The Chemical Nature of the Bound Nicotinic Acid of Wheat Bran: Studies of Partial Hydrolysis Products

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ABSTRACT

In samples of bound nicotinic acid (prepared in approximately 50% yield from wheat bran), at least 90% of the nicotinoyl moiety was in the tertiary form. A partial hydrolysis product of the bound nicotinic acid was isolated and identified as nicotinoyl glucose, by mass spectrometry, spectroscopic methods, and gas-liquid chromatography of the products of further hydrolysis. This partial hydrolysis product was obtained from bound nicotinic acid fractions that were both polysaccharide and glycopeptide in character. Bound nicotinic acid, therfore, contains nicotinoyl glucose as a subunit. The significance of this finding in relation to the limited nutritional availability of bound nicotinic acid is discussed.

The bound nicotinic acid of wheat bran is contained in a number of macromolecules, which are heterogeneous with respect to molecular weight, and contain both polysaccharide and glycopeptide (1). To investigate the linkage of nicotinic acid in these macromolecules, and hence to understand the reasons for the unavailability of bound nicotinic acid (2,3), it was necessary to isolate and characterize fragments of the macromolecules that contained bound nicotinic acid. Such fragments were formed by partial hydrolysis in acid under controlled conditions, and detected by their mobility on thin-layer chromatography (TLC); the compound next least polar to nicotinic acid, referred to as compound N, was studied since this was likely to be the simplest derivative.

Two preparations of bound nicotinic acid were used as a starting material for preparation of compound N. First, samples containing about 2% nicotinic acid, obtained in yields of about 10% by an acid extraction of wheat bran and referred to as niacytin preparations (1) were used. Second, preparations obtained by extraction of wheat bran with 50% aqueous ethanol followed by dialysis (1), referred to as nondiffusible nicotinic acid preparations, were also used; these samples contained 0.3% nicotinic acid but represented 47% of the bound nicotinic acid in wheat bran. Both preparations gave chromatographically identical samples of compound N.

MATERIALS AND METHODS

Bound Nicotinic Acid Preparations

Samples of bound nicotinic acid (niacytin and nondiffusible nicotinic acid preparations) were obtained as described by Mason et al. (1).

Estimation of Nicotinic Acid

Nicotinic acid was estimated as described by Mason and Kodicek (2).

Chromatographic Methods

TLC was carried out using thin-layers of both silicic acid (E. Merck, AG, Damstadt, Germany) and cellulose (Macherey Nägel & Co., Düren, Germany). Cellulose thin-layers (0.25 mm.) were prepared by mixing 15 g. of cellulose powder, containing binder, with 5 to 10 mg. of green fluorescent indicator (M Woelm, Eschwege, Germany) and 90 ml. of distilled water in a high-speed blender for 2

min., allowing it to stand for 1 min., and spreading it on five 20 × 20-cm. plates (or the equivalent number of smaller plates). The layers were dried at room temperature for 1 hr. and then at 37°C. for 12 hr. For preparative purposes 0.4-mm. thin-layers of both silicic acid and cellulose were used; twice the quantities given above were required for preparation of the latter.

The solvent mixtures employed were: system 1, butan-1-ol:acetic acid:water (4:1:1, by volume); system 2, butan-1-ol, saturated with water; system 3, butan-2-one:methanol:acetic acid (3:1:1, by volume); system 4, toluene:methanol:acetone:acetic acid (14:4:1:1, by volume); system 5, butan-1-ol:acetic acid:water (6:1:2, by volume).

The following methods of visualization were used. Compounds containing nicotinic acid were visualized by the method of Kodicek and Reddi (4), using cyanogen bromide gas and *p*-aminobenzoic acid. Iodine vapor and 50% aqueous (v./v.) sulfuric acid spray followed by heating at 60°C. for 1 hr. were used as nonspecific detection methods. Ehrlich's reagent (0.5%, w./v.) *p*-dimethylaminobenzaldehyde in ethanol containing 1% conc. HCl) at room temperature gave a yellow fluorescent compound with *o*-aminophenol. Amino acids were visualized using ninhydrin (5).

