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The Formation and Characterization of the Nicotine Analog of Triphosphopyridine Nucleotide†

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ABSTRACT: A nicotine analog of TPN (nicotine-TPN) was isolated and purified from an incubation mixture containing nicotine, TPN, and a rabbit liver microsomal fraction. Physical, chemical, and immunochemical evidence indicates that the nicotinamide moiety of TPN has been replaced by nicotine in this analog. Nicotine-TPN is considerably more stable than TPN in the presence of the liver microsomal fraction. The analog could also be formed in incubation mixtures containing pig brain or beef spleen DPNase instead of the liver microsomal preparation. Nicotine-TPN cannot substitute for TPN in the reactions carried out by TPN-specific isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, or glutamate dehydrogenase. Competitive inhibition with respect to TPN is observed with the last two enzymes.

Nicotine undergoes extensive metabolic changes in mammalian tissues to give at least eight known products (McKennis, 1965; Dagne and Castagnoli, 1972a,b). The liver is particularly active in the oxidation and demethylation reactions which produce metabolites such as cotinine (Hucker *et al.,* 1960), nicotine *N'*-oxide (Papadopoulos, 1964; Booth and Boyland, 1971), or nornicotine (Papadopoulos and Kintzios, 1963). However, little is known about intermolecular reactions of nicotine with other constituents of living cells. In particular, products resulting from the exchange of nicotine or its metabolites for the nicotinamide moiety in DPN or TPN have not been reported. Yet the enzyme, DPNase, exists in most animal tissues and is capable of exchanging the nicotinamide moiety of DPN and TPN for other pyridine containing compounds (Kaplan, 1960). For example, 3-acetylpyridine (Kaplan and Ciotti, 1956; Kaplan *et al.,* 1954) and 6-aminonicotinamide (Dietrich *et al.,* 1958) can replace the nicotinamide moiety of DPN and TPN to produce analogs of these coenzymes whose structures have been elucidated and whose physiological effects have been described. Nicotine has been shown to be an inhibitor for sheep brain DPNase (McIlwain, 1950) but not for beef spleen enzyme (Zatman *et al.,* 1954).

This paper describes the formation and characterization

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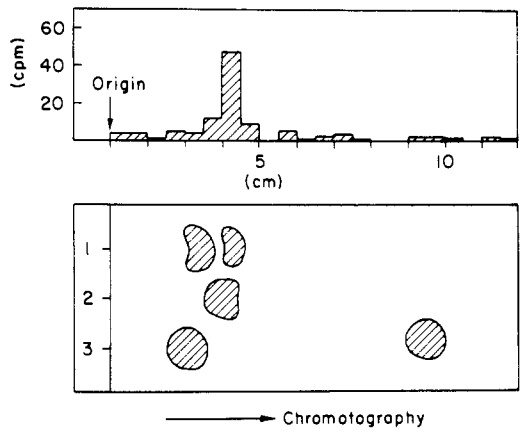

FIGURE 1: Separation of nicotine-TPN by tlc. The incubation mixture contained rabbit liver microsomes (9 mg of protein/ml of buffer), 0.05 M [^{3}H]nicotine, and 0.002 M TPN. The reaction conditions were the same as described in Methods. Samples were spotted on the plate (Eastman; 13181 silica gel with fluorescent indicator) and developed with the use of 2-propanol-concentrated NH_4OH-H_2O (6:3:1) as solvent. The plate was air dried, examined under uv light, and then sectioned and counted in a Packard liquid scintillation spectrometer. (1) Incubation mixture. Nicotine is absent in this particular chromatogram since the sample was desalted on a Sephadex G-15 column prior to application on tlc. (2) Purified nicotine-TPN. (3) TPN (R_F 0.18) and nicotine (R_F 0.77).

of a nicotine analog of TPN (nicotine-TPN) by a rabbit liver microsomal fraction, as well as by brain and spleen DPNase enzyme preparations. Evidence is presented which indicates that this analog is formed by a reaction catalyzed by DPNase in which nicotine is exchanged for the nicotinamide moiety of TPN.

