APIOSE REDUCTASE FROM AEROBACTER AEROGENES

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DONNA L. NEAL

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ABSTRACT

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by Donna L. Neal

This thesis is concerned with the characterization of a new enzyme, apiose reductase, present in a strain of A. aerogenes PRL-R3, selected for its ability to utilize D-apiose, as its sole source of carbon. The enzyme was not present when this strain was grown on D-glucose. Apiose reductase reduces D-apiose to its corresponding sugar alcohol, named apitol, in the presence of reduced nicotinamide adenine dinucleotide. The enzyme was characterized in both the forward and reverse direction with regard to pH optima, substrate specificity and Michaelis constants. In the forward direction (the reduction of D-apiose), studies were also conducted on the effect of sulfhydryl reagents, inhibition by p-chloromercuribenzoate and stability of the enzyme. Using paper chromatography the products of the forward and reverse direction were tentatively identified as apitol and D-apiose respectively. This study does not permit a conclusion to be reached as to whether this enzyme is directly involved in the dissimulation of D-apiose by this bacterium.

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By

Donna L. Neal

A THESIS

Submitted to
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in partial fulfillment of the requirements
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VITA

Donna L. Neal was born on March 19, 1942 at Seneca Falls, New York. She graduated from Mynderse Academy in Seneca Falls, New York in June 1960. In 1964, she received the Bachelor of Science degree in chemistry from Wheaton College in Wheaton, Illinois. Her graduate studies were pursued at Michigan State University in the Department of Biochemistry in September 1964 with the aid of a graduate assistantship. She will complete the requirements for the degree of Master of Science in the winter of 1967.

ACKNOWLEDGMENTS

I wish to express my gratitude to Dr. Paul Kindel for initiating my interest in this area of research and for the continued guidance, advice and encouragement which he has provided during the course of this investigation.

Much appreciation is also due to Dr. R. L. Anderson, Dr. W. A. Wood, and their graduate students who have given many helpful suggestions as well as technical assistance in various aspects of this study. I wish to thank Miss Roswitha Blohm for supplying part of the D-apiose used in this investigation. I am also grateful to many other graduate students and several other professors who have given various kinds of assistance and advice throughout my graduate studies.

The investigation was made possible through the facilities and financial assistance of Michigan State University and through the financial assistance provided by the National Institutes of Health.

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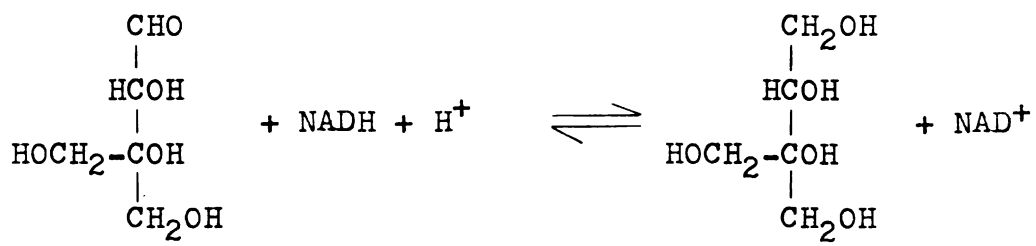
INTRODUCTION

Aerobacter aerogenes has been shown to be very versatile in its ability to utilize a wide range of sugars and sugar alcohols as its sole source of carbon for growth and energy. The data in this thesis further indicate the versatility of this bacterium by demonstrating its ability to utilize D-apiose, a branched-chain aldopentose, as its sole source of carbon.

This thesis is mainly concerned with a unique enzyme which has been found in extracts of A. aerogenes grown on D-apiose as a sole source of carbon. The enzyme, which I have named apiose reductase, reduces D-apiose[1] to the corresponding alcohol in the presence of NADH (reduced nicotinamide adenine dinucleotide).



[1]When apiose is drawn in the aldehydo or open chain form only carbon atom two is asymmetric. It is this carbon which determines whether the sugar is D or L. It has been shown that the naturally occurring sugar has the C-2 hydroxy group to the right when the Fischer projection formula is written and therefore has the D configuration (1). The corresponding enantiomorph has been chemically synthesized and called L-apiose (2). It should be noted that D-apiose in the furanose form has two additional asymmetric carbon atoms and can form two D-furanose and two L-furanose forms. Specific rules for naming branched-chain sugars have not yet been formulated. See references 3 and 4 for a more complete discussion of the nomenclature of branched-chain sugars.

Polyol:NADP oxidoreductase (aldose reductase) has been found in both filamentous fungi and mammalian tissues. The presence of an aldose reductase in bacteria has never been reported. In several species of fungi, the metabolism of certain aldopentoses involves an initial reduction to the corresponding alcohol, catalyzed by an aldose reductase. Chiang and coworkers (5, 6) have reported the presence of an NADPH (reduced nicotinamide adenine dinucleotide phosphate) dependent aldose reductase in extracts of Penicillium crysogenum. The enzyme, induced when the fungus is grown on D-xylose or L-arabinose, reduces D-xylose to xylitol and L-arabinose to L-arabitol. Veiga, Bacila, and Horecker (7) have reported the presence of a similar enzyme in Candida albicans. Chiang and Knight (8) have further shown the presence of this aldose reductase in several other species of fungi. They were unable to detect similar activity in several different bacteria grown on D-xylose or L-arabinose. On the basis of the studies on this enzyme by both groups of investigators, the activity observed with various aldoses is attributed to one enzyme.

In mammalian systems, the presence of an aldose reductase has been reported in several tissues. In all cases, the NADPH-dependent reductase shows a broad specificity for aldosugars. Hers (9) first demonstrated the existence of this enzyme in studies on the conversion of D-glucose to D-fructose in seminal tissue of sheep. The physiological function of the enzyme is thought to be in the conversion of D-glucose to

sorbitol, but several other aldoses are also reduced. Hers (10, 11) has also reported the presence of this enzyme in seminal vesicles of sheep and in placenta of sheep and cattle. Samuels and coworkers (12) have reported the presence of an aldose reductase in the male accessory organs of the rat, ram, bull, boar and stallion. van Heyningen (13) reported the ability of dialyzed extracts of rat lens to reduce D-xylose, D-galactose and D-glucose in the presence of NADPH. An accumulation of galactitol or xylitol was found in the lens of rats fed either D-galactose or D-xylose. Kinoshita and coworkers (14) have also reported the presence of this enzyme in the lenses of rats and rabbits. Hayman and Kinoshita (15) have purified and characterized the enzyme isolated from calf lens. These workers concluded that one enzyme is responsible for the activity obtained with a broad range of aldoses and aldehydes. More recently, Quan-Ma and Wells (16) have detected the presence of galactitol in several tissues in rats fed a diet containing 35% D-galactose. However, the presence of the enzyme in these tissues has not been reported.

There are two distinct differences between the enzyme reported in this study and the aldose reductases reported previously. These differences are in substrate specificity and in pyridine nucleotide specificity. Aldose reductases previously reported have all been NADPH-dependent enzymes with a broad specificity for aldose sugars. In contrast, apiose reductase is NADH-dependent and almost completely specific for D-apiose.

