The Thionicotinamide Analogs of DPN and TPN. I. Preparation and Analysis*

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The synthesis of the thionicotinamide analog of triphosphopyridine nucleotide catalyzed by pig brain transglycosidase is described. The thionicotinamide analogs of both diphospho- and triphosphopyridine nucleotide have been purified from the corresponding nicotinamide nucleotides by preparative gradient elution chromatography, and characterized by elementary analyses. The spectrophotometric constants reported are based on ribose, phosphate, and adenine analyses of the samples.

Anderson *et al.* (1959) and Anderson and Kaplan (1959) have reported the synthesis and some of the properties of the thionicotinamide analog of DPN.[1] Subsequently, a preliminary investigation on the synthesis of the corresponding TPN analog cast doubt on the purity of the original preparation and suggested that *Neurospora* DPNase was ineffective in the purification of this analog.[2] These studies prompted the reinvestigation of the problem of the purification of the thionicotinamide analog of DPN. The complete resolution of the thionicotinamide analogs of DPN and TPN from their respective parent compounds has been accomplished by gradient elution chromatography on columns of Dowex-1-formate. These procedures, as well as the analyses and determination of spectrophotometric constants, will be described in this article. The failure of the *Neurospora* DPNase to remove parent pyridine nucleotide from the transglycosidase reaction mixtures will be discussed in the accompanying paper (Anderson *et al.*, 1963).

EXPERIMENTAL

Materials.—DPN, glucose-6-phosphate, disodium salt, and yeast alcohol dehydrogenase were obtained from the Sigma Chemical Company. The preparation of TPN used in these experiments will be described separately.[3] Thionicotinamide was synthesized by the method of Karrer and Schukri (1945). The source of pyridine nucleotide transglycosidase activity was the acetone powder preparation of pig brain described by Windmueller and Kaplan (1962). The autolysis step was omitted; after washing, the insoluble residue was extracted further for one hour at 0° in 0.1 M phosphate buffer, adjusted with trichloroacetic acid to *p*H 2, to remove additional ultraviolet absorbing impurities. The precipitate was washed several times with dilute phosphate buffer. Glucose-6-phosphate dehydrogenase was prepared by the method of Noltman *et al.* (1961) to step V of their preparation.

Methods.—The exchange of the thionicotinamide free base for the nicotinamide moieties of DPN and TPN was carried out essentially as described by Kaplan and Ciotti (1956) for the synthesis of the 3-acetylpyridine analog of DPN. The details of the experiments are reported in the experimental section. The formation of the analogs was followed by assaying aliquots of the reaction mixtures with alcohol dehydrogenase and glucose-6-phosphate dehydrogenase for the DPN and TPN reactions, respectively. The criteria followed were the ratio of absorbancies at 400 mμ and 340 mμ, as well as the absolute absorbancy at 400 mμ. The reactions were stopped by chilling in ice and the addition of trichloroacetic acid to 10% (w/v) final concentration. The crude nucleotides were precipitated with acetone, washed first with acetone and then with ether. The dried precipitates were taken up in a little water, adjusted to *p*H 7 with 1 N sodium hydroxide, and applied to Dowex-1-formate columns.

Ion exchange chromatography was carried out as described by Hurlbert *et al.* (1954) and by Schmitz (1954) with the indicated modifications to effect the separations on a gram scale. The chromatographic fractions were analyzed with the suitable dehydrogenase reaction mixtures as well as by their absorbancy at 260 mμ; the pooled tubes were precipitated as described by Kaplan and Ciotti (1956) by adjustment of *p*H, precipitation with cold acetone, and washing with acetone and ether.

Analyses.—Nitrogen, sulfur, and water analyses were performed by the Galbraith Laboratories; water analysis was done on the sample, dried *in vacuo* at 100°. Adenine (Loring *et al.* 1952), ribose (Taylor *et al.*, 1948), and phosphate (Fiske and Subbarow, 1925) analyses were performed on standard solutions of the compounds, 20 mg/ml in water. The standard solutions were diluted 10-fold, and aliquots were taken for spectrophotometry. The molarity of the standard solutions was calculated from the adenine, ribose, and phosphate contents; the coefficients were calculated on this basis. Identical spectra were obtained on two Zeiss PMQ-II spectrophotometers located in different laboratories. The yeast alcohol dehydrogenase and the glucose-6-phosphate dehydrogenase reduction mixtures employed are described by Racker (1955) and Kornberg and Horecker (1955), respectively.

