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Preparation Of D-Glucose-6-Phosphate Dehydrogenase And Its Substrates Prior To Reactivity With Ozone

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PREPARATION OF D-GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND ITS SUBSTRATES PRIOR TO REACTIVITY WITH OZONE

Submitted in Partial Fulfillment of the Requirements for Graduation with Honors to the Department of Biology and Chemistry at Carroll College, Helena, Montana

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April 12, 1996
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF ILLUSTRATIONS</td>
<td>iv</td>
</tr>
<tr>
<td>INTRODUCTION AND LITERATURE REVIEW</td>
<td>1</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>11</td>
</tr>
<tr>
<td>RESULTS AND DISCUSSION</td>
<td>19</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>42</td>
</tr>
</tbody>
</table>
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ABSTRACT

Ozone is becoming more important in the area of disinfection of water and food. In order to determine how much ozone is needed to inactivate microorganisms that contaminate water and food supplies, the mechanisms of ozone toxicity must be understood. In a project in which ozone was used to oxidize an enzyme model, which in some experiments was encapsulated in liposomes, the activity before and after ozonation of the enzyme was recorded to determine how the enzyme affected the enzyme's rate of reaction. The enzyme, D-glucose 6-phosphate dehydrogenase (DG6P dehydrogenase), was studied to determine at what concentration it needed to be, as well as its substrates, NAD+ and D-glucose 6-phosphate. In addition to these parameters, any interfering substances needed to be discovered before any experiments with ozone commenced. One such substance, Triton x100, is a detergent that is used to break open the liposome membrane; the other, L-cysteine, is the amino acid that is most reactive with ozone, and thus it is used to terminate the ozone's oxidative attack on the enzyme. These experiments were accomplished with enzyme assays. The conclusions reached were that neither Triton x100 nor L-cysteine were inhibitive toward DG6P dehydrogenase. Furthermore, the concentration of NAD+ could be as low as 4.5 mM and that of DG6P equalling 3.0 mM so that neither one of these reagents was the limiting factor in the reaction. The enzyme, DG6P dehydrogenase, had a sufficient concentration at 3.0 mM. The enzyme could now be used in experiments involving ozone.
# LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Enzyme assays involving dilutions of NAD+ (undiluted through 1/40)</td>
<td>20</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Enzyme assays involving dilutions of NAD+ (undiluted through 1/192)</td>
<td>21</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Lineweaver-Burk plot from data in Fig. 2</td>
<td>22</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Lineweaver-Burk plot from data in enzyme assays using NAD+ dilutions (undiluted through 1/192)</td>
<td>24</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Enzyme assays involving different volumes of NAD+ in total reaction volume</td>
<td>25</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Enzyme assays involving dilutions of DG6P (undiluted through 1/40)</td>
<td>26</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Lineweaver-Burk plot from data using DG6P dilutions</td>
<td>28</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Enzyme assays involving dilutions of DG6P in total reaction volume</td>
<td>29</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Enzyme assays involving DG6P dehydrogenase dilutions (undiluted through 1/12)</td>
<td>30</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Enzyme assays involving DG6P dehydrogenase dilutions (undiluted through 1/96)</td>
<td>31</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Rate of enzyme activity</td>
<td>33</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Rate of enzyme activity with 0.8% Triton x100</td>
<td>34</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Rate of enzyme activity with 0.6% Triton x100</td>
<td>35</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Rate of enzyme activity with 0.4% Triton x100</td>
<td>36</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Rate of enzyme activity with 0.2% Triton x100</td>
<td>37</td>
</tr>
<tr>
<td>Figure 16</td>
<td>Lineweaver-Burk plot from data in assays involving Triton x100</td>
<td>38</td>
</tr>
<tr>
<td>Figure 17</td>
<td>Rate of enzyme reaction in assays involving L-cysteine</td>
<td>40</td>
</tr>
</tbody>
</table>
Ozone (O₃) is a multi-faceted molecule: it protects us from the harmful effects of cosmic radiation by forming a "blanket" in the upper atmosphere (stratosphere), but it is also becoming more abundant as a component of photochemical smog in the lower atmosphere (lower troposphere) (Mustafa, 1990). It is the latter observation that is raising several questions concerning the effects of ozone on biological systems: what levels of ozone are safe in the air that humans breathe, and what are the damaging effects of ozone in smog to humans and plants? In order for the nature of ozone toxicity to be understood, the structural and biochemical aspects of ozone must be explored.

Ozone is suggested to have little dipolar character because of its low solubility in water, a characteristic typical of other nonpolar gases, i.e. Xe, N₂ (Uppu and Pryor, 1993). Its formation in the stratosphere is thought to occur according to the Chapman cycle of photochemical dissociation of molecular oxygen by ultraviolet radiation and then a reaction between atomic oxygen and molecular oxygen, shown in reactions 1 and 2 (Mustafa, 1990).

\[
\begin{align*}
O₂ & \xrightarrow{hv (<242 \text{ nm})} O + O \quad (1) \\
O₂ + O & \xrightarrow{M} O₃ \quad (2)
\end{align*}
\]

\(M=\) a third-body molecule absorbing excess energy of reaction

The formation of ozone is balanced by its degradation, forming a natural equilibrium in the stratosphere. Several methods of ozone degradation are shown below in reactions 3, 4, and 5.

\[
\begin{align*}
O₃ & \xrightarrow{hv (<310 \text{ nm})} O₂ + O \quad (3) \\
O₃ & \xrightarrow{hv (<1140 \text{ nm})} O₂ + O
\end{align*}
\]
The rate of ozone degradation today is of major concern because it appears that certain anthropogenic chemicals (produced by humans), such as those resulting from the burning of fossil fuels, e.g. chlorofluorocarbons, are reacting with the ozone layer in the stratosphere and allowing destructive ultraviolet radiation to reach the surface of the earth (Mustafa, 1990).