METHODS AND MATERIALS

Source and identity of the bacterium: The bacterium, Aerobacter aerogenes, PRL-R3, source of the bacterial strains used in this study, was obtained from the laboratory of Dr. R. L. Anderson. A strain of this bacterium which would grow on D-apiose as its sole source of carbon was obtained by inoculating a tube containing a D-apiose-salts medium with cells from a peptone slant of wild type A. aerogenes PRL-R3. The tube containing the medium was inclined to 45°, and incubated at 30° on a reciprocating shaker. Growth occurred after a lag of 10 to 15 days. In three later experiments, the same strain was obtained by exactly the same procedure. The strain (designated strain A), after repeated transfer on D-apiose-salts medium, grew on this medium without a lag.

During the course of this study, strain A was inadvertently lost. Routine attempts to again obtain this strain from A. aerogenes PRL-R3, proved futile. When this happened, several factors were varied. Slants of A. aerogenes PRL-R3 from different individuals were used. Inoculations were made from wild type cells maintained on peptone and glucose slants as well as from new cultures grown from these slants. Fresh medium and different temperatures were tested. D-apiose isolated by a different procedure was tried. In addition, an induction experiment was carried out in which a starved

inoculum of glucose-grown cells was added to minimal medium containing D-apiose (17). The cultures were shaken and turbidimetric readings were made periodically for a period of five weeks. In another experiment, cells growing in D-glucose medium were transferred successively into tubes with decreasing concentrations of D-glucose and increasing concentrations of D-apiose. All experiments were without success.

A final experiment to reobtain this strain which utilized D-apiose was made by attempting to reisolate whole cells from a frozen broken-cell extract of strain A. A viable culture which grew on the D-apiose medium was eventually isolated from this extract. Since the strain obtained differed slightly from strain A, it was designated strain B. The bacterium was separated from one or more contaminating bacteria by repeated selection from D-glucose agar plates. On D-glucose agar plates, the colonies of this bacterium appeared very similar to colonies of A. aerogenes with the exception of size. The colonies of wild type A. aerogenes were larger than the colonies of strain B. Various observations and tests were used to determine if the bacterium isolated was A. aerogenes. The cells of strain B as viewed by phase contrast microscopy appeared as single, short, non-motile rods. The bacterium was observed to be Gram negative when compared to the Gram reaction of known bacteria; A. aerogenes (Gram negative) and Bacillus cereus (Gram positive) (18). Eosin methylene blue (EMB) agar facilitates the recognition of typical colonies of A. aerogenes (19). Colonies from strain B

appeared very similar to colonies of A. aerogenes on this agar. For further evidence, the IMVIC tests were carried out using A. aerogenes and Escherichia coli as positive and negative controls (20, 21). In all these tests, strain B and wild type A. aerogenes gave the same results. On the basis of these tests and observations, and considering that this strain was isolated from a broken-cell extract of A. aerogenes PRL-R3, strain A, the bacterium was identified as A. aerogenes (22).

The cell-free extract obtained from strain B also possessed the ability to reduce D-apiose in the presence of NADH. The data obtained from this strain with regard to substrate specificity, K_M, and pH optimum were the same as the data obtained from strain A. The only difference noted between the strains was in the specific activity of the enzyme. The specific activity of the enzyme from strain B was three times as great as the specific activity of the enzyme from strain A. These data give further proof that the strain isolated from the frozen, broken-cell extract was A. aerogenes.

Growth of bacteria and preparation of extracts: The bacterium was grown aerobically at 30° on a minimal medium. The medium consisted of 1.35% Na_2HPO_4, 0.15% KH_2PO_4, 0.3% $(NH_4)_2SO_4$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.0005% $FeSO_4 \cdot 7H_2O$ and 0.5% D-apiose or D-glucose (autoclaved separately).

Growth experiments were carried out to determine how readily the bacterium grew on D-apiose in comparison to its rate of growth on D-glucose. For standardized growth

experiments, tubes containing four ml of medium were inoculated with 2 x 10^6 cells per ml, inclined to 45°, and incubated at 30° on a reciprocating shaker. The inoculum consisted of cells from a culture grown on D-apiose (in the log phase). Turbidity measurements were made at time intervals with a Coleman Junior Spectrophotometer at 520 mμ. Total cell counts were made with a Petroff-Hausser counting chamber. Turbidity measurements, made at 520 mμ using a Bausch and Lomb Spectronic-20 colorimeter, were related to number of bacteria on a standard curve.

To obtain cell-free extracts, one ml of a culture in late log or early stationary phase in minimal media was used for the inoculation of 60 to 80 ml of minimal media. The cells, grown on a reciprocating shaker at 30°, were harvested by centrifugation at the end of the log phase (15 to 18 hrs after inoculation). The cells were washed once with distilled water, resuspended in five ml of distilled water, and exposed to sonic vibration in a Raytheon 10-kc oscillator for five min or broken in a French pressure cell. The broken-cell extract was centrifuged at 17,000 x g for 15 min, and the supernatant was called the crude extract. The supernatant obtained after the crude extract was centrifuged at 100,000 x g for one hr in a Beckman L-2 preparative ultracentrifuge was used in the spectrophotometric assays.

Chemicals: The sugar, D-apiose, was obtained by hydrolysis of the glycoside, apiin. Apiin was isolated from parsley seeds by the method of Gupta and Seshadri (23). Their procedure involved isolation by solvent extraction and

purification by preparation of the lead salt. The lead salt was decomposed with hydrogen sulfide and the glycoside crystallized from ethanol. Careful hydrolysis of apiin using 0.1N H_2SO_4 for 30 min at 100° removed virtually all the apiose along with only a small amount of D-glucose (24). The D-apiose was separated from the small amount of contaminating D-glucose by partition column chromatography on Celite 535 (25).

The corresponding sugar alcohol of D-apiose, to which I gave the common name apitol, was synthesized by catalytic hydrogenation with platinum oxide. Twenty-five mg of D-apiose was dissolved in 10 ml of redistilled 95% ethanol to which 10 mg of platinum oxide was added. The hydrogenation was carried out in a low pressure Parr pressure reaction apparatus under 45 lbs hydrogen pressure for 48 hrs at room temperature. In most cases, the D-apiose was completely reduced to the alcohol. Paper chromatography in ethylacetate-pyridine-water (120:50:40, by volume) and loss in reducing power as determined in the Nelson test (26) were used to determine extent of reduction. Apitol gave the characteristic reaction of a polyol with benzidine-periodate reagent. The apitol used in enzymatic assays was first separated from a faster moving impurity by paper chromatography in ethylacetate-pyridine-water (120:50:40). After elution from the chromatogram and concentration, the alcohol was treated with charcoal, filtered, and evaporated to dryness, and dried over phosphorus pentoxide.

All other chemicals used in this experimental work were commercial preparations.

