PREPARATION OF TRIPHOSPHOPYRIDINE NUCLEOTIDE*

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The report of Warburg et al. (1) on the isolation of triphosphopyridine nucleotide (coenzyme II, TPN) from blood cells is, so far as we know, the only published method for preparing this coenzyme. One of the laboratories most actively engaged in work with TPN enzymes is using this method essentially unchanged, except that their source material is liver rather than blood cells. While this procedure results in preparations of high purity, it is relatively complex and difficult and the yields are low, because of the frequency of steps with 60 to 70 per cent recoveries. In several trials of this method we were able to confirm the results reported.

Some earlier work on the preparation of diphosphopyridine nucleotide (DPN) (2) demonstrated that charcoal adsorption was useful as a purification step for this nucleotide. Through tests on charcoal adsorptions of TPN, a simplified method for the preparation of pure TPN has been developed. In this method, the TPN in heat-inactivated water extracts of pork liver is adsorbed on charcoal and eluted with a water solution of pyridine, the crude TPN being chromatographed on charcoal. Finally, pure material is obtained through the solution of the TPN in acid-methanol and precipitation with ethyl acetate, a step from the method of Warburg et al. (1).

EXPERIMENTAL

Facilities Required—In addition to the usual facilities of a chemical laboratory a power-driven meat grinder is needed. A high capacity (12 pounds per minute) model manufactured by the Toledo Scale Company was used in this study and also a pressure filter manufactured by the Sparkler Filter Company, though the latter is not essential and can be replaced by use of Buchner funnels and water-operated suction pumps. If the isolation is to be carried out on a large scale, large sized vessels are required. Cheap, galvanized steel ash-cans of 15 to 30 gallon capacity are very satisfactory.

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1 Personal communications from Dr. Santiago Grisolia and Dr. Severo Ochoa.
Assay—In this study it was necessary to have an assay method specific for TPN. This was accomplished by use of a preparation of glucose-6-phosphate dehydrogenase (Warburg's Zwischenferment). The enzyme was prepared by a method essentially the same as that of Warburg and Christian (3). A slurry of bottom yeast, obtained from a brewery, was washed three times by suspension in distilled water and filtration each time in a pressure filter. It was then broken up and spread in a thin layer in a fan-equipped drier and was air-dried at 30–35° for 48 hours.

400 gm. of this dried yeast were suspended in 1200 ml. of distilled water and allowed to autolyze for 10 hours at 37°. The suspension was then centrifuged, and about 300 ml. of supernatant were obtained. Of this a 150 ml. batch was diluted to 1500 ml. with distilled water and chilled to 0°. The solution was saturated with CO₂ by bubbling the gas through it for 30 minutes. The resulting sediment was removed by centrifugation at 0° and discarded. The supernatant fluid was diluted to 15,000 ml. with CO₂-saturated distilled water at 30°. This solution was allowed to stand at 30° for 30 minutes, then left at 0° overnight. A precipitate formed, the supernatant fluid was decanted and discarded, and the precipitate centrifuged. This precipitate contained the desired dehydrogenase. It was dried in vacuo over P₂O₅. The yield was 1.0 to 1.5 gm. Specificity was demonstrated by incubation with 1.0 mg. of an 80 per cent DPN preparation made by the method of LePage (2). No absorption was obtained at 340 μm. Glucose-6-phosphate was prepared from starch by use of a rabbit muscle preparation, according to the procedure of Fantl and Anderson (4), modified to include a 5 hour hydrolysis of the sugar phosphates at 100° in 1 N HCl. The glucose-6-phosphate was precipitated as the barium salt with alcohol, decolorized with charcoal, and reprecipitated with alcohol.

Assays for total TPN and DPN were carried out by the hydrosulfite reduction method described by LePage (2) for DPN. When pure TPN preparations were obtained, hydrosulfite assays of these agreed with results obtained by using the glucose-6-phosphate dehydrogenase. Measurements of DPN, when present, were obtained by difference.

For use, 60 mg. of the enzyme preparation were homogenized in 10 ml. of 0.1 per cent NaHCO₃ and left at room temperature 20 minutes. The insoluble residue was centrifuged and the resulting slightly opalescent fluid used. This solution retained its activity for at least 1 week when stored at 0°.