The formation of ozone in smog is quite different than the formation of ozone naturally occurring in the stratosphere. Ozone is formed in ambient air through several types of precursors, namely volatile organic compounds (VOC) such as vapor-phase hydrocarbons and halogenated organics, oxides of nitrogen (NOx), nitrogen oxide (NO) and other radicals, molecular oxygen, and sunlight (Mustafa, 1990). Cyclic ozone formation (reactions 6, 2, and 7) is the process of ozone formation that involves the breakdown and reformation of nitrogen dioxide. Notice in reaction 7 the regeneration of nitrogen dioxide and molecular oxygen; this steady-state condition occurs in the absence of any competing reactions (Mustafa, 1990).

Radical intermediates such as those found in smog result from certain chemicals undergoing photolysis. These radical intermediates participate in the conversion of NO to NO2, thus raising the ratio of [NO2]:[NO] (reaction 11 and 12). Ozone will thus build up in the air because the steady-state condition shown above in reaction 7 will take longer to achieve (Mustafa, 1990). Reaction 8, 9, 10, 11, and 12 show these pathways.
HCHO \quad \text{hv} (\textless 370 \text{ nm}) \rightarrow \quad H + \text{CHO} & (8) \\
H + O_2 \quad M \rightarrow \quad \text{HO}_2 \\
\text{CHO} + O_2 \quad \rightarrow \quad \text{HO}_2 + CO \\
\text{HONO} \quad \text{hv} (\textless 400 \text{ nm}) \rightarrow \quad \text{HO} + \text{NO} & (9) \\
\text{HO} + \text{CO} \quad \rightarrow \quad \text{CO}_2 + H & (10) \\
\text{HO}_2 + \text{NO} \quad \rightarrow \quad \text{NO}_2 + \text{HO} & (11) \\
\text{RO}_2 + \text{NO} \quad \rightarrow \quad \text{NO}_2 + \text{RO} & (12) \\

\text{Terminating reactions use up the supply of NO and NO}_2, and without this supply, the production of ozone will decrease and finally stop (reactions 13, 14, and 15). Photolysis of ozone, as shown in reaction 3, as well as the reaction between ozone and NO}_2 (reaction 16), also contribute to diminishing ozone in the lower atmosphere (Mustafa, 1990).

\text{HO} + \text{NO} \quad M \rightarrow \quad \text{HONO} & (13) \\
\text{HO} + \text{NO}_2 \quad M \rightarrow \quad \text{HONO}_2 & (14) \\
\text{HO}_2 + \text{NO}_2 \quad M \rightarrow \quad \text{HO}_2\text{NO}_2 & (15) \\
\text{O}_3 + \text{NO}_2 \quad \rightarrow \quad \text{NO}_3 + \text{O}_2 & (16) \\

\text{Ozone is considered an oxidant because it is able to remove electrons from other molecules, and this high reactivity allows it to react with a large variety of molecules (Uppu and Pryor, 1993), and indeed is one of the strongest known oxidants, with a standard redox potential of +2.07 V (Mustafa, 1990). Although its high reactivity would be characteristic of a species that was non-specific with what it reacts, the rate constants for certain organic functional groups with which ozone reacts varies more than } 10^{10}; \text{ this ranges from the amino acid cysteine, having one of the highest rate constants, to amides.}
and carboxylic acids, having the lowest (Pryor, 1992).

Ozone can attack biochemical molecules with various results. Every biomolecule is at risk for attack by ozone, including proteins, unsaturated fatty acids, nucleic acids, and electron donors such as thiols, ascorbate, and $\alpha$-tocopherol (Pryor, 1992). It is widely thought that ozone's toxicity is attributed to two types of action: a direct reaction causing oxidation by ozone itself and through the formation of radical intermediates (Mustafa, 1990). In lung tissue, for example, there would most likely be a direct reaction with ozone with susceptible molecules close to the air/tissue boundary. Secondary and tertiary products, such as radical intermediates, relay the damage ozone causes to more distant targets, e.g. internal tissues and organ systems (Pryor, 1992; Pryor and Church, 1991).

To begin with, lipid oxidation is a prime reaction of ozone, and important in ozone toxicity (Mustafa, 1990). This reaction occurs especially with the double bonds between carbons in the fatty acyl chains and has been demonstrated with model membranes, surfactant mixed micelles, monolayers at air/water interface, and aqueous emulsions (Uppu and Pryor, 1993). Other studies have involved ozone exposure to red blood cells (Mustafa, 1990). The bilayer found in liposomes has been shown to protect any biomolecules contained within while ozone is used up attacking the unsaturated fatty acids (UFA) found in the membrane (Pryor, 1992). The oxidation of these ethylenic bonds produces aldehydes, acids, and hydrogen peroxide, probably due to the Criegee mechanism of attack. This mechanism is the addition of ozone to the double bond, forming a 1-3 dipolar cyclo addition; this decomposes to carbonyl compounds and hydrogen peroxide (Bablon et al., 1991).

Carbohydrates are thought to react very little with ozone, because of their lack of strong nucleophilic sites. Aliphatic alcohols, on the other hand, could produce hydrogen peroxide, aliphatic aldehydes, acids, or ketones. Therefore, the ozone and carbohydrate reaction can produce hydroxyl intermediates which in turn can react with hydrocarbons (Bablon et al., 1991).
While purine and pyrimidine have very little affinity for ozone, the nucleotides that are formed from the purine and pyrimidine bases react very strongly with it. In a study by Doré et al., (1989), the reactivity sequence of the nucleotides in a batch system containing radical traps (very reactive free radicals) was as follows: thymine > uracil > guanine > adenosine > cytosine (Bablon et al., 1991). Another study by Ishizaki et al. (1984) demonstrated that in a system lacking radical traps, the reactivity sequence was as follows: G,T > U > C > A (Bablon et al., 1991).

