A dehydrogenase linked assay of DNA ligase activity
by Jerold Randall Morgan

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemistry
Montana State University

Abstract:
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Jerold Randall Morgan

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27 January 1987
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29 January 1987
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Signature Jerold R Morgan

Date 12/28/86

To my Grandfather

Rev. Jerry Sheldon Nelson

who taught me that life without
knowledge and wisdom is a
veritable desert

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I extend my gratitude to Dr. Pete Bartels for allowing me to use his equipment in Seattle. The use of this equipment permitted me to finish the necessary experiments proving the usefulness of the assay. I wish to also thank Dr. Gordon Julian, my advisor, for helping me through difficult phases of the project and teaching me how to handle problems. Without his extensive knowledge and creative ideas, I would have been unable to create the workable solutions necessary to bring the project to a successful conclusion.

I especially wish to thank my wife and family who believed in the values of effort and persistence.

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ABSTRACT

A novel dehydrogenase linked assay for prokaryotic DNA ligase activity is presented. This assay is based on the quantification of NAD^+ utilization by the DNA ligase. The ligase enzyme splits NAD^+ into NMN and AMP during the joining of DNA pieces. This assay is simple to use, inexpensive, and the reagents are stable for six months if stored in the dark. The assay avoids the use of radiolabels and mutagenic chemicals that are found in many DNA ligase assays. Finally, data is presented that indicates that the assay can be used to measure DNA ligase activity in crude extracts from prokaryotes.

EVOLUTION OF COLORIMETRIC ASSAY

Originally, the objective of the project was to purify a DNA ligase from the thermophilic bacteria T-2. However, the problems encountered with recommended assays for DNA ligase activity prompted a search for an alternative assay. The developed assay utilizes an enzymatic cycling of NAD^+, which in turn converts a dye from one oxidation state to another. This assay is sensitive and easy to use, and does not depend on the use of radiolabels. The following section of the thesis presents the problems encountered in the early stages of the project, and reveals the evolution of the new assay.

The first stage of the project involved familiarization with the ^{32}P labeled d(A-T)n ligase assay (1,2). Within the first three months, the procedure for the assay could be used routinely. The assay was complicated by the short half-life of the substrate (14 days) (3). Once the substrate was formed, it had to be used as quickly as possible to minimize the reduction in decay CPM (radioactive counts per minute). Also, the substrate had to be handled with special care to prevent exposing people to radiation. Additionally, all materials that came into contact with the labeled substrate had to be isolated until the radioactivity was sufficiently low to dispose of the

materials.

The main reason that the ^{32}P labeled d(A-T)n assay was not found to be suitable for the purification of the enzyme was not the hazardous nature of the radiolabel, nor the short half-life, but rather the costliness of the ^{32}P labeled ATP that was used to produce the substrate. The ^{32}P-ATP that was first utilized was kindly donated to Dr. Julian's lab by another research group. We used the donated ATP to produce the first lot of substrate. When it was time to order more of the labeled ATP, the full price became apparent to us. It was determined that the price of the substrate was too high to allow the use of the assay for the purification of the ligase. The purification stage of an enzymatic study requires a large number of activity assays to be run at each step. This decision not to use the ^{32}P-ATP assay was reinforced by the finding that there was endogenous phosphatase activity in the crude extract that would have to be removed or destroyed before the assay could measure any ligase activity. The phosphatase enzyme removes the radiolabel from the DNA before the ligase is able to join the fragments which lowers the measured ligase activity and gives false negative results. If there is a large excess of phosphatase activity, the activity of the ligase can be completely hidden. Thus, another assay was sought that would be useful during the purification of the ligase. Once the

enzyme had been purified, the ^{32}P labeled d(A-T)n assay was to be used in the kinetic studies of the enzyme if the other assay could not be used for kinetic studies.