Gas-liquid chromatography (GLC) was carried out on a 6-ft. column, 4 mm. i.d., packed with 3% E. 351 (J.J.'s [Chromatography] Limited, King's Lynn, Norfolk, England) on Diatoport S (80 to 100 mesh; Hewlett-Packard, Slough, Bucks.) using a F & M Scientific 402 (Hewlett-Packard) gas chromatograph with a flame ionization detector. The conditions were: carrier gas argon, 40 p.s.i.; flow rate, 20 ml. per min.; column temperature, 165°C., flash heater, 210°C.; detector, 190°C. Aliquots (1 μl.) of the samples dissolved in carbon tetrachloride were injected.

Trimethylsilylation

The trimethylsilyl (TMS) ethers of samples were formed by the method of Sweeley et al. (6) as follows. The sample was dried *in vacuo*, dissolved in redistilled pyridine (2 vol.), and hexamethyldisilazane (1 vol.; Koch-Light Laboratories Limited, Colnbrook, Bucks.)and trimethylchlorosilane (1 vol.; Koch-Light) were added. The reaction was carried out at room temperature for 5 min. For GLC the reagents were removed under a stream of nitrogen and the samples redissolved in carbon tetrachloride.

Physical Methods

The mass spectra of the TMS derivative of compound N and of TMS-glucose were determined using an AEI MS902 mass spectrometer, with direct insertion at 140°C. Infrared spectra were determined as potassium bromide microdiscs in a Unicam SP200 infrared spectrophotometer.

Hydrolysis of Compound N

Samples of compound N were hydrolyzed in 5M HCl at 100°C. for 30 min. The solution was taken to dryness under a stream of nitrogen and finally *in vacuo*. The residue was either trimethylsilylated or redissolved in 50% aqueous (v./v.) ethanol for chromatography.

EXPERIMENTAL AND RESULTS

Determination of the Proportion of Tertiary Nicotinic Acid in Bound Nicotinic Acid Preparations

Previous investigations of the nature of bound nicotinic acid (7,8) have estimated the nicotinic acid present only after complete hydrolysis. To estimate the proportion of the nicotinic acid in the tertiary form as opposed to the quaternary form (i.e., linked through the pyridine nitrogen atom), the specificity of the König reaction for tertiary pyridine compounds was utilized, the reaction failing to occur with quaternary nitrogen compounds (9). A comparison of the content of nicotinic acid in the bound nicotinic acid preparation before and after hydrolysis, therefore, gave an estimate of the proportion of the nicotinic acid in the tertiary form in the intact sample. The reaction proceeded more slowly with bound nicotinic acid, presumably because of hindered access of the reagent to the bound nicotinic acid; but the neutral conditions of the reaction did not cause cleavage of any N-C bond. This was confirmed by the absence of any reaction whatever of trigonelline (*N*-methylnicotinic acid). The total nicotinic acid content of samples of nondiffusible nicotinic acid was determined after hydrolysis, the amount being 3.1 γ per mg. Samples containing 15 γ of nicotinic acid were estimated, without hydrolysis, for nicotinic acid, and the time of reaction with cyanogen bromide was varied. The results (Fig. 1) showed that the reaction of intact bound nicotinic acid increased to 90% of that of the total nicotinic acid (i.e., determined after hydrolysis) with 15 min. reaction with cyanogen bromide. It was therefore concluded that at least 90%, and probably all, of the bound nicotinic acid in the nondiffusible nicotinic acid preparation was in the tertiary nitrogen form.

