Biochemical Pharmacology, Vol. 22, pp. 2645–2658. Pergamon Press, 1973. Printed in Great Britain.

BILIARY EXCRETION OF NICOTINAMIDE RIBOSIDE

A POSSIBLE ROLE IN THE REGULATION OF HEPATIC PYRIDINE NUCLEOTIDE DYNAMICS*

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(*Received* 26 *January* 1973; *accepted* 13 *April* 1973)

Abstract—Female rats fed approximately 5 mg/day/kg of body wt of nicotinic acid-^{14}C excreted 10 per cent of the daily ingested label in bile and 57 per cent in urine. Approximately two-thirds of the label in bile was present as nicotinamide riboside. Chemical analysis of nicotinamide riboside indicates that about 7 μmoles/day/kg of body wt are excreted into bile. Chemical and radiochemical analyses both indicate that biliary nicotinamide riboside excretion may account for a major fraction of the hepatic pyridine nucleotide turnover. Nicotinamide riboside was not detected in urine, while 1-methylnicotinamide was present in urine but not in bile. Of the daily dietary intake, 6·7 per cent was excreted in bile as nicotinamide riboside while 19·6 per cent was excreted in urine as 1-methylnicotinamide. After intraperitoneal administration of 150 mg/kg of nicotinic acid or 500 mg/kg of nicotinamide, the hepatic NAD^+ content increases 2 to 4-fold, accompanied by a marked increase in the turnover of this newly formed NAD^+. The biliary excretion of nicotinamide riboside increases up to ten times the normal rate during this period of increased hepatic NAD^+ turnover. The nicotinamide riboside excretion appears to be related to the elevation of NAD^+ and is independent of whether nicotinic acid or nicotinamide is used as the precursor. Comparison of the biliary nicotinamide riboside excretion with published values for the urinary excretion of NAD^+ metabolites during a similar hepatic NAD^+ increase indicates that the biliary route may be a major pathway for the elimination of hepatic NAD^+ metabolites. 1-Methylnicotinamide was not found in bile unless very large intravenous doses were given to animals with ligated renal pedicles. The possible role of the biliary system in pyridine nucleotide dynamics and the possible relation of biliary nicotinamide riboside excretion to the secretion of other organic cations into bile are discussed.

MANY ORGANIC cations are known to be efficiently excreted via the bile[1–3] and the large bile:blood concentration gradients observed, considered with other properties of their transport, have led to the proposal that the liver has a specific "active" transport system responsible for the secretion of many quaternary and ionized tertiary amines.[2,4,5] The observation of Schanker and Solomon[4] that the biliary excretion of procainamide ethobromide, a quaternary amine believed to be transported by this system, was inhibited by the injection of 1-methylnicotinamide led to our initial experiments designed to determine if 1-methylnicotinamide were itself excreted in bile. During these experiments it was found that normal rat bile contained a cationic nicotinamide derivative other than 1-methylnicotinamide. Since 1-methylnicotinamide was not present in normal rat bile and was only very poorly excreted in bile after intravenous

* This study was supported by USPHS Grant No. GM-00475-12.

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administration of doses of 1-methylnicotinamide equal to or considerably greater than those used by Schanker and Solomon, this suggested that some other intermediate in the pyridine nucleotide pathway is normally secreted into bile. Calculations of the possible amounts of the unknown compound raised the possibility that the biliary excretion of this compound might be a major pathway for the elimination of hepatic pyridine nucleotides and suggested that biliary transport might play a role in the regulation of hepatic pyridine nucleotide dynamics.

The above observations raised three important questions. First, does biliary excretion play an important role in pyridine nucleotide dynamics? Second, what is the nature of the compound(s) in bile *in vivo*? Third, if the cationic compound(s) described above comprises the major portion of the biliary derivatives, is this compound perhaps the endogenous substrate for the organic cation transport system proposed by Schanker? The following experiments provide partial answers to some of the above questions.

METHODS

Animals and diets

Female Sprague–Dawley rats (150–200 g) were purchased from Simonsen Laboratories, Inc., Gilroy, Calif. The normal diet consisted of a standard pelleted rat food (Berkeley Diet, Feedstuffs Processing Co., San Francisco, Calif.) which contained 179 ppm niacin and 0·27% tryptophan. The low niacin diet used in certain experiments consisted of 18% vitamin test casein (approximately 0·3 μg of niacin/g of diet), 68% sucrose, 10% vegetable oil and a salt mixture supplemented with a niacin-free vitamin fortification mixture (Nutritional Biochemicals Co., Cleveland, Ohio). A sufficient quantity of nicotinic acid labeled with nicotinic acid-^{14}C dissolved in water was mixed with this diet to provide approximately 1 mg/day of nicotinic acid to each rat and the resulting paste was available to the animals in stainless steel metabolism cages via a slatted tunnel which permitted collection of any spilled food.