TPN concentrates were, if not already in solution, made up in water as 0.15 to 1.0 per cent solutions and 0.1 to 0.3 ml. aliquots were used for each test. The sample was mixed with 0.4 ml. of 0.045 M glucose-6-phosphate, 0.5 ml. of fresh 1 per cent NaHCO₃, 0.10 ml. of enzyme solution,
and water to make 3.0 ml. This was mixed and incubated at room temperature 10 minutes (equilibrium was reached in approximately 5 minutes for 200 γ of TPN or less); then absorption was read at 340 μ in a Beckman quartz spectrophotometer against a blank containing all reagents except the glucose-6-phosphate. A separate correction (very small) could be made for absorption caused by the latter. The TPN content of the sample was calculated with the constant of Warburg (5) ($E = 5.65 \times 10^6$ sq. cm. per mole). In our experimental conditions we multiplied optical density by 396 to obtain micrograms of TPN per sample.

When batches of pork liver of 5 to 50 kilos were run in this study, the same yields and purity were obtained regardless of batch size. The process is described for an exemplary batch. This adheres to the findings as to yield and purity at the various stages obtained in a large number of separate experiments, several of which were run with the final and complete procedure.

**Isolation Procedure** — A 15 kilo batch of pork liver (eight livers) was obtained directly from the packing-house killing floor as soon after slaughter as available (approximately 20 minutes) and packed in chopped ice. It was transported to the laboratory as soon as possible (30 minutes). The meat grinder was set up over a 15 gallon can containing 15 liters of water at 90–100° with steam flowing into the water rapidly from a supply line (30 pounds pressure). The liver was weighed and run through the grinder directly into the hot water as rapidly as possible (5 to 7 minutes) with stirring of the water during the addition. When all the liver had been added (temperature now 80–90°), heating of the suspension was continued until the pink color, evident when a little of the suspension was dipped up in a beaker, disappeared and for 5 minutes thereafter. Then chopped ice and cold water were added to the vessel with stirring to make a final volume of approximately 60 liters, with the temperature at 25–35°. The suspension was filtered through cheese-cloth and the solid matter pressed as free of liquid as possible and discarded. An ordinary mop squeezer was found to be satisfactory as a press. A clear filtrate free of suspended meat and fat particles was obtained by filtration of the extract with the addition of approximately 5 gm. of Hyflo Super-Cel2 per liter. The filtrate now had a volume of 50 liters and was at pH 6.5. Without any further treatment, it was put into a 15 gallon can and 225 gm. of Nuchar C190 decolorizing charcoal3 were added. This was vigorously stirred with a mechanical stirrer for 15 minutes, then set aside at room

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2 A filter aid supplied by Johns-Manville.
3 Obtained from the Industrial Chemical Sales Division, West Virginia Pulp and Paper Company, Tyrone, Pennsylvania. It is necessary to specify the "unground" material in ordering the Nuchar C.
temperature for 2 hours to settle, after which time the supernatant fluid was decanted and discarded, leaving 95 per cent of the charcoal as a layer in the bottom of the can. The charcoal was transferred to a Büchner funnel and washed quickly with distilled water.

The charcoal cake was suspended in 2250 ml. of a solution of 10 per cent pyridine in water (by volume) and stirred mechanically for 20 minutes, then filtered with suction on a Büchner funnel. The charcoal was then discarded. The eluate was extracted with two successive 500 ml. portions of chloroform (technical grade, satisfactory). The chloroform layer was discarded each time. This procedure removed most of the pyridine, leaving the solution at approximately pH 6.3. After adjusting the pH to 2.0 with concentrated nitric acid, the water solution was mixed with 4 volumes of cold acetone and left at 0° overnight (or centrifuged immediately). In the morning the acetone solution was poured off and discarded. The crude nucleotides were caked on the sides and bottom of the vessel, and can be washed with acetone and dried if not to be used immediately. When dried, they contain approximately 10 per cent TPN and 20 per cent DPN. If the work is to be carried out immediately, the nucleotides are redissolved in a minimum of water (approximately 150 ml.), filtered at 0°, and assayed for TPN. The insoluble material is discarded. The solution is made to 3 per cent with pyridine and is then pH 5 to 6 and ready to be chromatographed. This solution shows no decline in TPN content at least for several days at 0°.

The columns for the chromatograms were constructed by attaching Pyrex glass tubes of the same diameter to fritted glass Büchner funnels of medium porosity. A large number of chromatograms were run with two sizes of these, one 21 mm., the other 46 mm. inside diameter, and the one with 5 times the area of the other. Since the results with these two sizes were identical as long as flow rates and depth of columns were the same, it seems likely that any size can be used. The procedure will be described for a 46 mm. column.

