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Title Biological Oxidation of Sorbitol

Abstract approved

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The oxidation of sorbitol by soluble extracts of A. suboxydans proceeds by two pathways depending upon which pyridine nucleotide is present. In the presence of TPN, sorbose is formed. In the presence of DPN fructose is produced. Both products of the oxidation of sorbitol have been identified by paper chromatography, optical rotation, crystalline form and melting point of the phenylosazones.

The fructose can then be phosphorylated and further oxidized by cell-free extracts by means of the Horecker pentose cycle. Both hexose and pentose were shown to accumulate in digestion mixtures, when TPP was removed from transketolase.

The oxidation of a number of synthetic polyhydroxy compounds has been shown to be catalyzed by non-specific dehydrogenase systems of A. suboxydans.

DPN-specific sorbitol dehydrogenase has been purified a minimum of 13 fold by a three step procedure. This enzyme has been shown to be sorbitol specific. Some of the more common sugar alcohols and a few compounds showing high dehydrogenase activity were not active toward the final enzyme preparation.

Mannitol dehydrogenase is TPN specific and may be the same enzyme as the TPN-sorbitol dehydrogenase. Both these

enzymes proportionately lose activity in the purification of the DPN sorbitol dehydrogenase. Mannitol is three times as active as sorbitol toward the preparations studied.

All attempts to separate the TPN-activity from the DPN-activity failed. Both these enzymes have a pH optimum of 8.5 and are stimulated by Mg^{++} and Mn^{++} . However, the TPN enzyme is more fragile, and the DPN enzyme has a much broader range of activity with pH.

A heat denaturation method involving protection of the enzyme by both the cofactor and substrate was used for the DPN linked sorbitol dehydrogenase. This effect has not previously been shown.

BIOLOGICAL OXIDATION OF SORBITOL

by

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BIOLOGICAL OXIDATION OF SORBITOL

INTRODUCTION

The oxidation of sorbitol to sorbose by species of Acetobacter was recorded many years ago in the biochemical literature. As early as 1852, Pelouze (32) allowed mountain ash berry juice to ferment spontaneously for 14 months and obtained an unknown sugar from the mixture. Forty-two years later, Bertrand (2) showed that this product, sorbose, was formed by the action of bacteria on the sorbitol occurring in the berry. Many species of the genus Acetobacter will convert sorbitol to sorbose, but Acetobacter suboxydans is superior, giving nearly 100% conversion of sorbitol to sorbose. (10)

1

The following abbreviations will be used:

ATP	adenosine triphosphate
ADP	adenosine diphosphate
CFE	cell free extract
DHA	dihydroxyacetone
DHA-P	dihydroxyacetone phosphate
DPN	diphosphopyridine nucleotide
DPNH	reduced DPN
mm	millimicron
TCA	trichloroacetic acid
TPN	triphosphopyridine nucleotide
TPNH	reduced TPN
TPP	thiamine pyrophosphate
Tris.	trishydroxyamino methane
TTZ	triphenyltetrazolium chloride
TTZH	reduced triphenyltetrazolium chloride (formazan)

Before 1935 this fermentation was only of academic interest, but since that time sorbose has become an important intermediate in the synthesis of vitamin C (ascorbic acid). Despite the great interest in this fermentation however, the chemical nature of the pathways concerned in the conversion of sorbitol to sorbose have not previously been determined. It is the purpose of this thesis to elucidate the biochemical nature of this and other similar dehydrogenations.

Sorbitol and its oxidation product, sorbose, have been studied in animal systems mainly because they are used as sugar substitutes in diabetes, and in other food preparations. Also they are of interest in animal studies, where they represent unusual substrates, and may on occasion, function as inhibitors. Embden and Griesbach (7) in 1914 showed that the liver would convert sorbitol to a compound whose derivative was glucosazone. Todd, Myers, and West (37) have investigated the metabolism of sorbitol and mannitol in dogs and have found that sorbitol increased the blood sugar and the deposition of glycogen; mannitol did neither. These authors state that sorbitol is much more readily converted to glucose than is mannitol. In animal systems, Blakley (3,4) has done significant work on the oxidation of sorbitol. He reports the existence of an enzyme in rat liver which oxidizes sorbitol to fructose.

The same enzyme oxidizes L-iditol to L-sorbose. This enzyme is DPN specific. Other sugar alcohols such as mannitol are not oxidized. A similar enzyme has been purified from rat liver (41), which in addition dehydrogenates L-arabitol and ribitol. Further indications of the metabolic fate of sorbitol have been given by Wick, et al (39), by measuring the expired air of rats fed uniformly labeled sorbitol. Their results suggest that sorbitol is converted to fructose and is then dissimilated by two pathways.

Although some experimentation has been performed with rat liver enzymes, the majority of the polyhydric alcohol studies have been carried out on Acetobacter species. The classical studies of Bertrand report the oxidation of eight sugar alcohols to give the corresponding keto sugars (2). Bertrand proposed a rule for these fermentations which states that in order for a sugar alcohol to be oxidized, its first and second hydroxy groups must be in the cis position (2). The best examples of this rule are found in oxidation of erythritol, meso-inositol, arabitol, perseitol, and mannitol to the corresponding keto sugars.

It should be noted that sorbitol oxidation is common to both animal and bacterial materials, liver oxidizing sorbitol to fructose, and A. suboxydans converting sorbitol to sorbose. Mannitol is oxidized only in bacterial systems. That similar enzymes performing both oxidations may exist

in a single organism has been suggested by Sebek and Randles (35). On the basis of compared rates of dissimilation with adapted Pseudomonas fluorescens cells, these workers state that two pathways exist, one leading to sorbose and the other forming fructose; however, they did not identify the fructose and based their conclusion only upon the failure of the adapted cells to utilize sorbose. Thus the discussion in the previous paragraphs indicates that the oxidation of polyhydric alcohols as a group, is of interest in any examination of sorbitol oxidation.

The entire phenomenon of dehydrogenation in A. suboxydans is truly interesting. Other oxidations besides those of the polyhydric alcohols seem to be non-specific and even the same substrate may be dissimilated by two pathways. This effect was found in glycerol oxidation by Hauge, King, and Cheldelin (14); in this case glycerol was either phosphorylated and oxidized to DHA-P¹ or oxidized to DHA without phosphorylation. Similar phosphorylative and non-phosphorylative pathways concerning glucose oxidation in cell free extracts have been shown by Klungsøyr (27). Many glycols are attacked by this organism. D-2,3-Butanediol and D-3,4-hexanediol are oxidized to the corresponding keto compounds as reported by Fulmer and Underkofler (10). Visser't Hooft has carried out a number of alcohol

oxidations. He has shown that: glycol is oxidized to glycolic acid, 1,2-pentanediol forms acetal, and that 2,3-butanediol produces both acetylmethyl carbinol and diacetyl (18). Some relationship is thus indicated between glycol oxidations and sugar alcohol fermentations in A. suboxydans.

Of the many problems which confront the biochemist when investigating the fermentation of sorbitol, the most important is: where does A. suboxydans get its carbon source for fashioning cellular structures? This question has arisen through the researches of several workers. Fulmer and Underkoffler have shown that this organism is capable of giving 100% yield of sorbose from sorbitol (10), yet sorbose cannot be fermented further (10). King and Cheldelin have emphasized this question by proving that resting cells of A. suboxydans may, in the absence of a nitrogen source, oxidize sorbitol with the consumption of over four atoms of oxygen per molecule of substrate (25). Since the step from sorbitol to sorbose requires only one atom of oxygen per molecule of substrate, it is obvious that other pathways, including the further oxidation of sorbose, also come into play.

Although some biochemical investigations have been made concerning the nature of these fermentations performed

by A. suboxydans, very few studies have been made on sorbitol oxidation. King and Cheldelin (24) have shown that the first step in sorbitol oxidation is non-phosphorylative in nature. Their conclusion was that this initial step does not supply energy at the coenzyme level and that sorbitol in some manner supplies carbon for cell growth. Thorough studies have been made by Hauge, King, and Cheldelin (14,15) concerning the oxidation of glycerol to DHA. Since glycerol is a polyhydric alcohol which also can function as the sole carbon source to growing cells, glycerol and sorbitol may likely have analogous modes of dissimilation.

The preceding studies on glycerol oxidation led to the elucidation of the main pathway of oxidation in A. suboxydans, where Hauge, King, and Cheldelin (15) showed that the Horecker pentose cycle is capable of oxidizing glycerol and glucose partly to CO_2 and water. This pathway not only can supply energy through oxidation, but can also manufacture those intermediates which could be used in the preparation of cellular materials. If some pathway in the oxidation of sorbitol could be connected to this cyclic mechanism, then a ready explanation would be available for the question previously proposed as to the source of carbon in A. suboxydans. Proofs will be given

herein for this important link, together with studies on sorbitol dehydrogenase.

METHODS AND MATERIALS

Organism

Acetobacter suboxydans ATCC no. 621 was used for all experiments. It was maintained on yeast-glycerol slants according to a method of Sarett and Cheldelin (34). The stock culture was transferred every thirty days and a forty-eight hour culture was used in cell growth preparation.

Preparation of Cells

The growth medium for the preparation of A. suboxydans cells was of the following composition per liter:

Sorbitol	50 g.
Yeast extract	10 g.
KH_2PO_4	5 g.
Dow-Corning anti-foam AF emulsion	0.5 g.