Chromatography: The solvent system, ethylacetate-pyridine-water (120:50:40) (27), designated Solvent 1, was routinely used for separation of apitol from D-apiose. In addition, the solvent system, butanol-pyridine-water (6:4:3 by volume) (28), designated Solvent 2, was also employed for identification of the reaction product. Washed Whatman No. 3MM paper (washed with 0.1M sodium citrate and distilled water) was employed for descending paper chromatography. Silver nitrate (29) and benzidine-periodate (30) were used for detection of sugars and sugar alcohols. In solvent 1, apitol has an R_A of 0.75 as compared to an R_A of 1.00 for D-apiose. In solvent 2, the R_A for apitol is 0.80 when compared to an R_A of 1.00 for D-apiose. R_A refers to the distance traveled by the alcohol divided by the distance traveled by D-apiose.

Spectrophotometric measurements: Absorbance measurements of reduced and oxidized pyridine nucleotides were made at 340 mμ in microcuvettes having a 1-cm light path, using a Gilford absorbance-recording spectrophotometer thermostated at 25°.

Protein was determined spectrophotometrically with the aid of a nomograph (courtesy of Calbiochem) based on the data of Warburg and Christian (31).

The standard assay for the reduction of D-apiose contained in a volume of 0.15 ml: 10 μmoles of glycylglycine buffer (pH 7.5), 3 μmoles of D-apiose and 0.05 μmole of NADH and enzyme.

The standard assay for the oxidation of apitol contained in a volume of 0.15 ml: glycylglycine buffer (pH 9.5), 14 μmoles; chemically synthesized apitol, 1.5 μmole; NAD, 0.2 μmole and enzyme.

A unit of enzyme is defined as the amount of enzyme giving an absorbance change of 1.0 per minute at 340 mμ in a reaction volume of 0.15 ml in the standard assay for either the forward or reverse reaction.

RESULTS

Growth characteristics: Initially, growth on D-apiose occurred after a lag of 10 to 15 days when four ml of salts solution supplemented with 0.5% D-apiose was inoculated with cells from a peptone slant of A. aerogenes PRL-R3. Following the first successful experiment, the experiment was repeated three more times with cells from other peptone slants of A. aerogenes PRL-R3. However, attempts to repeat this same experiment several months later were unsuccessful. After growth was once obtained, growth to the end of the log phase on subsequent transfers required 2 to 2.5 days with a standard inoculum of 2×10^6 cells per ml (Figure 1). After repeated transfer into medium containing D-apiose, growth was completed in less than two days (Figure 1). After 10 transfers on D-glucose medium, strain A still grew on D-apiose without a noticeable lag.

Enzymatic activity: The cell-free extracts from both strains were found to carry out the reduction of D-apiose in the presence of NADH. This activity could not be obtained when the cells from either strain were grown on D-glucose. The reaction is seen to be enzyme dependent in Figure 2. The reaction was studied in both the forward and reverse directions. Unless indicated otherwise, the data reported below is based on work done with strain A.

Fig. 1. Growth of A. aerogenes PRL-R3, strain A, on D-glucose- and D-apiose-salts medium. Inoculum for each tube consisted of 2×10^6 cells per ml from a culture grown on D-apiose.

a. Turbidity at 520 mμ plotted against time for two different cultures of cells grown on D-apiose-salts medium and one culture grown on D-glucose-salts medium.

b. A semilog plot of the data from Figure 1a for one culture grown on D-apiose.