Materials and Methods

(−)-Nicotine hydrochloride was purchased from K&K Laboratories. [^{3}H]Nicotine, prepared at New England Nuclear, Inc., by random catalytic tritium exchange on the pyridine ring, was purified by tlc (specific activity of 2.4 Ci/mmol).

Pig brain DPNase (specific activity, 0.009 unit/mg), beef spleen DPNase (specific activity, 0.044 unit/mg), baker's yeast glucose-6-phosphate dehydrogenase (specific activity, 350 units/mg), glucose-6-phosphate, and TPN were purchased from Sigma, while beef liver glutamate dehydrogenase and pig heart isocitrate dehydrogenase were obtained from Boehringer Mannheim Co.

Liver Microsomal Fraction. Livers obtained from New Zealand albino rabbits were homogenized in two volumes of cold 0.15 M KCl. The homogenate was centrifuged at 10,000*g* for 15 min and the supernatant fraction centrifuged again for 1 hr at 100,000*g*. The microsomal pellet was washed once with an equal volume of 0.15 M KCl and suspended in buffer. The protein content was measured by the procedure of Lowry *et al.* (1951).

Formation of Nicotine-TPN. A suspension of rabbit liver microsomes, pig brain, or beef spleen DPNase in 0.05 M sodium phosphate buffer (pH 7.3) was incubated at 37° with [^{3}H]nicotine and TPN in a Dubnoff metabolic shaking incubator. Aliquots of the incubation mixture were removed at time intervals and heated in a boiling water bath for 2 min to stop the reaction. The denatured protein was removed by centrifugation. After thin-layer chromatography, the quantity of [^{3}H]nicotine incorporated in the product was estimated from the specific radioactivity of [^{3}H]nicotine (Figure 1). The formation of nicotine-TPN in the presence of different concentrations of nicotine was determined in the same way.