Bile was collected under pentobarbital anesthesia from a cannula (Clay-Adams PE-10 polyethylene tubing) placed in the common bile duct. Rectal temperature was maintained between 36 and 38° with a heat lamp.

Chemicals and enzymes

Methyl ethyl ketone was obtained from Fisher Chemical Co., Santa Clara, Calif., and filtered through activated charcoal before use. Nicotinic acid, nicotinuric acid, nicotinamide hydrochloride and nicotinamide mononucleotide (monohydrate) were obtained from Nutritional Biochemicals Co., Cleveland, Ohio. 1-Methylnicotinamide, β-nicotinamide adenine dinucleotide (grade III, approximately 98%, from yeast), and CM-25 (carboxymethyl) Sephadex were obtained from Sigma Chemical Co., St. Louis, Mo. Nicotinamide adenine dinucleotide phosphate was obtained from Boehringer–Mannheim Corp., New York, N.Y. Nicotinamide-*N*-oxide and 1-methyl-2-pyridone-3-carboxamide were generous gifts of Drs. Sterling Chaykin and Patrick Collins. Nicotinic acid-6-^{14}C (45 mCi/mM), nicotinic acid-carboxyl-^{14}C (> 50 mCi/mM) and nicotinamide-carbonyl-^{14}C (> 50 mCi/mM) were obtained from Amersham/Searle Corp., Arlington Heights, Ill. Alcohol dehydrogenase (100 mg/3·4 ml of 2·4 M ammonium sulfate solution, pH 6·2, as a crystalline suspension, approximately 200 U/mg) was purchased from Boehringer–Mannheim Corp., and

bacterial alkaline phosphatase (*Escherichia coli*, 10 mg/ml, 40 U/mg) was obtained from Worthington Biochemical Corp., Freehold, N.J.

Preparation of nicotonamide riboside

Nicotinamide riboside was prepared by a modification of the method of Josse and Swartz.[6] Nicotinamide mononucleotide (2·45 mg in 0·5 ml of water) was added to 1·5 ml of 0·1 M Tris buffer, pH 8·0, containing 10 mM $MgCl_2$. Ten μl was removed for fluorescence determination and 0·15 ml was added to a 2-ml (14·5 cm) column of CM-25 Sephadex and eluted with 0·1 M ammonium acetate, pH 7·0, in 0·63-ml fractions. Alkaline phosphatase (10 μl) was added to the remaining solution, and after 30 min 10 μl was removed for fluorescence determination and the remainder was immediately added to a 20-ml (40 cm) column of CM-25 Sephadex and eluted with 0·1 M ammonium acetate, pH 7·0, to separate the nicotinamide riboside from residual nicotinamide mononucleotide and enzyme. The separation procedure was followed by taking aliquots of the effluent for fluorescence determination by the methyl ethyl ketone procedure described below and by monitoring the optical density at 260 nm. The volatile buffer was subsequently removed by lyophilizing.

Fluorometric determination of quaternary pyridine nucleotide derivatives

The method employed is based on the methods of Huff and Perlzweig[7] and Carpenter and Kodicek.[8] Bile (150 μl) or other solution to be analyzed was vigorously swirled with 150 μl of methyl ethyl ketone; 0·6 ml of 1 N NaOH was then added, and the mixture was shaken for 6 min at room temperature. Six ml of 1·7 N HCl was added, mixed and the flask placed on a 90° water bath for 5 min. The flask was then cooled to room temperature in another water bath and the fluorescence was determined on an Aminco–Bowman fluorometer (excitation max. = 370 nm, emission max. = 464 nm). Blanks were prepared by adding the methyl ethyl ketone after the HCl. This blank gave the same readings as blanks prepared by adding water in place of the methyl ethyl ketone, or by eliminating the NaOH, but was used because it contained all the ingredients present in the experimental sample. Standards were prepared in each material analyzed by adding known quantities of the compound to be measured. In the case of nicotinamide riboside, 1-methylnicotinamide was sometimes used as a standard in the analytical procedure to conserve the nicotinamide riboside.