The assay that was substituted for the ^{32}P labeled d(A-T)n assay was based on the electrophoresis of restriction fragments from lambda phage genomic DNA. The assay procedure involved digesting the lambda DNA with EcoR1 restriction endonuclease, exposing the fragments to the ligase sample, and then separating the resulting mixture by electrophoresis through agarose gels. Ligase activity was interpreted by witnessing an alteration in the band pattern in the gel. The monitored change was an increase in the size of all the fragments, which would be expressed as a slower rate of electrophoretic movement. Several catalogs showing activity of commercial ligase preparations showed gels that contained an increase in the size of restriction fragments after being exposed to the ligase at 16^{o} C. Many of these electrophoretic assays were run during the first year of the project. Several times the band pattern showed results that were interpreted as ligase activity.

However, a reinterpretation of the assay results became necessary late in the first year of research. A new culture of the thermophilic bacteria was received from ATCC, and an attempt was made to purify active material from several large growths of the bacteria.

Problems first appeared when the electrophoretic assay failed to reveal any bands in the gel at all. The assay was modified to include a digestion of protein with proteinase K before electrophoresis. The results from the assays showed no activity. A trial of material that had earlier shown activity was run as a control, and it did not show the change in band pattern that had been taken as characteristic of ligase activity.

At first the absence of the expected alteration in band pattern gave rise to the suspicion that the reagents used in the assay had gone bad. However, a check of the reagents as used in the old electrophoretic assay configuration showed that they were not the cause of the difficulty. When the samples were exposed to proteinase K, the fragments of DNA possessed the same mobilities as DNA that had not been incubated with ligase sample. If the proteinase K treatment was omitted, the samples showed activity. Also, samples of crude extract that lacked NAD^+ showed similar results as samples that had NAD^+ added prior to incubation with ligase. During the early stages of the project this result was justified by the suggestion that residual NAD^+ in the crude extract was responsible for the ligation activity. This explanation was no longer sufficient when more highly purified fractions that had been subjected to steps such as dialysis that should have removed any NAD^+.

A search of relevant literature was completed in order to find a possible explanation for the observed failure of the assay. It was found that the problem with the assay resulted from a misinterpretation of the data. At 37^{o}C the cohesive ends that were present on EcoR1 generated fragments did not possess enough hydrogen bonding attraction to remain joined together (4). Thus, the probability of ligating the fragments was low. However, the ends of the lambda genome possess a twelve base pair match that is joined during the replication stage of the phage . The two fragments that contain the matching end sequences are the only fragments to undergo an alteration in electrophoretic mobility (4). These two fragments are the largest and the smallest fragments that are formed by EcoR1 digestion. Thus, the expected result of the electrophoresis of ligated fragments should have been the disappearance of the smallest and the largest band, and the appearance of a band with an even larger size. In practice, the mobility of the new band was not sufficiently different from the mobility of the larger of the two unligated fragments to be separated from it. Thus, a disappearance of the smallest band would have to be interpreted as ligase activity. Since the recovery of DNA after the digestion with proteinase K was not quantitative, there was an inherent variation in the amount of the smallest fragment present during electrophoresis. This variation

rendered the assay unsuitable for determining ligase activity in crude samples where proteinase K digestion was necessary.

The artifact that had been interpreted as ligase activity was suspected to be caused by the binding of proteins to the DNA restriction fragments. The protein-DNA complex would have a greater molecular weight than the DNA alone. An increase in the protein concentration could give rise to larger complexes and thus increase the observed molecular weight. This is also the expected result from a sample containing ligase activity that would be ligating fragments into larger fragments.

Even before the mistaken interpretation of data from the electrophoresis assay was discovered, another assay had been explored. It had been anticipated that the other assay would prove to be more rapid than the electrophoresis assay. This assay involved the healing of nicks introduced into plasmids by S1 nuclease (5,6). The assay proved to be more difficult than was assumed. The production of large amounts of plasmid without random nicks was not successful. The assay was abandoned finally because of the presence of endogenous endonuclease activity in crude samples. The endonuclease activity presented the same problems that were present with the phosphatase activity when the ^{32}P labeled d(A-T)n was attempted on crude samples. A very small amount

of endonuclease activity would cover up any ligase activity present in a sample. This supression of observed ligase activity would be caused by the greater concentration of endonuclease substrate relative to ligase substrate. This greater relative concentration results from the fact that the endonuclease is able to use many different base pair sequences on the same plasmid as a substrate, whereas the ligase must find those sequences that possess a break in the phosphodiester backbone.