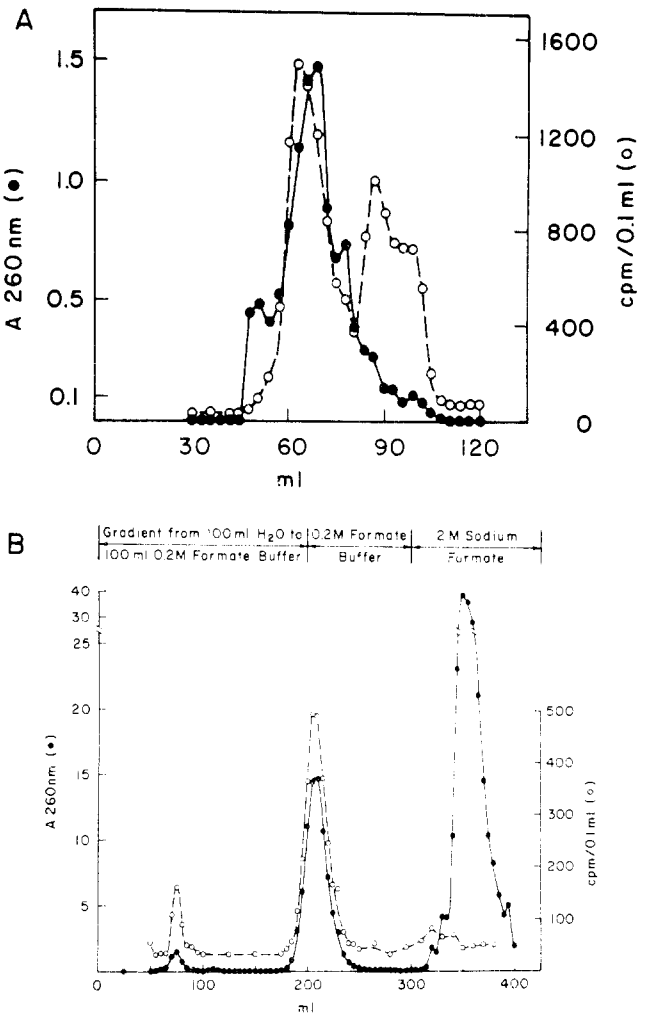

FIGURE 2: Purification of nicotine-TPN. The nicotine-TPN which had been prepared by a modification of the procedure of Kaplan and Stolzenbach (1957) as described in Methods was purified by gel filtration and ion-exchange chromatography. (A) Sephadex G-15 gel filtration. The acetone precipitate was dissolved in 3 ml of 0.05 M NH_4HCO_3, placed on a Sephadex G-15 column (2 × 30 cm), and eluted with 0.05 M NH_4HCO_3 solution at a flow rate about 1 ml/min; 3-ml fractions were collected. (B) Ion-exchange chromatography. The peak which emerged after gel filtration (from 60 and 75 ml) was pooled and lyophilized. It was dissolved in 20 ml of H_2O and loaded onto a column (1.5 × 12 cm), packed with Dowex-1 ion-exchange resin in the formate form. The column was washed first with 40 ml of H_2O. (Less than 5% of the material which absorbed at 260 nm and/or had radioactivity was found in this water eluate.) A linear gradient, from 100 ml of H_2O to 100 ml of 0.2 M formate buffer (pH 3.9) was then applied. At the end of the gradient, an additional 100 ml of 0.2 M formate buffer was added to the buffer reservoir. Nicotine-TPN emerged at the end of the gradient, while other uv absorbing material was eluted from the column only when 2 M sodium formate was added to regenerate the resin.

Large-Scale Preparation of Nicotine-TPN by Rabbit Liver Microsomal Fraction. A modification of the procedure reported by Kaplan and Stolzenbach (1957) for the preparation of DPN and TPN analogs was used. TPN (160 mg) was added to 36 ml of a solution containing rabbit liver microsomes (9 mg of protein/ml of buffer) and 0.05 M [^{3}H]nicotine. The mixture was incubated at 37° for 2 hr and then heated in a boiling water bath to denature the protein. After centrifugation, the supernatant solution was cooled and 200 ml of cold acetone was added slowly with stirring. After standing 20 hr at −27°, the supernatant fluid

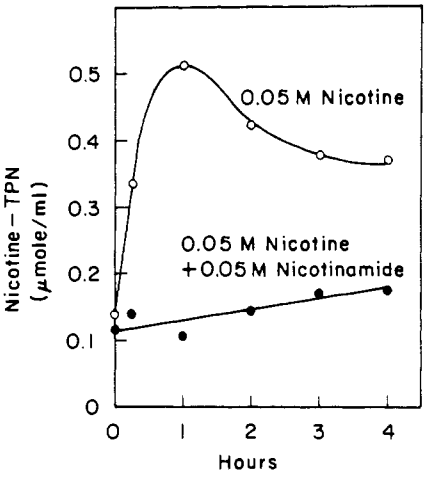

FIGURE 3: The formation of nicotine-TPN by the rabbit liver microsomal fraction in the presence and absence of nicotinamide. The reaction conditions were the same as described in Methods.

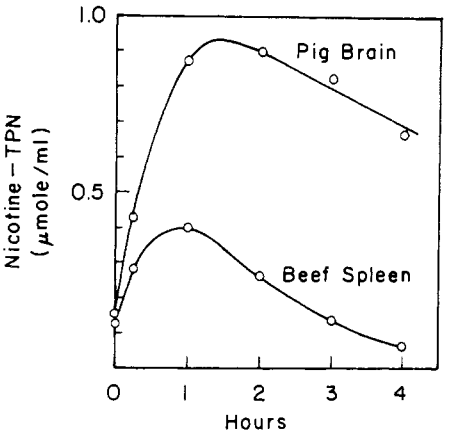

FIGURE 4: The formation of nicotine-TPN by pig brain and beef spleen DPNase. Enzymes were incubated with [^{3}H]nicotine and TPN as described in Methods.

was removed by decantation. The precipitate was washed once with 50 ml of cold acetone. After decantation of the supernatant fluid, the residual acetone in the precipitate was removed *in vacuo*. The sticky precipitate was dissolved in 0.05 M NH_4HCO_3 and chromatographed on a Sephadex G-15 column (Figure 2A). Fractions with high absorption at 260 nm and high radioactivity were pooled and lyophilized. The residue was then dissolved in H_2O and chromatographed on Dowex-1 ion-exchange resin (Figure 2B). Fractions containing nicotine-TPN were pooled (from 195 to 220 ml) and lyophilized, and the residue was dissolved in 3 ml of H_2O. The pH was adjusted to 8 by NH_4OH and the solution desalted on a Sephadex G-15 column. After the water and NH_4HCO_3 were removed by lyophilization, 12 mg of white powder remained. By assuming that the molar absorptions at 260 nm of TPN and the nicotine analog are the same, *i.e.*, ϵ_M 1.8×10^4, radioactivity measurements indicate that there is 0.94 mol of nicotine incorporated in each mol of the product.