In addition to UFA being strongly oxidized by ozone, proteins are especially susceptible to ozone attack; in fact, when proteins are studied with regard to reactivity with ozone when incorporated in a lipid bilayer, the proteins are oxidized while the UFA are left untouched (Uppu and Pryor, 1992). However, when studying liposomes, it is found that the bilayer protects species contained within, while the ozone is used up in the process of attacking the UFA found in the bilayer (Pryor, 1992). Uppu and Pryor (1992) elaborated on this study using reverse micelles made of sodium di-2-ethylhexylsulfo-succinate ["Aerosol OT" (AOT)]. These were suspended in a hydrocarbon solvent that held trace amounts of water containing the protein lysozyme. The micelles formed around these pools of water and the solution was subsequently exposed to ozone. They found that the UFA, whose hydrophobic tails were pointed out into the organic solvent and hydrophilic heads were facing the enclosed water pool, were oxidized first in a "sacrificial reaction" with ozone, while lysozyme was protected (Uppu and Pryor, 1992).

Ozone is especially reactive toward certain amino acids, cysteine, methionine, tryptophan, and tyrosine being at the greatest risk (Mustafa, 1990). These amino acids are susceptible to attack at two sites: the amine group and the R group. This reactivity is highly dependent on pH, especially regarding the amine group; a favorable pH for reactivity would be neutral or basic (Bablon et al., 1991). If the R group is an alkyl group, an increase of pH can increase the normally slow reactivity of this group. A sulfur alkyl group has a very high rate of reaction, even when the pH is acidic (Bablon et al., 1991).
The study of enzymes and their activity is an excellent way to determine the toxic effects that ozone has on a biological system. A simple system can eliminate interference from other compounds and allow one to focus exclusively on the enzyme. If comparisons of the enzyme before and after ozonation showed a decrease in activity, that is evidence that oxidation took place. In order to explain what preparatory work needed to be done and why, a review of enzyme kinetics is necessary.

Important factors involved in understanding and making calculations from enzyme-catalyzed reactions include enzyme concentration, ligand concentrations (substrates, products, inhibitors, and activators), pH, ionic strength, and temperature (Segel, 1968). Knowing these factors is extremely important in understanding the mechanism of a specific reaction.

There are certain values that are extremely important for comparing different enzymes or comparing the same enzyme at different concentrations of the enzyme and/or substrates or after being influenced by interfering compounds, activators, pH, temperature, and ionic strength. These values include the Michaelis constant (K_m), the maximum velocity (V_max), and the activity.

To begin with, the Michaelis constant, K_m, is an important constant that establishes a relationship between the velocity of an enzyme-catalyzed reaction and the concentration of substrate. Specifically, K_m equals the substrate concentration that produces one-half the maximum velocity (Equation 1) (Segel, 1968).

\[ v = \frac{[S] [V_{\text{max}}]}{K_m + [S]} \quad \text{when} \quad [S] = K_m: \quad v = \frac{K_m}{K_m + K_m} V_{\text{max}} = \frac{1}{2} V_{\text{max}} \quad (\text{Eq. 1}) \]

K_m is important for several reasons. First of all, it represents an approximate numerical value for the intracellular substrate level. In addition, K_m is a constant for each enzyme, allowing comparisons to be made from enzymes from different organisms or tissues or different stages of development (Segel, 1968). Another reason is that by knowing K_m,
assay conditions can be adjusted so that the concentration of substrate is much greater than $K_m$ and as a result the $V_{\text{max}}$ can be determined, and thus the total concentration of enzyme. Finally, this constant points out the most appropriate substrate for the enzyme, the substrate with the smallest $K_m$ being the most suitable because of its high affinity for the enzyme (Segel, 1968).

The $V_{\text{max}}$ can be defined as the maximum rate of reaction that is observed when all of the enzyme present in the reaction mixture is present as [ES], which is the complex consisting of the substrate bound to the active site of the enzyme (Segel, 1968). The Henri-Michaelis-Menten equation relates the $V_{\text{max}}$ with the instantaneous velocity ($v$) of the enzyme at any given substrate concentration. A graph called the Lineweaver-Burk plot can be obtained by taking the reciprocal of the Henri-Michaelis-Menten equation and the $V_{\text{max}}$ and $K_m$ of the reaction determined when plotting the inverse of the substrate concentration versus the inverse of the rate of the reaction (Rawn, 1983).

\[ \frac{1}{v} = \frac{1}{[S]} \cdot \frac{K_m}{V_{\text{max}}} + \frac{1}{V_{\text{max}}} \]  

(Eq. 2)

Here, $K_m$ and $V_{\text{max}}$ can be determined from the slope and the intercepts of the graph. This equation will form a straight line when plotting the inverse of the rate of the reaction and the inverse of the substrate concentration. The rate of the reaction is difficult to determine, as well as the $K_m$ and $V_{\text{max}}$, when the graph is the plot of substrate concentration and rate of reaction.

Enzymes are molecules, usually proteins, that have the ability to catalyze the biochemical reactions between certain molecules by lowering the activation energy of the reaction. They can accelerate reactions by at least a factor of $10^6$ (Stryer, 1995). They are highly specific molecules that have specific active sites for substrates which they catalyze. These reactions can be inhibited by a variety of molecules, either directly at the
active sites (competitive inhibitors) or at other sites on the enzyme molecule that somehow changes the active site to make it no longer compatible for the substrate (Stryer, 1995).