After a full year of experimental work, no usable assay had been found. At this point, a decision was made to develop an assay that could work during the purification of the ligase. A review of the literature revealed that many assays could not detect ligase activity in crude samples (4,7,8). The project was then altered to find an assay that could circumvent some of the problems encountered with the other assays that had been previously attempted.

In examining the ligase reaction in prokaryotes, (Figure 1), it is apparent that there are two major alterations in the molecular structures of the reactants. The first, and biologically most important, is that a phosphodiester bond is formed between two pieces of DNA. However, NAD^+ is also cleaved to form AMP and NMN (Figure 2). This cleavage is similar to the reaction in eukaryotes where ATP is cleaved to form PPi

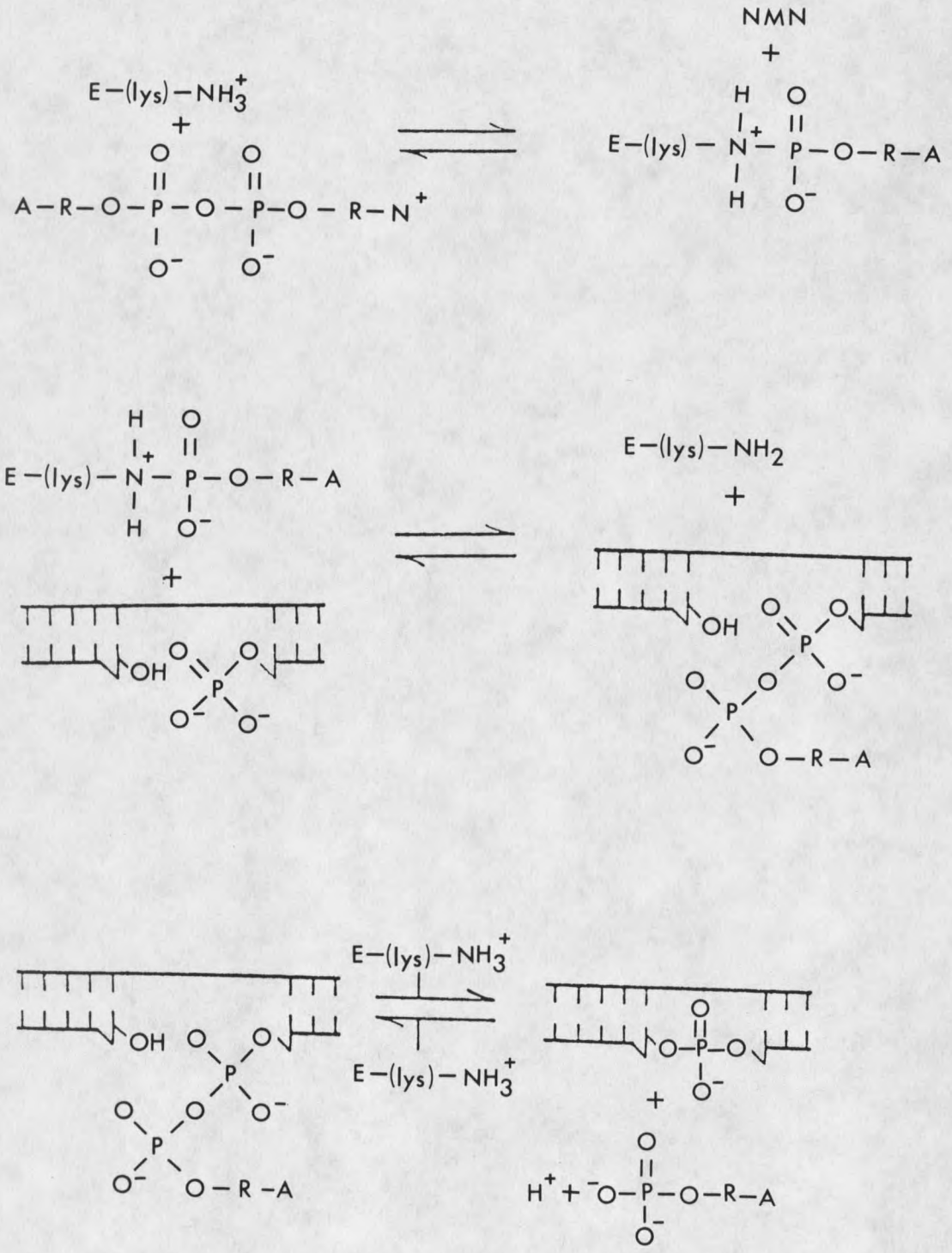
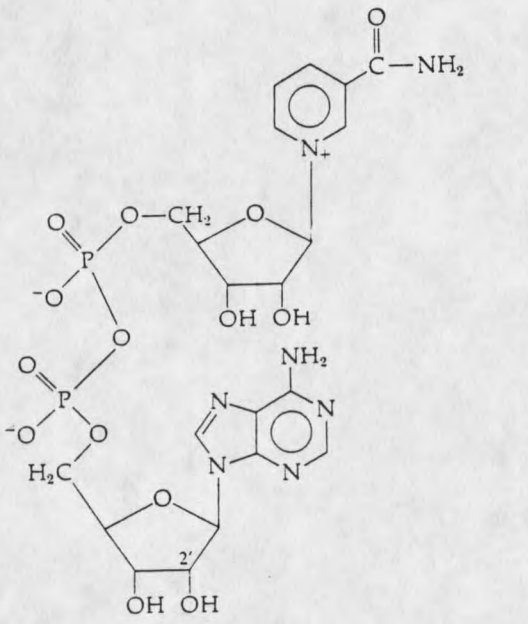
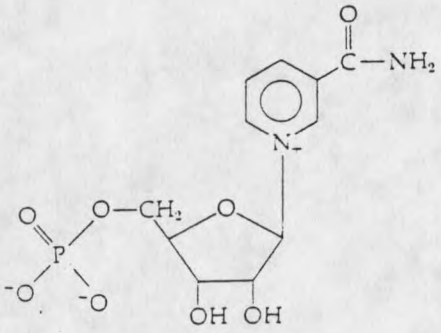


