distinct modes can be seen in these curves:

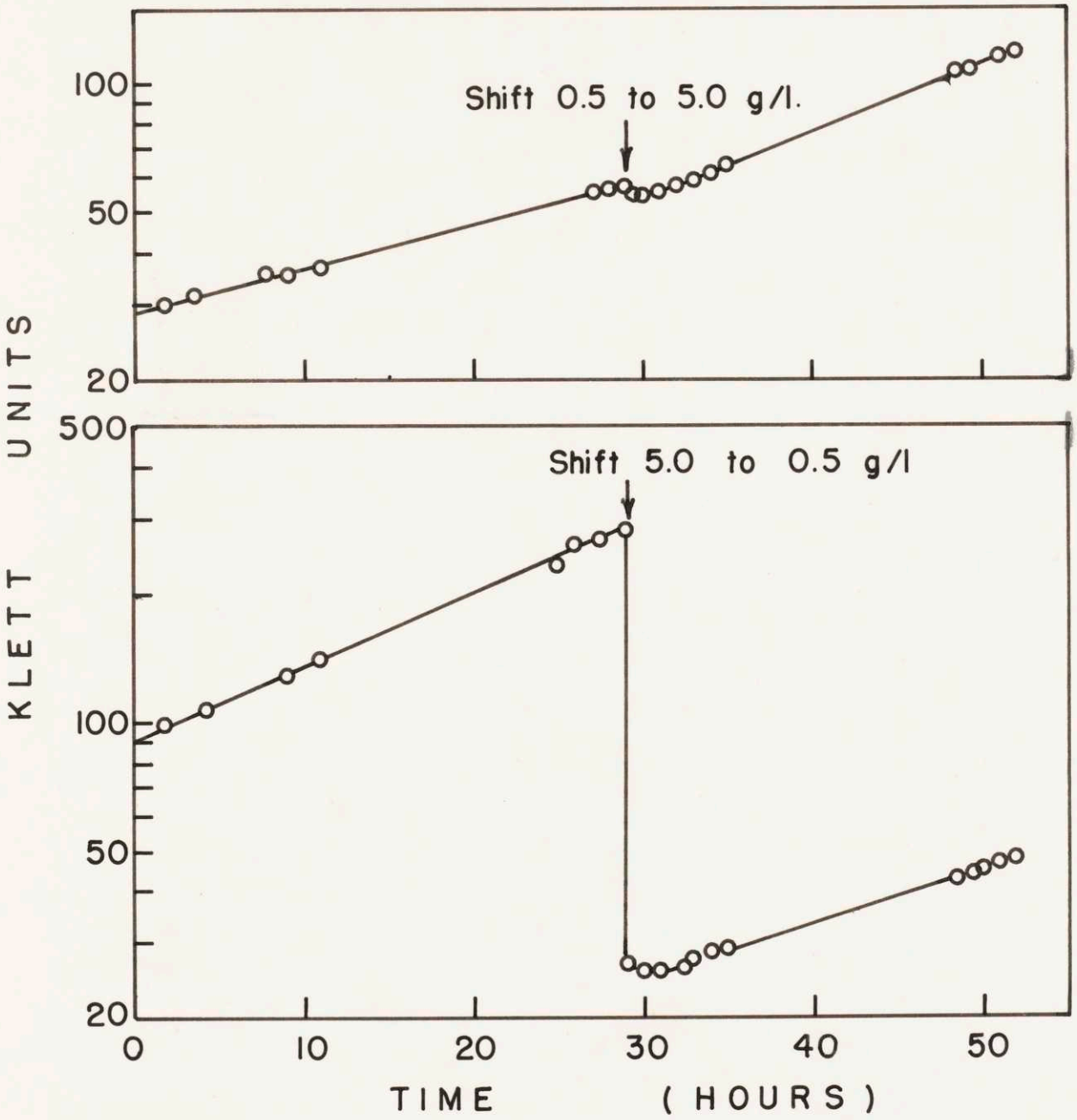


FIGURE 14.- SHIFTUP AND SHIFTDOWN OF L-GLUCOSE CONCENTRATION WITH RF-1.

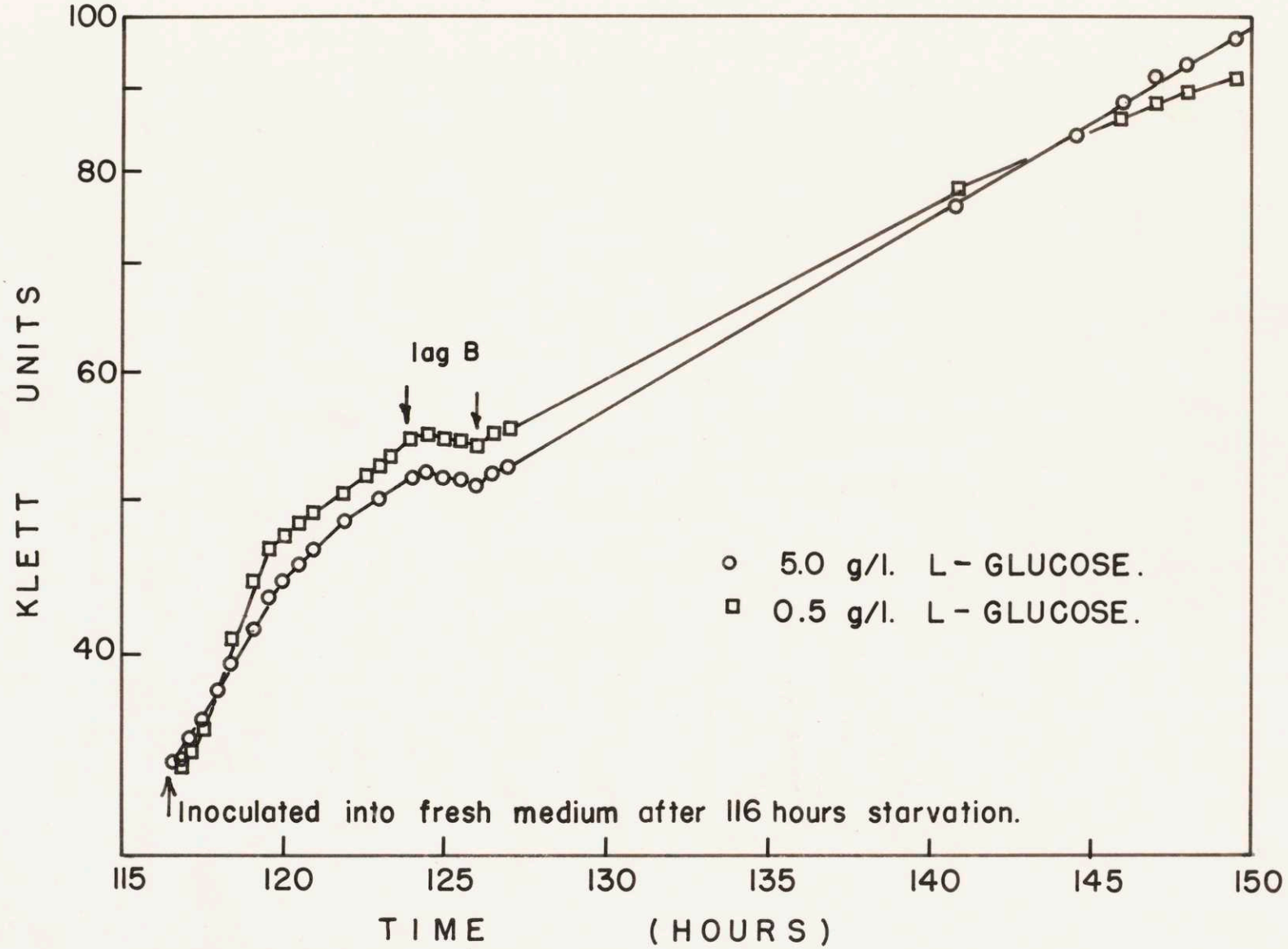


FIGURE 15.-Growth of a Mixed Culture of RF-2 and RF-11 after starvation.

1. The lag time is very short, probably less than 0.5 hour.

2. The initial growth rates are among the highest yet seen on L-glucose, about 5 hours doubling time at 0.5 g/L and about 8.5 hours doubling time at 5.0 g/L L-glucose.

3. A second lag period.

4. Steady state growth at the rates to be expected at these L-glucose concentrations.

These lag times will be designated "lag 1", since it is qualitatively the same as those previously measured in this study, and "lag B," since the second lag period is qualitatively different from those designated lag 2 in this study. Lag B will be measured from the point at which the initial growth phase reaches the minimum reading during the lag to the point at which steady state growth begins, determined by extrapolation of the best straight line representing growth. Lag 1, Lab B, and initial doubling time for three runs on this culture are plotted vs. starvation time in Figure 17. All three functions pass through a shallow inflection between 40 and 120 hours, then lag 1 and initial doubling time gradually increase and lag B drops at 166 hours starvation time. Cell number and mass (Klett absorption) of the starvation vessel are plotted in figure 16 as a function of time. The cells continue dividing for about one generation after removal of the carbon source. Visual microscopic counts were about 1.4x the plate counts at the beginning of the starvation and about 2.6x the plate counts at the end. Because the pseudomonads are small rods, the Petroff-Hauser cell was difficult to count at best, and nearly impossible after 100 hours of starvation because the cells continued to decrease in visual size. Furthermore, the amount of debris in the medium increases. At first count, the TSY plates had more than one order of magnitude fewer colonies

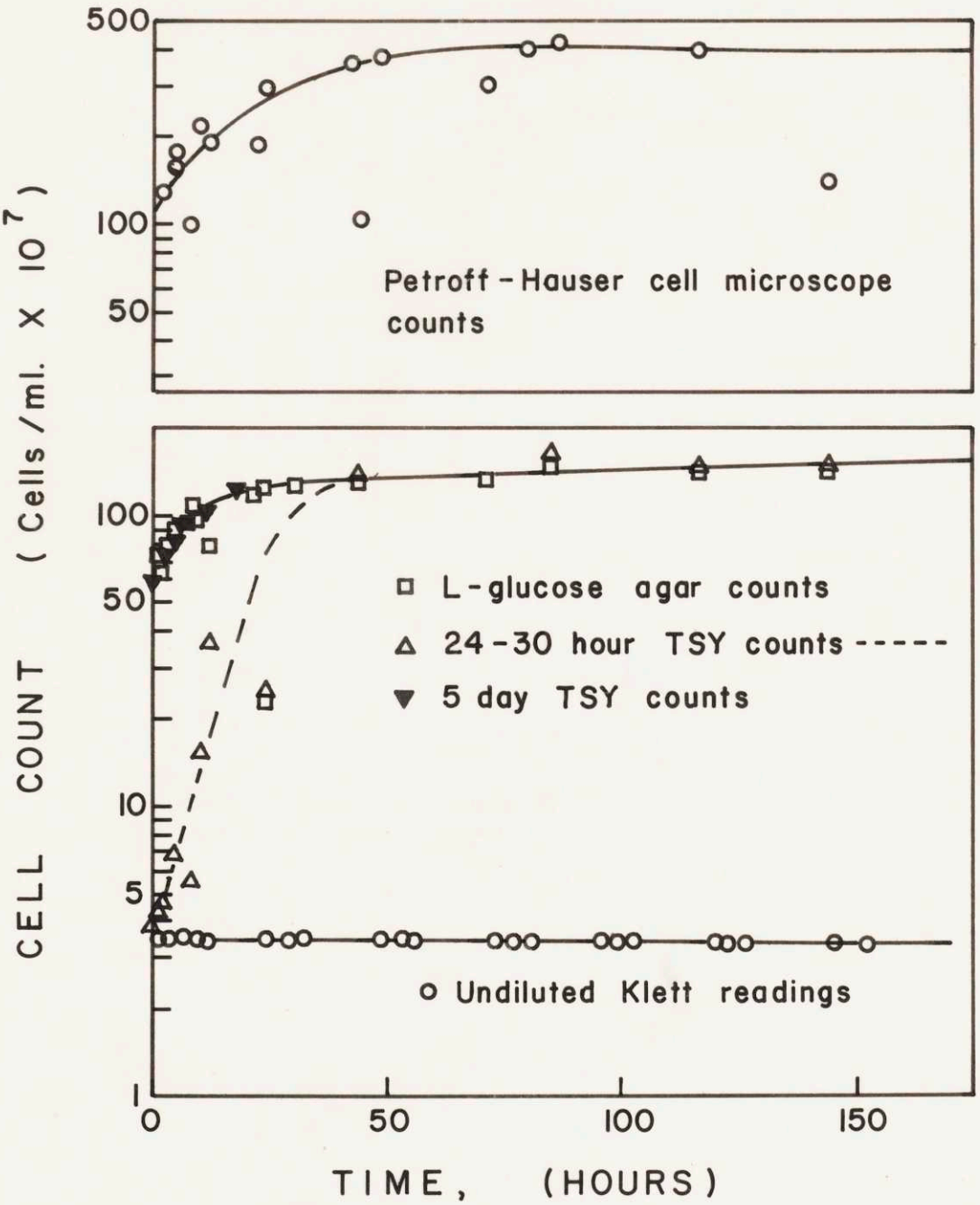


FIGURE 16.- CELL NUMBER AND MASS OF A MIXED CULTURE DURING CARBON STARVATION.