Ten liters of the medium were adjusted to pH 6 and placed in 20-liter carboys. These carboys were autoclaved at 15 lbs. per sq. in. pressure of steam (120°) for 45 minutes. Air, preheated to 30° was passed extremely rapidly through

the medium by means of spargers. The amount of air passing through was found to be the limiting factor in the yield of cells. An average yield of 1.2 g. per liter was obtained by this method both with sorbitol and glycerol grown cells.

Sorbitol was used, in most cases, as the carbon source instead of glycerol as in previous work by King and Cheldelin (23), because, as will be shown later, an increase in the enzymes studied was obtained.

100 ml. of inoculum, grown in the same medium, were incubated for 48 hours at 24° on a rotary shaker in a 500 ml. Erlenmeyer flask. Best growth was obtained if two successive liquid transfers were employed.

At the end of a 40 hour growing period, the cells were harvested by means of a Sharples supercentrifuge. The A. suboxydans cells were then starved in 200 ml. of phosphate buffer, 0.05 M, pH 6, and washed twice by centrifugation in a Servall centrifuge. In those experiments involving the preparation of enzymes, the wet cells were stored at 4° prior to sonic disintegration. In other experiments, the cells were lyophilized and stored at -20°C.

Cell Free Extracts (CFE)

Cell free extracts were prepared by either alumina grinding or sonic disintegration. The alumina method is

the same as that employed by King and Cheldelin (22), and was used in the majority of the experiments unless otherwise noted. This method consists in grinding the dried cells in a mortar and pestle to which has been added the following materials: 4 g. cells, 16 g. alumina (Alcoa No. 301), 6 ml. water. The grinding was done at 4° for 20 minutes. A total of 100 ml. of water was added and the preparation was centrifuged at 25,000 x g for 90 minutes at 0°. The supernatant liquid was dialyzed against distilled water for 8 hours, although in some experiments the dialysis was carried out against Dowex-50 for 20 hours to remove thiamine pyrophosphate (Kitos, Ph.D. Thesis, 26).

The sonic disintegration method was employed when the CFE was to be fractionated. 20 g. of wet cells were suspended in 30 ml. of distilled water and the suspension was sonicated in a 10 KC Raytheon magnetostrictive oscillator. The cup was cooled with tap water and the sonic digestion carried out for 45 minutes, with 5 minute intervals of vibration and cooling. The resulting homogenate was centrifuged at 25,000 x g.

Reagents

Table I lists the reagents used and their source. Unless otherwise indicated, these chemicals were purchased

and used without further purification. In only a few instances was the purity indicated by the manufacturer.

Sorbose-1-phosphate and sorbose-6-phosphate were prepared according to a method of Mann and Lardy (29). In the synthesis of sorbose-1-phosphate, the diisopropylidene derivative was made from sorbose and this compound was phosphorylated with diphenyl phosphoryl chloride (9) in cold, dry pyridine. The phenyl groups were then removed by hydrogen reduction with Adams' catalyst. $\text{Ba}(\text{OH})_2$ was added, and the barium salt precipitated with two volumes of acetone and then converted to the potassium salt by passing through an amberlite IR - 100 column charged with 1 M KCL. The product was shown by paper chromatography and enzymatic studies to be identical with a sample of sorbose-1-phosphate that had been kindly supplied by H.A. Lardy.

Sorbose-6-phosphate was prepared in a similar manner from 2,3-isopropylidene-L-sorbose. This compound was supplied by the Eli Lilly Co. Only an impure product was obtained and no pure sorbose-6-phosphate was available for comparison.

Attempts were made to phosphorylate sorbitol and sorbose with polyphosphoric acid in a manner similar to that of Seegmiller and Horecker (36), but no phosphorylated

Table I
Reagents and Their Source

Compound	Source
Adenosine Triphosphate (ATP)	Sigma Chemical Co., Inc.
Diphosphopyridine nucleotide (DPN)	" " " "
Triphosphopyridine nucleotide (TPN) (95.2%)	" " " "
Alumina C-γ	" " " "
Thiamine pyrophosphate (TPP)	Hoffmann-LaRoche & Co.
Sorbose	Pfanstiehl Laboratories,
Fructose	" Inc. " "
Adonitol	" " " "
Dulcitol (White Label)	" " " "
2-Methyl-2-nitro-1,3-propanediol (Practical)	" " " "
Diethyleneglycol (White Label)	" " " "
Cyclohexane-1,4-diol (Practical)	H & M Chemical Co., Ltd.
2,5-Dimethylhexyne-3-diol-2,5	Matheson Coleman & Bell,
2,5-Hexanediol	" " Inc.
Styrene Glycol	" " " "
Trimethylene Glycol	" " " "
Propylene Glycol	" " " "
2-Methylpentanediol-2,4	" " " "

The following chemicals were donated

Compound	Source
Diethanolamine	Carbide and Carbon
Hexylene Glycol	Chemicals Co.
Dipropylene Glycol	" "
2-Mercaptoethanol	" "
1,5-Pentanediol	" "
1,2,6-Hexanetriol	" "
Thiodiethylene Glycol	" "
1,2,4-Butanetriol	General Aniline & Film
2-Butene-1,4-diol	" " Corp.
2-Butyne-1,4-diol	" "
α-Thioglycerol (96.8%)	Evans Chemetics, Inc.
D-Perseitol	Dr. C.H. Wang

esters could be obtained. This may be due to a drastic hydrolysis step used to remove secondary phosphate esters.

Paper Chromatography

Whatman filter paper #1 was used. Phosphate esters were prepared according to the technique of Hauge, et al (14), and located according to a method of Hanes and Isherwood (12). Sorbose and fructose were identified from digestion mixtures containing the sugars concentrated to 0.1 ml. by lyophilization. 10 μ l. of this concentrate were placed on paper and developed with phenol-water (4:1). The sugars were located by the aniline phthlate method, Partridge (31). A more specific spray reagent employing thymol, 5%; HCl (12N), 95%; and FeCl_3 (.1 g.) was studied. The paper was sprayed, heated to 100° for 5 minutes, treated with ammonia vapor and the spots studied under ultra violet light. Sorbose appeared as a dark spot and fructose showed fluorescence. However, this method was not employed in most experiments because it was not as sensitive as the aniline phthlate method.

Preparation of Derivatives

The phenylosazones of sorbose and fructose were prepared in the following manner. 1 ml. of 50% trichloroacetic

acid was added to 20 ml. of the TTZ digestion mixture and then centrifuged at 10,000 x g. to remove the precipitated protein, TTZH and TTZ. The trichloroacetic acid was then removed by extracting with ether (three times). The resulting solution was treated in a manner according to Hamilton (11), to form the phenylosazone. The amount of oxidation was measured by proper dilutions with acetone of the centrifuged TTZH formed. Six times the calculated molar amount of phenylhydrazine hydrochloride was then added to the sugar solution. An equal weight of sodium acetate was added and the pH adjusted to 5.3. The resulting solution was heated in a water bath at 100° for 20 minutes and the osazone filtered. Sorbosazone was successively recrystallized from water, chloroform, and water. Fructosazone (glucosazone) presented more difficulties and was twice recrystallized from ethanol-water and the resulting impure compound passed through a talcum powder column according to a method of Jørgensen (20).

Sugar Determinations

For colorimetric determinations a Bausch and Lomb monochromatic colorimeter was employed. Ribose was determined by the orcinol test at 660 mμ, according to the method of LePage (28). The cysteine - H₂SO₄ method,

Axelrod, (1) was used to measure both sedoheptulose at 505 $m\mu$ and hexoses at 420 $m\mu$. Total sugar determinations were carried out by the anthrone test, Moris (30). Fructose and sorbose were also measured by the resorcinol method of Roe (33). In all experiments separate standard curves were run with sorbose and fructose, since only in the anthrone test were the extinction coefficients the same. The Fiske-Su-bbarow method (8) was employed for organic and inorganic phosphorus determinations.

Studies on the optical rotation of the oxidation products of sorbitol were carried out on a polarimeter. The $(\alpha)_D^{20^\circ}$ was calculated on the basis of sorbose and fructose determined both by the cysteine- H_2SO_4 and resorcinol methods mentioned above.

Electron Transfer

Experiments with oxygen as the final electron acceptor were performed by means of Warburg manometry. In all experiments, Warburg vessels were of approximately 18 ml. volume and 3 ml. of reaction mixture was used.

The reactions with triphenyltetrazolium chloride as the final electron acceptor were carried out as described previously by Hauge, King, and Cheldelin (14). Briefly, this method consists in incubating 1 ml. of the reaction

mixture in test tubes in vacuo and stopping the reaction with 4 ml. of acetone. The protein is centrifuged and the intensity of the red formazan (TTZH) compared to a standard curve. This standard curve was made up by reducing various amounts of TTZ with $\text{Na}_2\text{S}_2\text{O}_4$. This method was adopted for larger volumes in some experiments in order to obtain greater quantities of products.

The formation of TPNH and DPNH was followed spectrophotometricly at 340 m in a Beckman model B spectrophotometer. Matched pyrex cuvettes of 1 cm. light paths were used. In each cuvette the following were added to a total volume of 2.5 ml.:

MgCl ₂	0.5 M.	0.2 ml.
Tris buffer, pH 8.5	0.05 M.	1.2 ml.
Sorbitol	0.02 M.	0.1 ml.
Enzyme		1 ml.