Figure 1. Mechanism of prokaryotic DNA ligase reaction.





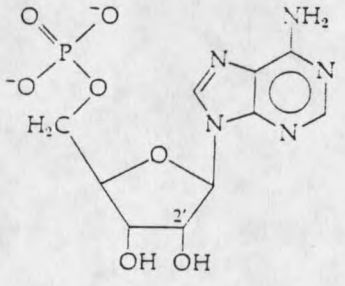


Figure 2. Structures of NAD^+ (top), NMN (middle), and AMP (bottom).

and AMP (9). The Wiess unit has been used for some time to measure eukaryotic DNA ligase activity (10), and the Wiess unit follows the conversion of ATP. The assays used to measure prokaryotic ligase activity have largely ignored the NAD^+ cofactor in favor of the DNA.

A method for measuring the use of NAD^+ by prokaryotic ligases was developed from other work that was accomplished during the project. When it was discovered that the culture of T-2 that had been used as a stock culture had become contaminated, a method for rapid identification of T-2 was developed. Dr. Julian had previously used a test that takes advantage of the lack of a pentose shunt in T-2. One of the enzymes in the pentose shunt is glucose-6-phosphate dehydrogenase. The test developed by Dr. Julian used the shift in UV absorbance that occurs when $NADP^+$ is converted to NADPH by glucose-6-phosphate dehydrogenase, in order to monitor activity of the enzyme. Since the enzyme should be absent in T-2, there should be no increase in the OD_{340} when glucose-6-phosphate and $NADP^+$ is mixed with a sample of disrupted cells.

The new test for T-2 culture purity was a colorimetric test for glucose-6-phosphate dehydrogenase activity developed from a scheme for measuring lactose dehydrogenase activity that had been found in an article (11). The test involves a dye and electron carrier. The $NADP^+$ is reduced by the enzyme, and, in

turn, the NADPH reduces an electron carrier, phenazine methylsulfate. The reduced form of PMS then reduces a dye, which alters the dye's absorbance spectrum. This colorimetric test is much more sensitive than the other test that involves UV absorbance.