There is potential to exploit the toxic effects of ozone on biological systems in ways that may benefit humans. Ozone, a highly reactive oxidant present in both polluted air as well as in the stratosphere, has been named as a potential alternative to chlorination in the treatment of water. Because low amounts of chlorine act as an effective bacteriostat, but not bactericide, and also are ineffective towards protozoan cysts, worm eggs, and viruses, chlorination is not necessarily sufficient in providing safe water for human consumption (Broadwater et al., 1973). Studies of ozone and its rate of reactivity, therefore, are valuable to the human population not only because of its potential benefits as a bactericide but also because of its known damaging effects to humans as a toxin.

There has been much research in this area to determine what microorganisms, i.e. viruses and bacteria, can be inactivated by treatment with ozone and for how long this treatment should last (Broadwater, W.T., et al., 1973; Burleson, G.R., et al., 1975; Foegeding, P.M., 1985; and Ishizaki, K., 1985).

The long-term goal of the project was to determine the effects, if any, that ozone has on a simplified enzyme system. If ozone has an inhibitory effect on enzyme activity, we would expect to see a decrease in the rate of reaction in this model system. An enzyme model was used, both with and without biological membranes. Because in living systems ozone attacks cells and not just suspended compounds, such as proteins, the biological membranes made the models more realistic.

The protein used for this series of experiments was D-glucose-6-phosphate dehydrogenase. In mammals, this enzyme catalyzes the first step in the hexosemonophosphate shunt, also known as the pentose shunt; it is known that glucose-6-phosphate dehydrogenase catalyzes the rate-limiting step and that careful control of this enzyme's activity is necessary to the control of the pathway, although the overall regulation of the shunt is not totally understood (Shreve, D.S. and Levy, R.L. 1980).
Because NADP+ is the electron acceptor, it is believed that it is the control in the irreversible step of the reaction, where glucose 6-phosphate is dehydrogenated by this enzyme. NAD+ can also be used; however, the phosphate group on NADP+ allows it to be tagged, and thus monitored (Stryer, 1995).

D-glucose 6-phosphate dehydrogenase was picked for several reasons. First of all, it contains no cysteine residues (Ishaque et al., 1974), and as stated previously, cysteine is the amino acid that reacts the most rapidly with ozone (Pryor, 1992). Secondly, this enzyme is a smaller molecule than other enzymes that were first used, having an approximate molecular weight of 103,700 (Olive and Levy, 1980). The smaller enzyme is advantageous when being encapsulated in liposomes, which will occur in the later stages of this study.

In addition to the enzyme and its substrates, other reagents were often found in the reaction system but were not essential to the reaction. These reagents, namely Triton x100 and L-cysteine, had specific uses in these experiments. Triton x100 is a detergent that is often used in experiments involving membranes, and, more specifically, micelles and liposomes. L-cysteine is the amino acid that is most reactive with ozone.

Before experiments of this caliber can be carried out, certain parameters must be determined. Because the mechanism of action for each individual enzyme isn't always completely understood, and all of the different promoters and inhibitors of the enzyme aren't always known, any chemical that the enzyme is exposed to must be "cleared" for use. What this entails is repetitious series of enzyme assays that enable one to calculate exactly how much and at what concentration the enzyme and substrate(s) should be in the total assay volume. Not only this, but any additional reactants in the assay, such as Triton-x, must be determined to be non-interfering to the enzyme; in order to study the effects of ozone on the activity of the enzyme, nothing else must be interfering with that activity, or else the experimental data are not valid. Some examples of previous research that have been performed on these parameters include: the effects that the ratio of
NADPH/NADP\(^+\) have on the activity on this enzyme (Oka, K., et al., 1981); kinetic mechanism of G6PDase (Levy, H.R., et al., 1983); and pH parameters in the reaction catalyzed by G6PDase (Viola, R.E., 1984);

For several weeks during the summer of 1995, I worked in the lab of Dr. R.L. Merson in the Department of Food Science and Technology at the University of California, Davis, under the direction of Theodoros G. Kallitsis. My goal was to continue the ground work preparing this enzyme, D-glucose 6-phosphate dehydrogenase, for further experiments using ozone. Specifically, several goals were to be accomplished in my experiments:

1. To determine the correct concentration of NAD\(^+\), glucose 6-phosphate, and G6PDase to be present in the reaction mixture so the substrates would not be the limiting reagents.

2. To determine the \(V_{\text{max}}\) and \(K_m\) of this enzyme.

3. To discover any interference to the activity of the enzyme caused by the two additional chemicals added to the system: L-cysteine and Triton-x.
MATERIALS AND METHODS

Preparation:

The buffer used throughout these experiments was a Tris-HCl buffer, pH 7.8, 100 mM. A 1.0 M Tris solution was diluted to a 100 mM solution by adding one part of the 1.0 M Tris solution to nine parts of deionized water. While stirring continuously, commercial reagent grade HCl 37% was added until the pH was 7.8. If the volume of HCl added was above 0.2% of the original Tris volume, it was corrected to the desired 100 mM by adding the appropriate amount from the 1.0 M Tris solution; then the pH was adjusted again.

The stock enzyme (0.5 mg/ml), available from Sigma, needed to be diluted in order to obtain what we called "application" enzyme. First, 200 µl of enzyme was diluted with 800 µl of Tris-HCl buffer (100 mM, pH 7.8). Next, one-half of the 1000 µl was diluted with 4500 µl buffer, making a total of 5000 µl of a 1/50 dilution. Finally, taking 2000 µl of this 1/50 dilution and diluting it with 6000 µl of buffer gave a 1/200 dilution; this was the application enzyme.