Readings were taken at 15 second intervals immediately after the addition of 0.5 ml. of TPN or DPN (concentration 1 mg./ml.).

A unit of enzyme activity is defined as that amount which causes an initial change in optical density of 0.001 per minute at room temperature. The initial change is calculated to be twice the change observed between 15 and 45 seconds. Although the linearity of enzymatic activity

with respect to enzyme quantity has not been established in crude CFE, it holds true with some limitations, in the $(\text{NH}_4)_2\text{SO}_4$ fraction, and has been verified after the heat purification step. Specific activity is expressed as units per milligram of protein. Protein is determined by the turbidimetric method of Bucher (5) standardized with crystalline bovine serum albumin.

Oxidations of some of the synthetic glycols, using whole cells, were performed on a rotary shaker in 100 ml. volumes. 500 ml. flasks were used and the time of reaction was usually 24 hours. This method gave larger amounts of products to characterize.

RESULTS AND DISCUSSION

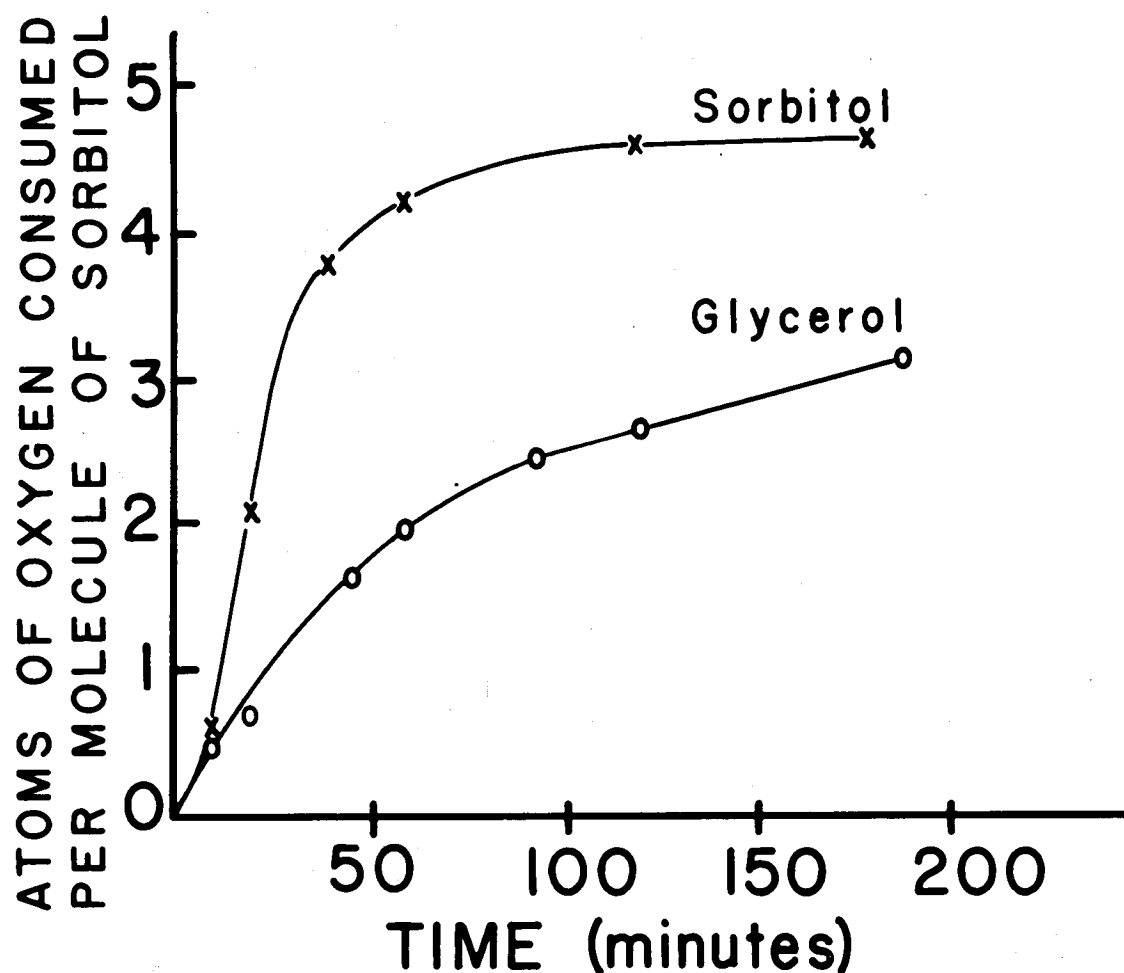
Activity of Sorbitol Grown Cells

Figure I compares the rate of oxidation of sorbitol catalyzed by sorbitol grown cells and glycerol grown cells. The rate of oxygen consumption was more than twice as great in the first hour for sorbitol grown cells. After this time period, the glycerol grown cells probably produced additional amounts of sorbitol dehydrogenase. Because of the delay in formation of enzyme in the glycerol grown cells, sorbitol cells were used in most experiments to obtain higher enzymatic activity. This experiment also indicates that the same enzymes are not catalyzing the oxidation of sorbitol and glycerol.

Sorbose Oxidation

Resting cells, grown on sorbitol in the presence of ATP, were able to oxidize sorbose with the consumption of three atoms of oxygen (see Figure 3), however, without ATP only one atom of oxygen was consumed and there was a two hour lag period before oxidation began. (See Figure 2). Sorbose oxidation without ATP was even poorer in glycerol grown cells with only 0.25 atom consumption after four hours. With cell-free extracts, either with O_2 or TTZ as

FIGURE 1
COMPARISON OF THE OXIDATION
OF SORBITOL BY GLYCEROL AND
SORBITOL GROWN CELLS OF
A. SUBOXYDANS



Manometric experiment with each Warburg vessel containing 20 mg. suspended cells, grown either on glycerol or sorbitol, 20 μ moles; $MgCl_2$, 6 μ moles ATP, 0.2 μ mole TPN, 6 μ moles sorbitol, 70 μ moles phosphate buffer, pH 6.0. Total volume 3.0 ml.; atmosphere air; temperature 29°C.

the final electron acceptor, no oxidation was obtained, even when ATP was supplied.

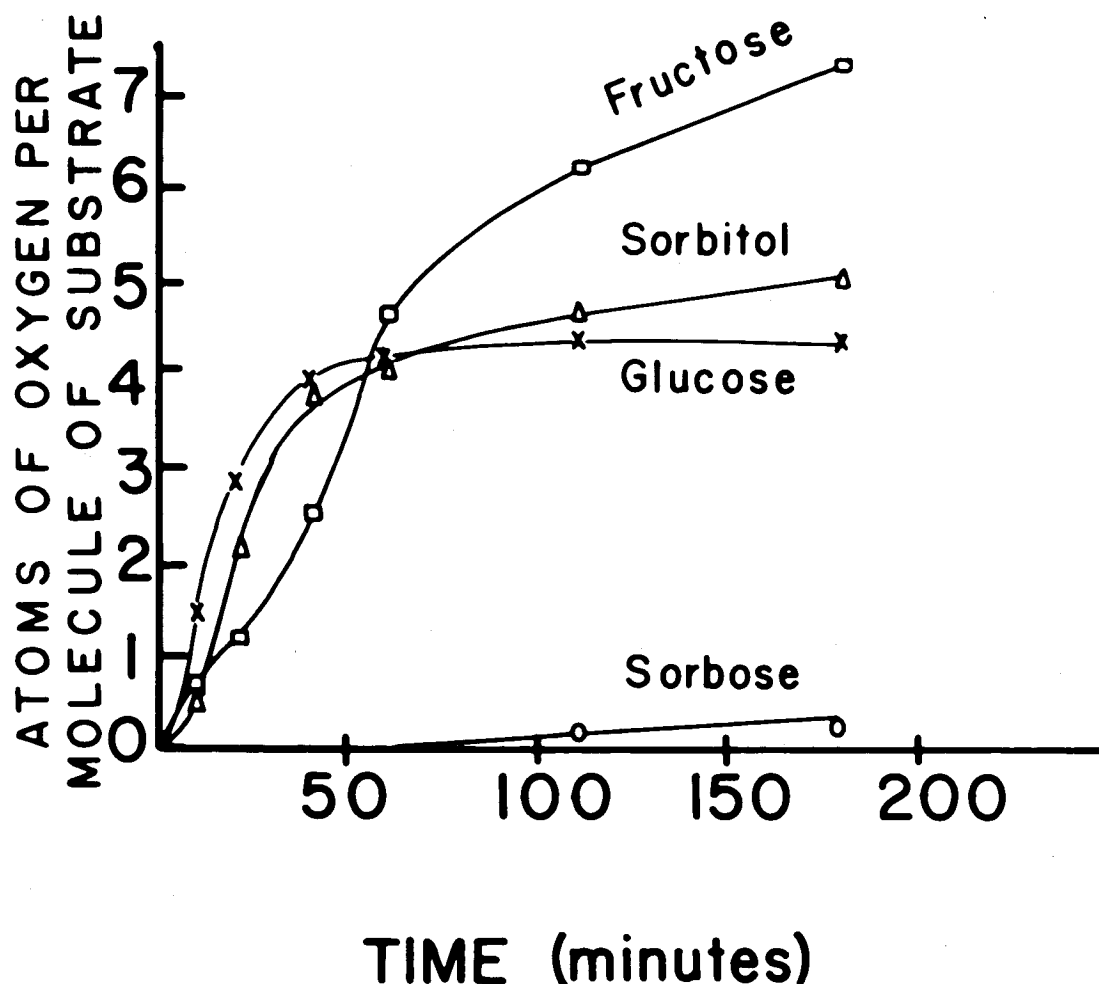
It is possible that ATP is required for the formation of phosphate esters. The most logical intermediates, sorbose-1-phosphate and sorbose-6-phosphate were prepared. Sorbose-1-phosphate was not significantly oxidized either by whole cells or CFE. Sorbose-6-phosphate was prepared in an impure state, and oxidation catalyzed by CFE using TTZ as the electron acceptor was indicated. Using paper chromatography, it was not possible to show the formation of any new phosphate esters after the incubation of sorbose with ATP and CFE in an anaerobic system; neither was any inorganic phosphate formed. Warburg manometry in bicarbonate buffer with sorbose, ATP, and CFE did not show any increase in acid that would have reflected a phosphorylation process.