The idea occurred that if the dye reaction was as sensitive to NAD^+ as it was to dehydrogenase, and the concentration of NAD^+ was made the rate limiting component, an assay for concentration of NAD^+ could be established. This test of NAD^+ concentration could then be incorporated into an assay for ligase activity. The assay would measure the decrease in the amount of NAD^+ present after incubation with a ligase sample. The more ligase activity present in a sample, the greater the decrease in NAD^+ concentration. A representation of the ligase activity assay is given in Figure 3.

The main aspects of such an assay that would need to be tested were sensitivity, linearity, and reproduciblity. Any useful assay for DNA ligasse would need to be sensitive enough to detect the small changes in NAD^+ concentration occuring in a reasonable sample size. The assay would also need to be linear,i.e. two units of DNA ligase should give double the measured NAD^+ usage that one unit of DNA ligase would use. Lastly, an useful assay needs to be reproducible over a wide range of samples, or have an internal standard built into the assay to calibrate the measurements.

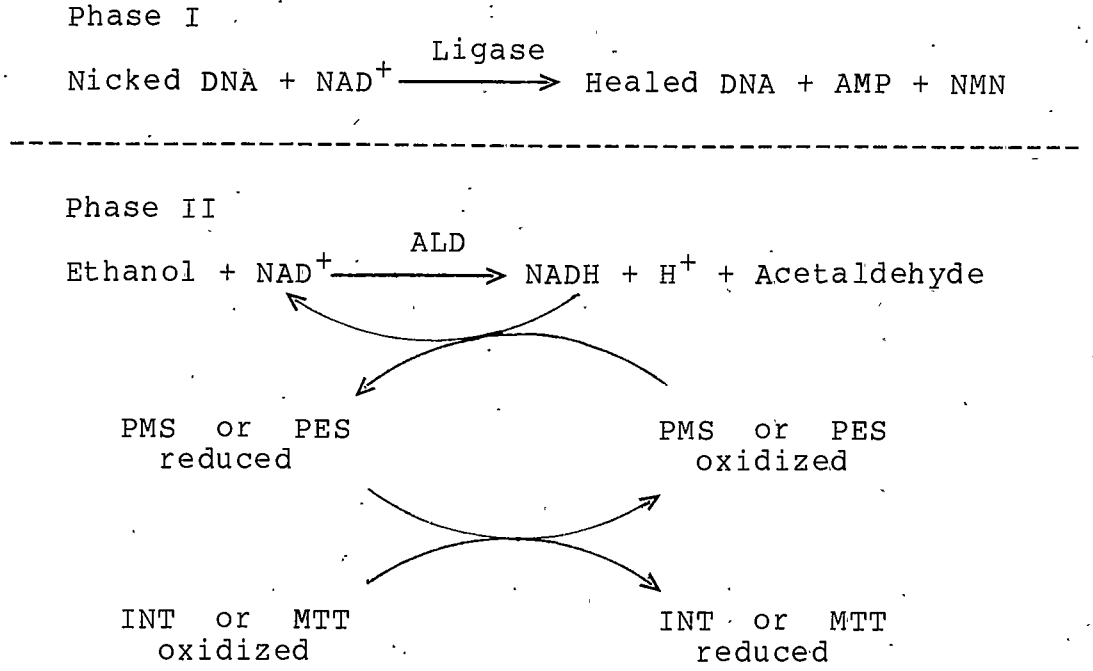


Figure 3. Representation of ligase assay. Phase I is the incubation of the DNA substrate with the ligase sample. This is followed by Phase II, which measures the amount of NAD^+ not utilized by the ligase.

The first step in forming a new assay was to determine if the dye conversion was sufficiently sensitive to detect the small amount of NAD^+ that would be present in a ligase reaction. By using a combination of a dehydrogenase and PMS to cycle the NAD^+ between its oxidized and reduced forms, a large amplification of the sensitivity of the assay for NAD^+ could be achieved.