Large-Scale Preparation of Nicotine-TPN by Pig Brain DPNase. Nicotine-TPN was prepared from pig brain DPNase by the same procedure except that 0.5 g of pig brain DPNase replaced the rabbit liver microsomal fraction. The final yield of nicotine-TPN was 30 mg.

Hydrolysis of the Pyridine Ribose Bond in TPN and Nicotine-TPN by Rabbit Liver Microsomes. Three milligrams of TPN or its nicotine analog were dissolved in small vials containing 9 mg of liver microsomes in 1.0 ml of 0.05 M sodium phosphate buffer (pH 7.2) and incubated with shaking at 37°. Aliquots of 0.1 ml were removed at each time interval and heated in a boiling water bath for 2 min to stop the reaction. After cooling, 0.9 ml of 0.05 M Tris-acetate buffer (pH 7.2) was added. The mixtures were centrifuged and the supernatant fluid was used for the assay of either TPN or nicotine.

TPN was assayed as follows. In a 2-ml cuvet, 0.5 ml of the supernatant solution was mixed with 1.3 ml of 0.05 M Tris-acetate buffer (pH 7.4) and 0.2 ml of 0.1 M glucose 6-phosphate. Then, 0.02 ml of glucose-6-phosphate dehydrogenase solution (35 units/ml) was added. The total increase of absorption at 340 nm was used as a measurement of the amount of TPN in the solution. The rate of hydrolysis of the nicotinamide ribose bond in TPN by the enzyme, DPNase, was assayed by the method of Colowick *et al.* (1951).

Nicotine released from nicotine-TPN was estimated by the radioimmunoassay procedure described by Langone *et al.* (1973).

Cleavage of Pyridine Ribose Bond by Alkaline Hydrolysis. Equal volumes of TPN or nicotine-TPN in water and 0.2 M NaOH were mixed in a small test tube and heated in a boiling water bath for 5 min. After cooling, the solution was neutralized with 0.5 M HCl and the products were identified by tlc.

Complete Acid Hydrolysis of TPN and Nicotine-TPN. One milligram of TPN or nicotine-TPN was dissolved in 1 ml of 1.0 M HCl and heated in a boiling water bath for 1 hr. AMP was hydrolyzed under the same conditions to serve as a standard for adenine. The products were identified by tlc.

Effect of Nicotine-TPN on TPN-Specific Dehydrogenases. Three TPN-specific enzymes, *i.e.*, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, and glutamate dehydrogenase, in 0.05 M Tris-acetate buffer (pH 7.4) and their respective substrates (0.005 M) were assayed in the presence of TPN and/or nicotine-TPN. In the case of isocitrate dehydrogenase, 0.002 M $MnSO_4$ was added to the incubation mixture. Activities were measured as the increase of absorption at 340 nm on a Gilford recording spectrophotometer with 0.1 expanded scale recorder.

Results and Discussion

Incubation of nicotine with TPN in the presence of rabbit liver microsomes produced a nicotine analog of TPN which could be separated from TPN by thin-layer chromatography (Figure 1). In large-scale preparations, the product was isolated and purified by a combination of gel filtration and ion exchange chromatography (Figure 2). Since the rate of nicotine-TPN production is almost linearly proportional to the concentration of nicotine up to 0.1 M, a high concentration of nicotine is required in the incubation mixture to get good yields of this compound.

The addition of nicotinamide to the incubation mixture markedly depresses the formation of analog (Figure 3), suggesting that the enzyme, DPNase, is responsible for the exchange reaction between nicotinamide and nicotine. In addition to the rabbit liver microsomes, commercially available DPNase from pig brain and beef spleen also catalyze the formation of the analog (Figure 4).