Figure 1a

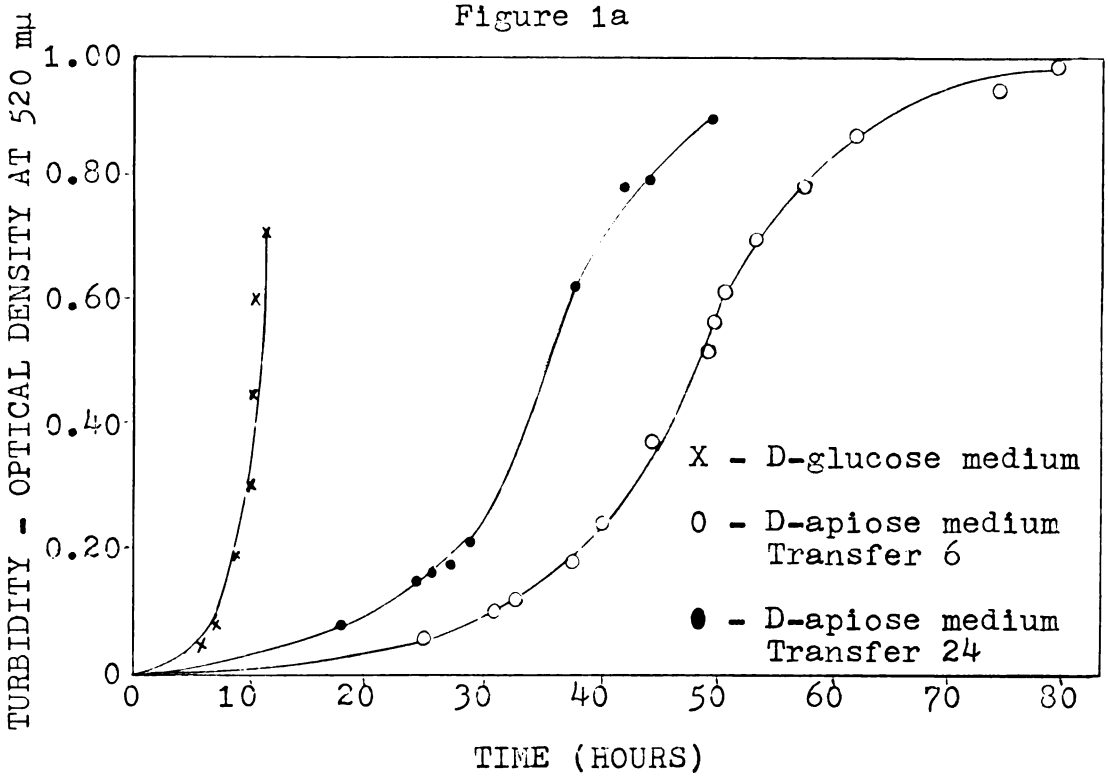


Figure 1b

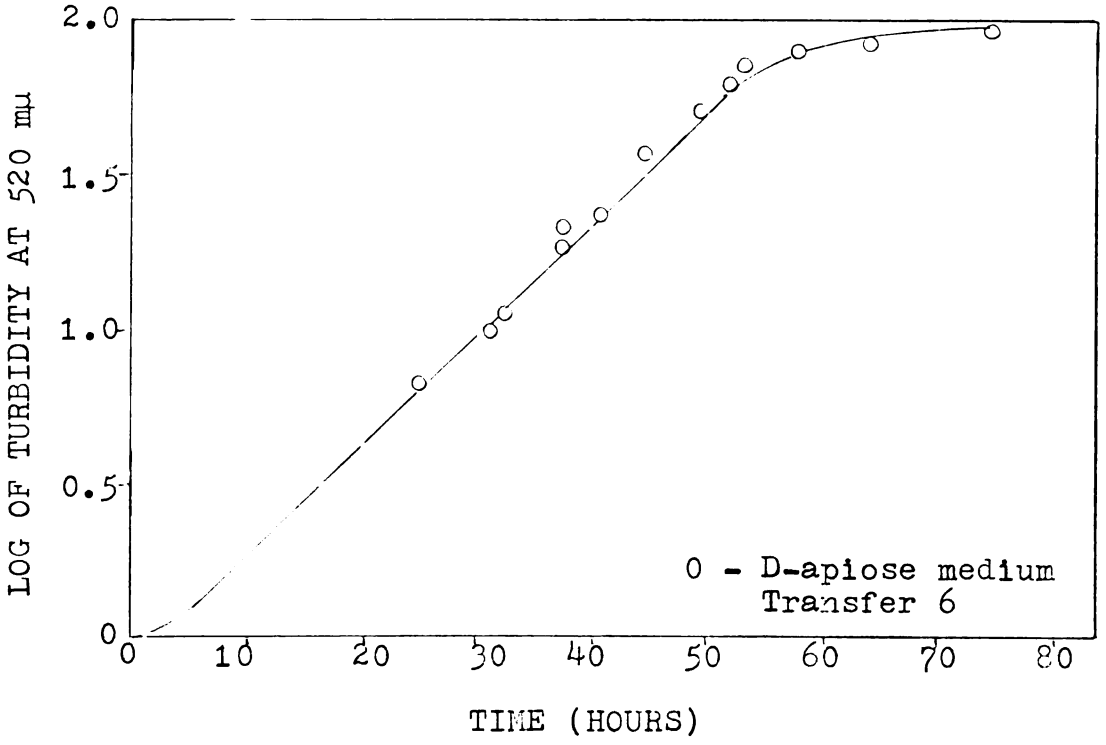


Fig. 2. The relationship of apiose reductase activity to enzyme concentration. Linearity of the assay is seen as applied to both the reduction of D-apiose and the oxidation of apitol in the standard assay conditions. The data plotted represent the average of triplicate determinations in Figure 2a and duplicate determinations in Figure 2b.

Figure 2a

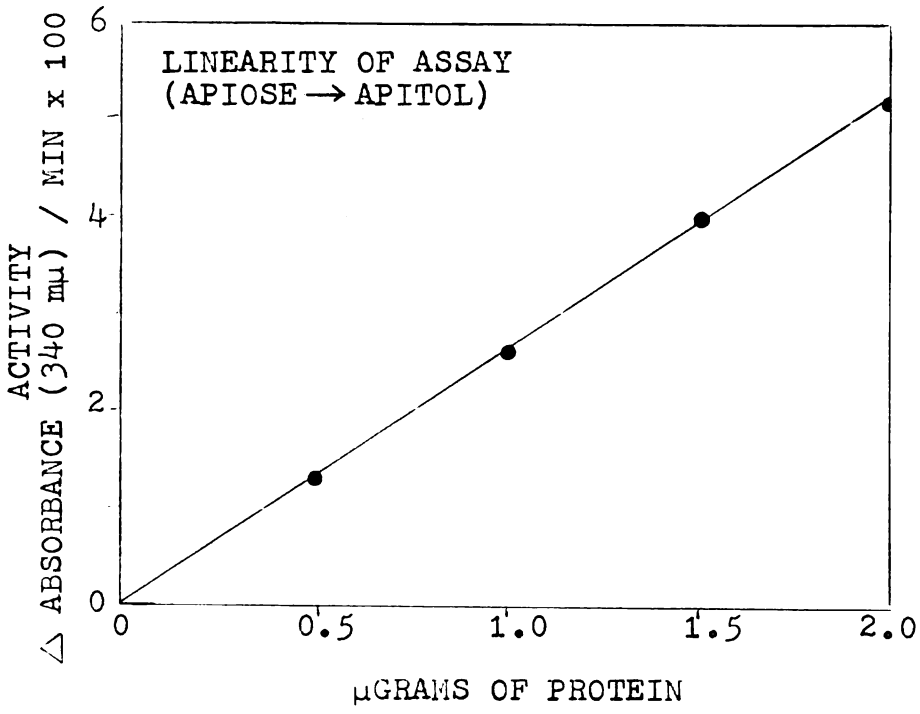
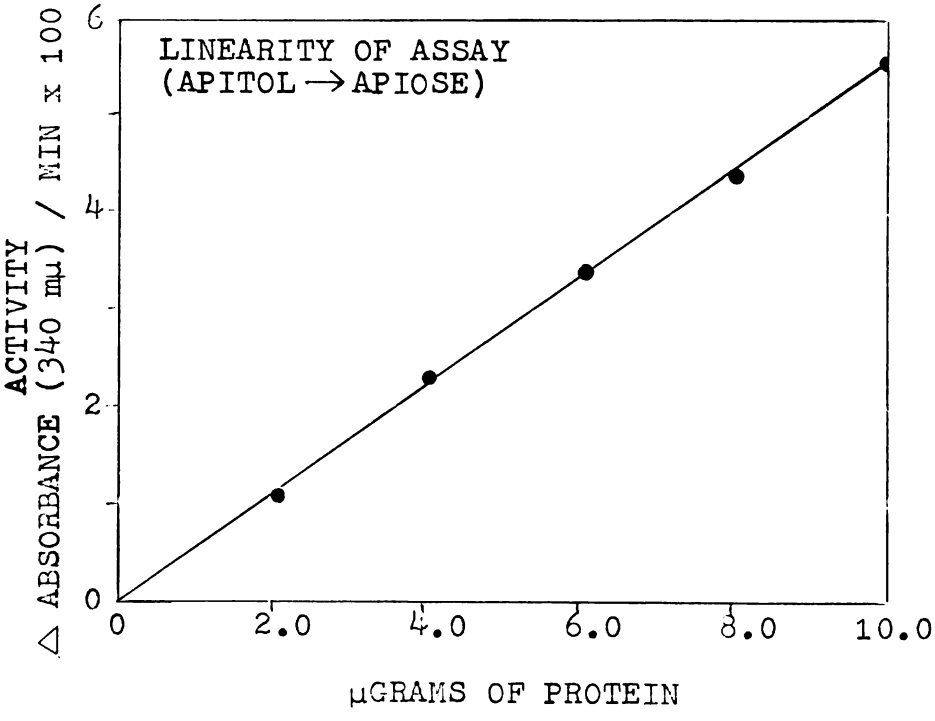


Figure 2b



Properties of the enzyme

Activity and stability: Extracts lost little activity after storage for 24 hrs at 0-4°. After 48 hrs, one half of the activity was lost. The extract showed no loss in activity when kept in the frozen state for two months. The extract could be refrozen two times before there was a significant loss in activity.

In the forward direction, the specific activity of the enzyme from strain A, averaged from three different extracts, was 28 units per mg (range: 26.6-33.6). In strain B, the specific activity obtained in one extract for the forward reaction was 100 units per mg.

pH optima: With D-apiose as substrate, the optimum activity was obtained at pH 7.5 in glycylglycine buffer and pH 6.5 in phosphate buffer (Figure 3). In glycylglycine buffer, the activity did not vary significantly between pH 6.0 and pH 8.0. In phosphate buffer, there was little change in activity between pH 5.5 and pH 8.0. With apitol as substrate, the optimum activity was obtained at pH 10.5 in glycine-sodium hydroxide buffer (Figure 4). In glycylglycine buffer, the activity was still increasing at pH 9.5. Activity obtained in glycine-sodium hydroxide buffer was less than in glycylglycine buffer.