Sorbitol Oxidation

Resting cells oxidized sorbitol with the consumption of up to 5.5 atoms of oxygen per molecule, either with or without ATP. This is in line with earlier observations (25, p. 584) on the non-phosphorylative character of the first sorbitol oxidation step, as contrasted to the oxidation of sorbose which is phosphate-dependent.

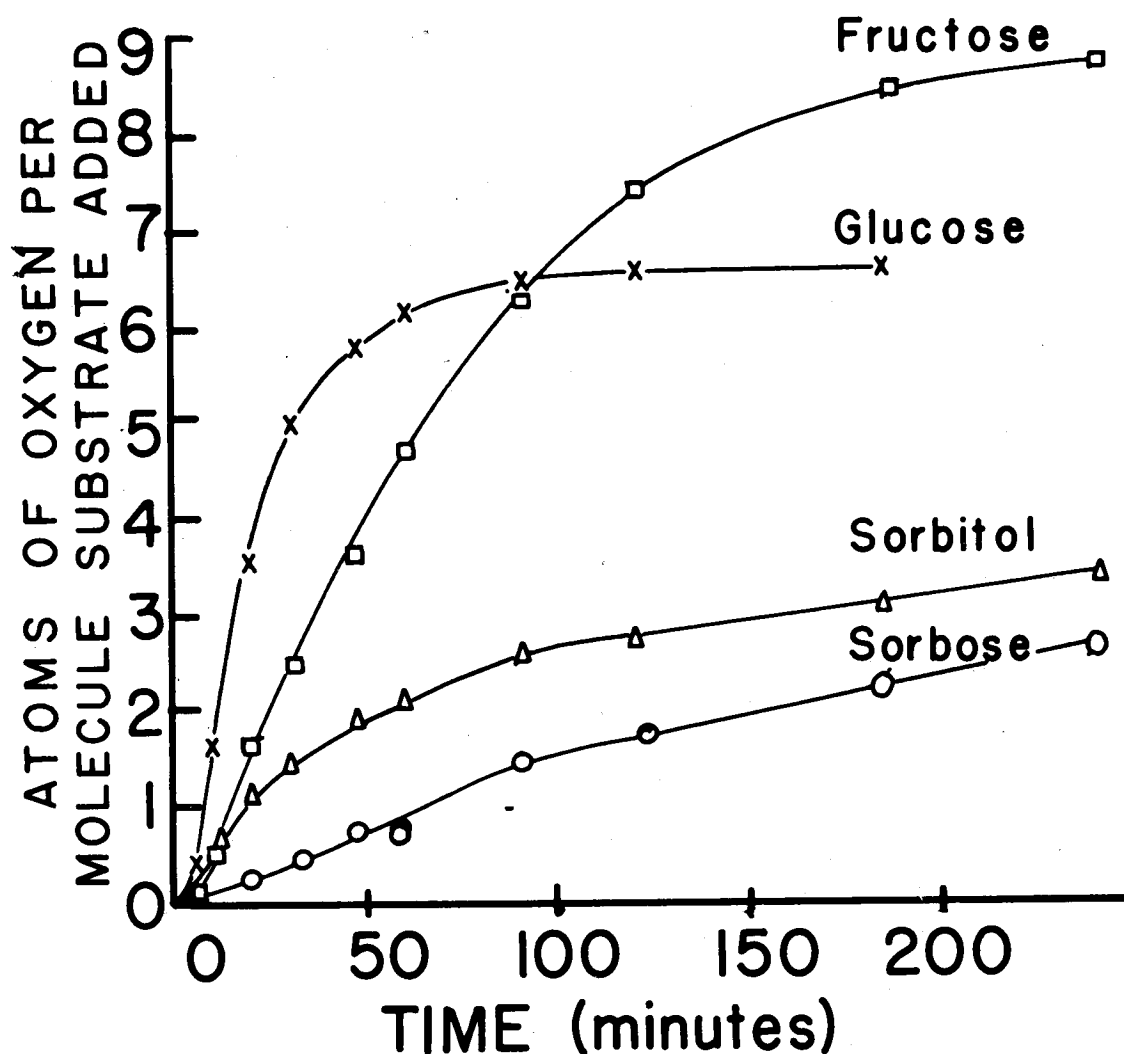
FIGURE 2

OXIDATION OF SUBSTRATES BY
A. SUBOXYDANS RESTING CELLS
 WITHOUT ADDED ATP



Manometric experiment with each Warburg vessel containing 6 μ moles of either fructose, glucose, sorbose, or sorbitol; 20 mg. of suspended sorbitol grown cells, 20 μ moles $MgCl_2$, 0.2 μ mole TPN, 70 μ moles phosphate buffer, pH 6.0. Total volume, 3.0 ml.; atmosphere air; temperature 29°C.

FIGURE 3
EFFECT OF ATP ON OXIDATION OF
SUBSTRATES IN A. SUBOXYDANS
RESTING CELLS



Manometric experiment with each Warburg vessel containing identical amounts of materials as in Figure 2, except that 6 μ moles of ATP were added.

Cell-free extract without ATP formed one molecule of TTZH per molecule of sorbitol, either with DPN or TPN. However, in the presence of ATP, DPN materially improved the oxidation over that of TPN (see Table II). This table also compares the behavior of these systems upon glucose and glycerol. It was apparent that this preferential enhancement of oxidation was peculiar to sorbitol.

Identification of the Products of Sorbitol Oxidation

Since the extent of the oxidation of sorbitol depended upon the pyridine nucleotide employed, it was thought that the products of DPN and TPN oxidation might be different. This proved to be correct as the chromatography of DPN and TPN enzyme digestion mixtures showed. Figure 4 is an illustration of one of these experiments, where fructose and sorbose were identified as the respective products. A small amount of fructose was formed along with sorbose in the TPN systems; this may have resulted from traces of DPN remaining in the enzyme preparations. Table 3 shows, in addition to the position constants, positive identification of the sugars by optical rotation, and melting points and crystalline form of the osazones.

Table II

Phosphorylative and Non-phosphorylative Oxidations
with DPN and TPN by Cell-free Extract of A. suboxydans

SUBSTRATE	TPN		DPN	
	-ATP	ATP	-ATP	ATP
Sorbitol	0.98	0.98	0.95	1.60
Sorbose	0	0	0	0
Glycerol	0	0.40	0.15	0.40
Glucose	1.30	2.40	1.10	1.95

The tubes were incubated in vacuo for four hours with 2 μ moles substrate; 2 μ moles ATP, 0.1 μ mole TPN or DPN, 50 μ moles $MgCl_2$, 100 μ moles Tris buffer, pH 8.5; 10 μ moles TTZ, 0.2 ml. of CFE. Total volume 1 ml., temperature, 30°.

FIGURE 4

PAPER CHROMATOGRAPHY OF SORBITOL DIGESTION MIXTURES

The reaction mixture contained 5 ml. dialyzed CFE, 180 μ moles sorbitol, 200 μ moles TTZ, 2 μ moles TPN or DPN as indicated, 500 μ moles $MgCl_2$, 400 μ moles Tris buffer, pH 8.5. Total volume 20 ml.; temperature 30°. After eight hours incubation, the mixture was chromatographed and developed as described in text. 1 - digestion mixture without TPN or DPN; 2 - digestion mixture with TPN; 3 - digestion mixture with DPN; 4 - fructose standard; 5 - sorbose standard.

