THE REDUCTION OF COZYMASE BY SODIUM BOROHYDRIDE* 

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Cozymase (diphosphopyridine nucleotide, DPN) has been assayed by a variety of biochemical and chemical methods (1). Among the simplest procedures has been the determination of the light absorption of reduced DPN at 340 m\(\mu\) after reduction by sodium hydrosulfite. Since special precautions are required to remove completely the excess reducing agent which absorbs in this region, it appeared advantageous to find another reducing agent which could be employed for the same purpose with greater ease.

A study of the action of sodium borohydride (NaBH\(_4\)) on DPN revealed that it filled the necessary requirements. The main advantage of this new reagent lies in its absence of light absorption at 340 m\(\mu\). Spectrophotometric measurement of DPN reduced by NaBH\(_4\) may therefore be made without removal of excess reagent. The assay procedure together with some observations on the properties of the reduction product will be described in this communication.

EXPERIMENTAL

Reagents—Sodium borohydride was obtained as a white powder of approximately 95 per cent purity.\(^1\) Its preparation and some of its properties have been previously described (2). In water solution, NaBH\(_4\) hydrolyzes slowly according to the equation NaBH\(_4\) + 2H\(_2\)O \(\rightarrow\) Na\(^+\) + BO\(_3\)^{-} + 4H\(_2\).

This reagent was selected from a variety of available metallo hydrides because it shows a moderate stability in water solution. The rate of hydrolysis was determined approximately by titrating NaBH\(_4\) with iodine, and it was ascertained that a 0.1 per cent solution in distilled water was completely hydrolyzed in 1 minute at 100°, but that at 4° only about 10 per cent of the available reducing ability of a 5 per cent solution was lost per day. These properties make it possible to destroy excess reagent completely when this is necessary, and also to prepare the reagent for use as a solution which may be kept in the cold for several hours.

Cozymase was purchased from the Schwarz Laboratories. Solid dihydricozymase was prepared according to Ohlmeyer (3). The assay of DPN

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\(^1\) Kindly furnished by Albert Stewart, Chemistry Department, University of Chicago.
with sodium hydrosulfite was carried out according to the procedure used by LePage (4).

All spectrophotometric measurements were made with a Beckman quartz spectrophotometer, model DU.

**Assay of DPN with NaBH₄**—The assay of DPN is accomplished by adding about 2 mg. of NaBH₄ (as solid or in solution) to 3.0 cc. of 0.1 M phosphate buffer, pH 7.0, containing about 0.5 mg. of DPN. These conditions are suitable for cells of 1 cm. width commonly used with the Beckman spectrophotometer. About 1 minute is allowed for the reduction. During this time the solution is shaken gently to aid removal of hydrogen formed by hydrolysis of the reagent. The optical density is then measured at once. Interference with the measurement by gas bubbles can readily be avoided by proper tilting of the cuvette immediately before the reading is made. Phosphate buffer may be used as a blank, but corrections must of course be made for any light absorption by impurities in the unreduced DPN preparation.²

<table>
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<tr>
<th>Assay of Cozymase Preparations</th>
<th>Per cent purity*</th>
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<tr>
<td></td>
<td>Lot 1</td>
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<tr>
<td>By reduction with sodium hydrosulfite (LePage (4))</td>
<td>37.6 ± 0.4</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; borohydride; spectrophotometric</td>
<td>38.1 ± 0.2</td>
</tr>
<tr>
<td>By reduction with sodium borohydride; iodine titration</td>
<td>39.1 ± 0.5</td>
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* Data are given with the average deviation from the mean of two or more determinations.

The validity of the method rests upon the fact that the spectrum of the reduction product is identical with that obtained with sodium hydrosulfite for the region 330 to 400 m. The absorption band with a peak at 265 m, characteristic also of adenine derivatives present as impurities in the DPN preparations used, was not appreciably changed by NaBH₄. Table I

² It is necessary to use a buffer of pH 7.0, since the reduction of DPN by NaBH₄ in solutions of pH greater than 7.5 leads to the production of a stable yellow color possessing an absorption peak at 340 m and a smaller peak at about 420 m. This does not occur at pH 7.0 to 7.1. A stable yellow product has been obtained by Adler and coworkers (5) on reduction of DPN with sodium hydrosulfite in highly alkaline medium. It has commonly been supposed to be a semiquinoid radical (6). The stable yellow substance obtained upon reduction of DPN with NaBH₄ in media of pH greater than 7.5 differs considerably from the above product in absorption spectrum and will be reported on more fully in a subsequent communication.
shows representative results obtained on assaying two different DPN preparations spectrophotometrically, by means of both the NaBH₄ and the hydrosulfite procedures. The percentage purity was calculated from an extinction coefficient of $6.27 \times 10^6$ sq. cm. per mole at 340 mμ (3).

Table I also shows the assay values obtained by iodine titration of the reduced DPN formed by NaBH₄. Titration of reduced DPN was carried out with 0.005 N iodine solution and starch as indicator. This method was first suggested by Karrer and Ringier (7) and has more recently been used by Drabkin (8) as a method for assaying solid dihydrocozymase. 1 mole of pure dihydrocozymase is quantitatively oxidized by 2 equivalents of iodine. For the determinations reported in Table I, the reduction was carried out as described for the spectrophotometric assay with a somewhat larger amount of the reactants, and excess NaBH₄ was then removed by heating to 100° for 1 minute.

**Biological Activity of Product**—The enzymatic reoxidation of reduced DPN was carried out with sodium pyruvate and lactic dehydrogenase obtained by dialysis of an extract of a pig heart acetone powder. In no case did enzyme alone or substrate alone produce any reoxidation. Reduced DPN obtained by use of sodium hydrosulfite was found to be 100 per cent reoxidizable by lactic dehydrogenase, as was demonstrated by Green and Dewan (9). Under similar conditions, DPN reduced by NaBH₄ was reoxidized only 45 to 51 per cent. Partial enzymatic reoxidation of NaBH₄-reduced DPN lowered the absorption band with a peak at 340 mμ in the same proportion at each wave-length over the range of 330 to 400 mμ.

It has been earlier observed that sodium bisulfite and hydrogen cyanide combine with DPN (but not with reduced DPN) to yield addition products absorbing at 340 mμ (10). That there was no interference from comparable addition products in the reoxidation of DPN reduced by NaBH₄ follows from the unchanged optical density obtained on the addition of a completely hydrolyzed solution of NaBH₄ to DPN.

**DISCUSSION**

The agreement between assay values appearing in Table I shows that sodium borohydride may be satisfactorily used for the assay of DPN.³ The products of reduction, however, though closely similar, are not entirely identical to those obtained by the use of sodium hydrosulfite.

The probability that reduction by sodium borohydride produced two isomeric forms of dihydrocozymase should be considered. Since, as

³ In applying NaBH₄ to the analysis of triphosphopyridine nucleotide, Mr. Eric Conn of the Department of Biochemistry, University of Chicago, has found that the results are in agreement with those obtained by the reduction of triphosphopyridine nucleotide with glucose-6-phosphate and Zwischenferment.
pointed out by Karrer and coworkers (11), the two possible isomeric dihydro products would be expected to differ in ultraviolet spectrum, it seems more probable that sodium borohydride acts upon DPN in a more complex manner.

SUMMARY

Sodium borohydride has been applied successfully to the assay of DPN. Since this reagent does not absorb in the near ultraviolet and the excess is readily hydrolyzed to sodium borate, the new assay procedures are more convenient than previous ones with sodium hydrosulfite. However, the reduced products obtained are only partially enzymatically active.

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BIBLIOGRAPHY

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