Nicotine-TPN was characterized by physical, chemical, and immunochemical procedures. Since both nicotinamide and nicotine have almost identical absorption spectra

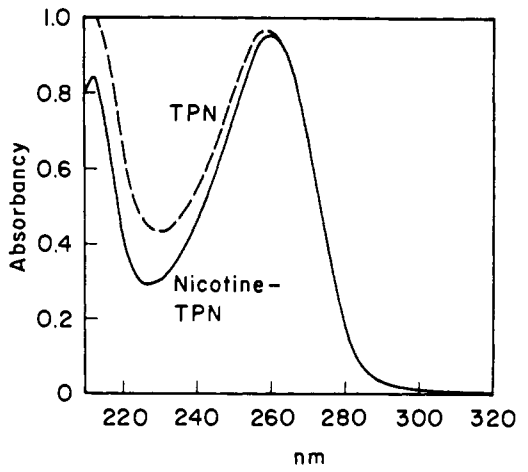

FIGURE 5: Uv absorption spectra of TPN and nicotine-TPN. Both compounds were dissolved in 0.05 M Tris-acetate buffer (pH 7.4). The spectra were recorded in a Cary 14 spectrophotometer.

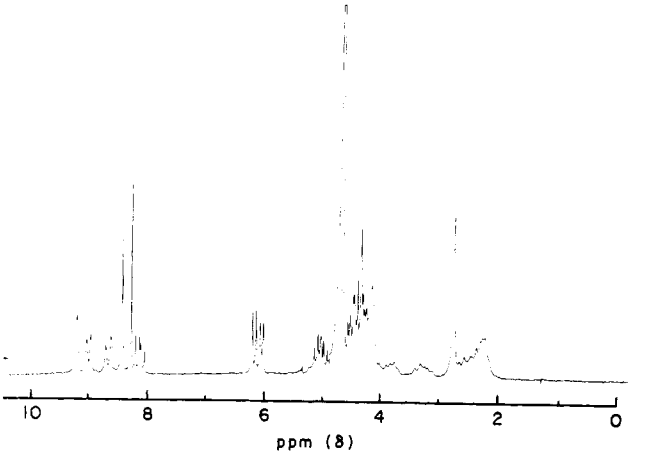

FIGURE 6: Proton magnetic resonance spectra of nicotine-TPN in acid. Nicotine-TPN (10 mg) was dissolved in 0.4 ml of D_2O containing 0.5% DCl. The spectrum was recorded in a WH-90 Bruker nmr Fourier transform spectrometer at a sweep width of 1200 Hz. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate was used as an external standard (3146 Hz).

around 260 nm, the similarities in the uv absorption spectra of nicotine-TPN and TPN at this wavelength are expected (Figure 5). However, λ_{min} for nicotine-TPN is at 226 nm compared to 230 nm for TPN and a difference in absorbance of about 30% is observed at this lower wavelength. This difference may be due to the fact that at 230 nm, the absorbance of nicotinamide is about 5.4-fold higher than that of nicotine. Since the uv spectrum of nicotine-TPN has no new peak formed around 310 nm, it is unlikely that the nicotone is incorporated by an addition reaction to the nicotinamide moiety of TPN. When *p*-aminobenzoic acid undergoes such an addition reaction on position 4 of the nicotinamide ring of DPN, the product shows a very high absorption peak at 310 nm (Guardiola *et al.*, 1958).

In the proton magnetic resonance spectrum of nicotine-TPN in D_2O-0.5% DCl (Figure 6), the presence of nicotine is indicated by the *N*-methylpyrrolidine protons from δ 2.0 to 3.8 ppm, and the nicotine-ribose bond by the doublet at 6.0 ppm for $C^1{}_1H$. The presence of adenosine is indicated by the singlets at 8.3 and 8.4 ppm for C_2H and C_8H and the doublet at 6.2 ppm for $C^1{}_1H$. Thin-layer chromatograms taken before and after the product was analyzed by nuclear magnetic resonance (nmr) showed no free nicotine or adenine. The proton magnetic resonance spectrum of nicotine-TPN taken at neutral pH still shows the *N*-methylpyrrolidine peaks, but the peaks which distinguish the adenosine moiety are broadened. This is possibly due to the presence of trace paramagnetic metal ions (Sarma and Mynott, 1973).