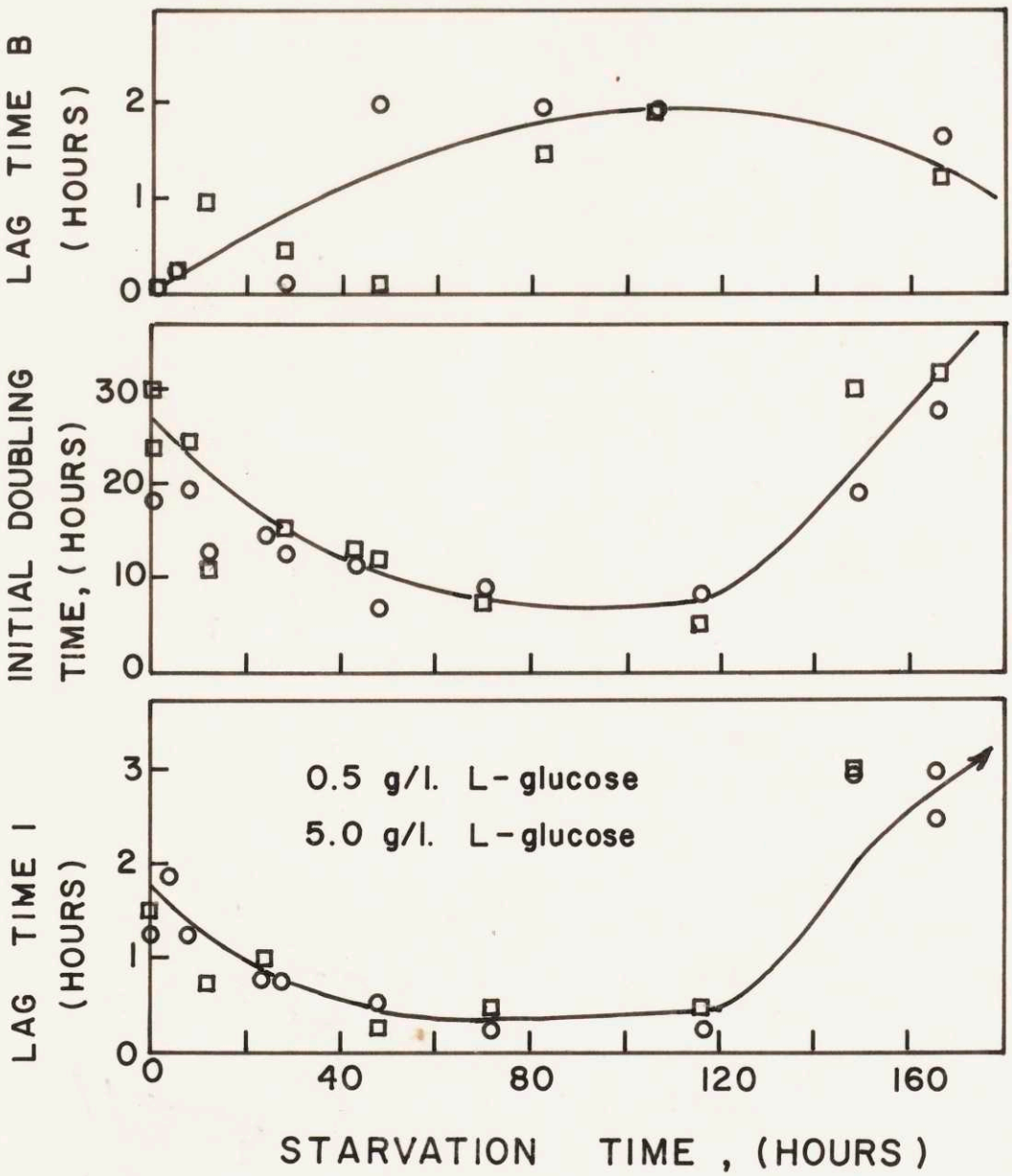


FIGURE 17.— GROWTH ON L-GLUCOSE AFTER CARBON STARVATION.

than the L-glucose plates. The TSY plates grew a crop of large (1 mm) colonies within 24 hours of spreading. These were used for the count until it was found that an additional 48 hours of incubation brought forth a second larger crop of apparently identical organisms. Use of 5 day counts on the TSY plates reconciled the L-glucose and TSY plate counts very well.

Similar carbon starvation studies on RF-1 showed that lag time and growth rates were unaffected for up to 12 hours starvation. After about 30 hours starvation, lag times gradually increased and growth rates decreased. There was no evidence of the phenomena demonstrated in the first study. As shown in Figure 18, the cell number (by plate count) increased by about 1 generation at 25-30 hours, and then decreased noticeably. The initial Petroff-Hauser cell count was about 1.2x those of the initial plates, but the cells in subsequent samples were impossible to count.

RF-1 cells growing exponentially in 5 g/L L-glucose were washed and resuspended at 2.0 g CDW/L in carbon-free medium. Samples of filtered medium after 1/2, 1, and 2 hours incubation at 30° C were analyzed for L-glucose. These samples contained traces of L-glucose, but all were below 0.5 mg/L.

G. Growth Kinetics of the Pseudomonad RF-1 on L-glucose

Growth rates were measured by plate counts and by Klett absorption on steady state growing cultures started at about 10^3 organisms/ml. Growths of the type plotted in Figure 19 were obtained. The cell number plots departed from straight-line exponential growth above about 5×10^7 cells/ml. The Klett plots remained linear up to Klett 125 (about 5.2×10^8 cells/ml) with undiluted brew and up to Klett 300 with 1:10 diluted brew samples. The diluted Klett readings, however, were considerably scattered.

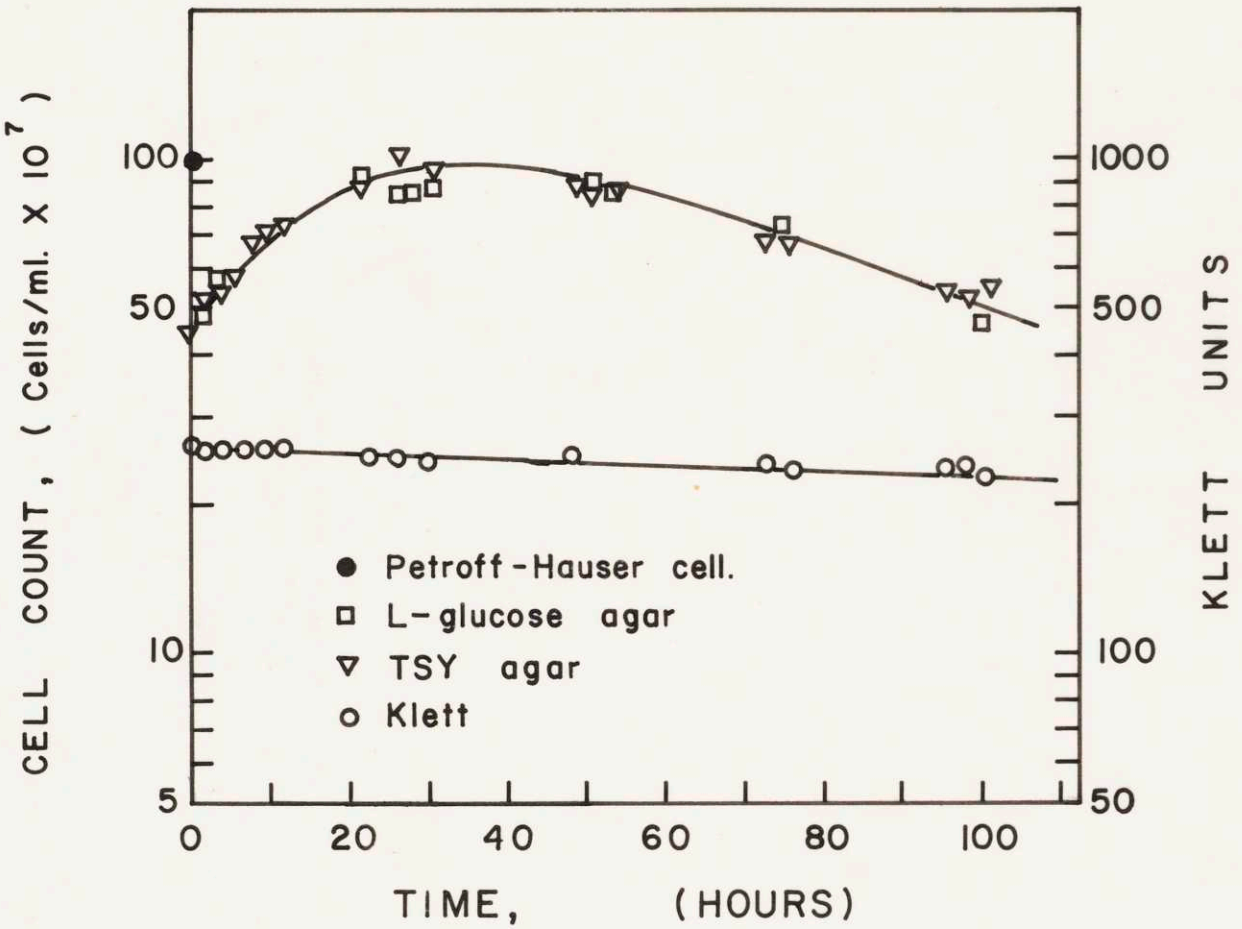


FIGURE. 18.— CELL NUMBER AND MASS OF RF-1 DURING CARBON STARVATION.

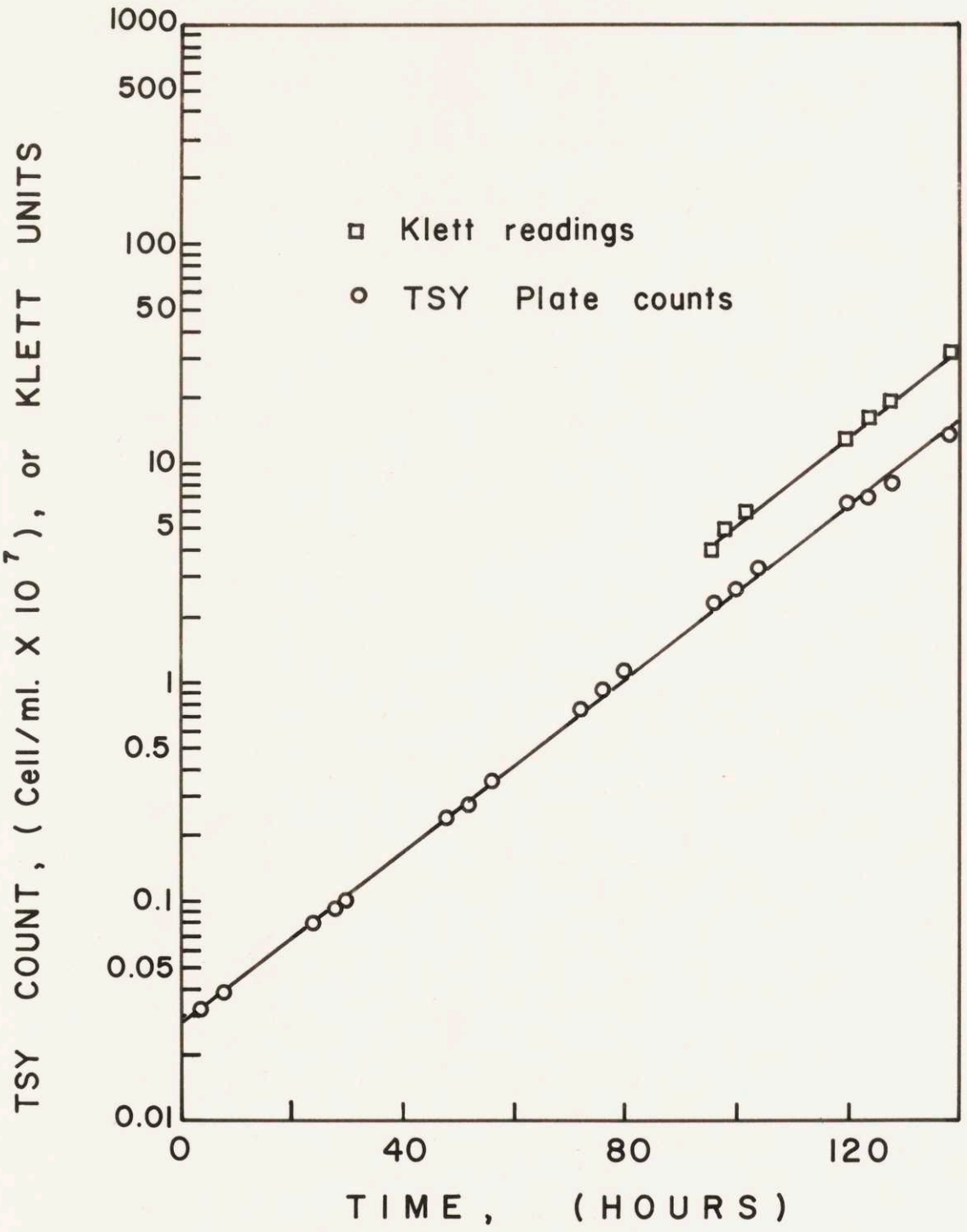


FIGURE 19.- GROWTH OF PSEUDOMONAD RF-1 AT 5.7 G./L. OF L-GLUCOSE.