Substrate specificity: The enzyme appears to be almost specific for D-apiose and its corresponding sugar alcohol. Little or no activity was obtained with 19 other sugars and

Fig. 3. pH Optima of apiose reductase (D-apiose → apitol). The standard assay was used except that the pH of the buffer and the buffer composition were varied as indicated, with the enzyme concentration constant. Data plotted represent the average of triplicate determinations at the same pH.

Figure 3

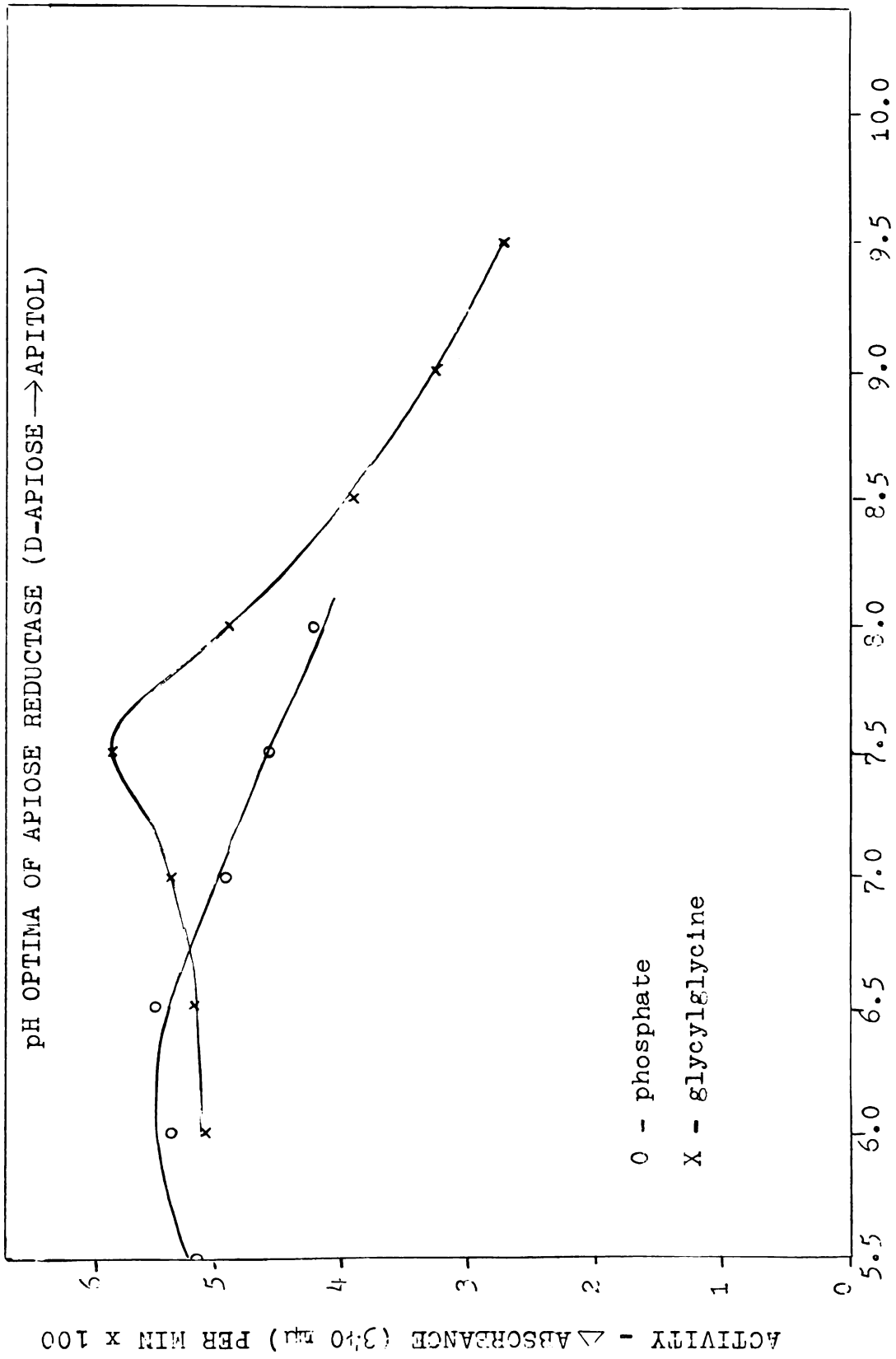
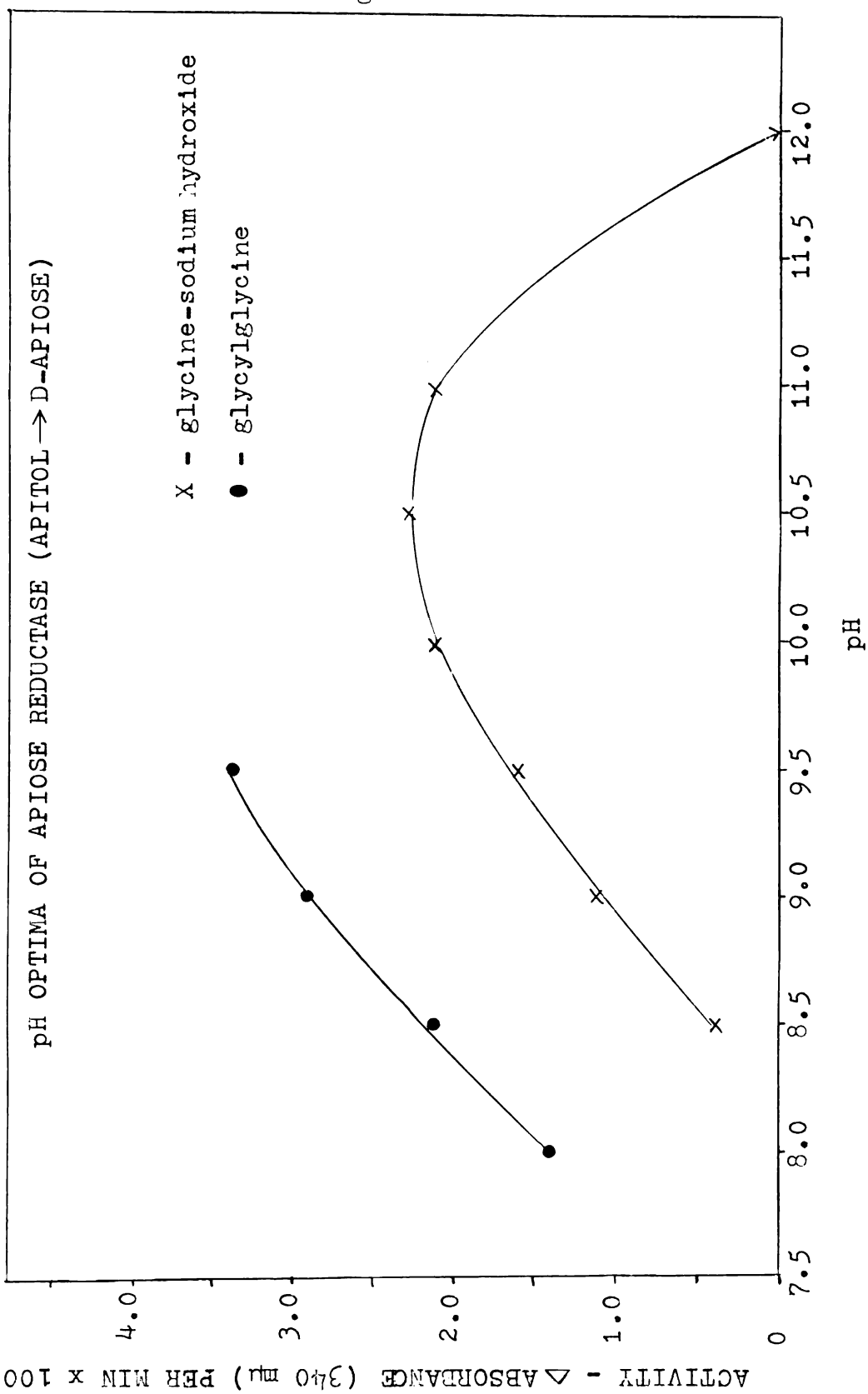