D-glucose 6-phosphate was simply diluted by taking 0.5 ml of the stock (200mM) and diluting it with 4.5 ml of buffer, making a 1/10 application solution. The NAD+ was diluted 2:1 by adding 0.5 ml of buffer to the 1.0 ml stock (45 mM); in further experiments the NAD+ was not diluted at all, but instead the stock solution alone was used as the application NAD+.

Enzyme Assays of NAD+ Dilutions:

In the first assays, the 2:1 application NAD+ solution was used and was further diluted in series. In other words, the application solution was diluted by 1/2, 1/4, 1/8, and 1/16; by 1/3, 1/6, 1/12, and 1/24; and by 1/5, 1/10, and 1/20. An undiluted NAD+ sample
was also assayed\(^1\). For each of these enzyme assays, varying amounts of the substrates, enzyme, and buffer were used; the total cuvette volume, however, always equaled 1.0 ml. In the case of the varying NAD\(^+\) dilutions, five series of assays were performed: one had a reaction volume containing the following:

\[
\begin{align*}
150 \, \mu l & \text{ DG6P (20 mM)} \\
50 \, \mu l & \text{ NAD\(^+\) dilution} \\
600 \, \mu l & \text{ Tris-HCl buffer} \\
& \text{ Vortex, and then add:} \\
200 \, \mu l & \text{ DG6P dehydrogenase} \\
& = 1000 \, \mu l \text{ total volume}
\end{align*}
\]

This reaction mixture was assayed for each NAD\(^+\) dilution at 25\(^\circ\)C at a wavelength of 340 nm (Olive and Shreve, 1971)\(^2\). The assay blank contained all of the above except for the enzyme; that volume normally held by the enzyme was replaced with buffer. The second assay used application NAD\(^+\) as well as application DG6P, but in this set three different NAD\(^+\) dilutions were assayed simply by adding different amounts to the assay reaction volume.

An additional assay for NAD\(^+\) dilutions was performed in the same manner. For each assay, 100 \(\mu l\) of enzyme and 150 \(\mu l\) of DG6P were used, but the application NAD\(^+\) and buffer amounts differed in the following manner:

---

\(^1\)In each of these assays in which dilution samples were used, an undiluted application sample was also assayed.

\(^2\)In all of the experiments discussed in this paper, the enzyme assays are performed at 340 nm at a constant temperature of 25\(^\circ\)C. This temperature was sustained in the spectrophotometer with a flow of water through the sample chamber from a regulated water bath.
NAD+ amount: 100 µl corresponding buffer amounts: 650 µl  
200 µl 550 µl  
300 µl 450 µl  
400 µl 350 µl  
500 µl 250 µl  
600 µl 150 µl

The fourth assay involving different concentrations of NAD+ was slightly more complicated than the first time. The application enzyme was prepared the same way as the glucose 6-phosphate, but the NAD+ application solution was simply the stock. With this stock, several dilution series were made: 1/2 up through 1/128, 1/3 up through 1/192, and 1/5 up through 1/160. An undiluted sample was also assayed. This reaction volume contained the following:

150 µl DG6P  
200 µl NAD+ dilution  
450 µl Tris-HCl buffer  
Vortex and then add:  
200 µl DG6P dehydrogenase  
= 1000 µl total volume

A final assay was performed using NAD+ dilution series in the same manner as above, but 100 µl of NAD+ was used rather that 200 µl, and 100 µl of enzyme was used instead of 200 µl. The amount of DG6P was the same (150 µl) and the buffer was increased to 650 µl.

Enzyme Assays of D-Glucose 6-Phosphate Dilutions:

The same procedure was performed for different concentrations of DG6P, and the data from these assays were calculated in a similar way as well. The application DG6P (a one-tenth dilution) was diluted in a 1/2, 1/3, and 1/5 dilution series, the highest dilution...
being 1/40. An example of the procedure is as follows:

150 µl DG6P dilution  
100 µl NAD+ (stock)  
650 µl buffer  
Vortex and then add  
100 µl DG6P dehydrogenase  
= 1000 µl total assay volume

Enzyme Assays of D-Glucose 6-Phosphate Dehydrogenase Dilutions:

Finally, the stock enzyme itself was diluted in several series of enzyme assays, each one varying slightly from the previous. These assays are important not only in order to determine the appropriate concentration of enzyme needed to calculate the activity, but we will use them to also determine the effects, if any, of any additional reagents.

The enzyme assays were in a similar format as the previous involving NAD+ and DG6P. The application DG6P dehydrogenase was used to make 1/2, 1/3, and 1/5 dilution series, with a 1/192 dilution being the highest dilution. The protocol for the assay was the following:

150 µl DG6P  
100 µl NAD+ (stock)  
650 µl buffer  
Vortex and then add  
100 µl DG6P dehydrogenase dilution  
= 1000 µl total assay volume

Enzyme Assays Involving the Addition of Triton x100:

Another set of assays was performed in which an additional reagent was present in the reaction volume. This reagent was the detergent Triton x100, and its presence in the reaction volume was to determine whether it had any inhibitory effects on the enzyme's activity. Triton breaks open membranes, releasing the protected contents contained inside and exposing them to the surrounding medium. The same protocol was used as previously
described, but this time the enzyme dilutions only extended to 1/32, and 30 µl of 10% Triton x100 were added to the reaction volume, while only 620 µl buffer were used. As usual, the total reaction volume was 1000 µl. Another assay set was performed, the purpose of which was to ascertain whether it was indeed the enzyme being inhibited, and not NAD+ or DG6P. Stock NAD+ was diluted in 1/2, 1/3, and 1/5 series up to 1/16, and this same dilution procedure was done for application DG6P. Then the assay was performed using 10% Triton x100 in the reaction mixture; first the assays involving the NAD+ dilutions and constant DG6P were performed, and then the varying DG6P dilutions and constant NAD+.