The growth rates obtained from these runs are plotted as the solid curve on Figure 20 as a function of substrate concentration. The curve goes through a maximum growth rate of about 0.049 hr^{-1} at 4.5 g/L L-glucose. It then decreases gradually to 0.041 hr^{-1} at 16 g/L L-glucose.

Before we discovered that inoculation of exponentially growing cells into new medium resulted in slightly lower growth rates, a large body of growth rate data had been collected using an older procedure. Under this procedure, cells were grown at 2.5 g/L L-glucose. When the cell density reached about 0.5 g/L, this culture was used to inoculate fresh medium containing the desired L-glucose concentration. The growth rate at the new concentration was measured over at least two generations and invariably yielded the straight logarithmic plots of steady state exponential growth. The data obtained in this fashion are plotted on the dotted curve in Figure 20. The entire dotted curve shows lower growth rates, even at 2.5 g/L. Substrate inhibition at 10 g/L appears to be more severe.

The data in the solid curve of Figure 20 are plotted in classical Lineweaver-Burk fashion in Figure 21. This curve also shows substrate inibition quite clearly. However, if a straight line through the points 1/S=0.16 is extrapolated, the intercepts show an apparent μ_{max} of 0.0506 (13.7 hr. doubling time) and an apparent K_s for overall growth of 0.14 g/L L-glucose.

A mixed culture of RF-1, RF-2, and RF-11 was washed, resuspended in carbon-free medium, starved 48 hours, then grown on L-glucose at 5 g/L for four cycles. The result was enrichment of RF-2 and RF-11, with RF-11 receiving some advantage, as shown in Table XXIV.

H. L-Glucose Transport in the Pseudomonad, RF-1

Measurement of the inital velocities of L-glucose uptake over the

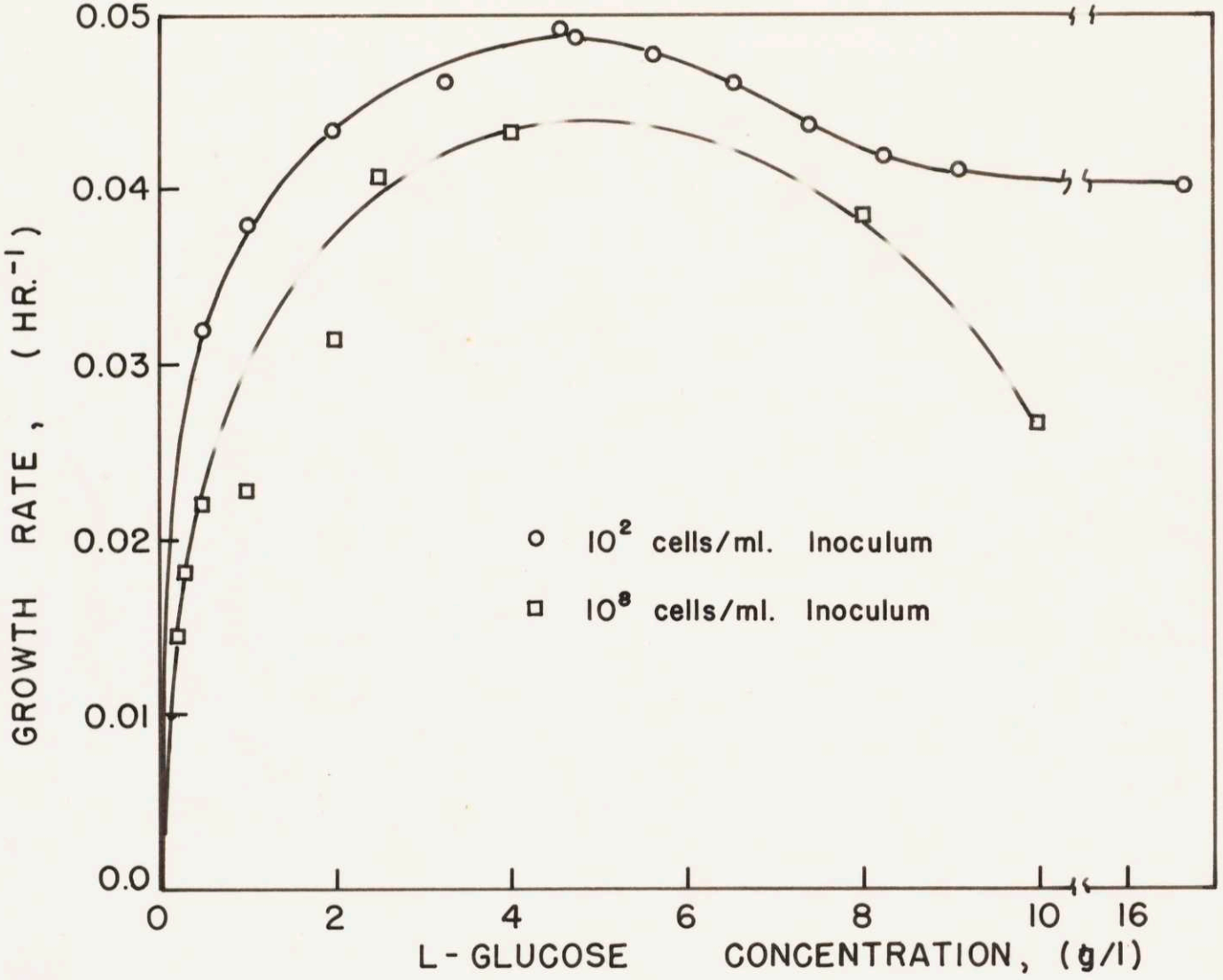


FIG. 20.- GROWTH RATE OF RF-I vs. L-GLUCOSE CONCENTRATION.

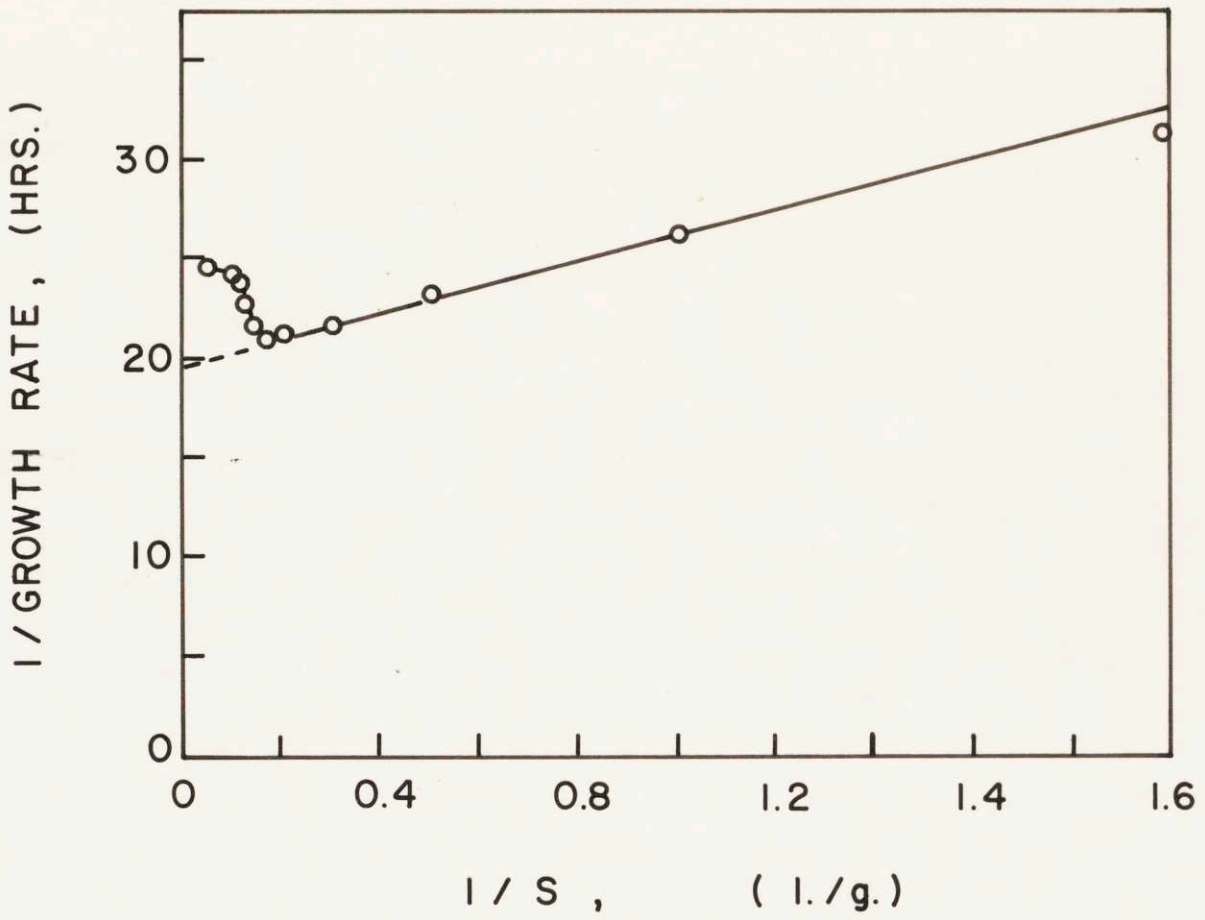


FIGURE 21.- LINEWEAVER-BURKE PLOT OF GROWTH ON L-GLUCOSE.

TABLE XXIV

EFFECTS OF CYCLIC CARBON STARVATION ON A POPULATION OF L-GLUCOSE UTILIZERS

Cycle[a]	Cell Counts per ml RF-1	RF-2	RF-11
0	4.2×10^8	1×10^7	$\sim .5 \times 10^7$
1	7.2×10^6	6.5×10^6	2.0×10^6
2	3.4×10^5	4.7×10^7	4.2×10^7
3	1.4×10^5	9.6×10^6	2.8×10^8
4	$\sim 5 \times 10^4$	1.3×10^7	2.6×10^8

[a] One cycle consists of washing the cells, resuspending in 6-CFM, starving 48 hours, adding 5g/L L-glucose, and permitting the culture to grow before the next wash.

range of 0.5 to 10 g/L L-glucose concentrations is reported in Table XXV. The rates ranged from 1.24×10^{-3} mg L-glucose/(mg CDW-min) at 0.5 g/L broth concentration to 2.41×10^{-3} mg L-glucose/(mg CDW-min) at 10 g/L broth concentration. A typical uptake curve is shown in Figure 24 (p.115). Samples after 10 minutes of uptake all showed equivalent L-glucose concentrations below those in the medium.[6] A Lineweaver-Burk plot of the rate data is shown in Figure 22. The intercepts of the best straight line through the points yield $V_{max}=2.63 \times 10^{-3}$ mg L-glucose/(mg CDW-min) and $K_s=0.65$ g/L (3.6 mM).

The sodium azide-poisoned cells transported L-glucose at essentially the same rate as the unpoisoned cells. It was found that the 0° C medium wash in the original procedure led to little or no labelling being found in the azide-poisoned cells, so this run and one of the 4.54 g/L runs were made with a 30° C dilution and washes replacing the 0° C wash in the procedure. No significant difference was found between the 30° C and 0° C washes with unpoisoned cells.

A batch of cells grown at 0.5 g/L L-glucose had an initial L-glucose uptake velocity of 2.42×10^{-3} mg L-glucose/mg CDW-min, essentially the same as the uptake rate of cells raised at 5.0 g/L L-glucose.

Control experiments with uniformly labelled radioactive D-glucose produced the uptake curves shown in Figure 23. The data from D-glucose with healthy cells and with 30 mM sodium azide-poisoned cells are listed in Table XXVI. The D-glucose was transported by the healthy cells at an initial rate of 23.6×10^{-3} mg D-glucose/(mg CDW-min) and reached an intra-

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[6] A cell density of 1.08 g CDW/ml was used for this calculation (Aiba et al, 1965).

TABLE XXV

L-GLUCOSE TRANSPORT RATES IN THE PSEUDOMONAD, RF-1

L-glucose g/l	Initial Velocity mgL-g/(mg CDW-min)x10^3	S_{IC10}[a] mg/ml
0.50	1.24	0.33
0.98	1.58	0.38
2.38	2.24	0.72
4.22[b]	2.42	3.8
4.54	2.35[c]	3.9
10.02	2.41[d]	4.1

TABLE XXVI

D-GLUCOSE TRANSPORT IN THE PSEUDOMONAD, RF-1

D-glucose g/l	Initial Velocity (mg D-g/(mg CDW-min)x10^3	S_{IC10}[a] g/l
4.54	23.6	25.5
4.22[b]	7.04	3.9

[a] Equivalent intracellular glucose concentration after 10 minutes uptake.

[b] Cells preincubated 10 minutes with 30mM NaN_3.

[c] ± 0.13 at 95% confidence, 4 run average.

[d] ± 0.20 at 95% confidence, 2 run average.