Determination of NAD^+ in liver

The entire liver was removed, quickly weighed, and homogenized with 9 volumes of 10% trichloroacetic acid (TCA). After centrifugation, the NAD^+ in the supernatant was determined by the enzymatic method of Greengard *et al.*,[9] using 10 μl of the alcohol dehydrogenase suspension described above. Standards were prepared by adding known amounts of NAD^+ to a 20% liver homogenate. After addition of the standards, the homogenate was mixed with an equal volume of 20% TCA and the supernatant was then treated identically with the experimental samples.

Determination of radioactivity

Aliquots of bile, urine and column eluates were counted directly in 10 ml of Bray's solution on a Packard Tri-Carb scintillation spectrometer. Up to 150 μl of bile did

not produce severe quenching. Strips of Whatman No. 1 filter paper used for paper chromatography and paper electrophoresis were cut up and placed directly into the bottom of a scintillation vial. The radioactivity present in both bile and urine was almost completely recovered from the paper after swirling with Bray's solution. The recovery from the filter paper was always checked by comparing the recovered counts with the counts present in the solution spotted on the paper. Radioactivity in liver was determined in Bray's solution after digestion of the liver in a commercial tissue solubilizing agent (NCS, Amersham/Searle Corp., Arlington Heights, Ill.). The total counts present in liver were closely approximated by the counts present in the supernatant of a homogenate of liver with 9 volumes of 10% TCA. In one experiment (recovery of metabolites of nicotinic acid during nicotinic acid-6-^{14}C feeding), the radioactivity present in the TCA supernatant was used as a measure of the radioactivity present in the liver. In all determinations of radioactivity, internal ^{14}C standards were used to correct for quenching.

Chromatography and electrophoresis

The cationic derivatives in bile and urine were separated on columns of CM-25 Sephadex. Columns had a bed volume of either 2 or 20 ml and were 14·5 or 40 cm respectively. Usually 0·1 M ammonium acetate, pH 7·0, was used as the eluant and 0·63 ml on 6·3 ml fractions were collected from the 2 or 20 ml columns, respectively. In some cases the Sephadex was equilibrated with water and after the application of bile the column was washed with one column volume of water, then one-half column volume of 0·05 M formic acid (adjusted to pH 2·4 with NH_3) and finally with 0·5 M formic acid (adjusted to pH 2·2 with NH_3) until all radioactivity and fluorescence yielding material were recovered.

Paper electrophoresis was carried out on Whatman No. 1 paper in 0·075 M sodium acetate buffer, pH 5·0, for 30–60 min at 1500 V. Quaternary pyridinium derivatives were visualized under long wavelength u.v. light after standing over a 1:1 mixture of methyl ethyl ketone–concentrated NH_3 for 1 hr. Other nicotinyl compounds were visualized by their u.v. absorption under short wavelength u.v. light. The cationic fraction from bile or urine was obtained from CM-25 Sephadex columns and concentrated by lyophilizing before application to the paper strips.

Paper chromatography was carried out on Whatman No. 1 paper developed with 60% (v:v) acetone in water or 60% (v:v) isopropanol in water.

Experimental design

Excretion of nicotinic acid-^{14}C metabolites in bile. Two experiments were carried out to determine the rate of excretion of nicotinic acid metabolites in bile and urine simultaneously with the elimination of its metabolites (primarily pyridine nucleotides)[10] from liver. In the first experiment, nicotinic acid-6-^{14}C (1 mg of nicotinic acid = $2·6 \times 10^5$ counts/min) was fed to six female rats for 9 days at an intake of approximately 1 mg/day of nicotinic acid per rat. The radioactive tracer was then removed while the diet with the same content of unlabeled nicotinic acid was fed. The disappearance of the label could then be followed without altering the chemical dynamics of the system. Urine was collected each day. After the first night on unlabeled food, bile was collected from three of the rats and the livers were then removed. Three days later this procedure was repeated on the remaining rats. The second experiment was

similar in design. Twelve rats were used, the specific activity of the nicotinic acid was $2{\cdot}7 \times 10^6$ counts/min/mg of nicotinic acid, and nicotinic acid-carboxyl-^{14}C was used as a tracer. Animals were housed in pairs in metabolism cages for this experiment. The labeled diet was fed for 12 days and bile and livers were collected from four rats each on days 1, 3 and 5 after the label was removed from the diet. Urine was collected throughout the experiment.