A granular charcoal known as Nuchar C was chosen for the chromatography. The coarse material was removed by discarding matter that would not pass through a 40 mesh standard sieve. The material used is essentially all retained on a 200 mesh sieve, and the sieved material was acid-washed and dried before use.

4 150 gm. of Nuchar C, which had been sieved to obtain the material which would pass through 40 mesh, were suspended in 2 liters of 10 per cent hydrochloric acid and heated to boiling, then filtered dry on a Büchner funnel with suction. The use of metal equipment such as metal spatulas was scrupulously avoided. The charcoal cake was resuspended in 2 liters of distilled water, filtered, and the process repeated. Then the cake was dried for 8 to 10 hours in an oven at 85–95°.
Nuchar C was poured into the column dry and tamped down gently with a piece of filter paper on top until a 10 inch depth was obtained. This requires approximately 100 gm. of the charcoal. A light vacuum was applied at the outlet and water added to the column to displace the air. A solution of the nucleotides in 3 per cent pyridine (100 to 150 ml.) containing approximately 5 gm. of the crude material (500 mg. of TPN) was poured onto the column. When this had just entered the charcoal, a solution of 3 per cent pyridine in water was added, and addition of this continued as long as the column was in operation. A head of approximately 3 inches of solution above the charcoal was sufficient to maintain the desired rate of flow (5 to 10 ml. per minute). The fluid which emerged from the column at first was colorless and below pH 3.0, as indicated by

**Table I**

Results of Charcoal Chromatograph to Purify TPN

A solution of 3 per cent pyridine was the eluant. 500 mg. of TPN were run onto the column in a solution also containing 1000 mg. of DPN.

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Volume (ml.)</th>
<th>TPN (mg.)</th>
<th>DPN (mg.)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>500</td>
<td>0</td>
<td>0</td>
<td>Colorless pH 3</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>1</td>
<td>0</td>
<td>&quot; &quot; &quot; 4.0</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>125</td>
<td>0</td>
<td>Slight color pH 6.2</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>160</td>
<td>0</td>
<td>&quot; &quot; &quot; 6.2</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>130</td>
<td>0</td>
<td>&quot; &quot; &quot; 6.2</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>34</td>
<td>0</td>
<td>&quot; &quot; &quot; 6.2</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>10</td>
<td>0</td>
<td>Colorless pH 6.2</td>
</tr>
<tr>
<td>8</td>
<td>50</td>
<td>8</td>
<td>5</td>
<td>&quot; &quot; &quot; 6.2</td>
</tr>
<tr>
<td>9</td>
<td>100</td>
<td>12</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td><strong>Total...</strong></td>
<td><strong>850</strong></td>
<td><strong>480</strong></td>
<td><strong>305</strong></td>
<td></td>
</tr>
</tbody>
</table>

* The remaining DPN could be obtained by continuing the elution.

Spot plate tests with bromoresol green indicator. The pH was tested every few minutes until it suddenly became pH 4.0 or over. The liquid collected up to this point was termed Fraction 1. Then collection of the fluid was started for Fraction 2 and cuts made every 50 ml. No TPN emerged until this pH change occurred, which was usually after approximately 500 ml. of fluid had filtered through the column. The TPN was present largely in the first four or five cuts after Fraction 1. This is illustrated in Table I. It will be noted that a 96 per cent recovery is obtained from the chromatogram and 90 per cent of the TPN is in Fractions 3 to 6 inclusive, which contain no DPN.

Fractions 3 to 6 inclusive were extracted twice with 50 ml. of chloroform (U. S. P.), treated with 4 ml. of concentrated nitric acid, and pre-
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cipated with 5 volumes of acetone at 0°. The suspension was centrifuged in the cold, washed with acetone, and dried in vacuo over sulfuric acid. The material obtained was a white preparation weighing 0.435 gm. and assaying 87 per cent TPN. No DPN was present. Recovery was therefore 76 per cent of the 500 mg. used. An additional 6 per cent present in Fractions 7 to 9 can be added to later columns.

The following operation was carried out with cold reagents, entirely in a room at 0°. A 400 mg. aliquot of the above preparation (ground in a mortar to a fine powder) was suspended for 15 minutes, with mechanical stirring, in 250 ml. of anhydrous methanol containing anhydrous hydrochloric acid (0.1 N). The suspension was filtered through a fritted glass funnel with suction and the filtrate precipitated with 3 volumes of anhydrous ethyl acetate. The flocculent precipitate was centrifuged and dried in vacuo, and the supernatant discarded. The precipitate, when dry, was 325 mg. of white material which assayed 107 per cent TPN (by using Warburg constants (1)). The recovery here was 100 per cent. When this preparation was put in a vacuum oven for several hours under high vacuum at 60°, it lost approximately 4 per cent of its weight. However, it is apparently unable to withstand this treatment and the assay dropped to 85 per cent.