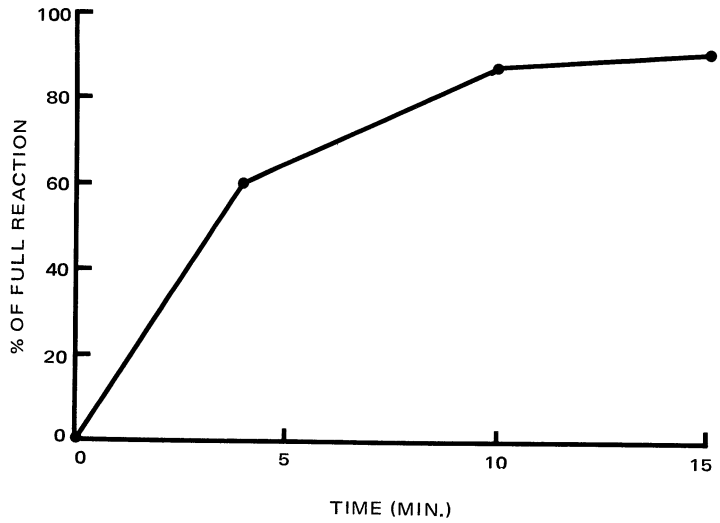

Fig. 1. Intact samples of bound nicotinic acid were estimated colorimetrically for nicotinic acid, varying the time of reaction with cyanogen bromide. Results are expressed as a percentage of the reaction of hydrolyzed bound nicotinic acid.

Preparation of the Nicotinoyl Derivative, Compound N

It was determined in preliminary experiments that hydrolysis of bound nicotinic acid proceeded rapidly to completion under alkaline conditions, whereas under acidic conditions a number of nicotinic acid-containing compounds were formed that could be readily detected after TLC on silica gel or cellulose thin-layers in solvent systems 1 and 2. These compounds could also be detected, although in smaller amounts, after alkaline hydrolysis. Compounds containing *o*-aminophenol were similarly obtained from samples that contained bound nicotinic acid (10), but these could be differentiated from the nicotinic acid-containing compounds by TLC. The R_f values of these compounds are given in Table I. It was observed that the nicotinic acid- and *o*-aminophenol-containing fragments had similar R_f value (N and A, N′ and A′ in Table I) on paper chromatography in system 5, as reported by Kodicek and Wilson (11), but it became clear from these and other experiments (1) that *o*-aminophenol was not closely associated with nicotinic acid.

The optimum conditions for formation of compound N were investigated as follows. Samples (10 mg.) of niacytin preparations were dissolved in 2.5 ml. of 0.1M, 1.0M, and 5.0M HCl, and the solutions were heated at 100°C. Aliquots (0.5 ml.) were taken at intervals up to 3 hr., neutralized, precipitated with 3 vol. ethanol, and centrifuged (1,000 × g. for 10 min.), and the supernatant reduced to dryness under nitrogen. The residue was suspended in ethanol (0.1 ml.), and centrifuged (100 × g. for 10 min.), and samples (10 μl.) of the supernatant were chromatographed on TLC in system 1 on silica gel. The nicotinic acid-containing compounds were visualized. The amounts of nicotinic acid in the compounds were estimated from the chromatogram using a Chromoscan densitometer (Joyce-Loebl & Co. Ltd., Gateshead) with a Wratten No. 47 filter; the response from the samples was compared with known amounts of nicotinic acid, for which the response was linear. The results are shown in Fig. 2. The optimum conditions found thus for formation of compound N were hydrolysis in 1M HCl at 100°C. for 1 hr.

Samples of nondiffusible nicotinic acid preparations, and of fractions of this material which were either polysaccharide or glycopeptide in character prepared by ion-exchange chromatography (samples D1–D4; see Mason et al., 1), also gave a

TABLE I. R_f VALUES OF COMPOUNDS CONTAINING NICOTINIC ACID AND o-AMINOPHENOL[a]

Compound	System					
	1		2		3	5
	Silica TLC	Cellulose TLC	Silica TLC	Cellulose TLC	Silica TLC	Paper chromatog.
Nicotinic acid	0.52	0.70	0.12	0.61	0.45	0.72
N	0.35	0.54	0.29	0.50	0.16	0.49
N′	0.16	0.29	0.13	0.20	0.07	0.24
o-aminophenol	0.57	0.69	0.70	0.85	0.38	0.74
A	0.30	0.44	0.43	0.49	0.13	0.43
A′	0.15	0.20	0.25	0.21	0.06	0.28

[a]Compounds N, N′, A, and A′ were obtained by partial acid hydrolysis of wheat bran extracts containing bound nicotinic acid; experimental details are given in the text. Compounds N and N′ contained nicotinic acid, and compounds A and A′ contained o-aminophenol. The solvent systems are given in the Methods section.