The concentration of NAD^+ to be monitored must lie in the range that would be used by a ligase sample in a reasonable length of time. It was determined that 50 to 500 pmoles of NAD^+ would be a usable range. This range was chosen because it was able to detect a significant change in the amount of NAD^+ present, after incubation

of the NAD^+ and one unit of DNA ligase at 37°C for two hours. One unit of ligase activity is defined as the amount of enzyme required to give 50% ligation of Hind III digested lambda DNA in 30 minutes at 16°C in a final volume of 5 ul and a 5'termini concentration of 0.12 uM (12). One unit would use 4.8 pmoles of NAD^+ in two hours under the assay conditions listed above. If a 5 pmole alteration in the amount of NAD^+ present could be assayed consistently, then the assay would be usable for reasonable sample sizes.

The first trial of the colorimetric assay used ethanol as the dehydrogenase substrate and alcohol dehydrogenase as the enzyme responsible for cycling the NAD^+ to NADH. A range of NAD^+ concentrations that began at 500 pmoles, and decreased to 100 pmoles was used to test the linearity of the relationship between the dye conversion and the NAD^+ concentration. The correlation coefficient for the plotted data was calculated by Hewlett Packard linear regression as 0.977. The low value can be attributed to the inaccuracy of the delivery of alcohol dehydrogenase by the micropipetter. The replacement of the pipetter with a more accurate one resulted in an increase in the correlation coefficient to 0.988 (Figure 4).

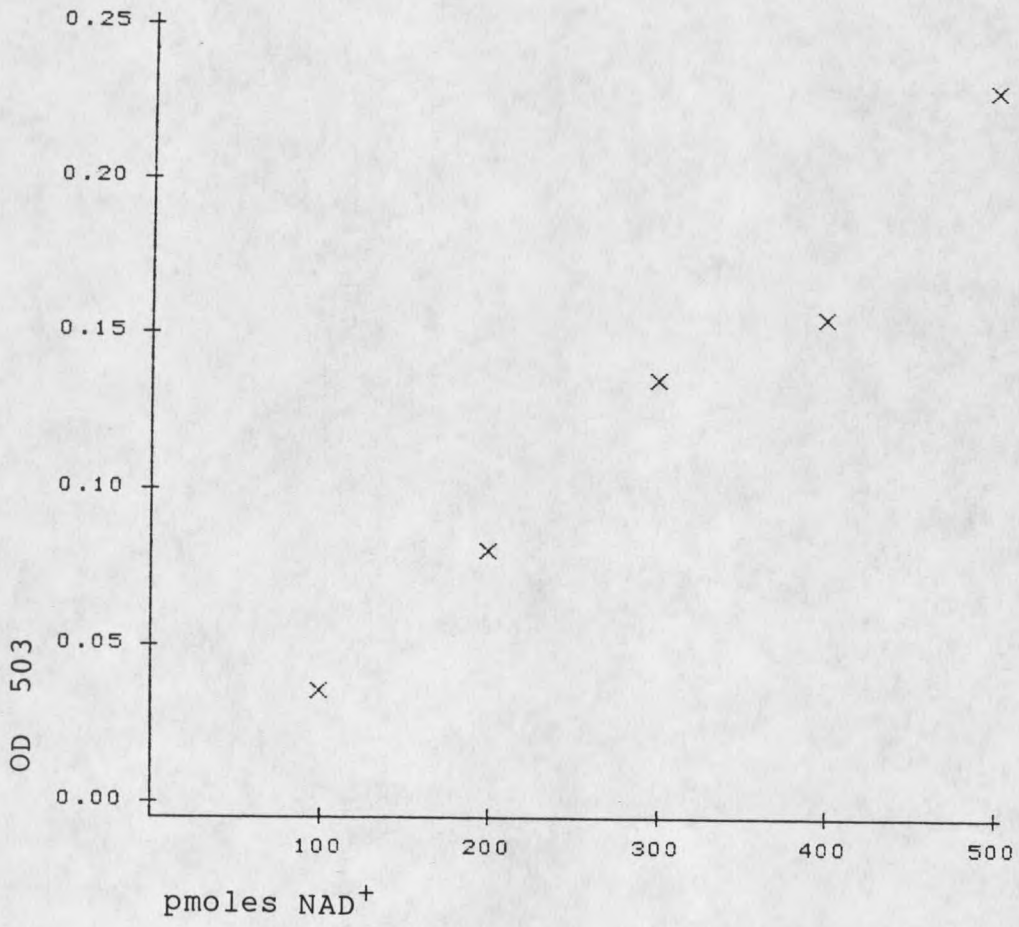


Figure 4. Test of absorption at 503 nm versus pmoles of NAD^+. Color development time was 1800 sec. Dye and electron carrier were INT and PMS respectfully.