pH OPTIMA OF APIOSE REDUCTASE (D-APIOSE → APITOL)
O - phosphate
X - glycylglycine
ACTIVITY - Δ ABSORBANCE (340 mμ) PER MIN x 100
pH
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
0
1
2
3
4
5

Fig. 4. pH Optima of apiose reductase (apitol → D-apiose). The standard assay was used except that the pH of the buffer and the buffer composition were varied as indicated, with the enzyme concentration constant. Data plotted represent averages of duplicate determinations at the same pH.

Figure 4


pH OPTIMA OF APIOSE REDUCTASE (APITOL → D-APIOSE)
X - glycine-sodium hydroxide
● - glycylglycine
ACTIVITY - ΔABSORBANCE (340 mμ) PER MIN x 100
4.0
3.0
2.0
1.0
0
7.5
8.0
8.5
9.0
9.5
10.0
10.5
11.0
11.5
12.0
pH

sugar alcohols (Table 1). NADH functioned much more effectively than NADPH in the reduction of D-apiose. Likewise, the oxidation of apitol was almost specific for NAD (Table 2).

Kinetic constants: From a Lineweaver-Burk plot, the K_M value for D-apiose was determined to be 2×10^{-2}M (Figure 5). For apitol, the K_M was determined to be 9.6×10^{-3}M (Figure 6). The V_{max} was calculated to be 1.27 µmole/min/mg in strain A and 4.45 µmoles/min/mg in strain B. With apitol, the V_{max} obtained from strain A was 0.39 µmoles/min/mg.

Inhibition by p-chloromercuribenzoate: p-Chloromercuribenzoate inhibited the apiose reductase from strain B 100% at 3×10^{-3} M (Table 3). The activity of the enzyme could be restored in the presence of 0.01M cysteine or 0.033M mercaptoethanol. In preparations diluted to a protein concentration of one mg per ml, the presence of 0.06M mercaptoethanol increased the stability of the enzyme when stored at 0-4°.

Metal ion requirement: Enzyme activity was not appreciably affected by the presence of 0.02M EDTA (ethylenediamine tetraacetic acid), indicating the enzyme does not have a metal ion requirement.

Product identification: The product of the D-apiose reduction was tentatively shown to be a sugar alcohol with the benzidine-periodate spray reagent. The product of the apiose reduction was detected from the following mixture incubated for 30 min at room temperature in a total volume of 0.5 ml: 5 µmoles

TABLE 1

Substrate specificity of apiose reductase

The standard assays were used except that the 3 μmoles of D-apiose or 1.5 μmole of apitol were replaced by equivalent quantities of the substrates indicated.

Sugar substrates	Relative activity	Sugar alcohol substrates	Relative activity
	%		%
D-apiose	100	apitol	100
D-glucose	0	D-mannitol	1
D-mannose	0	galactitol	0
D-fructose	0	sorbitol	0
D-galactose	0	ribitol	8.6
L-rhamnose	0	xylitol	0
L-fucose	0	L-arabitol	0
D-ribose	0	erythritol	1
D-xylose	0	glycerol	0
L-arabinose	0		
D-erythrose	1.5		
D-glyceraldehyde	0		

TABLE 2

Pyridine nucleotide specificity of apiose reductase

The reaction mixtures were those of the standard assay. For NADPH or NADP, 0.05 μmole and 0.2 μmole were used respectively.

Substrate	Relative activity			
	NADH	NADPH	NAD	NADP
	%	%	%	%
D-apiose	100	1.5		
apitol			100	0.9

Fig. 5. Lineweaver-Burk plot relating apiose reductase reaction velocity to D-apiose concentration. The standard assay was used at pH 7.5 except that the D-apiose concentration was varied as indicated while the enzyme concentration remained constant. At the concentrations indicated, the points plotted represent averages of duplicate determinations. The K_M given represents the average of the data from three such plots.