Once again, enzyme assay sets were performed, but this time not only was the enzyme diluted and Triton x100 included in the assay volume, but other sets included a constant concentration of enzyme with different dilutions of the detergent. DG6P dehydrogenase was assayed in several different concentrations: in the undiluted application concentration, 1/2, 1/4, and 1/6. Triton x100 was normally present in the previous experiments as a 10% solution, representing 3% of the total reaction volume, but here we assayed total reaction volumes composed of 8%, 6%, 4%, and 2% Triton x100. Each assay was performed three times, totaling 60 assays. The assay set without Triton x100 was a repeat of the protocol of the first assay set using enzyme dilutions. The assay sets with Triton x100 differed slightly from the previous ones. The amounts of DG6P and NAD+ were the same, as was the amount of enzyme, but the amount of Triton x100 was either 60 or 80 µl, depending on what concentration of Triton x100 was needed in the reaction volume, and the corresponding amount of buffer was 590 µl and 570 µl. In every case, the total reaction volume was 1000 µl.
Enzyme Assays Involving the Addition of L-Cysteine:

Several assays were done to test the effects of another reagent, L-cysteine, on the activity of the enzyme, DG6P dehydrogenase. L-cysteine was normally added to the assay reaction mixture when performing enzyme assays in solutions that contained enzyme samples, the substrates, and ozonated buffer. Because cysteine is the amino acid that reacts most rapidly with ozone, by adding it to the reaction mixture at specified time intervals, the reaction between ozone and the enzyme will be terminated immediately as ozone turns its attack on the more susceptible amino acid. In this way, one can "quench" the remaining ozone present in a reaction system at any time after the enzyme and ozonated buffer have been mixed just by adding L-cysteine. The stock L-cysteine was 100 mM, and it was diluted by 1/10 making the application L-cysteine 10^{-2} M. By adding 50 \mu l of L-cysteine to the total assay volume, this reagent will be at a final concentration of 5 \times 10^{-4} M. The following dilution series was made of the application L-cysteine: 1/2 through 1/8, and 1/10 through 1/80. A few dilutions were made of the application enzyme as well, those being 1/2, 1/4, and 1/6. The stock NAD+ was used, and the application DG6P was a 1/10 dilution of the stock. The protocol of the assay was as follows:

\[
\begin{align*}
150 \mu l \text{DG6P} \\
100 \mu l \text{NAD+} \\
50 \mu l \text{L-cysteine dilution} \\
600 \mu l \text{buffer} \\
& \text{Vortex and then add} \\
& \text{100 \mu l DG6P dehydrogenase dilution} \\
= & \text{1000 \mu l total assay volume}
\end{align*}
\]

Liposome Preparation:

In addition to the above assays, one other preparation was needed before any experiments with ozone were to be performed. This was the making of liposomes, which
were the models representing actual cells enclosing enzymes. The phospholipid used was a mixture of 10% 18:1 dioleoyl-phosphotidylcholine, which corresponds to 10% of this phospholipid having a hydrocarbon tail 18 carbons long with one double bond (18:1) in an organic solvent of which 90% was a 45% dipalmitoyl-phosphotidylcholine. This phospholipid solution was dried in sterile test tubes and placed in an airtight container.

When ready to use, a sample was removed from the container and the enzyme suspension was added to it. This suspension was prepared by adding 1300 μl of buffer to 200 μl of the stock enzyme, DG6P dehydrogenase, making a 1/7.5 dilution.

After adding the enzyme solution to the dried phospholipid, the sample was placed in a 45°C waterbath, which helps the lipid dissolve, and then vortexed. When the lipids dissolved, they immediately formed liposomes because of the hydrophobic carbon tails. The sample was then run through an extruder approximately ten times. This pressurized container forced nitrogen gas through a compartment, pushing the lipid/enzyme solution (liposomes) through a series of filters, beginning with very thin paper filters with a pore size of 0.4 μm and ending with large stainless steel filters with pores approximately 2-3 mm in diameter. This process forced the liposomes to reduce their diameter to that of the smallest pores; in effect, making them similar to biological cells. After running the liposomes through the extruder the first five times, a clean Pasteur pipette (used to transfer the liposomes from the test tube back to the opening at the top of the extruder) and clean test tube replaced the first ones, to remove any traces of liposomes that had accidentally not been placed in the extruder. The remaining liposomes were run through the extruder an additional five times. The extruder was hooked up to the 45° waterbath, so the liposomes were kept at a constant temperature.

The remaining extruded liposome solution was then run through a chromatography column, made either of Sepharose CL-6B or Sephacryl S 400 HR, to separate the liposomes that encapsulated the enzyme from the surrounding medium containing just enzyme. The column allowed the liposomes to run through the column first, and this
effluent could be collected easily because it was cloudy and therefore easily distinguishable from the surrounding medium. The effluent containing the enzyme alone was also collected.