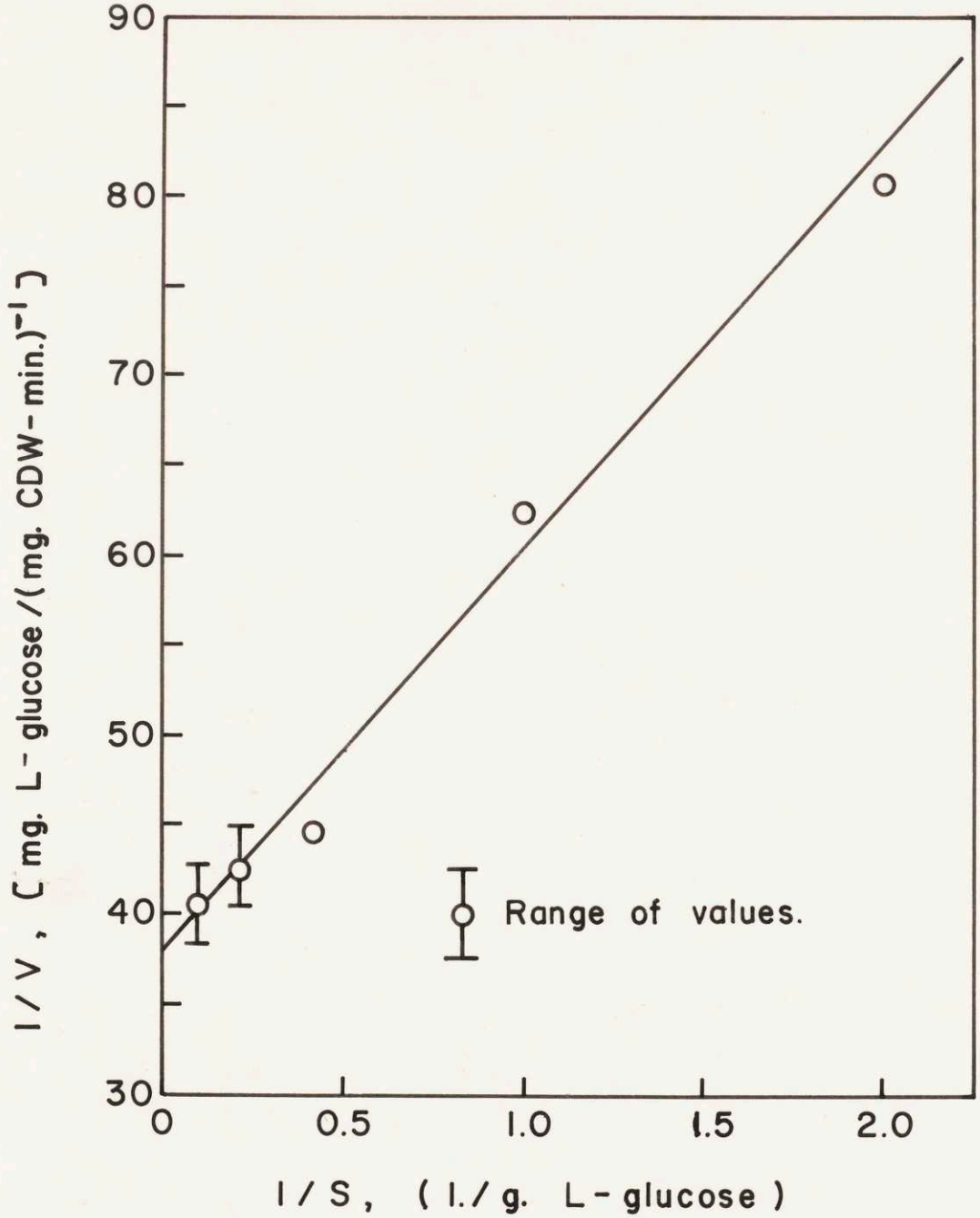

FIGURE 22. - LINEWEAVER-BURKE PLOT OF L-GLUCOSE UPTAKE IN RF-1.

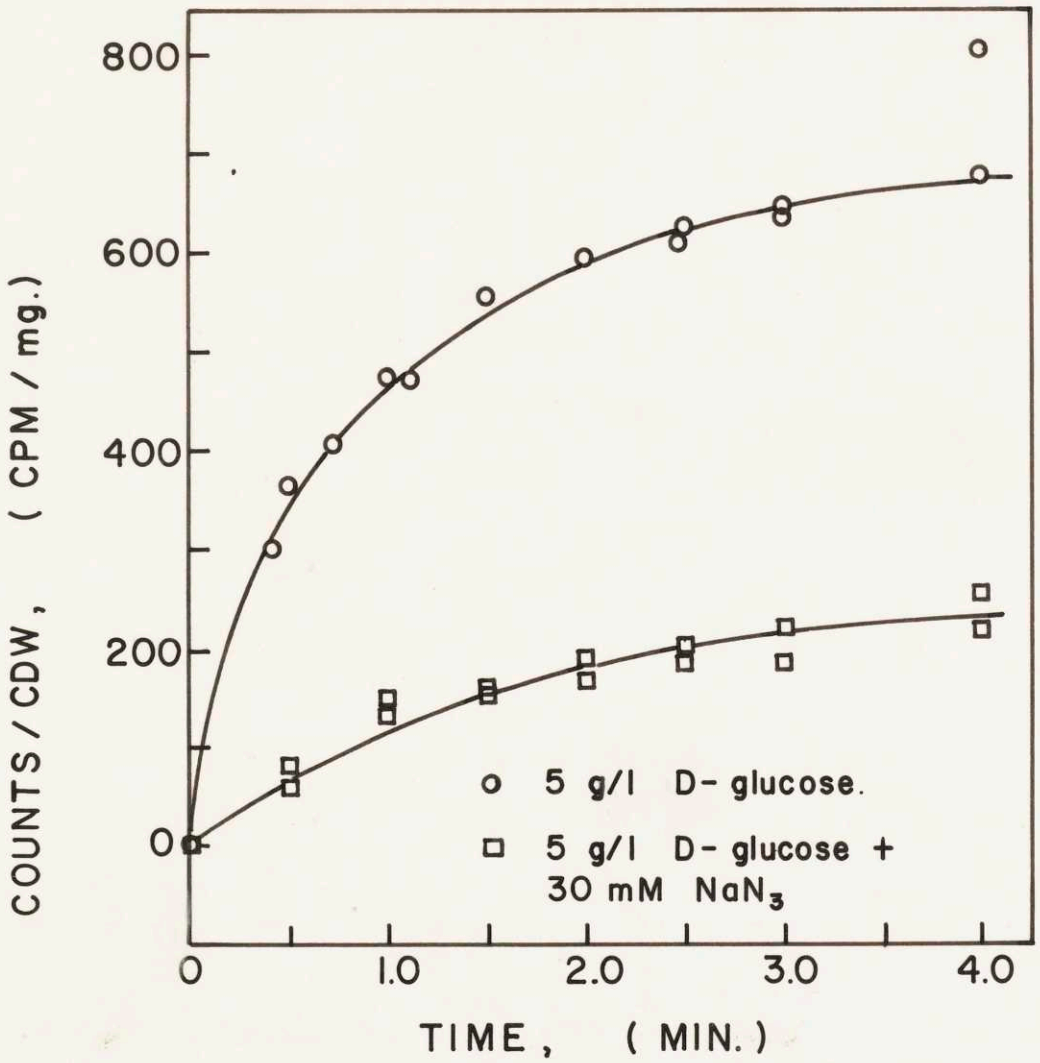

FIGURE 23.- D-GLUCOSE UPTAKE IN PSEUDOMONAD RF-1.

cellular concentration equivalent to 25.5 mg/ml D-glucose in 10 minutes. The azide poisoned cells transported D-glucose at an initial rate of 7.04×10^{-3} mg. D-glucose/(mg CDW-min) and the equivalent intracellular D-glucose concentration reached 3.9 g/L in ten minutes.

When 0.1 g/L D-glucose was added simultaneously with 4.54 g/L labelled L-glucose, L-glucose uptake was retarded compared with the absence of D-glucose. Uptake curves of this run are shown in Figure 24. L-glucose uptake was not eliminated, however, and the cell radioactivity was equivalent to about 3.7 mg/ml L-glucose after ten minutes uptake.

I. Disposition of Carbon 1 in L(1-^{14}C)-Glucose

When the radioactivity of the cells was monitored over a longer time span, it was found that the cells took up the labelled carbon at a very rapid rate for the first 120 minutes, then continued to take it up at a rate approximately parallel to the growth rate. See Figure 25. If the cell yield were less than 0.5 g CDW/g L-glucose, and if all carbon 1 were being incorporated into cell mass, the radioactivity should be increasing at twice the rate of cell mass.

When cells were washed clean of the radioactive medium and resuspended in unlabelled growth medium, the behaviour depicted in Figure 26 was observed. Cells grown in 10 g/L radioactive L-glucose 20 hours have a high initial count (20,300 cpm) and gradually lost more than 50% of it. About 8400 counts were permanently incorporated. A small amount of the label built up in the medium, but the 700 or 800 counts in solution was small compared to the loss. When the loss from the cells lined out, the radioactivity gradually disappeared from the medium. The 260 counts in the last medium sample were reduced to 140 by acidifying and boiling a sample of the medium.

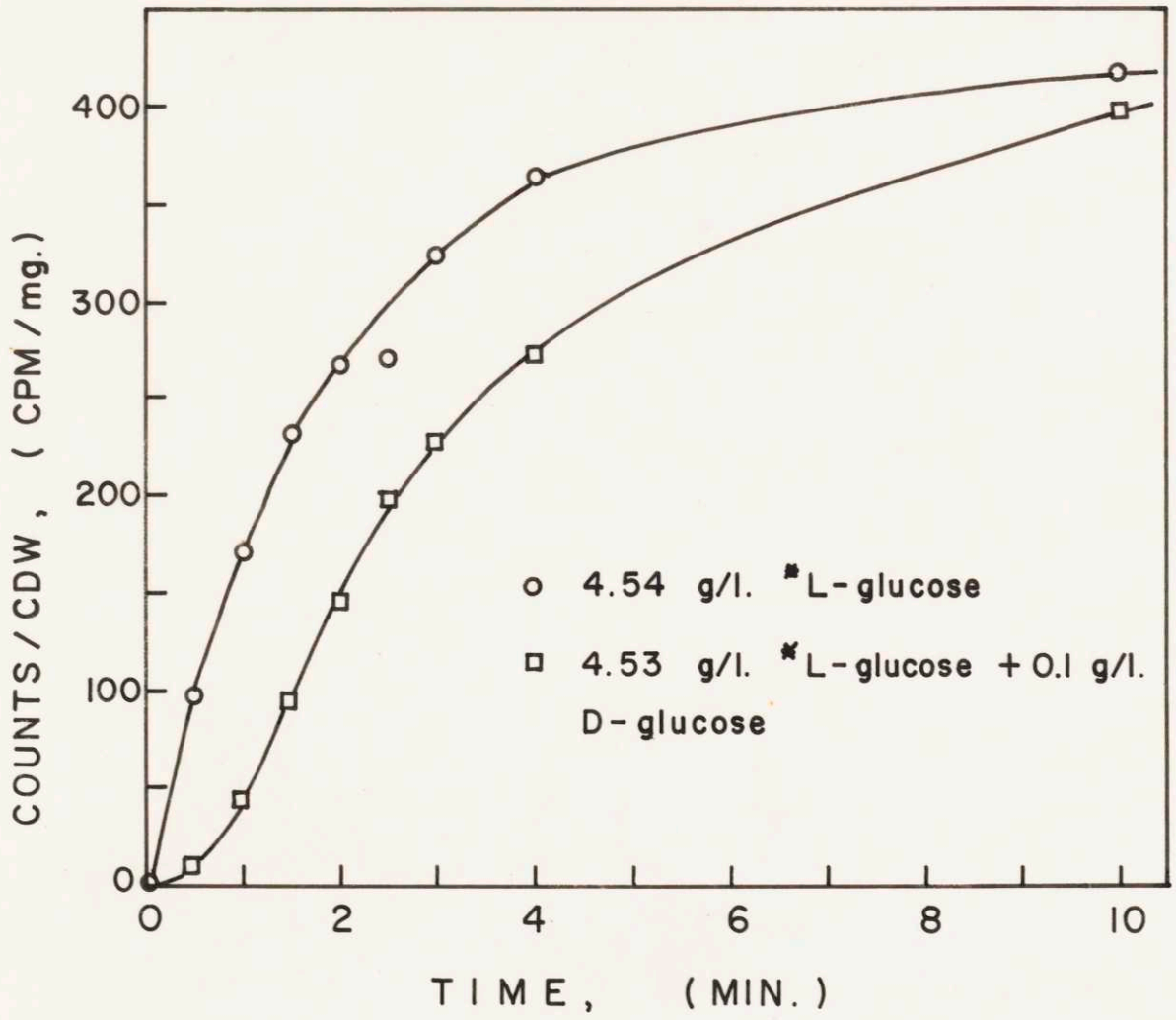

FIGURE 24.- L-GLUCOSE UPTAKE IN THE PSEUDOMONAD RF-1.