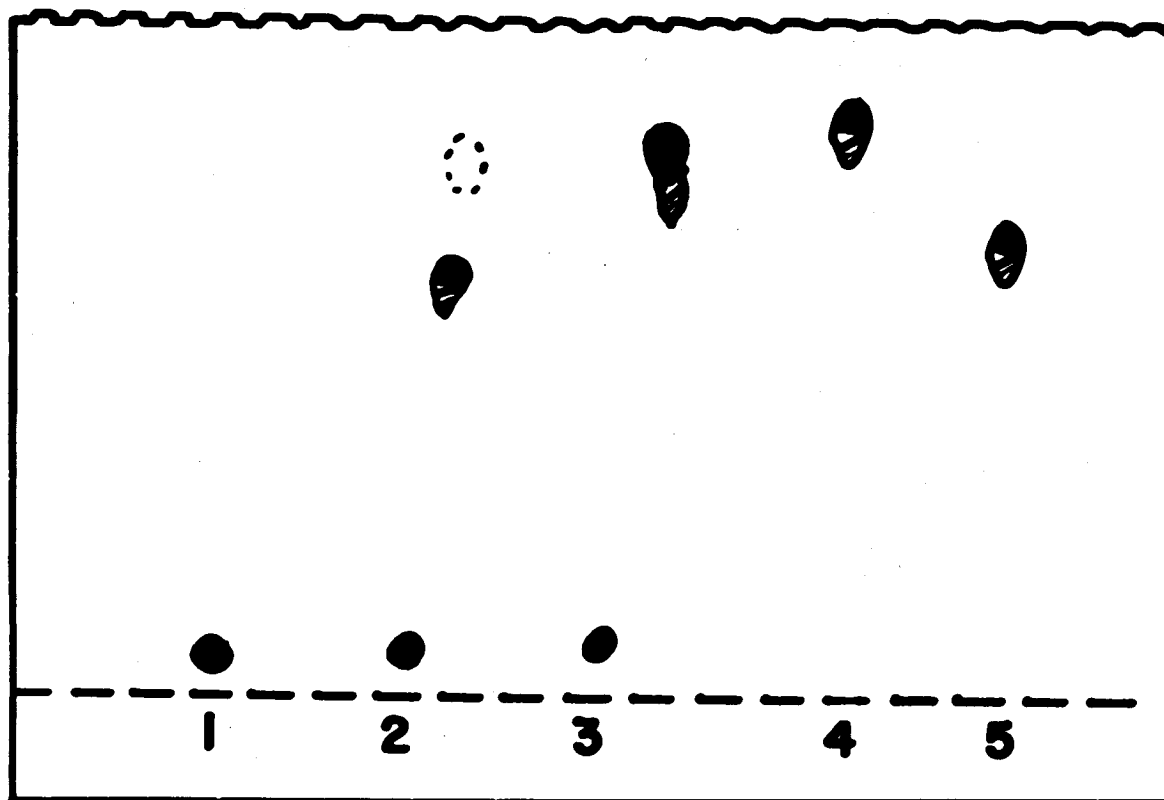


Table III

Identification of Products of Sorbitol Oxidation

	Pyridine Nucleotide added		D-Fructose Standard	L-Sorbose Standard
	DPN	TPN		
Position constant in Phenol-H ₂ O (4:1)*	0.93	0.69	1.00	.73
$[\alpha]_D^{20}$ (16, Vol. II, p. 565)	-102°	-48°	-92°	-42°
Crystal Form Osazone (13, p. 684)	Rosettes	Amorphous	Rosettes	Amorphous
Melting Point of Osazone (16, Vol. IV, p. 366)	206°	161.5-163°	206°	163°

*The position constant is the distance traveled by the compound divided by the distance traveled by fructose.

The reaction mixtures containing the following: 180 μ moles sorbitol, 10 μ moles TPN or DPN, 3 ml. CFE, 100 μ moles TTZ, 500 μ moles MgCl₂, 1 μ mole tris buffer, pH 8.5. Total volume 10 ml., temperature 30°. Time, 4 hours. 1 ml. of 50% TCA was added, the mixture centrifuged and extracted with ether. The $[\alpha]_D^{20}$ was calculated on the basis of sorbose and fructose determined both by the cysteine-H₂SO₄ and resorcinol methods.

Fructose Oxidation and Pentose Cycle Activity

In contrast to sorbose, which was not oxidized in cell-free extracts, fructose was dissimilated to the extent of about 3 atoms of oxygen per molecule in soluble extracts, and up to 8.8 atoms by whole cells, in the presence of ATP. Without ATP, however, there was a definite lag in fructose dissimulation (see Figure 2), indicating a requirement of energy for the formation of ATP which subsequently phosphorylates fructose. The intermediate oxygen consumption by resting cells in the sorbitol oxidation (5.5 atoms) is presumably due to the summation of the dual pathways of which a part of the substrate goes to sorbose with only one atom oxygen consumed per mole of sorbitol.

Upon oxidation of sorbitol with DPN, the fructose formed could be dissimilated via the pentose cycle, once phosphorylated by ATP. Table IV demonstrates the quantitative formation of pentose and hexose for both the DPN and TPN oxidations. Pentose accumulated in the digestion mixture because TPP had been removed from the enzyme (by dialysis against Dowex-50) and transketolase could therefore not function. The observed oxidation agrees fairly well with the calculated amount of TTZH based on the quantities of hexose and pentose formed if one pathway goes

Table IV
Formation of Hexose and Pentose from Sorbitol
in the Presence of ATP

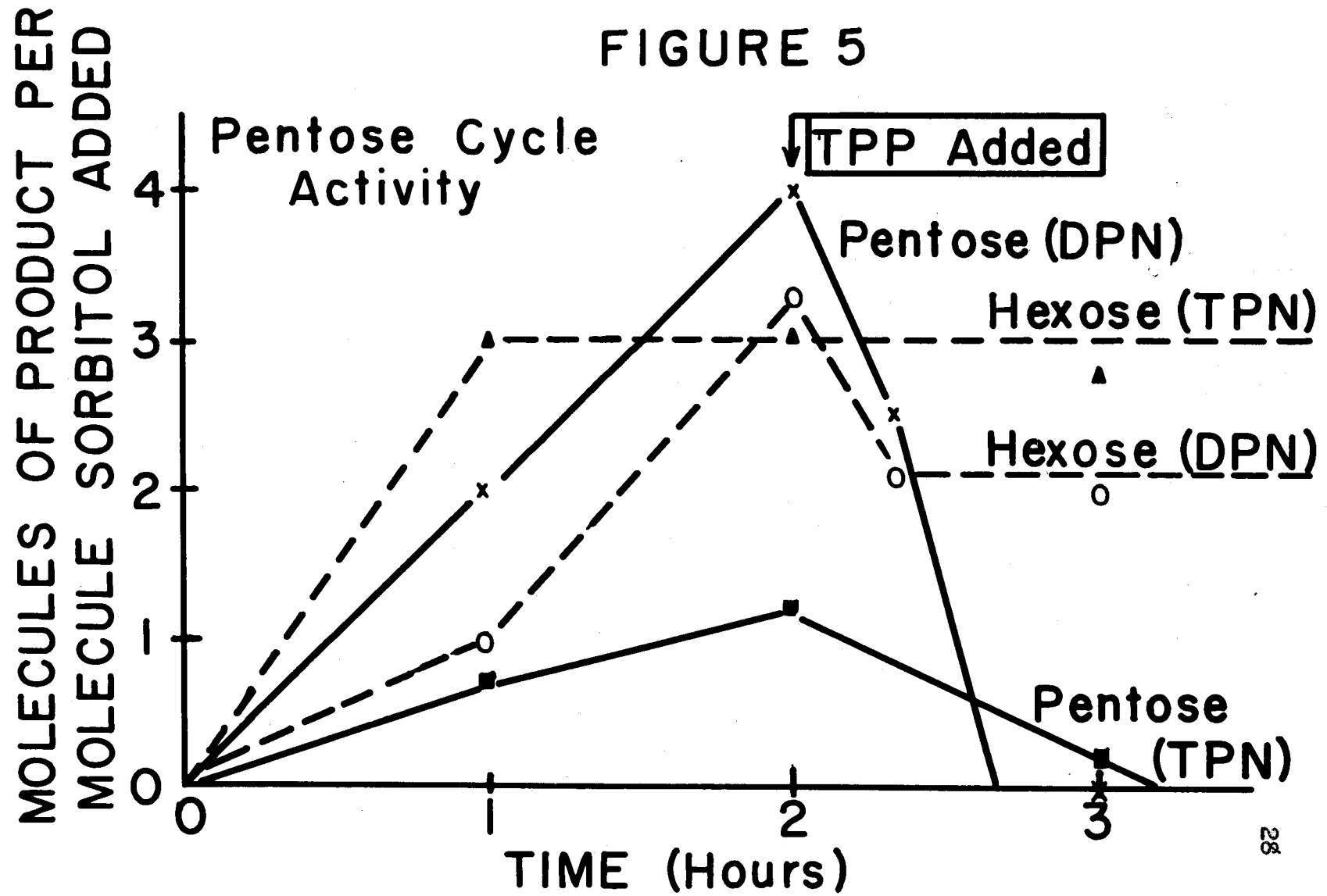
	observed μ moles	calculated* μ moles	observed μ moles	calculated* μ moles
TTZ reduced	31	25	7	7
Hexose formed	1		7	
Pentose formed	8		0	

*Based on quantities of hexose and pentose formed (3 moles TTZ should be reduced per mole of pentose obtained).

The tubes were incubated in vacuo with 20 μ moles sorbitol, 20 μ moles ATP, 1 μ mole TPN or DPN, 0.5 μ mole of $MgCl_2$, 1 μ mole Tris buffer, pH 8.5; 100 μ moles TTZ, 3 ml. CFE. Total volume 10 ml., temperature 30°. Time, 2 hours. The reaction was stopped with 1 ml. 50% TCA. Pentose and hexose were assayed as described in the text, with ribose and fructose (or sorbose) as standards.