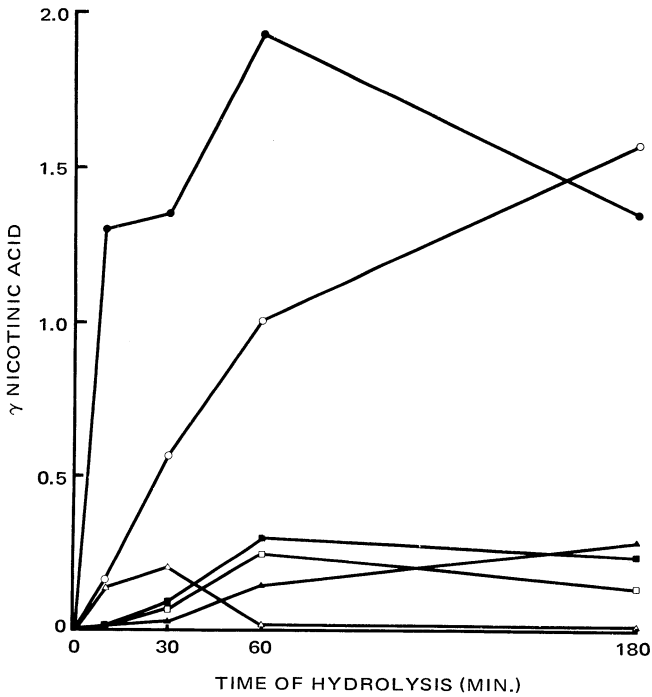

Fig. 2. Samples of bound nicotinic acid were hydrolyzed at 100°C. Aliquots were taken at time intervals as shown, and the amounts of nicotinic acid, compound N, and compound N′ present determined as described in text. ●, nicotinic acid, from hydrolysis in 5.0M HCl; ○, nicotinic acid, 1.0M HCl; ▲, nicotinic acid, 0.1M HCl; △, compound N, 5.0M HCl; ■, compound N, 1.0M HCl; □, compound N′ 1.0M HCl.

substance that cochromatographed with compound N, obtained from niacytin preparations, in system 1 on cellulose and systems 2 and 3 on silica gel thin-layers. It was concluded that compound N was obtained from niacytin or nondiffusible nicotinic acid preparations, or from fractions of the latter.

Compound N was prepared from niacytin preparations and isolated by the procedure shown in Fig. 3. For preparation of compound N from nondiffusible nicotinic acid preparations, the quantities used were: 1 g. of nondiffusible nicotinic acid preparation, hydrolyzed in 100 ml. 1M HCl, partially neutralized with 47 ml. 2M NaOH, extracted with three 250-ml. volumes of ethyl acetate. The solution was adjusted to pH 7 with NaOH and freeze-dried. The solid was extracted with three 25-ml. volumes of ethanol. This extract was reduced to dryness *in vacuo*, redissolved in 0.5 ml. of 50% aqueous (v./v.) ethanol, and then purified by TLC as before. Approx. 400 γ compound N were obtained from 1 g. of starting material, a yield of about 7% of the nicotinoyl moiety.

Samples prepared from both niacytin and nondiffusible nicotinic acid preparations were chromatographically pure, no other substance being detectable in systems 1, 2, and 3 on silica gel thin-layers, using all the methods of visualization given in Methods. As described above, the samples of compound N from either

starting preparation were chromatographically identical in systems 1 (on cellulose), 2, and 3 (on silica).

The TMS derivative of compound N (see Methods) could be detected on TLC in system 4 at R_f=0.71, by the usual cyanogen bromide-*p*-aminobenzoic acid method of Kodicek and Reddi (4).