The post-column liposome sample was enzymatically assayed to be used as a control when comparing experiments where ozonated buffer was included in the assay. The liposomes, containing the enzyme, were diluted by a 1/2 dilution series, up through 1/16. Each dilution was assayed both in the presence and absence of Triton x100. The two protocol are as follows:

<table>
<thead>
<tr>
<th>With Triton x100</th>
<th>Without Triton x100</th>
</tr>
</thead>
<tbody>
<tr>
<td>150 µl DG6P (application)</td>
<td>150 µl DG6P (application)</td>
</tr>
<tr>
<td>100 µl NAD+ (stock)</td>
<td>100 µl NAD+ (stock)</td>
</tr>
<tr>
<td>30 µl 10% Triton x100</td>
<td>650 µl buffer</td>
</tr>
<tr>
<td>620 µl buffer</td>
<td>Vortex and then add</td>
</tr>
<tr>
<td>Vortex and then add</td>
<td>100 µl liposome solution</td>
</tr>
<tr>
<td>100 µl liposome solution</td>
<td>= 1000 µl total assay volume</td>
</tr>
</tbody>
</table>

= 1000 µl total assay volume

Now the liposomes were ready to be treated with ozonated buffer, in a different set of experiments than the one focused on in this paper.
RESULTS AND DISCUSSION

The purpose of the first set of experiments was to determine the appropriate amount and concentration of NAD+ to use in the total assay volume. Assays were carried out using different dilutions of NAD+ in the total reaction volume and those data were reviewed and processed. The data received from the NAD+ dilution experiments were first graphed to determine whether the data were accurate for each dilution series; the data for Figure 1 were from the experiment using 50 µl of application NAD+ dilution and 200 µl of DG6P dehydrogenase in the total assay volume. The equation next to each line gives the slope of the line or the rate of the reaction. The R² (regression) value evaluates the accuracy of the data, through which a line is extrapolated. The optimum R² value that a line can receive is a 10.0 x 10⁻¹, or 1. We considered a value above a .99 to be accurate enough for our research. Figure 2 shows another experiment of stock NAD+ dilutions used in an enzyme assay, this time diluted to 1/192, using 200 µl of NAD+ and 200 µl of DG6P dehydrogenase in the total assay volume. A Lineweaver-Burk plot was made for the data in Figure 2 to determine the K_m and V_max of the reaction (Figure 3). By comparing these values one can determine any differences in activity and substrate concentration. This shows the V_max to be equal to 0.260 µmol NADH per minute. The K_m value is equal to 0.390 mM NAD+. In order to determine whether NAD+ was the limiting reagent in the reaction, an additional Lineweaver-Burk plot graphed for the second assay using dilutions of NAD+ up to 1/192 is shown on Figure 4. This time both the NAD+ and DG6P dehydrogenase volumes were decreased to 100 µl in the total assay
Figure 1—Enzyme assays involving dilutions of application NAD+ (undiluted through 1/40). The $R^2$ values illustrate the accuracy of the data collected.
Figure 2—Enzyme assays involving dilutions of stock NAD+ (undiluted through 1/192). The $R^2$ values again show the accuracy and validity of the data.
Figure 3--Lineweaver-Burk plot from the data in figure 2. From this data the $K_m$ and $V_{max}$ were determined. In the total assay volume, 200 µl of both NAD+ and DG6P dehydrogenase were included.
volume of 1000 µl. Because the concentration of the enzyme had been halved, the $V_{\text{max}}$ equals 0.128 µmol NADH per minute. The $K_m$ value is 0.308 mM; this value is over half that of the previous value of 0.390 mM, which means there is an abundance of NAD+ in the sample relative to the amount of enzyme. Thus, the NAD+ will not be the limiting reagent.

Several enzyme assays using varying NAD+ dilution were performed to determine if NAD+ was the limiting reagent. This experiment involved different assays performed using varying volumes of NAD+ in the total reaction mixture; Figure 5 shows a graph made to determine the amount of NAD+ needed in the assay mixture that would give the maximum initial rate of enzymatic reaction. The NAD+ used was the stock (45 mM) and no dilutions of this application NAD+ were made before assaying. This graph shows that the NAD+ needed can be as little as 100 µl and still give a constant rate of reaction before running out for up to one minute. This is, therefore, the amount we used.

The same procedure used for NAD+ dilutions was also used for the other substrate, D-glucose-6-phosphate (DG6P), to assure that it also would not be the limiting reagent. In the experiment where the application DG6P was diluted up to 1/40, Figure 6 shows the rates of reaction using different dilutions of DG6P and the corresponding $R^2$ values. A Lineweaver-Burk plot was calculated to determine the $V_{\text{max}}$ and $K_m$ values; they were 0.833 µmol NADH per minute and 0.0554 mM, respectively (Figure 7). Notice here that the $K_m$ value for DG6P is almost $10^2$ lower than that of NAD+; therefore, the hypothesis that the NAD+ determines the rate-limiting step could be correct. Figure 8
Figure 4--Lineweaver-Burk plot from data in enzyme assay using NAD+ dilutions (undiluted through 1/192); from this the $K_m$ and $V_{max}$ were determined. This set of assays included 100 μl of both NAD+ and DG6P dehydrogenase in the total assay volume.
Figure 5--Enzyme assays involving different volumes of NAD+ in total reaction volume. All of the R² values show that the data fits perfectly on the line.
Figure 6--Enzyme assays involving dilutions of DG6P (undiluted through 1/40). The $R^2$ values for this set of data show that the experiments resulted in accurate data.
Figure 7--Lineweaver-Burk plot from data using DG6P dilutions; the $K_m$ and $V_{max}$ were determined. This assay contained 100 µl of both NAD+ and DG6P dehydrogenase, and 150 µl of DG6P in the total assay volume.
shows results similar to those in Figure 5, however, a different volume of DG6P was added to the total reaction volume in each assay performed. This procedure was done three times and the corresponding equations were derived from the data. Once again, it was found that the minimum amount assayed, 150 mmols of DG6P, was sufficient to keep the reaction rate constant for recordings timed up through one minute.

Finally, enzyme assays were performed that contained different dilutions of the application enzyme, DG6P dehydrogenase. Figures 9 and 10 are graphs of enzyme dilutions used in the total assay volume. Because of the density of the lines running through the data points, the assays were separated into two different graphs: Figure 9 contains the data up to the DG6P dehydrogenase dilution of 1/12, and Figure 10 contains the data from 1/16 through 1/96. Equations were written for several of the dilutions, giving the rates of the reactions, as well as the $R^2$ values.