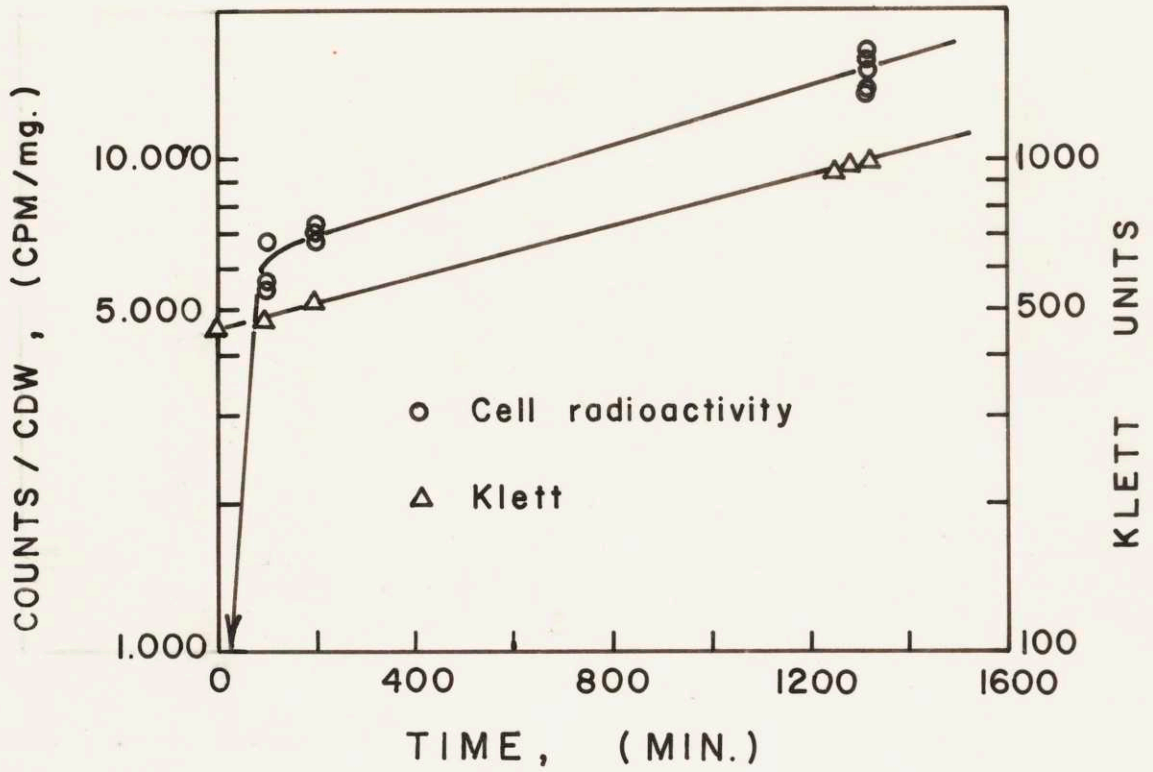

FIGURE 25.- RADIOACTIVE L-GLUCOSE UPTAKE DURING GROWTH.

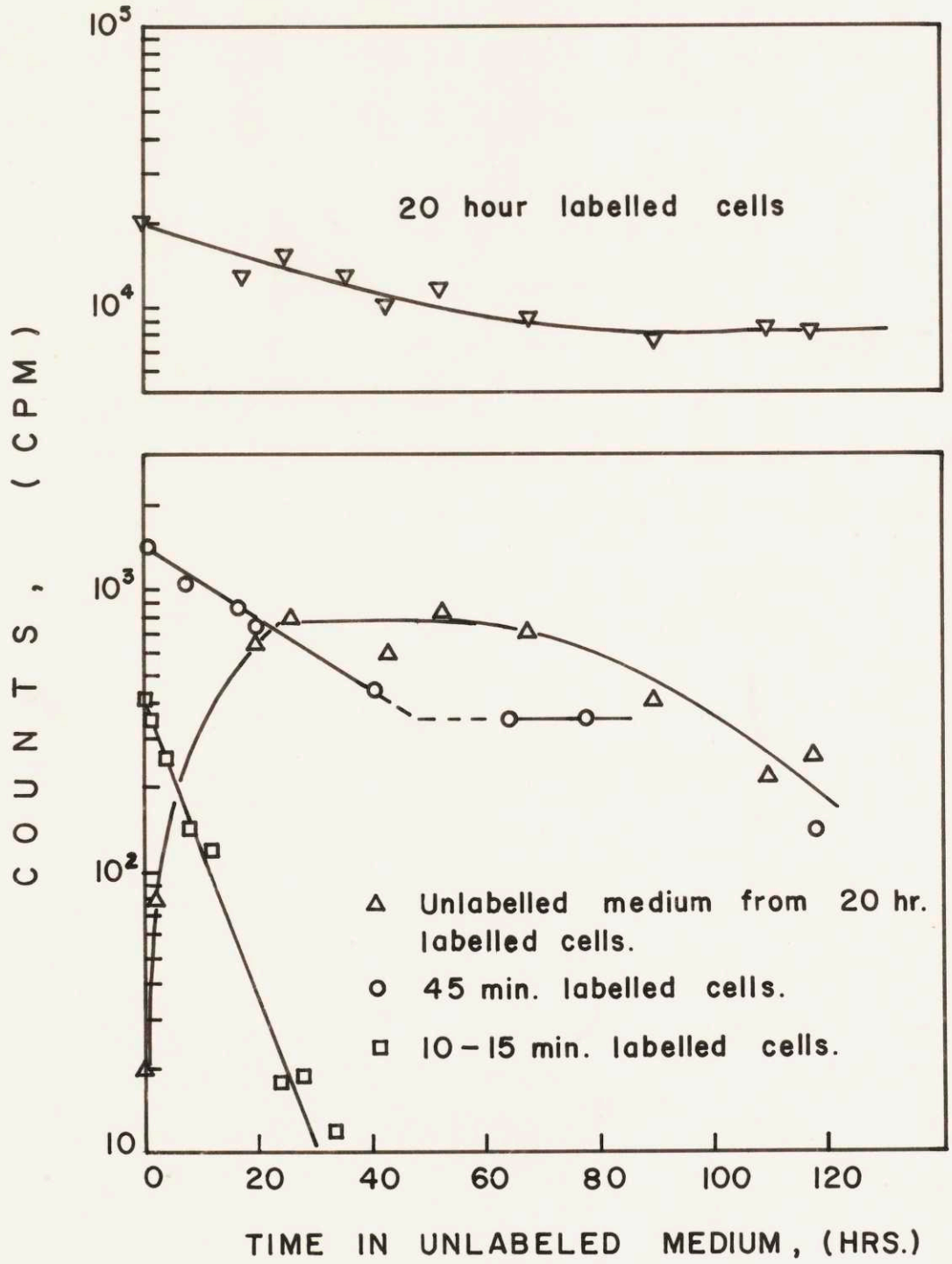

FIGURE 26.- RADIOACTIVE L-GLUCOSE DISPOSITION DURING GROWTH.

Cells grown in radioactive medium about 45 minutes behave qualitatively the same, but permanently incorporate only about 25% of the radioactive carbon.

Cells centrifuged from the radioactive medium within a few minutes after the start of the label uptake are in contact with the radioactive L-glucose about 10-15 minutes. These start at 415 cpm/g CDW and the radioactivity falls to undetectable levels in about 31 hours.

During the course of the ^{14}C labelling studies, it was determined that water quenches about 8% /ml in a Triton X - toluene scintillant. It was also determined that the 25 mm and 50 mm millipore filters and the 24 mm glass fiber filters do not quench detectably up to 3 filters per scintillation vial.

VI DISCUSSION

A. Enrichment and Cultivation of L-Glucose Utilizers

One outstanding result is the occurrence of potential L-glucose utilizing microorganisms in eight of the eleven wild microbial populations examined. To our knowledge, L-glucose has never been reported to be utilized as carbon or energy source in any organism.

The failure of previous attempts to observe utilization of L-glucose might be attributed to insufficient incubation time. Resting cultures of three weeks or more in age did not begin to grow during the first 24 hours when subcultured to L-glucose and often required one to three weeks incubation before growth began. Even growing inocula have substantial lag phases and steady state doubling times of 13 or more hours. Bruton (1967) incubated Streptomyies griseus 24 hours. Rudney did not state his incubation time, but the context implies that his Bacterium (Escherichia) coli and Bacterium (Aerobacter-Klebsiella) aerogenes were incubated for 24 hours. Emil Fischer (1890) incubated brewer's yeast with L-glucose for 24 hours. Both Kotyk (1967) and Cirillo (1968) used L-glucose as a non-metabolizable D-glucose analogue. Mortlock (1971) reported very long lag times of 35-56 days for adaption of Aerobacter aerogenes PRL-R3 to L-xylose (5-decarbohydroxy L-glucose).

Mortlock's experience with L-xylose might be compared to our obtaining the first L-glucose utilizer in about 25 days. These results plus our own lack of success in isolating L-glucose utilizers by direct enrichment of soil samples on L-glucose medium suggest that the addition of small amounts of natural carbon sources may have some merit. These results are not significant however, and a systematic study would be required to establish the value of this technique.

Via Dr. R.P. Mortlock (1971), we have a verbal report that an L-glucose utilizing microorganism was in the posession of Dr. W.A. Anderson. It apparently lost its ability to metabolize L-glucose during storage on agar, a fact which agrees with our experience. We are maintaining our library of organisms in minimum broth culture, changed every six to eight weeks. Several samples of RF-1 were lyophilized, but a three week incubation to revitalize one of these samples has so far been unsuccessful. Two attempts to grow RF-1 on a silica gel agar substitute based on Dupont "Ludox"[1] were likewise unsuccessful. Neither of these approaches have been ruled out yet.

Classification of bacterial isolates yielded five gram nagative rods in genera taxonomically related to the pseudomonads and none from the enteric group. Previous work on rare sugar utilization with Aerobacter aerogenes has characterized growth on unnatural pentoses, L-mannose and L-fructose, and D-allose. Since A. aerogenes is a ubiquitous soil organism (Alexander, 1961) we might have expected to find it. It must be remembered, of course, that our procedures do not guarantee that a potential L-glucose utilizing A. aerogenes was not in our original samples nor that one is not now being maintained in our mixed cultures. Incubation of one of our own strains of A. aerogenes for 90 days produced negative results.

The fact that pseudomonads were isolated from these cultures is not surprising since these organisms are known to be ubiquitous in soil (Alexander, 1961), hay (Alexander, 1961) and aerial (Shaw, 1956) enviroments and are known to have very versatile carbon requirements (Ornston, 1971). The detailed taxonomic study of the pseudomonads by Stanier,

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[1] One quart of Ludox silica gel suspension and instructions for making the plates were kindly supplied by Dr. R.P. Mortlock, University of Massachusetts-Amherst.

Palleroni, and Doudoroff (1966) points out that the standard methods of bacterial species classification were developed for the enteric groups and are unsatisfactory for pseudomonads. Even the Hugh and Leifson test is not necessarily readily interpretable, and we found this to be the case. They used a medium without vitamins. We have not explored this point thoroughly, but cultures of RF-1 on several sugars did not grow without the addition of our vitamin mixture. It is possible that the vitamins are required only for initiation of growth under the adverse conditions of L-glucose utilization. Stainer et al (1966) found that yellow, red, or no pigments were made by the same strain in different media. None of the strains in their collection were found to utilize hexadecane after 5 days incubation, although Iizuka (1964) reported pseudomonads in crude oil isolates. Our RF-1 grew slowly on hexadecane after a 140 hr. (almost 6 days) lag.

The Bacilli, RF-9 and RF-10 were isolated at 55° C and refractile bacillary spores have been observed in the 30° C cultures. Their erratic growth and sporulation patterns on L-glucose, however, make them less amenable to straightforward metabolic analysis than the pseudomonads. The growth rates of RF-9 were badly scattered in successive cultures and "steady state" populations of motile vegetative rods, sporulating rods, and spores could be maintained for days with slow disappearance of L-glucose from the medium and no increase in optical density or (measurable increase) in cell mass. It is quite possible that the self-inhibition phenomenon first observed in B. stearothermophilus by Fuchs (1969) is responsible. The inhibitory metabolite (or one of them, at least) has since been isolated and is under study at MIT. The vegetative cultures could be maintained, even if not increased in broth culture. It is also possible that catabolism of

L-glucose is so slow that the cells are in the carbon starved situation that normally triggers sporulation.

Attempts to germinate spores in L-glucose were unsatisfactory. They could be germinated by D-glucose but sporulated when the D-glucose was exhausted without utilizing any L-glucose. Two samples of spores in ten germinated in L-glucose broth after a 15 minute heat treatment at 80° C. One sample in eight similarly germinated after brief boiling over a bunsen burner.

Repeated attempts to isolate eukaryotic forms from the thermophilic cultures failed. It is possible that this organism(s) was actually growing on lytic products of bacillary sporulation, that it required some growth factor produced by the bacilli, or that it was actually a pleiomorph of the bacillis. The first seems unlikely, however, since a white cottony mold was found in isolated colonies on two of the 55° C L-glucose agar plates inoculated from the broth. This mold(s) would not grow out when reinoculated into L-glucose broth, so the plate growth may have been due, at least in part, to saccharides from thermal or enzymatic agarolysis. The thermophile plates were incubated at 55° C and significant agarolysis was probably occurring. Once the organisms have grown several generations on other monosaccharides, they appear to lose their ability to utilize L-glucose. The growth factor requirement is still a possibility, since such a factor could be carried from broth to plate and then diluted below critical concentration when a colony was returned to broth.

The pleiomorph is also a likely explanation since hyphae and small yeastlike cells occasionally reappear in low pH (3.0) Bacillus cultures. We have since found, by personal communication (Alroy, Fuchs, Tannenbaum, 1971) that Bacillus stearothermophilus often exhibits filamentous growth

under nutrient-limited conditions. Brock (1967) and Friedman (1968) have observed B. stearothermophilus in filamentous branching growth with the spontaneous appearance of spheroplasts with unusually strong osmotically and thermally resistant membranes. A picture of these filaments and spheroplasts published by Brock (1969) looks very much like the hyphae and yeastlikes observed in this study. Darland (1970) describes a free-living procaryote isolated from burning coal refuse piles which lacks cell walls and grows as pleiomorphic spheroplasts or filaments. He classified the organism as Mycoplasma sp.