Factors Affecting Yields and Recoveries—The most critical factor affecting the yield of TPN is the handling of the liver tissue. It is desirable for the liver to be chilled as soon as possible after the animals are slaughtered. Once the liver is chilled and intact, the coenzyme content is well stabilized. However, in experiments in which the liver was not ground directly into the hot water, yields were reduced 80 to 90 per cent. Destruction of the TPN is apparently very rapid in the ground tissue.

On several occasions, when the heating period was evidently not sufficiently prolonged, a pink color remained in the liver filtrate. Very little TPN was obtained in these cases. Evidently the TPN was not split off the protein, or was destroyed by enzymes not inactivated in the heat treatment.

When the charcoal was filtered from the suspensions, a period of several hours was required for this operation. The added 5 per cent TPN recovered, over that obtained as described, was considered too small an amount to justify this effort.

Tests were made to determine how much charcoal was required to adsorb all the TPN from the liver extracts and to assure that none remained in the filtrate. For this, two approaches were used. Aliquots of the filtrate were precipitated with mercuric acetate as in the procedure of Warburg et al. (1), and the mercury salts decomposed with hydrogen sulfide and tested for TPN. None was found when the specified amount
of charcoal had been used. A better check was obtained by use of a second charcoal adsorption and assay of eluates of this adsorption for TPN. It was established in separate experiments that added TPN could be completely recovered from the extracts by charcoal adsorption.

The solubility of TPN in the acetone supernatant was tested by using a charcoal adsorption and testing the eluate, after it had been demonstrated that charcoal would adsorb TPN from 80 per cent acetone. No TPN was recovered, though some DPN was found present in these solutions.

A number of charcoals were studied in order to choose the most suitable one for the initial charcoal adsorption from the liver extracts. This study was carried out by stirring 2 liter aliquots of a liver extract with 10 gm. quantities of the charcoals for 15 minutes, filtering, and eluting each charcoal with 100 ml. of pyridine-water, extracting the pyridine from each with chloroform, and assaying enzymatically for TPN. The charcoals were evaluated in terms of three properties, clarity and color of eluate (an indication of purity), capacity to adsorb TPN, and ability to settle after suspension in the extract. On this basis, either Nuchar C190 or Nuchar W would be suitable. The results are shown in Table II.

With Nuchar C190, a brief study was made of the effect of variations in the concentration of pyridine used for elution. Three concentrations were tried, 5, 10, and 20 per cent pyridine (by volume) in pilot batches. The eluates from these three tests contained 7.7, 10.3, and 7.2 mg. of TPN respectively. On precipitation and drying in vacuo the preparations were 6.0, 8.0, and 5.9 per cent TPN respectively. The obvious choice was made to use 10 per cent pyridine.

The same six charcoals were studied as to suitability for use in chromatograms, with the exception of Nuchar WA. It was necessary to use

<table>
<thead>
<tr>
<th>Charcoal sample</th>
<th>TPN in eluate (mg.)</th>
<th>Eluate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norit A.</td>
<td>24.0</td>
<td>Clear, highly colored; charcoal settled well</td>
</tr>
<tr>
<td>Nuchar C.</td>
<td>29.8</td>
<td>&quot; &quot; &quot; &quot; mainly floated on surface</td>
</tr>
<tr>
<td>&quot; C190</td>
<td>31.6</td>
<td>Low in color but opalescent; charcoal settled well</td>
</tr>
<tr>
<td>&quot; C115</td>
<td>18.0</td>
<td>&quot; &quot; &quot; &quot; clear; charcoal settled fairly well</td>
</tr>
<tr>
<td>&quot; W</td>
<td>31.6</td>
<td>&quot; &quot; &quot; &quot; &quot; &quot; &quot; well</td>
</tr>
<tr>
<td>&quot; WA</td>
<td>8.7</td>
<td>&quot; &quot; &quot; &quot; &quot; &quot; &quot; fairly well</td>
</tr>
</tbody>
</table>
approximately 60 per cent as much Hyflo Super-Cel by weight as charcoal in all cases, except that of Nuchar C, in the columns, as a mixture and suction had to be applied to the receiver to obtain adequate flow rates in these cases. For all these charcoals, the same distribution of TPN and DPN was found in the eluates. However, recoveries were low (34 per cent) in the case of norit A and were only 72 to 85 per cent with the others. When they were given the acid washing and drying treatment, all gave increased recoveries (90 to 96 per cent), and a colored impurity which came through with the TPN from neutral charcoals was completely retained.