Figure 5

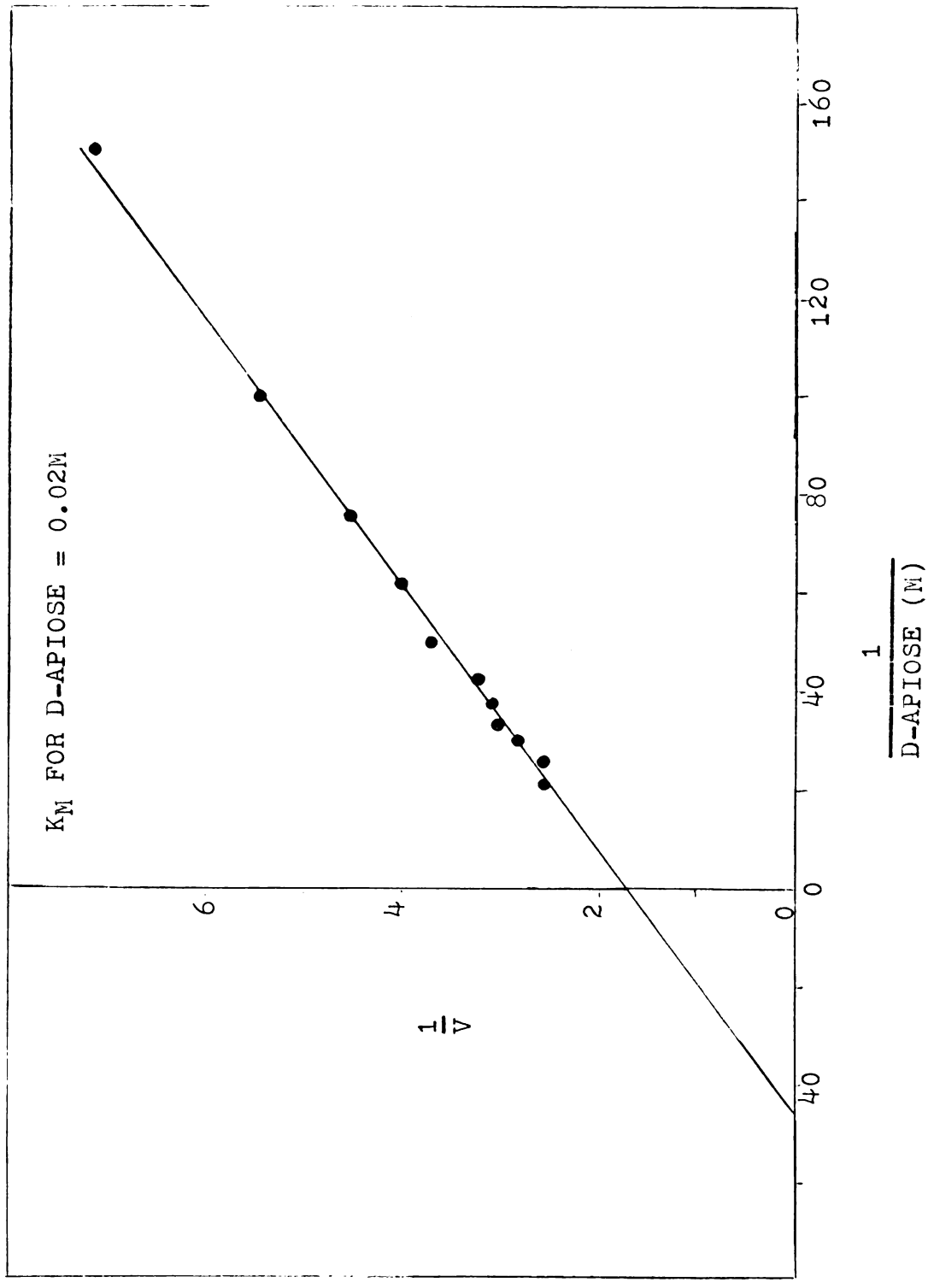

K_M FOR D-APIOSE = 0.02M
$\frac{1}{V}$
6
4
2
0
40
0
40
80
120
160
$\frac{1}{\text{D-APIOSE (M)}}$

Fig. 6. Lineweaver-Burk plot relating apiose reductase reaction velocity to apitol concentration. The standard assay was used at pH 9.5 except that the apitol concentration was varied as indicated while the enzyme concentration remained constant. At the concentrations indicated, the points plotted represent averages of triplicate determinations. The K_M calculated represents the average from three such plots.

Figure 6

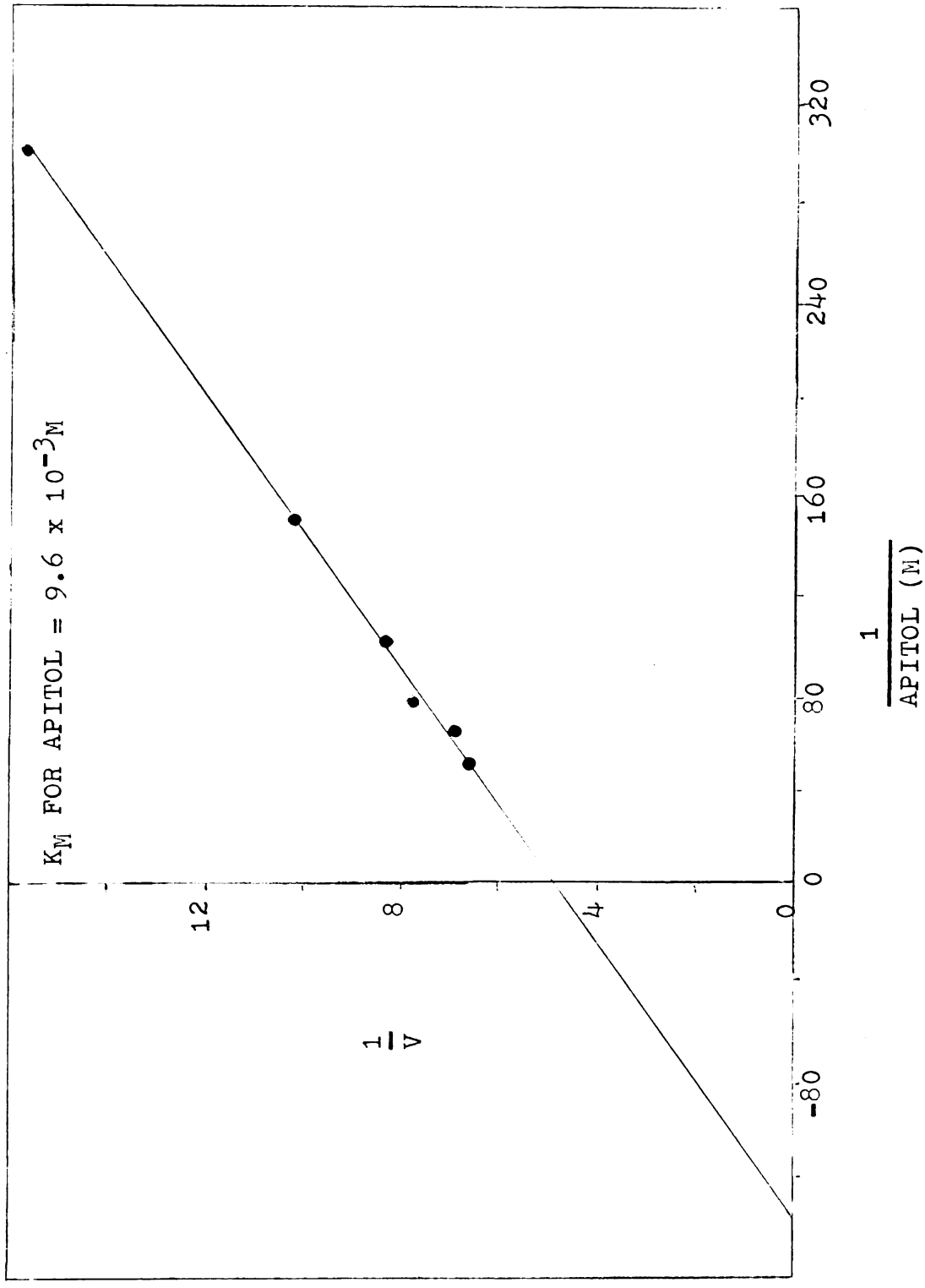

K_M FOR APITOL = 9.6 x 10^{-3}M
$\frac{1}{V}$
12
8
4
0
-80
0
80
160
240
320
$\frac{1}{\text{APITOL (M)}}$

TABLE 3

Inhibition of apiose reductase by p-chloromercuribenzoate

The standard assay was used with the addition of p-chloromercuribenzoate as indicated.

p-Chloromercuribenzoate	Absorbance (340 mμ) / 5 min
M	
0	0.37
1×10^{-6}	0.36
1×10^{-5}	0.36
1×10^{-4}	0.32
6×10^{-4}	0.20
1×10^{-3}	0.08
3×10^{-3}	0.00
5×10^{-3}	0.00

of D-apiose, 0.5 μmole of NADH, and approximately 15 units of enzyme. The reaction was terminated by boiling for 3 min, the solution concentrated, and applied to Whatman No. 3MM paper. The chromatogram was developed with either solvent 1 or solvent 2 and the alcohol was located with benzidine-periodate or silver nitrate. In both solvent systems, the product of the reaction had the same R_A as the sugar alcohol chemically synthesized from D-apiose.