RESULTS AND DISCUSSION

Preparation of Compounds.–Figure 1 shows the course of formation of TNTPN in the pig brain DPNase system. The protocol of the reaction mixture is indicated in the legend of the figure. Similar results are obtained with DPN; however, a lower $A^{400}_{\mathrm{mM}}/A^{340}_{\mathrm{mM}}$ ratio appears to be approached as a limit. Figure 2 shows the resolution of the two analogs from their respective parent pyridine nucleotides. The chromatographic separation of α- and β-DPN in this system has been demonstrated;[3] the mobility of α-DPN is such that,

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[1] The abbreviations used in this paper are DPN and TPN, di- and triphosphopyridine nucleotide, respectively; TNDPN and TNTPN, the 3-thiocarbamidopyridine analogs of DPN and TPN, respectively; Tris, tris(hydroxymethyl)aminomethane.

[2] Carried out by two of us (A.M.S. and B.M.A.) in the laboratory of Dr. N. O. Kaplan.

[3] Stein, A. M., Lee, J. K., Anderson, C. D., and Anderson, B. M., unpublished experiments.

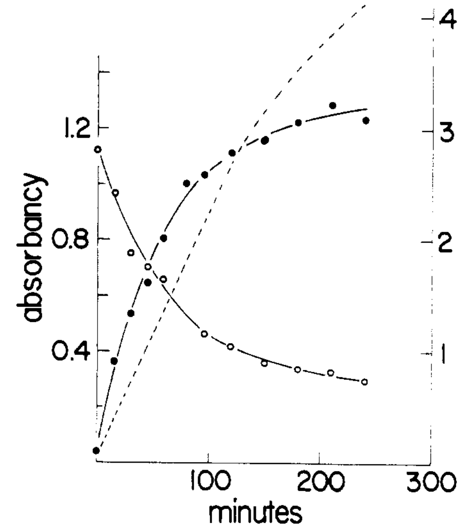

FIG. 1.—Synthesis of TNTPN. The following were added to the reaction mixture: TPN, 1 g, neutralized to pH 7.5; DPNase preparation from 2.5 g pig brain acetone powder (555 μmoles DPN split per hour); 3140 μmoles potassium phosphate, pH 7.5; 1 g thionicotinamide; water to 68 ml final volume. The mixture was incubated in a shaking bath at 37°. Aliquots of 0.01 ml, withdrawn at the indicated times were added to 1 ml of the glucose-6-phosphate dehydrogenase reaction mixture. Net absorbancies at 340 mμ (open circles) and 400 mμ (solid circles) were obtained after addition of glucose-6-phosphate dehydrogenase in sufficient amount to reduce the nucleotides in 30 seconds. The dotted line represents the ratio of absorbancy at 400 mμ to that at 340 mμ.