Identification of Compound N

TLC and Color Reactions. As described above, compound N was separated by

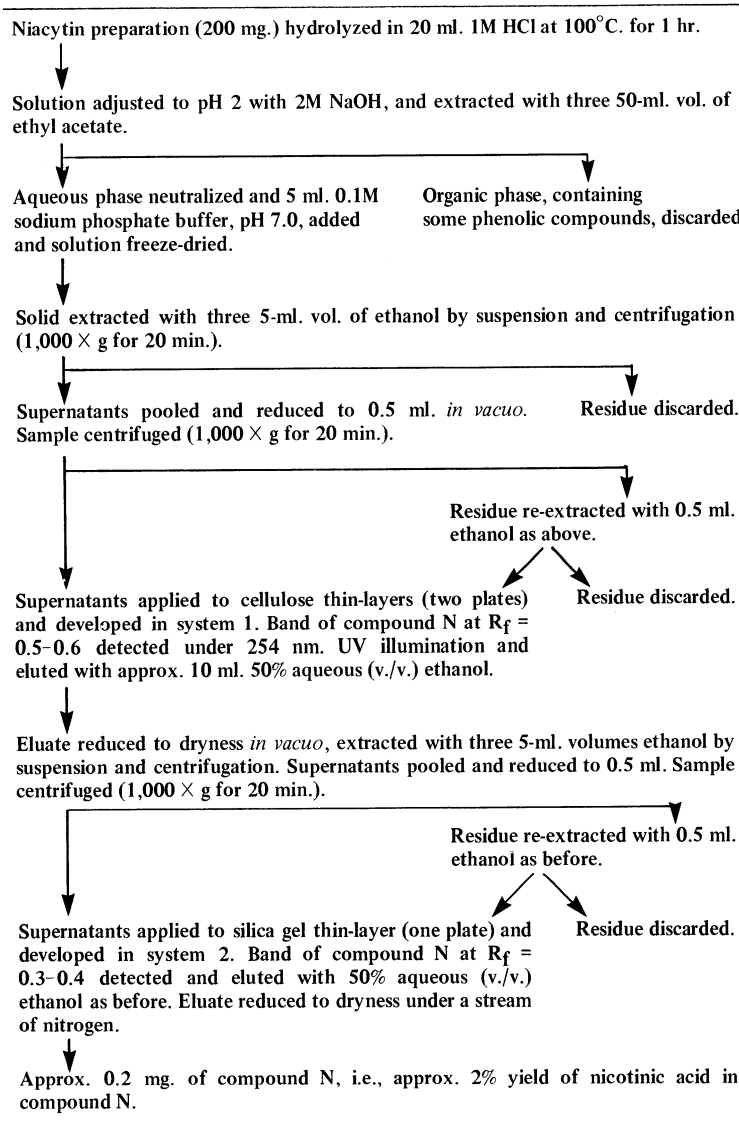

Fig. 3. Scheme of preparation of compound N from bound nicotinic acid (niacytin preparation).

TLC from substances containing *o*-aminophenol. Compound N gave a positive reaction by the cyanogen bromide-*p*-aminobenzoic acid method of Kodicek and Reddi (4) for tertiary pyridine derivatives, to give an orange-yellow fluorescent compound; it was also visualized with sulfuric acid. Iodine gas, ninhydrin, and Ehrlich's reagent gave no reaction with compound N.

UV Spectroscopy. The UV spectrum of compound N in 50% aqueous (v./v.) ethanol had λ_{max} of 258 (sh), 264 and 272 (sh) nm. For comparison, methyl nicotinate had λ_{max} of 258 (sh), 264 and 270 (sh) nm.

Mass Spectrometry. The mass spectrum of the TMS-ether of compound N is shown in Fig. 4a, and for comparison that of the TMS-ether of glucose is shown in Fig. 4b. In the spectrum of the TMS derivative of compound N, the molecular ion was detected at *m/e* 573, of low abundance (0.02% relative abundance). The loss of a methyl group from the molecular ion to give the more intense (0.3%) relative abundance) ion at *m/e* 558 is typical of the first fragmentation of a TMS-ether (12), and the presence of this ion confirms that the molecular ion is at *m/e* 573. This ion