This product was also sent to Dr. N. O. Kaplan for proton magnetic resonance spectrum analysis on a 220-MHz nmr spectrometer. At pH 6, the spectrum taken in his laboratory appears as a typical spectrum for pyridine dinucleotides indicating that the conformation of nicotine-TPN is similar to that of TPN and other TPN analogs.

When cyanide was added to TPN and nicotine-TPN, the formation of a new absorption peak at 325 nm was observed only with TPN. The inability of nicotine-TPN to give the addition reaction is expected since this test depends upon the inductive effects of the group in the 3 position (Kaplan, 1960). For example, while cyanide addition to DPN and the DPN analogs of 3-benzoylpyridine and 3-pyridylacrylamide (Anderson *et al.*, 1959) results in an increase around 325 nm, there is no change in the spectra of the DPN analogs of

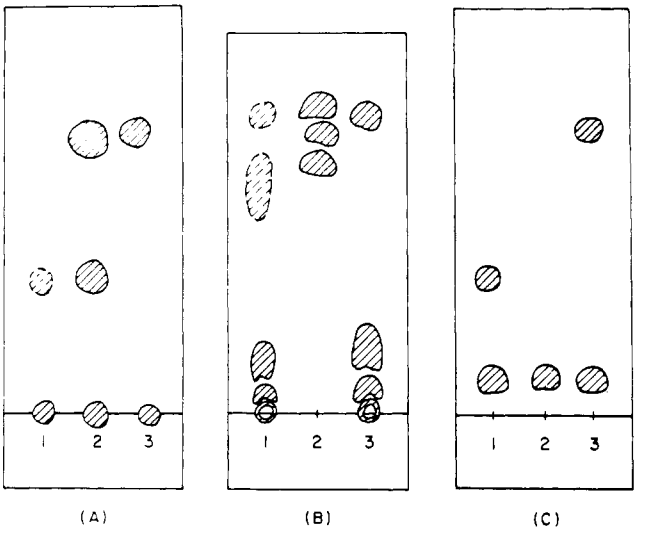

FIGURE 7: Thin-layer chromatography of the alkaline (A,B) and the acid (C) hydrolysates of TPN and nicotine-TPN. Solvents: (A and C) ethanol-acetone-benzene-concentrated NH_4OH (1:8:10:1); (B) 2-propanol-concentrated NH_4OH-H_2O (6:3:1). Samples: (A and B) (1) alkaline hydrolysate of TPN, (2) mixture of nicotine, nicotinamide, and nicotinic acid listed in decreasing R_F values; (3) alkaline hydrolysate of nicotine-TPN. (C) (1) acid hydrolysate of TPN; (2) acid hydrolysate of AMP; (3) acid hydrolysate of nicotine-TPN.

3-aminopyridine (Anderson *et al.*, 1959) and 3-methylpyridine (Kaplan and Ciotti, 1956).

As shown by tlc (Figure 7A,B), all components in the alkaline hydrolysates of nicotine-TPN and TPN are identical, with the exception of nicotine and nicotinamide (or the nicotinic acid which results from the hydrolysis). Furthermore, both nicotine and adenine are found in the acid hydrolysate of nicotine-TPN (Figure 7C). These results indicate that the structure of nicotine-TPN and TPN are identical, except that nicotine has replaced the nicotinamide moiety of TPN.