One lot of norit A gave low recoveries and this was not remedied by acid washing. One batch of TPN concentrate which had been subjected to a mercury precipitation, and the mercury removed as sulfide, gave low recoveries on chromatograms. Traces of metals are therefore to be avoided.

On one occasion, a liver extract was treated with charcoal and left overnight at 0° to settle, rather than the usual 2 hours. The eluate from this charcoal was highly colored and much difficulty was subsequently encountered in the purification of the TPN concentrate obtained.

The TPN could be assayed by the enzymatic method with either the fractions obtained directly from the chromatographs, in which case 3 per cent pyridine was present, or the solutions from which some of the pyridine had been extracted with chloroform, since neither the pyridine nor the chloroform affected the enzyme appreciably. No TPN is lost in the chloroform-pyridine layers which are discarded, as shown by identical assays obtained with solutions before and after extraction.

Studies were made of the yield of TPN obtained from beef and pork liver processed in identical manner. The yield in terms of TPN found present in the charcoal eluates was 42 mg. of TPN per kilo for pork liver and 32 mg. per kilo for beef liver. As a rule, pork liver is cheaper and more readily available.

When the chromatographs are run with 3 per cent pyridine, the DPN remains on the column until the TPN is almost all eluted. However, the DPN can be completely recovered if more 3 per cent pyridine is run through the column. We chose to ignore the DPN here, since it is much more easily obtained from yeast (2). However, on one occasion the DPN was precipitated and dried, without any attempt at fractionation, and a white preparation obtained which assayed 70 per cent DPN.

Some experiments conducted with yeast which had been heat-inactivated and filtered showed that the direct application of charcoal adsorption to the filtrates was equally successful with yeast. For example the filtrate (100 liters) from 200 pounds of yeast was treated with charcoal, as de-
scribed above for liver, and the charcoal eluted with pyridine. The eluates were extracted and the water layer precipitated with acetone. Material was obtained which assayed 45 per cent TPN-DPN. It contained 8 gm. of DPN and 0.6 gm. of TPN. When this concentrate was extracted with 0.5 N HCl in methanol and precipitated with 3 volumes of ethyl acetate, the TPN was destroyed and the DPN completely recovered. In the final step of our procedure, use was made of a phase of the method of Warburg et al. (1) in which anhydrous methanol containing 0.1 N HCl is used. The latter report a TPN recovery of 95 per cent. We found that recoveries were complete (100 per cent) if the whole operation was carried out rapidly in a refrigerator (0°). However, even if only the filtration was carried out at room temperature, recoveries dropped to 81.6 per cent.

**DISCUSSION**

The procedure described here represents a considerable simplification compared with the methods previously available. The reagents required (charcoal, acetone, pyridine) are relatively cheap and the over-all yield of the TPN in the initial extracts is approximately 80 per cent when final purity is reached. The yields obtainable with the procedure formerly available were in the vicinity of 26 per cent.

TPN made available by this preparative procedure has already made possible the discovery of a TPN-specific enzyme system involved in the destruction of carcinogenic azo dyes by rat liver. It should make possible more widespread research on TPN-linked enzymes.

The assays for TPN have all been computed with the constant used by Warburg (5). However, some calculations made recently by Horecker and Kornberg (6) indicate that pure TPN should have an extinction coefficient approximately 11 per cent higher than that of Warburg. Our findings would seem to substantiate these calculations. It would appear that this is the first report in which pure TPN has actually been obtained.

**SUMMARY**

A procedure is described for preparation of pure triphosphopyridine nucleotide (TPN) from pork liver. This is accomplished by inactivation of the liver in hot water, adsorption of the TPN from the water extract with charcoal, elution with pyridine-water, and precipitation of the TPN with acetone. The preparation is further purified by chromatographing on charcoal and by dissolving in acid-methanol and precipitating with ethyl acetate. The recovery of TPN present in the liver extracts is 80

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*Unpublished data from Dr. Gerald C. Mueller and Dr. J. A. Miller.
per cent. Approximately 0.500 gm. of pure TPN is obtained from 15 kilos of pork liver.

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