The higher value of the correlation coefficient encouraged the trial of the colorimetric assay of NAD^+ concentration in a ligase activity assay measuring the decrease in NAD^+ that results from the cleavage of NAD^+ into NMN and AMP by the ligase. The DNA substrate used in the assay was prepared by digesting salmon sperm DNA 10ug/ml with Mlu-1 overnight at 37^oC. This enzyme was

used due to the fact that the recognition and cleavage sequence is composed of G-C base pairs (13), which are more temperature stable(14). The ligase sample consisted of a commercial preparation of E. coli ligase purchased from New England Biochemicals. (The results are tabulated in Table 1.) Also, a test was conducted to determine the optimal temperature at which to run the assay. (The results from this test are also presented in Table 1.) The results from these two tests show that the assay for ligase activity is feasible. The optimal temperature was determined to be 37°C using Mlu 1 digested DNA.

Tube #	Temperature/Time of ligation	OD_{503}
1	0°C / 0 hours	0.326
2	20°C / 4 hours	0.169
3	25°C / 4 hours	0.170
4	30°C / 4 hours	0.163
5	37°C / 4 hours	0.153

Table 1. Test of optimaltemperature for ligation reaction. Color development time was 1800 sec. Dye and electron carrier were INT and PMS respectfully. All tubes contained 10 units ligase and 500 pmoles NAD^+. Substrate consisted of 1 mg digested DNA.

A test of the linearity of dye conversion versus time of alcohol dehydrogenase reaction was performed to determine the precision of the assay, which could be highly dependent of the accuracy in the timing of the colorimetric incubation period (Figure 5). The plot was