A gram + nonacid-fast, non-sporulating rod was the major population in one stage 3 culture, but we were unable to isolate it in pure culture on L-glucose. There are several possibilites for this organism(s) even if the observed separate, unbranched morphology is assumed to be representative. Lactobacillus, Propionobacterium, Erysipolethrix, Cellulomonas, and Brevibacterium, are all good condidates. Listeria does not have versatile carbon requirements and Kurthia generally requires amino nitrogen. Cellulomonas is of some interest because it is common in soil and decomposing plant tissue, pleiomorphic, and gram variable. If a single strain with the proper morphology were isolated, it could be very similar to a Pseudomonas. It has peritrichous flagella. Brevibacterium is a "catchall" taxon for this type of bacterial rod, similar in function to the genus Torulopsis for nonfermenting yeasts.

We recognize the fact that the enrichment method does not guarantee that all potential L-glucose utilizers in the original samples were present at stage 3. Among those not isolated might be:

1. those very slow to start which are overgrown.
2. those sensitive to inhibitors produced by other organisms.

3. late bloomers similar in appearance to the original population which would be overlooked by the observer

4. those requiring a growth factor not supplied in the medium

Similarly stage 3 could contain organisms unable to grow on L-glucose in subsequent pure culture, including:

1. those actually growing at the expense of the primary population rather than on L-glucose.

2. those requiring a growth factor not supplied in the medium, but produced by accompanying organisms

3. those capable of growth on CO_2 ($CO_3^=$) non-photosynthetically.

Finally, the mixed cultures still in our maintenance library may have L-glucose utilizers not yet isolated.

B. Identification of L-Glucose Utilizers

Tentative identifications of ten of the twelve organisms isolated are summarized in Table XVIII.

The classification of the bacterial rods is not definitive because the organisms are in the very difficult taxonomic areas of the Pseudomonadaceae-Achromobacteraceae and because one important criterion, flagellation, is uncertain. The Pseudomonadaceae and Achromobacteraceae are in the Pseudomonadales and the Eubacteriales respectively. These orders are very large taxonomic groups and they are normally separated on the basis of trichomous growth. However, trichomes may never be observed in most taxa of the Pseudomonadaceae, and the phenotype of this family differs from that of the aerobic members of the Achromobacteraceae mainly in having polar flagellation rather than peritrichous. Our Leifson stain method of flagella determination was often inconclusive, and electron microscopy was not available at the time of this study. With this precaution, we propose

the identification listed in Table XVIII. The yellow pigments in RF-1 and RF-2 were checked for the uv absorption characteristic of Xanthomonas sp. (Starr & Stephens, 1964), but none was found. The pigment in RF-1 is slightly diffusible, and in RF-2 it is definitely not.

RF-1 utilizes D-arabinose, but not D-fucose, so it might be excluded from the P. pseudomallei and fluorescent groups described by Stanier et al (1966). Since these organisms were isolated on the basis of L-glucose metabolism, however, carbohydrate utilization patterns may not be good taxonomic indices.

RF-3 could be either Pseudomonas or Achromobacter, depending on flagellation. In consideration of slightly darkened zones from a tan pigment diffusion on agar plates, we have classified it as Pseudomonas.

RF-11 has a pink-coral red slightly diffusible (?) pigment. The organism does not grow anaerobically and is Voges-Proskauer negative, so we have tentatively assigned it to genus Pseudomonas.

RF-4 is a gram + coccus which grows as well or better at 37° C as at 30° C. It uses ammonium nitrogen, so the genera Staphylococcus and Pediococcus can be eliminated. It is obligately aerobic, does not occur in chains, and neither grows nor lyses in 40% bile salts over a two week period. On these bases, we have assigned it to genus Micrococcus in spite of its preference for 37° C. Williams et al (1953) have described a genus Aerococcus not listed in Bergey's (Breed et al, 1957) that grows well at 37° C and is not inhibited by 40% bile salts. Most cocci lyse in 40% bile salts, and it is possible that our RF-4 would be reclassified as Aerococcus in the future.

The 45° to 65° C temperature range of RF-9 with no growth at 30° or

37° C definitely places this organism as Bacillus stearothermophilus. The swollen sporangia with central to terminal spores observed are consistent with this identification. Anaerobic growth is poor, but present.

RF-10 does not grow at 60° C and the sporangia are nonswollen with subterminal, ellipsoidal spores and could be either Bacillus subtilis or B. licheniformis. Since we have not seen anaerobic growth, we have classified it as B. subtilis.

RF-14 is still a taxonomic enigma. Black yeasts are reported (Skinner, 1947; Pfaff et al, 1966; Rose & Harrison, 1969; Lodder, 1971), but they all turn black after aging of lighter colored colonies. Reflection plating showed no evidence of ballistospores, so the Order Sporobolomycetaceae cannot be proven. Cells growing on L-glucose look like Sterigmatomyces, but 0 to 3% NaCl has no effect on growth and the sterigma disappear in culture on richer media. Some of the sterigmate cells are reminiscent of Debaryomyces hansenii with exaggerated necks on the ascospores. Interestingly, RF-13 keys directly to Torulopsis candida, suspected to be an imperfect form of D. hansenii (Lodder, 1970). RF-13 and RF-14 were single colony isolates from the same culture. Other than this tenuous circumstantial evidence, however, there is no reason to believe that the organisms are related. Growth on L-glucose is poor enough to precipitate ascosporulation in most yeasts and it is remotely possible for a mutant to be "locked into" a phenotype that is normally a very minor morphological form. For the same reason, RF-14 could be a variant of any of a number of perfect fungi.

C. L-Glucose Metabolism

1. L-glucose Transport

The initial step in utilization of any carbon source is its transport to the cell interior. The transport rates measured for RF-1 can be converted to the growth rates that they will support under transport limitation if we assume a yield (Y) of 0.5g CDW/g L-glucose. If we then plot these points on the Lineweaver-Burk plot for growth on L-glucose as in Figure 27, it can be seen that L-glucose uptake is not obviously limiting growth rate over the concentration range examined. In this case, μ_{max} could be .0833 (t_d = 8.3 hr.). If a lower yield were assumed, the uptake line would be closer to the measured growth line, but even at Y = 0.4, as in the dotted line, uptake could support a higher growth rate if the L-glucose were immediately metabolized. Still, L-glucose uptake rates are on the order of the growth rates. It is quite possible, perhaps even likely, that the K_m of one or more subsequent reactions is high enough that the growth rate is limited by the intracellular L-glucose concentration or that of its immediate metabolites. In this sense, L-glucose transport may be limiting growth rate. If so, there is at least one other limitation involved.

At 2.63×10^{-3} g L-glucose/(g CDW-min), our V_{max} for L-glucose transport is about 2.6x that measured by Kotyk (1967) for Saccharomyces cerevisiae grown on 30 g/L D-glucose. Our K_m = 0.65 g/L is about 3.6x higher than Kotyk's 0.18 g/L. These data from two such disparate organisms are interestingly close to each other.

Cells grown at 0.5 g/L L-glucose had essentially the same uptake rate at 5 g/L L-glucose as those grown at 5 g/L. Thus, the hypothesis of Gaudy et al (1971) that initial substrate concentration determines the maximum

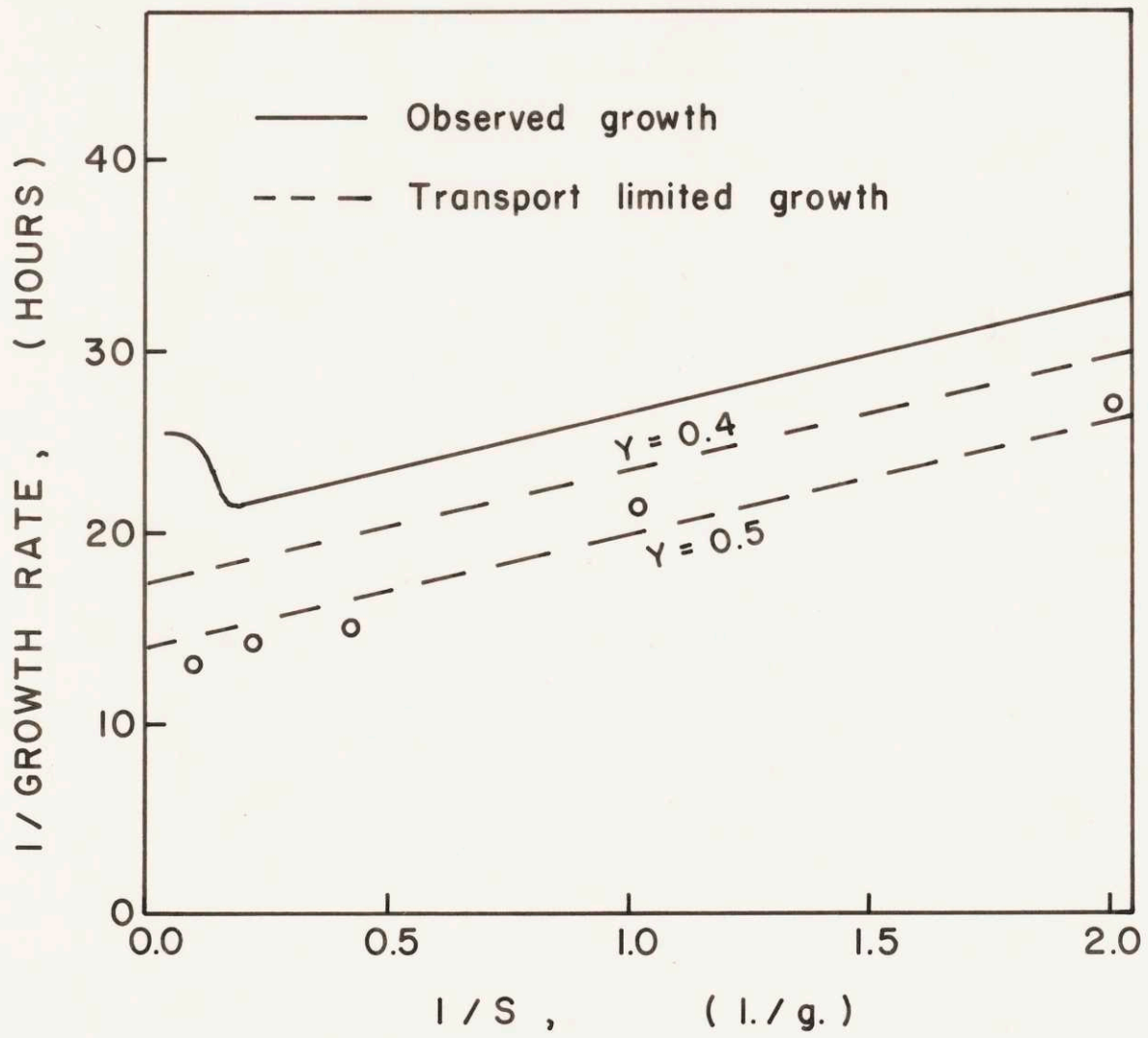

FIGURE 27.- EQUIVALENT GROWTH OF RF - I UNDER TRANSPORT LIMITATION.

growth rate of Flavobacterium sp. on D-glucose, D-fructose and sucrose by control of the number of permeation sites does not apply to the uptake of L-glucose by RF-1. The same experiment shows that Harvey's, (1970) precautions against measuring specific enzyme rates on cells grown under rate-limiting conditions do not apply to the concentration range and accuracy of our transport study. In any case, it would have been impossible to grow RF-1 (or RF-2 or RF-11) under other than carbon-limited conditions on L-glucose because of the substrate inhibition and high overall K_m observed.

Leder (1972) found that Escherichia coli can lose substantial portions of its low molecular weight metabolite pools when subjected to cold and/or osmotic shock. His work seriously challenges the use of the 0° C washes in our procedure. Additional experiments showed that the cold shock had no significant effect on the radioactivity retained by the healthy cells. The azide-poisoned cells, however, retained about 3x more radioactive label when washed at 30° C than they did when washed at 0° C. Thus, it appears that the 0° C washes in our procedure were not only unnecessary, but potentially incorrect. Fortunately, healthy RF-1 cells do not release their intracellular pool easily and our results are not compromised.

L-glucose transport in RF-1 is evidently a process of facilitated diffusion. This is not surprising in retrospect because of the rarity of L-glucose, but organisms from environments with low carbon source concentrations often use active transport for carbohydrate assimilation (Romano, 1972). Our results show that RF-1 has an active transport system for D-glucose (probably constitutive). It can also take up D-glucose by facilitated diffusion but only 2.7x faster than it takes up L-glucose. This is a much lower selectivity between the two racemes than one would expect if

the system were normally used for D-glucose transport. One very interesting aspect of D-glucose uptake is that it apparently does not shut off L-glucose transport. When 0.1 g/L D-glucose and 5 g/L L-glucose were presented simultaneously, L-glucose uptake appeared to be delayed, and the uptake curve was not a typical hyperbola. There was enough D-glucose in the medium to make the intracellular concentration 46 mg D-glucose/ml. These results suggest that the active transport of the D-glucose has a transient negative effect on L-glucose uptake, but diffusive transport of the L-glucose is only slightly hindered. Furthermore, the equilibrium intracellular concentration of L-glucose may be independent of intracellular D-glucose.