Figure 5: CFE was dialyzed against Dowex 50 for 20 hours. The reaction mixture contained 3 ml. dialyzed CFE, 20 μ moles sorbitol, 20 μ moles ATP, 100 μ moles TTZ, 2 μ moles DPN or TPN as indicated, and 200 μ moles Tris buffer, pH 8.5. Total volume, 20 ml.; temperature, 30°. After two hours incubation, 5 μ moles TPP were added to each tube. 3 ml. aliquots were removed at the indicated time intervals, and the reaction stopped with 0.5 ml. of 50% TCA. Pentose and hexose were assayed as described in the text with ribose and fructose (or sorbose) as standards. A blank of the digestion mixtures containing no sorbitol was subtracted to give the plotted values.

FIGURE 5



to sorbose and the other pathway goes to fructose and then the pentose cycle.

Figure 5 indicates the rate of formation of hexose and pentose by the cell-free extract during sorbitol oxidation and in the presence of ATP, with TTZ as electron acceptor. TPP deficient enzyme was used and the TPP added back after two hours. In the oxidation of sorbitol with DPN and ATP, pentose formed to the extent of 0.4 mole per mole of sorbitol, then dropped to zero after the addition of TPP; hexose accumulated in the mixture until TPP was added, then dropped slightly to a constant value. In the incubation of sorbitol with TPN and ATP, 0.1 mole of pentose was formed per mole of sorbitol. This also decreased to zero upon the addition of TPP, whereas hexose reached a peak of 0.3 mole per mole of sorbitol and was unaffected by the addition of TPP. Because of the large amount of hexose in these systems, interference and unreliable values were obtained in sedoheptulose determinations. The small amount of pentose formed during the TPN oxidation probably is due to the same factors that caused fructose formation in the corresponding chromatographic experiments. Whether this effect is due to traces of DPN in the system or in part to as yet unidentified intermediates (related to sorbose?) has not been determined.

Table V

Oxidation of Some Sulfur Compounds Catalyzed by A. Suboxydans

Compound	(1) $\frac{\mu\text{atoms oxygen consumed}}{\mu\text{mole substrate}}$	(2) $\frac{\mu\text{mole TTZH formed}}{\mu\text{mole substrate}}$	Remarks
Monothioglycerol $\text{CH}_2\text{OHCHOHCH}_2\text{SH}$	1.62	0.42	Paper chromatography showed a single product, positive to both acid and azo-iodide (SH) spray reagents. (42)
2-Mercaptoethanol $\text{CH}_2\text{OHCH}_2\text{SH}$	1.98	0.90	Paper chromatography showed single azo-iodide spot (42). Readily formed 2,4-dinitrophenylhydrazones (mp. 245-248°)
Thiodiethyleneglycol $(\text{CH}_2\text{OHCH}_2)_2\text{S}$	1.45	0.42	

- (1) Each Warburg vessel contained 50 μmoles MgCl_2 , 0.2 μmole DPN, 5 μmoles substrate, 100 μmoles phosphate buffer, pH 6.0; 20 mg. suspended fresh glycerol grown cells. Total volume 3.0 ml.; atmosphere air; temperature 29°; time five hours.
- (2) Each 3" tube contained 2 μmoles substrate, 0.1 μmole TPN, 50 μmoles MgCl_2 , 100 μmoles Tris buffer, pH 8.5; 10 μmoles TTZ, 0.2 ml. CFE. Total volume 1 ml.; temperature 29°; time four hours.

Table VI

Synthetic Glycol Oxidations Catalyzed by A. Suboxydans

Compound	(1)	(2)	Comments
	$\frac{\mu\text{moles O}_2}{\mu\text{mole sub-strate}}$	$\frac{\mu\text{mole TTZH}}{\mu\text{mole sub-strate}}$	
2-Butyne-1,4-diol	0	0.20	Decomposes in soln.
1,2,4-Butanetriol	1.20	0.20	Does not support growth
1,2,6-Hexanetriol	1.45	0.36	*Supports growth 18%
2-Butene-1,4-diol	2.70	0.70	
2,3-Butanediol	0.95	0	
Propylene glycol	1.55	0.28	*Supports growth 21%
1,5-Pentanediol	1.80	0.62	
Hexylene glycol	0.83	0	
Cyclohexane-1,4-diol	0.97	0	
2,5-Hexanediol	1.80	-	
1,3-Pentanediol	1.30	-	
2-Methyl-2-nitro 1,3-propanediol	0	0.31	

The following compounds were not oxidized: diethylene glycol, pentaerythritol, dipropylene glycol, styrene glycol, and 2,5-dimethyl-hexyne-3-diol-2,5.

* Compared to the optical density of a 24 hour culture grown on glycerol.

(1) Same experimental conditions as (1) of Table V

(2) Same experimental conditions as (2) of Table V

These intermediates may also be responsible for the partial dissimilation of sorbose in resting cells, in the presence of ATP.

Oxidation of Synthetic Polyhydroxy Compounds

Tables V and VI give oxidative data, both with whole cells and CFE, on some commercially available synthetic organic hydroxy compounds. The results of these studies indicate that the enzymes of A. suboxydans which catalyze the dehydrogenation of alcohols are relatively non-specific, at least to unnatural compounds. Only initial attempts were made to identify the products of these reactions. 2-Mercaptoethanol probably is oxidized to thioglycolic acid since the reaction proceeds very rapidly to 2 atoms oxygen consumed per molecule. The product is acidic and contains a thio group. It is possible that the oxidation is catalyzed by alcohol dehydrogenase which is very active in A. suboxydans (25). Likewise, since glycerol is so readily converted to DHA by this organism, the final product of oxidation of monothioglycerol could be thio-dihydroxyacetone. This product is a carbonyl compound and contains a thio group. Its 2,4-dinitrophenylhydrazone was obtained, but it was not sufficiently pure to give the correct

carbon and hydrogen analysis. (Found: Carbon - 39.2%, Hydrogen - 2.84%; calculated: Carbon - 37.6%, Hydrogen - 3.84%)

Compounds such as 1,2,4-Butanetriol and 1,2,6-Hexanetriol have structures sufficiently similar to sorbitol so that they may be attacked by sorbitol dehydrogenase. The high value of 3 atoms of oxygen consumed per molecule of 2-Butene-1,4-diol is worthy of further investigation.

Purification of DPN - Specific Sorbitol Dehydrogenase

Step I. Preparation of Soluble Extracts - Fresh sorbitol grown cells were prepared in the manner described in the methods and materials section and subjected to sonic oscillation by the method also described earlier in the text. The unbroken cells, cellular debris, and other particulate matter were discarded after centrifugation at 25,000 x g. for 90 minutes at 0°C.

Step II. Ammonium Sulfate Fractionation - The crude CFE (100 ml.) from the preceding step was dialyzed against 60% saturated ammonium sulfate (3 liters), adjusted to pH 7; temperature 4°C., time 9 hours. The very viscous suspension resulting was centrifuged and the supernatant liquid discarded. The precipitate was dissolved in a minimum amount of water (75 ml.) and dialyzed against 40%

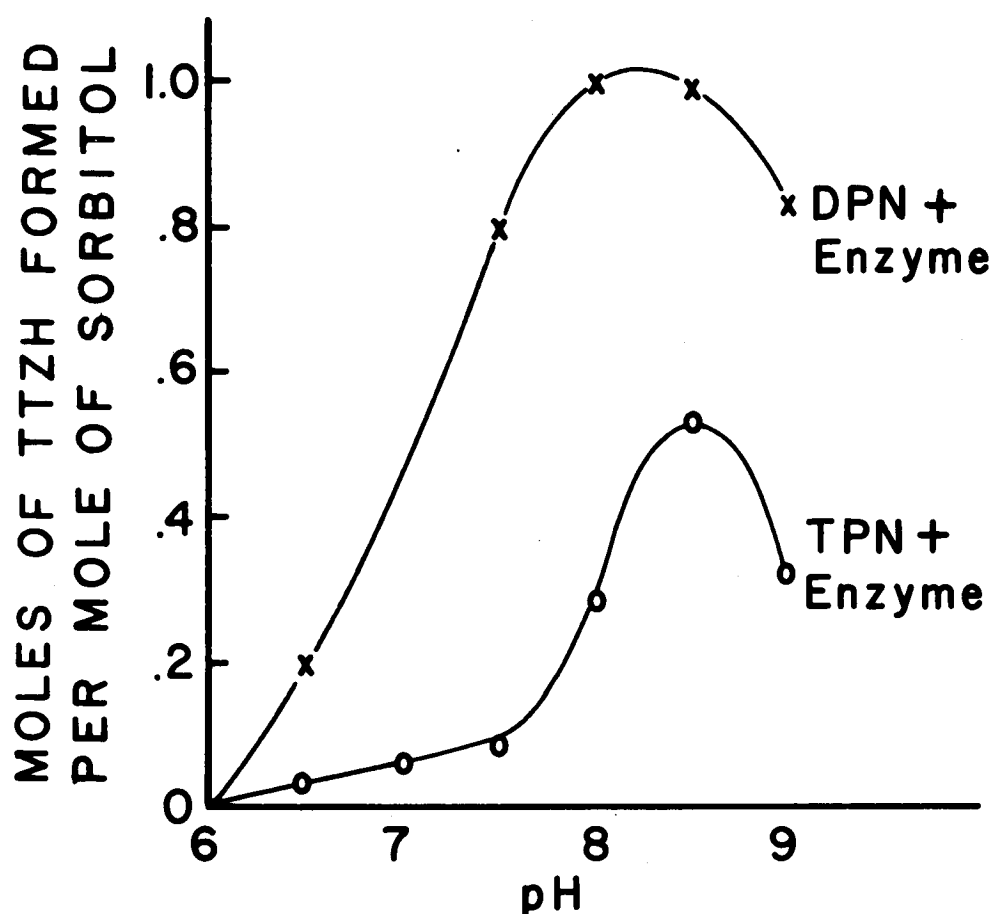
Table VII
Purification of Sorbitol Dehydrogenase from A.
suboxydans

Trial I					
	mg./ml.	units/mg.	mg./ml.	units/mg.	units/mg.
Step I Crude CFE	28	*9	32	*4	30
Step II (NH ₄) ₂ SO ₄ 40-60% Saturated	4.8	*25	4.4	*15	140
Step III Heat De- natured	0.2	900	0.39	165	390

* Enzyme activity was not a linear function of protein concentration in these fractions.