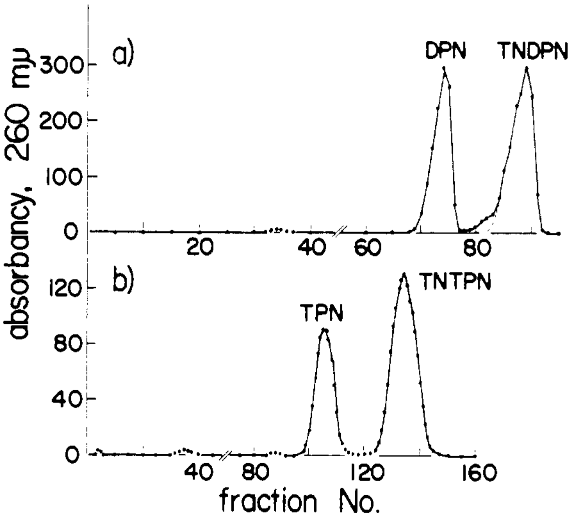

FIG. 2.—Separation of nicotinamide and thionicotinamide nucleotides. Chromatography of crude nucleotide mixtures on Dowex-1-formate, X2, 200–400 mesh, development by gradient elution. (a) DPN-thionicotinamide transglycosidase reaction mixture, starting with 1.5 g DPN. Column, 2.3 × 50 cm, mixer, 6 liters water; reservoir, 3 N formic acid; fraction volume, 12 ml. The dotted line (comprising fractions 82–84) is the calculated absorbancy at 260 mμ for the TNDPN component. (b) TPN-thionicotinamide transglycosidase reaction mixture (see Fig. 1). Column, 2.2 × 21 cm; mixer, 2 liters 0.1 M ammonium formate; reservoir, 1.0 M ammonium formate; fraction volume, 10 ml.

TABLE I

ANALYSES OF THE THIONICOTINAMIDE ANALOGS OF DPN AND TPN

Formula	Thionicotinamide–DPN·$3H_2O$		Thionicotinamide–TPN·$3H_2O$, NH_4 Salt		
Weight:	733		831		
				Found	
	Theory	Found	Theory	I	II
% N	13.40	13.11	14.41	—	14.00
% S	4.37	4.44	4.21	—	4.30
% H_2O	7.37	7.20	6.51	—	7.15
Adenine	1	0.99	1	0.985	0.970
Ribose[a]	2	2	2	2	2
Phosphate	2	2.10	3	3.06	3.21

[a] The adenine and phosphate contents are calculated on the basis of 2 moles of ribose determined in the standard solutions.

under these conditions there would be little or no contamination with the early portion of the TNDPN peak. Unlike TNTPN, which appears as a homogeneous component in the chromatogram, the early portion of the TNDPN peak is contaminated with a small, yellow impurity absorbing at 260 mμ. Analysis of the individual fractions is required to insure pooling of the homogeneous portion. The over-all yields of the reaction and isolation procedures were 390 mg of TNDPN and 420 mg TNTPN, respectively, from 1 g of the corresponding nicotinamide nucleotides.

Analyses.—The isolated compounds were hydrolyzed by the pig brain DPNase preparation and the products were examined by paper chromatography. In the system 95% ethanol—0.1 M acetic acid, 1:1, the only nucleotides detected were adenosine diphosphoribose and adenosine triphosphoribose for the products of TNDPN and TNTPN digestion mixtures, respectively; in the system propanol-water, 80–20, thionicotinamide was the only pyridine base detected.

The analytical data for the compounds are shown in Table I. Moisture analysis indicates the compounds precipitate from acidic 80% acetone as the trihydrates; thionicotinamide-TPN is recovered as the ammonium salt. The presence of one atom of sulfur is a sensitive index of the purity of the compounds; it is found to be 1.7% and 2.1% in excess of theory for TNDPN and TNTPN, respectively. Figure 3 shows the spectrum of the oxidized and reduced forms of TNDPN. The corresponding data for the TPN analog are virtually superimposable on these, and therefore are not presented. Careful examination of the spectrum of the oxidized compound in the 300-mμ region reveals the

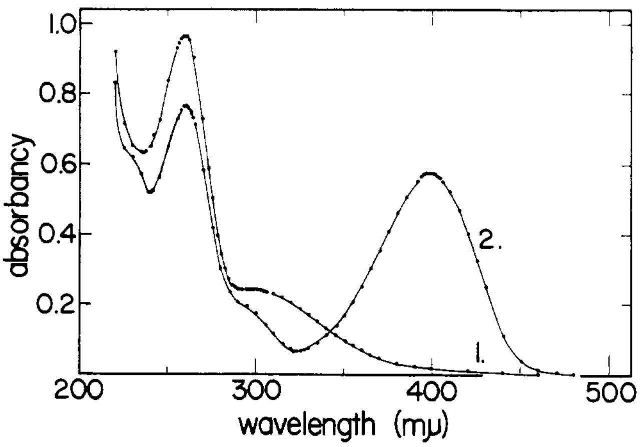

FIG. 3.—Absorption spectrum of the thionicotinamide analog of DPN: (1) oxidized form, in 0.05 M potassium phosphate buffer, pH 7.5; (2) reduced form, in the yeast alcohol dehydrogenase reaction mixture. The concentration of nucleotide is 0.037 mg/ml.