2. Adaptation of Resting Cultures to Growth on L-Glucose

The growth rates, plating efficiencies, and yields of resting cultures on L-glucose (Figure 10) suggest that there is an appreciable fraction of dead (non-reproducing) cells. These non-reproducers are not induced to growth on L-glucose, but are gradually outgrown by cells able to use it. In fact, a substantial fraction of the cells reproducing must have daughters unable to utilize L-glucose to account for suboptimal growth after twelve generations (4000x). If the capacity to utilize L-glucose is a result of a phenotypic mutation, it is a remarkably unstable and reproducible one, since the phenomena of adaptation to L-glucose are reestablished if a growing culture is held in the resting state for a few weeks. The cultures behave as though there are a limited number of molecules of an essential enzyme or cofactor and many daughter cells are left without it until the culture reaches a high enough growth rate (energy level?) to generate a sufficient supply. RF-11 will not adapt to L-glucose from rest if our standard 6-CFM is used, but it will grow as well as RF-1 if it is started on sterile used medium. Transfer of L-glucose cultures to fresh medium

always results in a measurable lag even if the inoculum was growing exponentially at a good rate. This lag does not appear when growing cells are transferred to L-glucose broth from D-glucitol or D-arabitol cultures and possibly D-mannitol. The growth rate data on RF-1 was significantly lower when measured over the first two generations from a heavy inoculum than when started from very low inocula and permitted to grow six or more generations at steady state. These observations all point to an unknown growth factor, possibly excreted into the medium during relatively healthy growth, that is made in insufficient quantities at low metabolic levels. It is possible, of course, that the above observations are unrelated. The slow adaptation for example, could be caused by inefficient enzyme induction, the behaviour of RF-11 by inducing carbon compounds in used medium, and the transfer lag by suboptimal vitamin levels. If optimal growth in RF-1 required a level of dissolved CO_2 in the medium above that obtained from the .03 atm. ambient partial pressure, some of this behavior might be explained. Thus, the mysterious growth factor could be an enzyme, cofactor, vitamin, inducer, or simply CO_2. It could also be a combination of these. If CO_2 is responsible, it should be easy to incorporate a starting "shot" of Na CO_3 into the procedure for future use. Our procedures up to this point have employed agitation in baffled flasks, which might not be good for startup. The substance required for initiating growth of RF-11 on L-glucose is likely to be medium soluble, but is probably not volatile.

3. Growth of L-Glucose Utilizers on Other Carbohydrates

When the growth of L-glucose-adapted RF-1 on other sugars is considered, the most striking point is the short lag times and high growth rates on the polyols, D-glucitol, D-arabitol, and possible D-mannitol. It is tempting to propose utilization of L-glucose on this basis. The three

polyols have a D-glyceraldehyde configuration at carbons 4, 5, and 6 in common. D-arabitol and L-glucose share an L-glyceraldehyde configuration at carbons 1, 2, and 3. There are no other obvious points of correspondence. If L-glucose were hydrogenated and fed into an oxidoreduction network like that of figure 5, it is conceivable that any of eight structures could arise by dehydrogenation or by successive oxidoreduction resulting in epimerization at carbons 2 or 5. Thus, the L-glucose structure could lead to L-glucitol, L-fructose, L-mannitol, L-mannose, D-sorbose, D-iditol, D-gulose, or D-idose if suitable activity were available on each intermediate. Microbial growth on L-fructose and L-mannose have been reported, and enzymes with good activity on L-glucitol, D-iditol and D-idose have been isolated. The polyol dehydrogenases are typically nonspecific and simultaneously induced. We feel that this mechanism is a prime suspect in the search for the metabolic pathway for L-glucose. It must be remembered, however, that polyol shunts also have regulatory functions on NAD^+, NADH, $NADP^+$, and NADPH levels (Hollman, 1964; Wimpenny & Firth, 1972) and that cultures growing on L-glucose are undoubtedly under abnormal metabolic stresses. Thus, the observed responses to polyol substrates may be incidental to side reactions of L-glucose utilization. In particular, if the organism must fix CO_2 to grow well (see Section 5, p.), it must generate some reducing potential. A high level of CO_2 fixation may not be a preferred route when the organism is growing rapidly on a good carbon source.

Another interesting carbohydrate with a growth rate close to that on L-glucose is L-xylose (5-decarbohydroxy L-glucose). This sugar is rare in nature, and if the 6 hour lag is due to transport problems, it could be being utilized via the same pathway as L-glucose. L-xylose utilization typically occurs by isomerization to D- or L-xylulose, free or enzyme bound,

through xylitol followed by phosphorylation. This again implicates an oxidoreduction reaction. Pseudomonads utilize several pentoses and hexoses via their bionic acids, however, so no conclusion can be drawn.

The shiftback experiment from D-glucitol, D-arabitol and D-glucose to L-glucose is of interest because it strengthens the implication that the polyol pathway(s) is involved in L-glucose utilization. Even more interesting, is the fact that D-glucose did not shut down L-glucose utilization. The 4 1/2 generations of growth on D-glucose should have been enough to seriously slow the organism's return to normal growth if the necessary enzymes needed reinduction or if they were affected by catabolite repression at these growth rates. In addition, the D-glucose shiftup and D-glucose plus L-glucose uptake experiments support the conclusion that the presence of D-glucose does not stop L-glucose transport or metabolic activity. On the other hand, the deadaption of L-glucose utilizers on agar or after 18 generations of growth on D-glucose support our previous conclusion that the organism is synthesizing an essential factor during growth on L-glucose. While 100-fold dilution has only a minor temporary effect on this factor, extended monsynthesis is lethal to the L-glucose utilizer.

It will be interesting to see if the late-blooming populations on TSY plates are associated with these phenomena or with the relatively long generation time (6 hours) of this organism on D-glucose. There is no obvious connection at this time. Note that, during carbon starvation, the number of cells producing visible colonies did not increase as much as the total cell number (Figures 16 and 18).

4. Cell Response to Carbon Starvation

The carbon starvation experiments were initiated because it was felt that the organisms might require depletion of an internal L-glucose pool before

maximum initial uptake rates could be acheived. In association with this requirement, we did not want to perform the uptake experiments with cells so damaged that they would be unable to operate normally. Therefore, we also wanted to establish how long the cells could be starved and still display a normal response to subculture on L-glucose. The first experiment ran only eight hours. It established that the cells were virtually unchanged in response during this time, and that they continued to divide without increase in cell mass after removal of the extracellular carbon source. The third experiment confirmed this behaviour in RF-1 and showed that this organism can be carbon-starved 24 to 30 hours without changing its growth or lag time responses. The experiments were of value in that they indicated relatively large intracellular pools of low molecular weight carbohydrate metabolites. They implied that large groups of uptake runs, spanning several hours could be made. The latter conclusion was borne out by similar results for uptake rates at 5 g/L L-glucose at the beginning and the end of a nine hour group of runs.

The second starvation experiment was inadvertantly run with a mixed culture of RF-2 and RF-11. It yielded the surprising information that some L-glucose utilizers can be starved for more than 7 generation times without imparing their viability or their ability to grow on L-glucose. In fact, after about 40 hours of carbon starvation, initial mass accumulation rates began to increase, peaking between 80 and 120 hours. Furthermore, a lag B phase correlating with starvation time appeared after inital growth, followed by growth at a rate normal for the L-glucose concentration.

We interpret these results to indicate that initial L-glucose transport proceeds at a rate higher than the steady-state growth rate of the cells. Then L-glucose, or an intermediate derived from it, builds up an intra-

cellular concentration so high that it seriously inhibits one of the reactions in the sequence. Whereupon L-glucose or the intermediates involved build up in concentration until the facilitated diffusive transport of L-glucose stops. When enough material has passed through the inhibited step, the inhibition is partially relieved, and the growth lines out at the steady state rate. This hypothetical sequence is consistent with the transport and growth rates measured on RF-1 and with the observed substrate inhibition on RF-1 growth rate. If the L-glucose molecule were cleaved into two 3 carbon moieties, as in D-glucose in Emden-Meyerhoff-Parnas (EMP) glucolysis, one of them would be L-glyceraldehyde, and L-glyceraldehyde is known to be toxic to some organisms. (Lardy, 1950; Cutinelli et al, 1967) Lardy's work showed that the actual inhibitor in mammalian tissue was L-sorbose-1-phosphate, a product of the condensation of L-glyceraldehyde and dihydroxyacetone phosphate. The use of the EMP pathway is not consistent with evidence of elimination of carbon 1 as CO_2 in the radioactivity chase experiments.

5. The Fate of Carbon 1 of L-glucose

Chasing the radioactivity of cells preloaded with labelled L-glucose and grown in unlabelled medium (Figure 26) shows that essentially all of the label from a short pulse is eliminated from the cells. We take this to indicate that carbon 1 is not incorporated into macromolecules. Concommitant to this is the conclusion that it is part of a pool of soluble low molecular weight metabolites.

Cells preloaded with labelled L-glucose for longer periods of time show a higher degree of label incorporation. An earlier experiment with unlabelled L-glucose showed that RF-1 does not readily release it into the medium. The radioactivity in the medium at its highest accounts for less

than 10% of the label in the cells, and it slowly disappears from the medium after it stops declining in the cells. Furthermore, the radioactivity in the medium is substantially reduced by acidifying and boiling. Therefore, the carbon 1 is released from the cells as a volatile substance. The yields of this organism have regularly been 0.44 to 0.48 g CDW/g L-glucose and anthrone-sulfuric acid analyses have shown quantitative removal of reducing carbohydrates from the medium. Therefore we interpret these results as CO_2 generation from carbon 1 of the L-glucose accompanied by simultaneous reassimilation and evaporation of the CO_2 in the medium. Pseudomonas oxalaticus is known to fix CO_2 (Doelle, 1969). In fact, when growing on formate as sole carbon source, all of the formate is oxidized to CO_2 and reassimilated via phosphoglycerate or malate. CO_2 can also be fixed into pyruvate or phosphenol pyruvate. Since Wood and Werkman found a net increase of carbon during a Propionobacterium arabinosum fermentation in 1936, hetero-trophic CO_2 fixation has been observed in a variety of organisms. The subject is reviewed in detail by Doelle (1969). These organisms typically will not utilize CO_2 as sole carbon source. It is very difficult to measure the radioactivity in the broth accurately below 200 cpm because only 0.1 of the sample is read.

6. Growth Rates on L-glucose

The growth rate-concentration data presented in Figures 19 and 20 show a complex type of substrate inhibition. We interpret these curves as representing two or more rate limiting steps in a reaction sequence. The first step (transport) approaches V_{max} as the L-glucose concentration goes above 8 g/L. One or more subsequent steps is noticeably inhibited at equivalent extracellular L-glucose concentrations lower than this. As a result, the inhibition effect becomes growth limiting well before the transport does.

As substrate concentration is increased, the transport limitation results in essentially unchanged intracellular concentrations, so the inhibition does not increase further and the cells are growing transport-limited in a steady state of inhibition.

The placement of the points on the Lineweaver-Burk plot of growth rates (Figure 21) indicates that the reciprocal growth rate function might be a convex curve as 1/S increases beyond the range of our data. Indeed, the data from the high inoculum runs (dotted line in Figure 20) describe just such a curve. If a pure enzyme were involved, this curvature would indicate competition with another reaction for a scarce substrate (Webb, 1963). The behavior can also be qualitatively explained in terms of decreased cell viability at the low growth rates imposed by low L-glucose concentration. Since we suspect cell death at low growth rates and since the curves are not those of a single reaction, we suspect that the latter is the explanation.

Dean and Hinshelwood (1966) have analyzed experiments on inoculum size in detail. Using Aerobacter aerogenes, they concluded that apparent lag times and submaximal but exponential growth rates could be completely explained by cell viability. Plate count and Klett data from our low inoculum growth rate experiments (Figure 19) show that the number of cells producing countable colonies begins to depart from exponential growth at about 0.18 g/L CDW, but the rate of mass accumulation remains exponential up to about 1 g/L.

VII CONCLUSIONS

L-Glucose utilizers occur in natural soil, hay, and aerial environments. The ones isolated are typically aerobic organisms with very versatile carbon source requirements and are resistant to death under carbon starvation.

L-glucose adaptation from resting culture is slow and probably does not involve mutation. It probably does involve one or more scarce growth factors; enzyme, vitamin, cofactor, or CO_2, and at least one of these growth factors is probably soluble and excreted during steady state growth of fully adapted cultures.

The organisms which have been fully adapted and grown for extended periods have yields of 0.43 to 0.48 g CDW/g L-glucose and are probably utilizing the entire molecule. Adapted organisms have doubling times of 13 to 20 hours at 2 to 5 g/L L-glucose.

Growth of RF-1 exhibits substrate inhibition resulting in a maximum real growth rate of 0.0478 $hr.^{-1}$ (14.5 hour doubling time), but the apparent μ max by Lineweaver-Burk extrapolation is 0.0506 $hr.^{-1}$ (13.7 hour doubling time). The apparent overall K_m of growth is 0.14 g/L L-glucose. The inhibition is a complex one, clearly relieved somewhat at L-glucose concentrations above 8 g/L. Cell viability and possible self-produced soluble positive growth factors in the medium also affect growth kinetics.