The product of the reverse reaction, the oxidation of apitol, was tentatively identified as apiose from the following mixture incubated for 30 min in a total volume of 0.5 ml: 5 μmoles of chemically synthesized sugar alcohol, 0.5 μmole of NAD, 20 μmoles of glycylglycine buffer (pH 9.5), and 15 units of enzyme. The product gave the same R_A as D-apiose when the chromatogram was developed in either solvent 1 or solvent 2 and then treated with silver nitrate.

DISCUSSION

This study shows the presence of a unique enzyme in Aerobacter aerogenes when this bacterium utilizes D-apiose as its sole source of carbon. The uniqueness of this enzyme is seen in its substrate specificity, its pyridine nucleotide specificity, as well as its occurrence in bacteria. Apiose reductase, as is the case with other reductases, shows a low affinity for its substrates. An important question concerns the role of this enzyme in the metabolism of D-apiose. The fact that the enzyme was only induced when either strain was grown on D-apiose would suggest that the enzyme is necessary to the bacterium.

In other organisms, aldose reductases are known to function in various roles. In Candida albicans (7) and Penicillium chrysogenum (5), the aldose reductase is implicated in the metabolism of pentose sugars. In these fungi, the only mechanism found for the conversion of D-xylose to D-xylulose, an intermediate in their metabolism, involves the reduction of the aldopentose to its corresponding sugar alcohol, followed by the oxidation of the sugar alcohol to the ketopentose. The presence of a NAD-dependent dehydrogenase which is necessary for the conversion of the polyol to the ketopentose has been demonstrated in both species of fungi. The presence of a D-xylose isomerase, the enzyme which

catalyzes the direct conversion of D-xylose to D-xylulose has not been detected in fungi. However, this enzyme is present in bacteria.

The significance of the aldose reductase which occurs in mammalian tissues is not fully understood. Samuels (12) has suggested that those tissues which are able to produce fructose, have an aldose reductase and a sorbitol dehydrogenase which function to convert D-glucose to D-fructose. The relative importance of this nonphosphorylative route for fructose formation versus the phosphorylative route is not known. Although these tissues have high levels of D-fructose, the function of this D-fructose is not understood. Aldose reductase is also present in the lenses of several animals. In the case of a high sugar diet, the corresponding polyol accumulates in the lens and may be a factor in the formation of sugar cataracts. The enzyme, present in the lenses of animals on normal diet, functions in the conversion of D-glucose to D-fructose (14). The importance of this route and the fate of the fructose which is formed are not known.

It was thought that the metabolism of D-apiose by A. aerogenes perhaps involved a mechanism similar to that suggested for the metabolism of aldopentoses by fungi, namely reduction to apitol and reoxidation to the ketosugar. In studies with whole cell preparations of A. aerogenes, Tattrie and Blackwood (32) suggested that erythritol was formed in the fermentation of D-erythrose, D-threose, and D-erythrulose. Hiatt and Horecker (33) found that a strain of Alcaligenes

faecalis, grown on D-erythrose, consumed erythritol and L-erythrulose nearly as well as D-erythrose. However, in both cases, the data is not sufficient to suggest that erythritol is directly involved in the metabolism of the aldosugar. In A. aerogenes, polyol dehydrogenases, induced in the presence of the pentitols (34, 35) and erythritol (36), catalyze the oxidation of the polyols to the corresponding ketosugars. These dehydrogenases are NAD-dependent, as in fungi. Since the reduction of D-apiose requires NADH, it was thought a reoxidation would most likely involve a NADP-dependent enzyme. In the cell-free extracts from strain A, no reduction of NADP could be obtained with apitol and NADP. Repeated attempts to demonstrate this reaction were negative and it was concluded that the apitol is not reoxidized to the ketosugar under the conditions of the assay.

Another possibility for the metabolism of the alcohol would involve its phosphorylation. Barker and Lipmann (37) have reported the phosphorylation of glycerol and erythritol by ATP (adenosine triphosphate) in Propionibacterium pentosaceum. The phosphorylation of apitol in strain A was not explored sufficiently to exclude it.

It is possible that apiose reductase may not be directly involved in the dissimulation of D-apiose by the bacterium, but may function in a less obvious role. The major path of D-apiose dissimulation may involve its conversion to a compound other than apitol. In preliminary stages of this study, experiments showing phosphorylation of D-apiose with ATP, isomerization,

or direct oxidation of D-apiose with a pyridine nucleotide could not be detected in the cell-free extract. However, the search for these reactions was not extensive enough to exclude their presence.

To continue the study of the metabolism of D-apiose in A. aerogenes, further investigation for various enzymatic activities should be made with the cell-free extract. The investigation should include tests for enzymatic reactions which involve D-apiose or apitol. The most obvious reactions to be examined include: phosphorylation of either D-apiose or apitol with ATP or another phosphoryl donor, isomerization, or direct oxidation of D-apiose. If it is impossible to detect any further enzymatic reactions in a cell-free extract, the investigation may have to be approached differently. The dissimulation of D-apiose may proceed by a less obvious mechanism, one which may not be found readily by testing for enzymatic activities.

At least two other approaches could be made if the enzyme studies with the cell-free extract do not yield positive results. By using radioactive D-apiose and different cofactors in the cell-free extracts, labeled intermediates might accumulate. The identification of any labeled intermediates could be very helpful in proposing a mechanism operative in the dissimulation of D-apiose. A second approach would be a fermentation study with whole cells, assuming the bacterium ferments D-apiose. Such a study should yield information about types and quantities of products

which accumulate. From the products of the fermentation, it might be possible to suggest a pathway for the degradation of the sugar and then test for the postulated reactions.

From my study, it is not possible to state the physiological role for apiose reductase in A. aerogenes. However, further exploration for enzymatic activities or labeled intermediates should give information which would suggest a function for this enzyme.

SUMMARY

A strain of A. aerogenes PRL-R3 was obtained which utilized D-apiose as its sole source of carbon. An enzyme which reduced D-apiose to its corresponding sugar alcohol in the presence of NADH was found in extracts from this strain. The enzyme was not present when this strain was grown on D-glucose. The enzyme, named apiose reductase, was almost specific for D-apiose and its corresponding alcohol, apitol. Little or no activity was obtained with 19 other sugars and sugar alcohols. The K_M for D-apiose was 2×10^{-2} and the K_M for apitol was 9.6×10^{-3}. The enzyme was completely inhibited in the presence of 3×10^{-3}M p-chloromercuribenzoate. Activity could be restored by 0.01M cysteine or 0.033M mercaptoethanol. No metal ion was required for activity. The optimum activity for D-apiose reduction was obtained at pH 7.5 in glycylglycine buffer. In the reverse direction, optimum activity was obtained at pH 10.5 in glycine-sodium hydroxide buffer. The product of the reduction of D-apiose was tentatively identified as apitol.

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