TABLE II
SPECTROPHOTOMETRIC CONSTANTS OF THE THIONICOTINAMIDE ANALOGS OF DPN AND TPN

	Thionicotinamide-DPN		Thionicotinamide-TPN	
	mμ	A_{mM}[a]	mμ	A_{mM}
		Maxima		
Oxidized[b]	259	19.7	259	19.5
	296[b]	5.0	296[c]	4.9
Reduced[d]	259	16.2	260	15.8
	398	11.9	399	11.7
Cyanide[e]	257	25.1	257	25.6
	359	10.0	359	10.0
		Minima		
Oxidized[b]	236	12.9	236	12.5
Reduced[d]	240	10.8	240	10.7
	324	1.3	324	1.5
A_{400}/A_{340}[f]	5.25		5.34	

[a] Millimolar absorbancy coefficients at the designated wavelengths. [b] Determined in 0.1 M potassium phosphate buffer, pH 7.5. [c] This maximum has been demonstrated by using dense solutions in the region of the maximum (A_{296} = 1.3). [d] Reduced enzymatically; see under Methods. [e] Values determined 20 minutes after the addition of molar potassium cyanide. [f] Absorbancies are for the reduced compounds.

existence of a second maximum at 296 mμ. This peak is altered on reduction.

The spectrophotometric constants of the analogs are shown in Table II. The spectral data for the reduced forms are obtained directly on freshly reduced samples in the dehydrogenase reaction mixtures, the reference cuvet containing all additions save pyridine nucleotide. Complex spectral changes ensue upon addition of the cyanide to the thionicotinamide analogs, consequently the spectra are not presented here. Following a 20-minute interval after addition of the cyanide solution, constant values are obtained and these are presented in Table II. In agreement with the Pabst Laboratories commercial literature,[4] reaction with cyanide markedly increases the ultraviolet absorbancy of the thionicotinamide analogs. This effect appears to be unique among the pyridine nucleotides. The values obtained for the coefficients of the reduced analogs at 400 mμ are about 4–5% higher than those reported by the Pabst Laboratories.[4] We have investigated this problem further by chromatographing a sample of Pabst TNDPN and have estimated the lot analyzed to contain about 4% β-DPN. A comparison of the spectral properties of their product, either published by them or determined by us, with the corresponding sample reported here, leads to the same conclusion.

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The Thionicotinamide Analogs of DPN and TPN. II. Enzyme Studies*

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The reactivity of the thionicotinamide analogs of DPN and TPN in various dehydrogenase and DPNase systems is described. The K_m values of the nicotinamide and thionicotinamide coenzymes obtained in the various dehydrogenase reactions compare very closely, whereas the V_{max} values may show large divergence. In the transglycosidation reaction with pig brain DPNase, the thionicotinamide analogs are converted quantitatively to the corresponding nicotinamide compounds. The thionicotinamide analogs are shown to be reactive with the DPNase from *Neurospora*, either as weak substrates or as very powerful competitive inhibitors of the cleavage of DPN.

The preceding paper in this series (Stein *et al.*, 1963) describes the preparation of the thionicotinamide analog of triphosphopyridine nucleotide (TNTPN)[1] as well as the methods of purification for both this analog and the previously reported (Anderson *et al.*, 1959) thionicotinamide analog of diphosphopyridine nucleotide (TNDPN). The present paper represents a further study of the enzymes employed in the preparation and characterization of the thionicotinamide coenzyme analogs, to provide justification for their use and to provide evidence for the coenzymatic activity of the

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[1] The abbreviations used are: TNDPN, the thionicotinamide analog of DPN; TNTPN, the thionicotinamide analog of TPN; Tris, tris(hydroxymethyl)aminomethane.