RF-1 eliminates carbon 1 of L-glucose as a soluble, volatile compound and reassimilates a portion of it...probably as CO_2. L-glucose transport in RF-1 proceeds via facilitated diffusion and has $V_{max} = 2.63 \times 10^{-3}$ mg L-glucose/(mg CDW-min) and $K_m = 0.65$ g/L L-glucose. It shows classical Michaelis-Menton kinetics without visible evidence of inhibition from

0.5 to 10 g/L L-glucose. If transport is a rate-limiting step in growth of RF-1, it is not the only one. It is affected, but not inhibited by the presence of D-glucose and intracellular concentrations of L-glucose appear to be independent of D-glucose.

The enzymes required for D-glucitol catabolism are being synthesized in cells growing on L-glucose. Thus, a metabolic pathway normally devoted to polyol metabolism may be involved in L-glucose utilization. Because of the stresses of growth on L-glucose, it is not clear whether this role is direct, ancillary, or irrelevant.

The properties of RF-1 relating to its utility as a protein source from L-glucose containing feeds are:

1. The yields averaging 0.46 g CDW/g L-glucose are near those normally expected from a hexose. Thus, carbon recovery is quite good.

2. The growth rates at 5 g/L L-glucose average 17 hours doubling time and the lower substrate concentrations of continuous culture would seriously reduce this. Thus, a faster growing mutant would be desirable.

3. L-glucose utilization is not repressed by D-glucose. Thus, RF-1 could probably grow in continuous culture with traces of D-glucose present.

4. One or more possible positive growth factors have not been identified. It is not clear how this might affect continuous culture, but medium recycle or CO_2 addition might cure problems if they exist.

5. Cells have been grown to 2.8 g/L CDW in shake flasks with good growth rates and yields with no serious pelleting problems. Thus, RF-1 would probably be amenable to high cell densities in agitated vessels.

6. The cells are small, 0.4 to 0.9 by 0.6 to 4.0 μ even when growing well on L-glucose. Thus, cell recovery by conventional methods would be relatively expensive.

Other properties of a protein source, such as nucleic acid and amino acid content, amino acid profile, etc. have not been considered in this work.

VIII RECOMMENDATIONS

The next steps in this research must be evaluated in terms of the objectives.

A. L-Glucose Utilizing Microorganisms

The value of adding small amounts of natural energy sources to enrichment media should be determined. A straightforward, but logistically demanding, experiment would be the screening of several duplicate samples for L-glucose (L-xylose, L-ribose?) utilizers with and without natural sugar addition. If it is really effective, this technique could be very useful for the future isolation of rare substrate utilizers.

One major question concerning the identities of our organisms is flagellation. Electron microscopy of suitable cultures should be quite helpful.

B. Pathway(s) of L-Glucose Metabolism

Our study indicated that L-glucose utilizers have substantial intracellular pools of low molecular weight metabolites. Radioactively labelled pools can be extracted with boiling water, deionized (with attention to the possibility of phosphorylated intermediates), chromatographed, and radiographed (Kotyk, 1967; Falcone and Romano, 1971; Eagon & Phibbs, 1971). Pulse labelling of starved or growing cells should establish the order in which intermediates appear. The time scale of our results indicates that sequential sampling is feasible.

Polyol metabolism appears to be related to L-glucose metabolism. L-glucose reducing activity could be examined in crude cell extracts (Shaw, 1956; Arcus & Edson, 1956; Mortlock, 1965). If such activity can be demonstrated, it could be the basis of an assay for enzyme isolation.

C. RF-1 as a Single Cell Protein Source

A faster growing mutant would be desirable. RF-1 could be mutagenized and selected for fast growth on L-glucose. Continuous culture is normally a good technique for selection, but in this case, high purity L-glucose is very expensive. Thus, sequential transfer of growing cells in small batch cultures might be better.

The effects of the simultaneous presence of other sugars should be studied. A practical process based on artificially generated carbohydrates will probably be a mixed stream. Even later stages of a multistage fermentation could be expected to receive a feed containing traces of other sugars. If the enzymes necessary for L-glucose metabolism are repressed or inhibited by any of them, residence time might be extended. The present research indicates that this would not be a problem as long as an L-glucose-adapted population is present.

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APPENDICES

APPENDIX A

Nomenclature

Definitions of carbohydrate nomenclature important to this study are in Section III - A.

CDW.....cell dry weight, g.

K_m.....Michealis-Menton saturation constant, g/L.

K_s.....Monod substrate saturation constant, g/L.

Lag 1.....standard growth lag as defined in Methods, p.64 , and fig. 12, hr.

Lag 2.....extended growth lag as defined in Methods, p.64, and fig. 12, hr.

Lag B.....lag occurring during growth as defined in Results, p. , and fig. 15, hr.

S.....substrate concentration, g/L.

uv.....ultraviolet radiation.

V.....initial reaction velocity, mg substrate/(mg CDW-mim).

Y.....yield as defined in Methods, p. , g CDW/g substrate.

V_{max}.....maximum reaction velocity by extrapolation to $S = \infty$, mg substrate/(mg CDW-min).

3-CFM.....carbon-free medium pH 3.2 to 3.5.

3-LGM.....L-glucose medium pH 3.2 to 3.5.

6-CFM.....carbon-free medium pH 6.4 to 6.8.

6-LGM.....L-glucose medium pH 6.4 to 6.8.

$(\alpha)_D^{20^\circ}$.....molar optical rotation at 20° C with sodium light, degrees of angle.

APPENDIX B

Correlation of cell dry weights and Klett readings is shown in Figure 28. These data were all obtained at doubling times between 14 and 30 hours. Klett values above 100 were obtained at 10x dilution with 6-CFM. Diluted and undiluted Klett values began to differ between 90 and 125 Klett units. Figure 29 shows a correlation between diluted and undiluted readings. Theoretically this should be a hyperbola, but for practical purposes within the reproducability of the data, the curvature is slight.

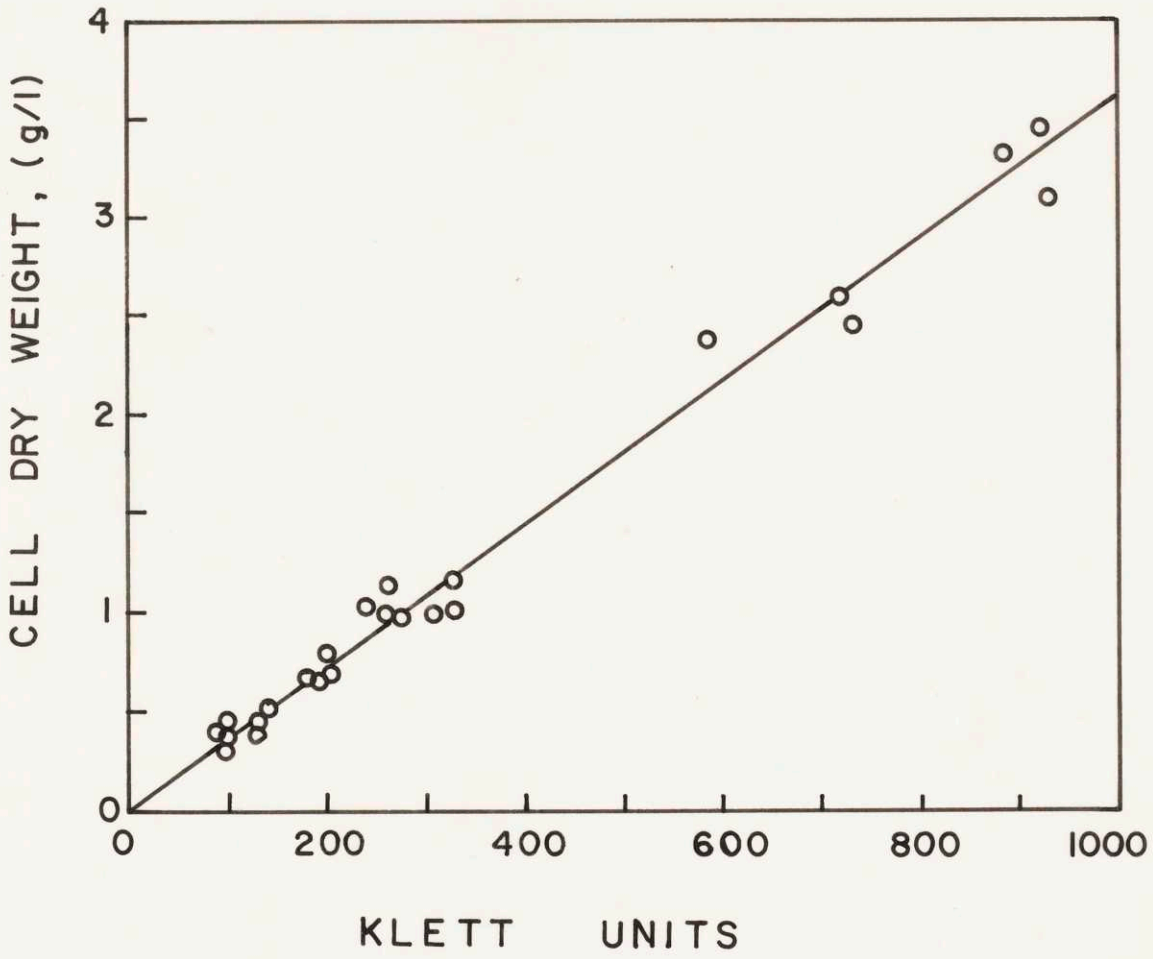

FIGURE 28.- CORRELATION OF CELL DRY WEIGHT WITH KLETT UNITS.

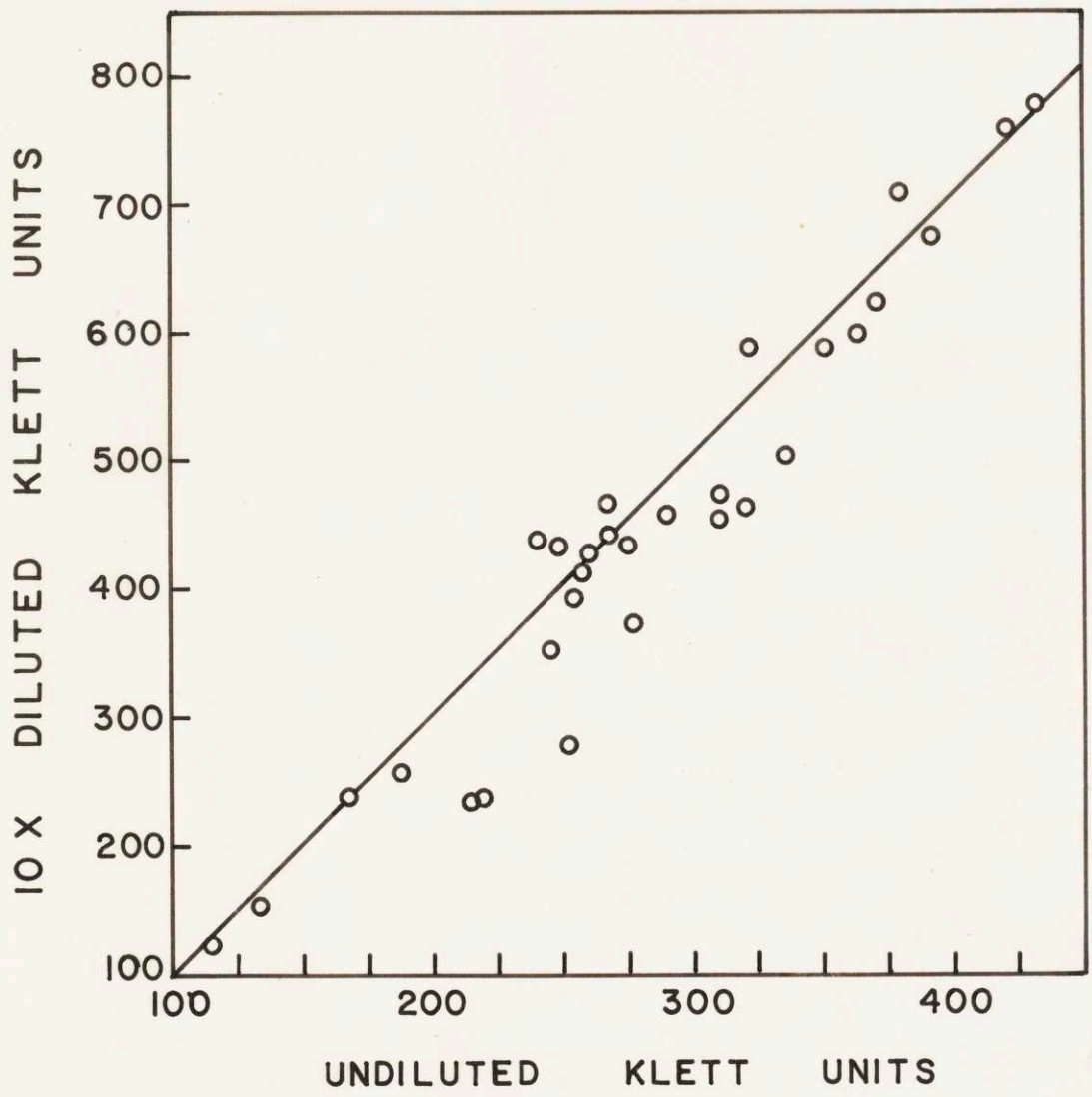

FIGURE 29.– CORRELATION OF 10 X DILUTED KLETT READINGS WITH UNDILUTED READINGS.