Compared to the nicotinamide ribose bond of TPN, the nicotine-ribose bond of the analog is much more resistant to hydrolysis by the rabbit liver microsomal fraction. The enzyme, DPNase, is probably responsible for the cleavage of the glycoside bond. At the same concentration (2.4 ×

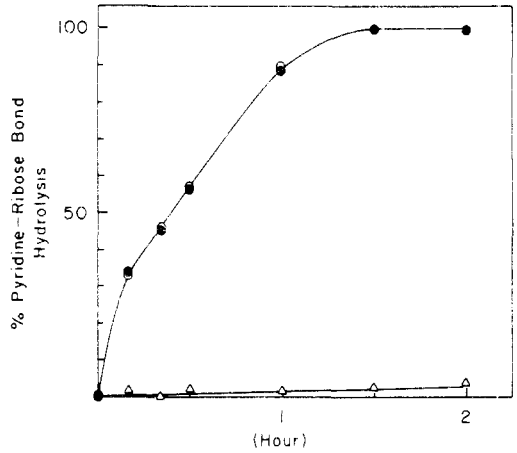

FIGURE 8: Hydrolysis of TPN and nicotine-TPN by the rabbit liver microsomal fraction. Hydrolysis of TPN was measured as: (●) disappearance of TPN in glucose-6-phosphate dehydrogenase assay; (○) disappearance of nicotinamide-ribose bond in cyanide addition reaction. Hydrolysis of nicotine-TPN was measured by estimating the released nicotine (△) by the radioimmunoassay procedure of Langone *et al.* (1973).

10^{-3} M), the nicotinamide moiety of TPN was almost completely released in 1.5 hr, compared to only about 5% release of the nicotine (Figure 8). Such a low yield of nicotine would also be obtained if nicotine was released at the same rate as the nicotinamide moiety of TPN but were metabolized subsequently to other products by the liver microsomes. However, this possibility was ruled out since alkaline hydrolysis of the solution even after 6-hr incubation gave almost 100% recovery of nicotine as measured by the same radioimmunoassay method. It should be emphasized that the radioimmunoassay for nicotine permits estimation of the alkaloid even in the presence of closely related metabolites since the specificity of the antibody is directed toward *both* the pyridine and *N*-methylpyrrolidine rings (Langone *et al.*, 1973). In these experiments, 50% inhibition of the antigen-antibody reaction was obtained with 0.06 nmol of nicotine and 1.0 nmol of nicotine-TPN, respectively. The liberation of nicotine was determined after alkaline or enzymatic hydrolysis by comparing the per cent inhibition obtained in a known aliquot of solution with the standard nicotine curve.

The action of pyrophosphatase could not account for the destruction of nicotine-TPN. The low pyrophosphatase activity in our preparation of rabbit liver microsomes was demonstrated by the fact that the rate of destruction of TPN as measured by glucose-6-phosphate dehydrogenase was essentially the same as the cleavage of nicotinamide ribose bond as measured by the cyanide addition reaction (Figure 8). Jacobson and Kaplan (1957) also showed rabbit liver microsomes had low pyrophosphatase activity.

The physiological significance of nicotine-TPN is not clear at the present time. However, animals are known to suffer adverse physiological effects with some pyridine analogs formed *in vivo*, *e.g.*, the analogs of 3-acetylpyridine (Kaplan *et al.*, 1954) and 6-aminonicotinamide (Dietrich *et al.*, 1958). Our results indicate that nicotine-TPN cannot be used as a coenzyme by glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, or isocitrate dehydrogenase *in vitro*. Nicotine-TPN has no effect on the activity of isocitrate dehydrogenase when present with TPN at four times the molar concentration of the coenzyme. It does, however, competitively inhibit the activity of both glucose-6-phosphate dehydrogenase and glutamate dehydrogenase. The dissociation constants, K_i's of nicotine-TPN for glucose-6-phosphate dehydrogenase and glutamate dehydrogenase, as determined by the double reciprocal plots of the reaction velocities *vs.* the concentrations of TPN in the presence of different amounts of nicotine-TPN, are 1.2×10^{-4} and 2.1×10^{-4} M, while the K_m's for TPN measured under the same experimental conditions are 1.2×10^{-5} and 4.4×10^{-5} M, respectively. The competitive inhibition of either glucose-6-phosphate dehydrogenase or glutamate dehydrogenase may not be important by itself, since the K_i value is at least fivefold higher than the K_m for TPN at the same conditions. However, this observation suggests the possibility that nicotine-TPN, as a structural analog of TPN, may block one or more metabolic pathways where TPN is required as cofactor. If nicotine-TPN cannot substitute for TPN in physiological reactions, its formation might be considered as a decrease of the amount of TPN in living cells. Compared to TPN, nicotine-TPN was relatively stable to degradative enzymes in rabbit liver microsomes. If nicotine-TPN proves to have similar stability toward other intracellular enzymes, then accumulation of this analog is possible.