- Trial I - Steps carried out with approximately 35 ml. of CFE.
- Trial II - Steps carried out with 150 ml. of CFE, equal to 12 g. dry cells.
- Trial III - Performed by M.K. Devlin in a manner similar to Trial II except that 10 μ moles of sorbitol were used in the assay procedure and the reaction was started by the addition of sorbitol. Steps I and II were corrected for non-linearity by extrapolating the observed values to the zero concentration of the enzyme.

FIGURE 6
pH OPTIMUM OF TPN
AND DPN ENZYMES



TTZ reduction experiment, in 3" test tubes, at 30°, for two hours, in vacuo. Each tube contained 2 μ moles sorbitol, 0.1 μ moles of TPN or DPN, 50 μ moles $MgCl_2$, 0.2 ml. CFE, 10 μ moles TTZ. 100 μ moles buffer; phosphate buffer employed at pH 6.0, 6.5, and 7.0; Tris buffer used at pH 7.5, 8.0, 8.5, and 9.0.

ammonium sulfate (3 liters), pH 7, for eight additional hours, and then restored to its original volume with 40% ammonium sulfate. This mixture was then centrifuged and the precipitate discarded. The preparation was dialyzed against water until it was free of sulfate.

Step III. Heat Denaturation - To the dialyzed liquid from the foregoing step, sorbitol and DPN were added to 50% and 0.05% concentration respectively. This mixture was heated at 52° for three or four minutes on a water bath, then dialyzed at 4° until the enzyme assay showed no sorbitol. This required up to 30 hours with frequent changes of water in the dialyzing bath. The precipitated protein was centrifuged and discarded.

Attempts at further purification with calcium phosphate gel, (Keilin, 21, p. 399), and alumina C-γ were not very successful. Adsorption on calcium phosphate gel and elution with 0.2 molar phosphate buffer improved activity in some experiments, but in others it did not.

Attempted Separation of the Activity of TPN
from DPN

The TPN-specific enzyme which oxidized sorbitol to sorbose appeared to be fragile and most experiments were

Table VIII
Comparison of the DPN and TPN
Enzymes with Mannitol and Sorbitol

Fraction	SPECIFIC ACTIVITY			
	Sorbitol units/mg.		Mannitol units/mg.	
	TPN	DPN	TPN	DPN
Step I (crude CFE)	12	9	35	0
Step II ((NH ₄) ₂ SO ₄ ppt.)	8	65	16	0
Step III (heat denatured)	0	330	0	0

Units per mg.: assayed by procedure given in methods and materials section.

met with a loss in activity. Attempts to eliminate all the DPN activity from the TPN enzyme preparation were not successful; however, at least 75% of the TPN enzyme could be precipitated at a 60% saturation of ammonium sulfate. This suggests that it is a smaller molecule. The TPN activity was very strongly absorbed on calcium phosphate gel and efforts to elute it resulted in a 90% loss in activity. The activity of this enzyme was lost completely when 50% acetone or 50% ethanol were used to concentrate the activity.

Properties of the DPN and TPN Specific Sorbitol
Dehydrogenases

Figure 6 shows the pH optimum of the DPN and TPN enzymes. Although these occurred at about the same pH values (8.0 - 8.5), the range of activity for the DPN enzyme was much broader. Stimulation of the activity of both enzymes was obtained with Mg^{++} and Mn^{++} but not with Zn^{++} or Ca^{++} .

Table VIII compares the specificities of the TPN and DPN enzymes in the three fractions with respect to the oxidation of mannitol and sorbitol. DPN shows no activity toward mannitol, whereas the heat denatured enzyme shows no TPN-sorbitol activity. Mannitol is more readily

oxidized than sorbitol by the TPN enzyme and the loss of activity toward the two sugars during purification is proportional. It seems likely that TPN-sorbitol dehydrogenase and mannitol dehydrogenase may be the same enzyme.

Table IX reveals the specificity of the DPN-linked dehydrogenase toward various substrates. None of the naturally occurring compounds except sorbitol were attacked in the presence of the purified enzyme. Likewise, this preparation was only feebly active toward 2-Butene-1,4-diol in spite of the fact that whole A. suboxydans cells extensively oxidized this substrate.

Mannitol was found to be oxidized as completely as fructose by whole cells (8.7 atoms oxygen per molecule). This appears reasonable in view of the reported conversion of mannitol to fructose by this organism. (10, p.262) On the other hand, the slight oxidation of ribitol by whole cells was somewhat unexpected, as was also the failure of CFE to dissimilate this substrate. This enzyme may possibly be lost or destroyed in the sonic treatment of the cells.

Table X summarizes conditions which protect the DPN-sorbitol dehydrogenase in the heat denaturation step (Step III). High concentrations of a polyhydroxy compound and a pyridine nucleotide appear necessary to prevent denaturation of the enzyme. The more closely these compounds are

Table IX

Oxidation of Sugar Alcohols and Other Substrates Catalyzed
by Whole A. Suboxydans Cells, CFE, and Purified Sorbitol
Dehydrogenase (DPN-linked)

Substrate	⁽¹⁾ $\frac{\mu\text{moles oxygen}}{\mu\text{moles sub-}} \frac{\text{strate}}{\text{strate}}$	⁽²⁾ $\frac{\mu\text{moles TTZH}}{\mu\text{moles sub-}} \frac{\text{strate}}{\text{strate}}$	⁽³⁾ specific activity units/ mg.
Sorbitol	4.50	0.95	142
Mannitol	8.70	0.99	0
Ribitol	0.95	0	0
Dulcitol	0	0	0
Perseitol	0.83	-	0
Glycerol	-	.15	0
Ethanol	-	-	0
Acetaldehyde	-	-	0
2-Butene-1,4-diol	2.70	0.79	2

(1) Whole Cells: conditions as in Table V.

(2) CFE: conditions as in Table V.

(3) Purified DPN - Sorbitol Dehydrogenase: see definition of specific activity in the text. The enzyme used was Step III described in text.

Table X

The Effect of Polyhydric Alcohols and Pyridine Nucleotides
on the Heat Denaturation of the
DPN-Linked Sorbitol Dehydrogenase

Addition	Specific Activity units/ mg.
None (unheated)	*15
None (heated)	26
50% Sorbitol	0
50% Mannitol	0
50% Sucrose	0
0.05% DPN	0
0.05% TPN	0
50% Sorbitol 0.05% DPN	165
50% Sorbitol 0.05% TPN	96
50% Mannitol 0.05% DPN	80
50% Mannitol 0.05% TPN	52
50% Sucrose 0.05% DPN	63
50% Glycerol 0.05% DPN	90

The above compounds were added to the enzyme solution and this mixture heated to 52° for 3 minutes. The resulting solution was cooled, dialyzed against water for 24 hours, and the denatured protein discarded after centrifugation. The specific activity was determined by the method described in the text.

* Enzyme activity non-linear with protein concentration.

related to the proper substrate and cofactor, the better the recovered activity. Thus sorbitol gave the best activity.

A possible explanation (considering a three-way combination of protein-coenzyme-substrate to be involved) may be as follows: that the compound in question combines with its normal receptor site on the protein, at the same time inducing an exposure of the other appropriate site, (unattached, since the proper cosubstrate is absent); and that this results in denaturation of the protein. With both active sites occupied, the dehydrogenases for structurally unrelated compounds, as well as other proteins are presumably denatured indiscriminately so that sorbitol dehydrogenase is effectively concentrated by this treatment.

Attempts were made to purify other enzymes by this method. Ethanol and acetaldehyde dehydrogenases showed no activity after the heat denaturation step employed in Table IX. Likewise, glycerol and mannitol dehydrogenases were inactivated after heating with a 50% solution of the appropriate solution and 0.05% of the corresponding pyridinenucleotide.