Triton x100 is a detergent that is used in ozone experiments to break open the liposome membrane, releasing the enzyme contained inside and exposing it to the ozonated buffer. To determine whether Triton x100 has any inhibitory effect on the enzyme, enzyme assays were performed that included Triton x100 in the total assay volume. If Triton x100 did have an inhibitory effect on the enzyme then any data derived from ozone experiments would be invalid. The next set of graphs (Figures 11 through 15) are results of the experiments involving Triton in the assay total volume to determine the effect, if any, that Triton has on the enzyme's activity. Each of these assays was performed in triplicate for the purpose of having reliable data; this was essential to know before continuing on with ozone experiments. For many of the different dilutions
Figure 8—Enzyme assays involving dilutions of DG6P in total reaction volume. The R^2 values show the accuracy of the data.
Figure 9--Enzyme assays involving DG6P dehydrogenase dilutions (undiluted through 1/12.)
Figure 10--Enzyme assays involving DG6P dehydrogenase dilutions (1/16 through 1/96.)
there are no discrepancies between the three sets of data. Figure 11 involves the assay of undiluted application glucose-6-phosphate dehydrogenase without any Triton-x added. The following four figures (Figures 12-15) show the same concentration of the enzyme in the reaction volume, but with decreasing concentrations of Triton-x. The letters "A", "B", and "C" represent the first, second, and third trial of each dilution of Triton x100 and/or DG6P dehydrogenase. Additional graphs of the dilutions of DG6P dehydrogenase with different concentrations of Triton x100 were designed; similar results were found. By comparing the rate of reaction from Figure 10 showing undiluted DG6P dehydrogenase (7.60 x 10⁻¹ increase in absorbancy per minute) as opposed to the rate of reaction of the same amount of undiluted enzyme in the total assay volume in Figure 12 containing 10% Triton x100, there is a small decrease in the values (difference equals 0.5 x 10⁻¹). Figure 16 illustrates that the V_max of DG6P dehydrogenase did not decrease substantially with the addition of Triton x100 in the total assay volume (equaling 0.123 μmol NADH per minute), compared with that of an assay without any Triton x100 (equalling 0.128 μmol NADH per minute). It was therefore decided that the Triton x100 does not inhibit the enzyme enough to look for a replacement detergent.

L-cysteine is used in the ozone experiments to "quench" the ozone; it reacts with the excess ozone in the reaction vessel to terminate the reaction of ozone with the enzyme. Just as Triton x100 was included in the total assay volume to determine the inhibitory effects it might have on DG6P dehydrogenase, the same protocol had to be followed for L-cysteine to determine any inhibitory effects it might have on this enzyme. Figure 17
Figure 11—Rate of enzyme activity in an enzyme assay set containing undiluted DG6P dehydrogenase and no Triton x100.
Figure 12--Rate of enzyme activity with 0.8% Triton x100
Figure 13--Rate of enzyme activity with 0.6% Triton x100.
Figure 14--Rate of enzyme activity with 0.4% Triton x100.
Figure 15--Rate of enzyme activity with 0.2% Triton x100.
Figure 16--Lineweaver-Burk plot from data in assays involving Triton x100.

\[ f(x) = 2.09E+0^x + 8.10E+0 \quad R^2 = 9.94E-1 \]
illustrates the effect of L-cysteine on the activity of the enzyme. "Cyst A" simply represents the first round of assays performed, since this series was done in duplicate. This graph is a sampling of the first few dilutions of both the enzyme and cysteine, since the enzyme was diluted up to 1/80 with each dilution of L-cysteine, and two assays were run for each. This graph basically illustrates that L-cysteine has little effect on the enzyme, and the decrease in activity is primarily due to the more diluted samples of the enzyme.

This research was aimed at obtaining some basic data about substrate concentration, enzyme concentration, and interfering substances, and these simple goals were accomplished. The results did not reveal any major advances in understanding this enzyme, D-glucose 6-phosphate dehydrogenase, but helped in preparing the research to move to an advanced level in the overall project discussed in the introduction.

The assays involving NAD+ dilutions and its role in the reaction revealed that the concentration of NAD+ needed was the minimum with which we experimented. This amount turned out to be the 45 mM stock, and since we used 100 μl in the total assay volume of 1000 μl, the concentration of NAD+ in the total assay volume was 4.5 mM.

The D-glucose6-phosphate was determined to be at an adequate concentration at the 150 μl minimum used. This corresponds to a concentration of 3.0 mM in the total assay volume.

The Triton-x needed for the opening of the liposomes was at first thought to interfere in this enzymatic reaction. In subsequent assays, however, it was discovered that Triton-x did not negatively affect the DGPDase and decreases in the rate of reaction were
Figure 17--Rate of enzyme reaction in assays involving L-cysteine. This graph illustrates the independence of the activity of DG6P dehydrogenase on the level of L-cysteine.
due to more dilute enzyme concentrations. It was therefore decided to continue to use the Triton-x detergent at its normal 10% concentration.

L-cysteine was likewise found to not interfere with the DGPDase and it was again thought that the decrease in the rate of reaction was due to decreased enzyme concentration. The concentration of L-cysteine in the total assay volume that will be used to quench the ozone treatment is 0.5 mM.

These experiments will allow others to continue his experiments of applying ozone to an enzyme or enzyme-liposome suspension and to attribute the decrease in the activity of the enzyme to the ozone treatment itself. While these assays were repetitious and usually unvarying, they were necessary to perform before accurately interpreting any data on the effects of ozone on the enzyme, D-glucose-6-phosphate dehydrogenase.
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