Although it has been generally considered that nicotine is not a substrate for DPNase in the exchange reaction with DPN (McKennis, 1965), it is unlikely that nicotine will exchange with TPN but not with DPN if pyridine analog exchange reactions for both coenzymes are catalyzed by the sole enzyme, DPNase (Kaplan, 1955; Windmueller and Kaplan, 1962). Nicotine has been reported to increase the biosynthesis of DPN from its natural precursors in the liver of rats (DeClerq and Truhaut, 1962). We have recently isolated the nicotine analog of DPN from an incubation mixture containing nicotine, DPN, and pig brain DPNase. Nicotine-DPN cannot substitute for DPN in the horse liver alcohol dehydrogenase reaction. It inhibits this dehydrogenase more effectively than its TPN counterpart (W.-C. Shen and H. Van Vunakis, 1974). In addition, since several known metabolites of nicotine still contain the pyridine moiety, *e.g.*, continine and nicotine *N'*-oxide, the ability of these compounds to form DPN and TPN analogs is under investigation.

Nicotine is the most abundant alkaloid in tobacco and is present in cigarettes in amounts ranging from 0.5 to 2.0 mg of nicotine/cigarette. The extent to which nicotine nucleotide analogs are formed *in vivo* and whether their formation is related to any of the clinical problems associated with smoking remain to be determined.

Acknowledgment

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Properties of RNA in Formamide†

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ABSTRACT: Procedures for the purification of formamide and preparation of RNA solutions are described, and details are given of buffers which function within the required range in this solvent. It is shown that for single-stranded RNA and homopolynucleotide species formamide is an effective denaturant at room temperature, and, as judged by spectroscopic criteria, annihilates both base pairing and single-stranded stacking. Further, though smaller, optical changes are observed on heating these solutions, and considerable changes in the circular dichroism, including, in the case of poly(rA), for example, inversion of the ellipticity, occur when all traces of water are removed. It is shown by proton magnetic resonance that even at a water concentration 10^{-3} that of the formamide there is preferential solvation of RNA by the water. Two-stranded RNA does not melt spontaneously in moist formamide at room temperature, but does so on warming, and the melted chain remains indefinitely stable after cooling; this represents an unusual hysteresis effect. When the formamide is completely dry the double-stranded RNA melts spontaneously on dissolution. Sedimentation studies of RNA species and fractionated poly(riboadenylic acid) samples have been performed in formamide. It is found that to a first approximation all conform to a single sedimentation coefficient-molecular weight relationship, and can therefore within these limits be regarded as conformational homologs in this solvent. This provides justification for the application of empirical methods of molecular weight determination to RNA in formamide solution, in particular polyacrylamide gel electrophoresis.

In searching for solvent conditions which would allow zone electrophoresis of RNA, but in which the chain would be devoid of all secondary structure, we found that polyacrylamide gels could be set and run in pure formamide (Staynov *et al.,* 1972). Work by Ts'o and his coworkers (Helmkamp and Ts'o, 1961, 1962; Ts'o *et al.,* 1962) had shown that DNA and single-stranded RNA were devoid of optically detectable structure in this medium, although at sufficiently high ionic strength (Ts'o *et al.,* 1963) some structure of low thermal stability appeared, which, it was suggested, might be unrelated to that present in the native state in water. There is less certainty about two-stranded, fully base-paired RNA, which is reported to be incompletely melted in formamide at room temperature (Strauss *et al.,* 1968). We have examined the conformation and other relevant properties of RNA and its constituents in formamide under the conditions used in electrophoretic experiments. The results are described below, as also is evidence for a strong preferential interaction of RNA with water.

Materials and Methods

Escherichia coli rRNA was prepared from packed MRE 600 cells by the method of Robinson and Wade (1968). Rabbit reticulocyte rRNA was extracted by the phenol-sodium dodecyl sulfate method from purified ribosomes

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