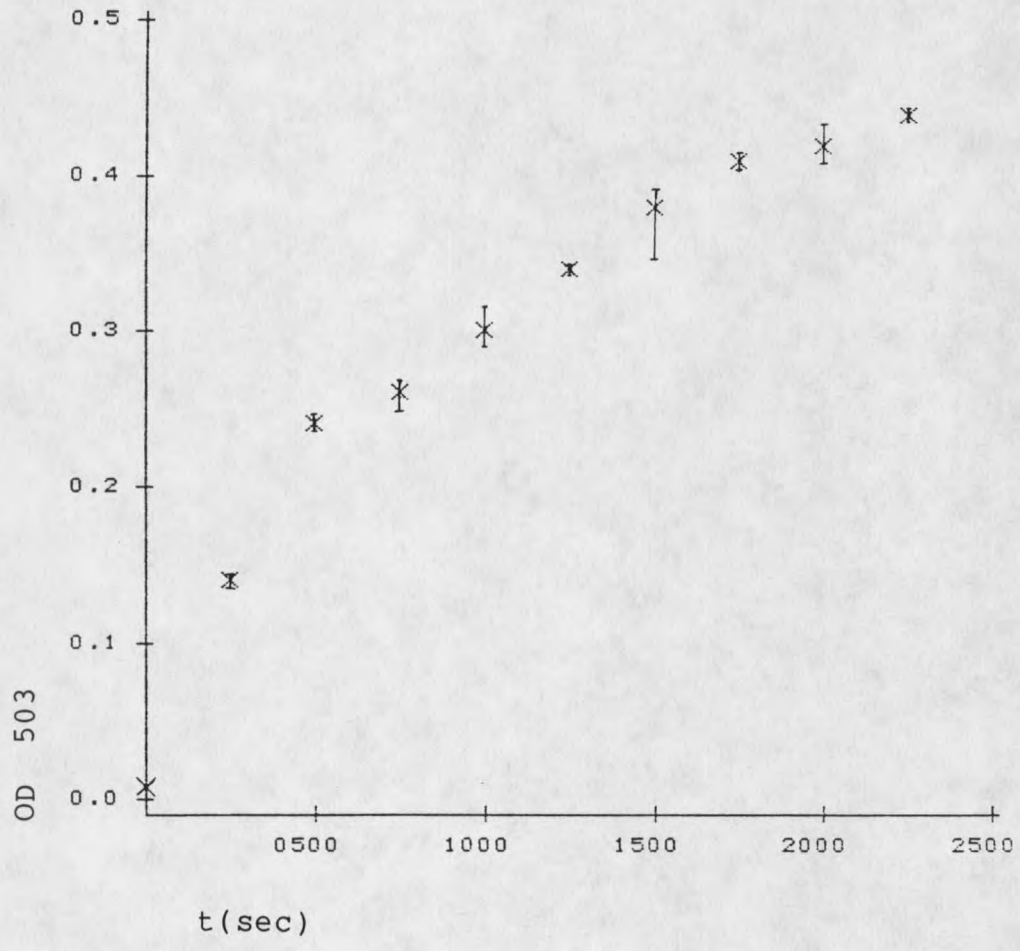


Figure 5. Test of linearity of dye conversion versus time. All samples contained 500 pmoles NAD^+. Dye and electron carrier were INT and PMS respectfully.

obviously nonlinear, which might indicate that the reaction rate of the alcohol dehydrogenase reaction is not limited by the concentration of NAD^+. The assay was modified to include the addition of 3% BSA to the alcohol substrate. The BSA stabilized the dye, and

prevented the precipitation of the dye, which dramatically increased the linearity of the dye conversion (Figure 6) (11). However, the BSA also increased the background considerably (Figure 7).

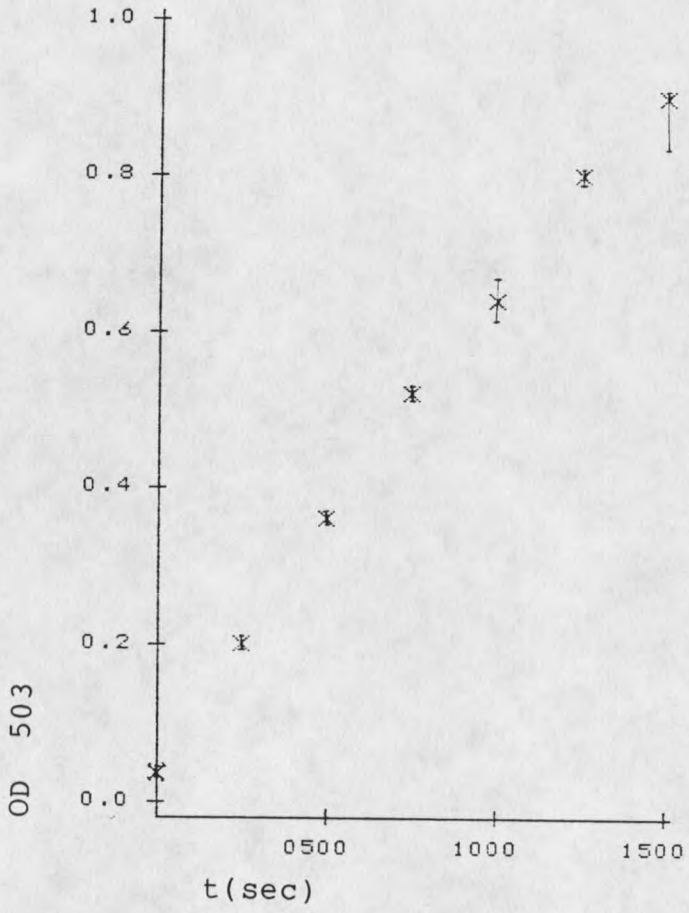


Figure 6. Test of linearity of dye conversion using 3% BSA to stabilize the dye. Dye and electron carrier were INT and PMS respectfully. All samples contained 500 pmoles NAD^+.