CONCLUSION

Figure 7 depicts the two pathways for the oxidation of sorbitol by A. suboxydans CFE. An additional pathway has been found by Widmer, King, and Cheldelin (40) in the particulate matter. This particulate dehydrogenase catalyzes a one-step oxidation of sorbitol (presumably to a hexose). Enzymatic studies of purified DPP-specific sorbitol dehydrogenase showed that this enzyme was specific for sorbitol and may be related to the sorbitol dehydrogenase found in rat liver by Blakley (3). The TPN specific dehydrogenase has not been purified, so no conclusions can be made that it is the same enzyme which catalyzes the oxidation of many cis - hydroxy compounds by A. suboxydans. If this were true, mannitol is probably the natural substrate, and thus this organism may possess enzymes for converting both mannitol and sorbitol to fructose. Both these enzymes fit into categories proposed by Edson (6), where this worker assumes three types of sugar alcohol dehydrogenases; mannitol dehydrogenase being non-specific.

Further dissimulation of the fructose is accomplished by phosphorylation by ATP. The Horecker pentose cycle is depicted in Figure 8. Fructose-6-phosphate enters the pentose cycle and is oxidized in a manner proposed by

FIGURE 7

BIOLOGICAL OXIDATION OF SORBITOL BY
CELL FREE EXTRACTS OF A. SUBOXIDANS

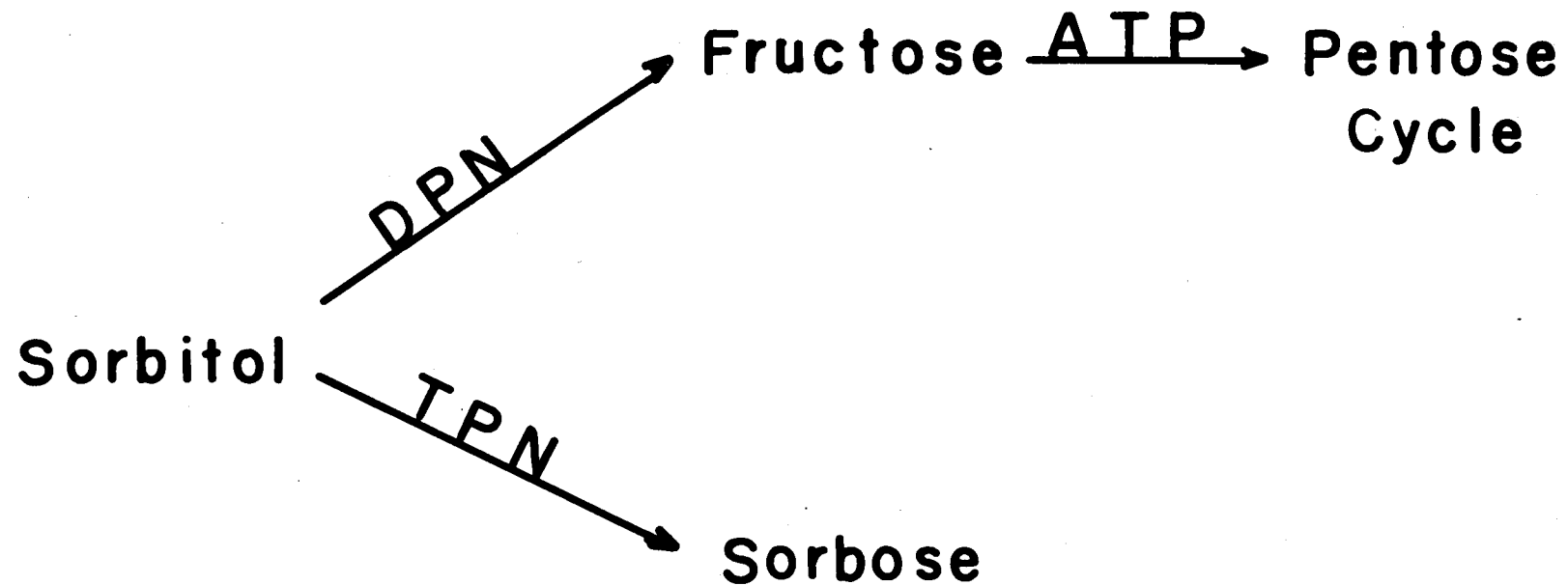
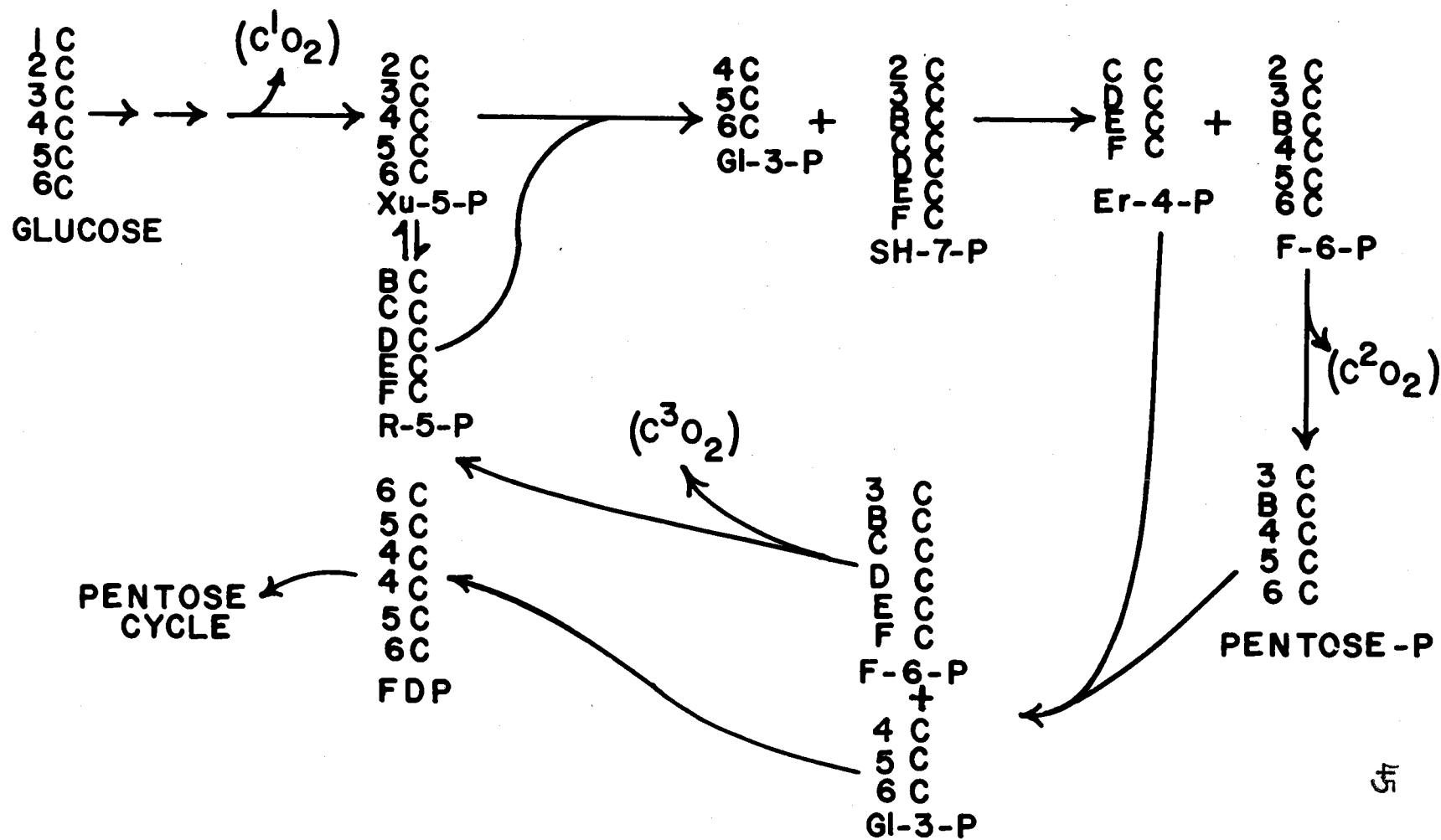


FIGURE 8
FATE OF GLUCOSE CARBON IN THE PENTOSE CYCLE



Horecker (19) and by this organism according to Hauge, King, and Cheldelin (15).

This unexpected influence of pyridine nucleotide in guiding metabolic "traffic" has also been noted in certain animal systems both in this laboratory and by Wenner and Weinhouse (38).

Sorbose, on the other hand, cannot be further phosphorylated or oxidized with CFE. Only in whole cells in the presence of an energy source such as ATP can this sugar be extensively dissimilated. Whether this process is a reduction of sorbitol by TPNH, a phosphorylation by fructokinase in a manner similar to that found by Hers (17) in rat tissue, or some still unknown process has not been determined.

SUMMARY

The oxidation of sorbitol by soluble extracts of A. suboxydans proceeds by two pathways, depending upon which pyridine nucleotide is present. In the presence of TPN, sorbose is formed. In the presence of DPN, fructose is produced. The fructose can then be phosphorylated or further oxidized by cell-free extracts. DPN-specific sorbitol dehydrogenase has been purified a minimum of 13 fold and is sorbitol specific. Mannitol dehydrogenase is TPN specific and may be the same enzyme as the TPN-sorbitol dehydrogenase.

A heat denaturation method involving protection of the enzyme by both the cofactor and substrate was used for the DPN linked sorbitol dehydrogenase. This effect has not previously been shown.

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