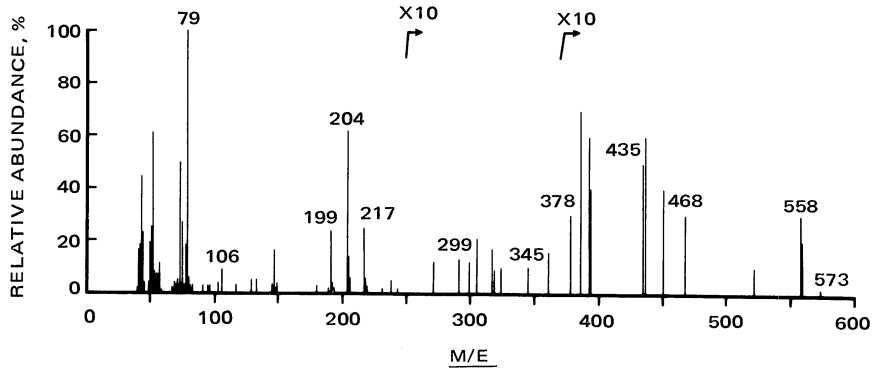

Fig. 4a. Mass spectrum of the TMS derivative of compound N. The scale is expanded twice, by a factor of 10 each time, at *m/e* 250 and 370 as indicated.

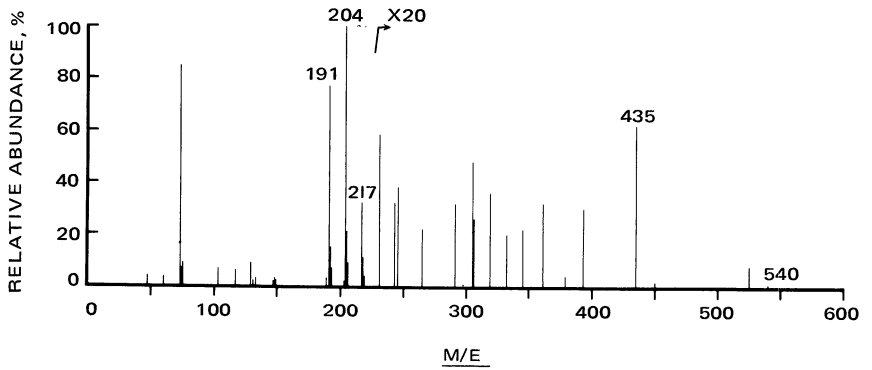

Fig. 4b. Mass spectrum of the TMS derivative of glucose. The scale is expanded by a factor of 20 at *m/e* 220, as indicated.

corresponds with the tetra-TMS derivative of a nicotinoyl hexose ($C_{24}H_{47}NO_7Si_4$). Loss of two successive trimethylsilanol residues (mass 90) from the ion at *m/e* 558 gives rise to the ions at *m/e* 468 and 378. The ions present in the spectrum of the TMS derivative of compound N that are absent in that of TMS-glucose (Fig. 4b) are likely to contain the nicotinoyl moiety. This is confirmed for the ions at *m/e* 558 and 468 by the loss of the neutral fragment of mass 123 corresponding to nicotinic acid ($C_6H_5NO_2$) to form the ions at *m/e* 435 (present in the spectrum of TMS-glucose, see Fig. 4b) and 345 respectively; also by the transition *m/e* 378 to 299, which represents the loss of C_5H_5N, the pyridinium cation, which itself is the base peak in the spectrum. Furthermore, the peak at *m/e* 106 is probably due to C_6H_4NO, which is the base peak in the mass spectrum of ethyl nicotinate (unpublished observation). The ions at *m/e* 199, 204, and 217 are typically formed in the fragmentation of the TMS-glucose moiety and are present in both spectra. The identities of these and other ions in the mass spectrum of TMS-glucose are discussed by Chizhov et al. (13).

The mass spectrum of the TMS derivative of compound N therefore provided further evidence that compound N was a nicotinoyl hexose.

IR Spectroscopy. In the IR spectrum of compound N, determined as described in Methods, the following bands below 2,000 $cm.^{-1}$ were observed (wave numbers given in $cm.^{-1}$): 1,723 (m), 1,600 (s), 1,445 (m), 1,430 (m), 1,395 (sh), 1,350 (w), 1,300 (m), 1,200 (sh), 1,120 (s), 1,080 (s), 1,060 (s), 870 (w), and 745 (w). The carbonyl stretching band at 1,723 $cm.^{-1}$ is consistent with the presence of a nicotinoyl ester, methyl nicotinate, having this band at 1,730 $cm.^{-1}$; however, the free carbonyl band of nicotinic acid itself is at 1,720 $cm.^{-1}$. The bands at 1,300 $cm.^{-1}$ and 1,120 $cm.^{-1}$ are typical of the C-O stretching vibration of an aryl ester (14), and are present in the spectrum of methyl nicotinate (at 1,290 $cm.^{-1}$ (s) and 1,110 $cm.^{-1}$ (s)). However, glucose also has a series of strong bands between 1,140 and 1,010 $cm.^{-1}$ due to C-O stretching vibrations, which could account for the bands at 1,120, 1,080, and 1,060 $cm.^{-1}$ in the spectrum of compound N. The band at 1,600 $cm.^{-1}$ was probably due to contamination with silica (from preparative TLC) which has an absorption band around this frequency.

Hydrolysis and Rechromatography. TLC of samples of hydrolyzed compound N in system 1 on silica gel demonstrated the presence of nicotinic acid and of a sugar that had the same R_f value (0.27) as glucose. The identity of this sugar was established by GLC of the TMS derivatives of the hydrolysis products of compound N (see Methods). The retention times of the major peaks were 13.3 and 20.2 min., identical to those for the TMS derivatives of α- and β-glucose. The system used gave good resolution of glucose from other hexoses tested (galactose, mannose, fructose), pentoses, and methylpentoses, as suggested by Davison and Young (15) who first described this system. GLC of the TMS derivative of samples of intact compound N did not show these peaks. These findings positively identified the hexose to which nicotinic acid is esterified as glucose.

DISCUSSION

Recent work has shown that the nicotinic acid in wheat bran is bound in a number of macromolecules that contain both carbohydrate and glycopeptide (1). This bound nicotinic acid has now been shown to be present as a tertiary nicotinoyl derivative. Partial hydrolysis of bound nicotinic acid preparations gave rise to a

fragment that was more polar than nicotinic acid on TLC; this fragment, referred to as compound N, has been isolated and identified. The identity of compound N has been established as a glucose ester of nicotinic acid. The mass spectrum of the TMS derivative showed that it contained nicotinic acid and hexose only. The UV and IR spectra also indicated the presence of a nicotinoyl ester, and further hydrolysis gave a sugar that was identified as glucose by GLC of its TMS derivative. The position of substitution of the glucose has not been determined because of insufficient material for study.

From these results it is concluded that nicotinoyl glucose is a subunit of bound nicotinic acid, the nicotinoyl moiety being linked through the carboxyl group as an ester. It is unlikely that this ester is an artifact produced during hydrolysis, since no fragments that might correspond to, for example, a nicotinic acid-amino acid complex were found (unpublished results indicated that compound N′ was a disaccharide linked to nicotinic acid); moreover, nicotinoyl glucose was produced under both acid and alkaline conditions. In samples of bound nicotinic acid that contain largely glycopeptide, nicotinoyl glucose was also found to be present, and hence in these molecules also there is no need to postulate more than one basic form, although this nicotinoyl glucose unit is linked to a number of different macromolecules.

If a basic unit in which bound nicotinic acid is contained were nicotinoyl glucose, the question arises as to why this should be unavailable, since nicotinoyl glucose itself is readily utilized (E. Kodicek, unpublished observation). Recent work has shown that the unavailability is by no means absolute: nicotinic acid-deficient rats utilize 15 to 20% of orally administered bound nicotinic acid, and normal rats are able to utilize more than this, possibly up to 40% of bound nicotinic acid as the free vitamin (16). Studies on intact preparations of bound nicotinic acid (1) have shown that the nicotinic acid is largely in cellulose- or hemicellulose-type molecules, some or all of these containing β1-4 linked glucose molecules (11). Other experiments have shown that *in vitro* treatment of bound nicotinic acid preparations with pancreatic enzymes, α- and β-glucosidases, and cellulase did not produce any free nicotinic acid, nor any of the nicotinic acid-containing partial hydrolysis products described above (J. B. Mason, unpublished observations). In view of the limited availability of bound nicotinic acid and the absence of enzymes capable of hydrolyzing hemicelluloses and particularly cellulose (β1-4 linked glucose) in the gastrointestinal tract, it is likely that the partial unavailability is due to restricted access of gastrointestinal esterases to a nicotinoyl ester bond contained in molecules that are particularly resistant to the action of digestive enzymes.

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