Samples of bile obtained from these experiments were subjected to column chromatography on CM-25 Sephadex, paper electrophoresis, and paper chromatography as described above to determine the nature of the labeled material in bile derived from the nicotinic acid-^{14}C in the diet.

Test for 1-methylnicotinamide excretion in bile after intravenous administration. After cannulating the common bile duct and collecting a sample of normal bile, 1-methylnicotinamide was administered via the left external jugular vein and the biliary excretion of quaternary pyridinium compounds was followed. Experimental animals were paired with saline controls. The renal pedicles were ligated in these experiments to eliminate renal accumulation and excretion of 1-methylnicotinamide. The methyl ethyl ketone procedure described above was used to determine the quaternary pyridinium compounds in bile. Since this method determines both nicotinamide riboside and 1-methylnicotinamide, there is an endogenous blank due to the nicotinamide riboside normally excreted in bile. This blank is equivalent to slightly less than 4 μmoles of 1-methylnicotinamide/day/kg of body wt, or about 0·17 μmole/hr/kg of body wt.

Effect of increased liver NAD$^+$ on nicotinamide riboside excretion in bile. After cannulation of the common bile duct and a rest period of approximately 30 min, a sample of control bile was collected for 1 hr. Animals were then given either 150 mg/kg of nicotinic acid, 500 mg/kg of nicotinamide, or saline, i.p. Bile was then collected each hr for 7 more hr. At the end of the experiment, the NAD$^+$ content of liver was measured. The NAD$^+$ content was also measured in a group of sham-operated control rats at a time when the bile collection would have begun had the experimental procedure been carried out. In some of these experiments, 25–30 μCi/animal of nicotinic acid-^{14}C (150 mg/kg) or nicotinamide-^{14}C (500 mg/kg) was administered i.p. Biliary excretion of label and nicotinamide riboside was measured each hr for 7 hr and the liver content of NAD$^+$ and radioactivity was then determined. Nicotinamide riboside excretion reached peak values about 5 hr after precursor administration. Samples of bile collected between 5 and 7 hr after precursor administration were subjected to CM-25 Sephadex chromatography and paper electrophoresis to determine the radioactive nicotinamide riboside in bile.

RESULTS

Excretion of nicotinic acid-^{14}C metabolites in bile

Figure 1a shows a CM-25 Sephadex separation of the labeled material in the bile obtained from the experiment feeding nicotinic acid-carboxyl-^{14}C. Sixty-eight per cent of the radioactive material in bile is retained by the cation exchange column and is associated with the quaternary pyridine derivative yielding a fluorescent compound after reaction with methyl ethyl ketone. The proportion of radioactivity in this cationic fraction was virtually identical on days 1 and 5 after the nicotinic acid-^{14}C was

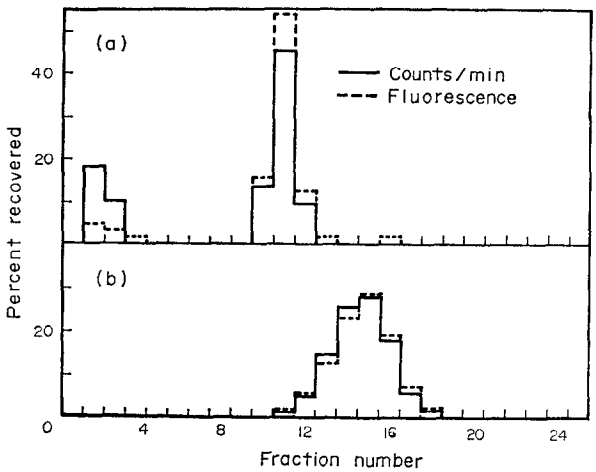

Fig. 1. (a) Sephadex CM-25 chromatography of bile from rats ingesting 1 mg/day of nicotinic acid-^{14}C ($2{\cdot}7 \times 10^6$ cpm/mg) for 12 days. Three ml of bile (1 ml from each of three rats) was added to a 40 cm column and eluted with 0·1 M ammonium acetate. (b) Co-chromatography of nicotinamide riboside standard and radioactivity from cationic fraction of bile. Fractions 10–12 from separation (a) (above) were lyophilized, dissolved in a small volume of water, mixed with a standard solution of nicotinamide riboside, and eluted from a 14·5 cm column of Sephadex CM-25 with 0·1 M ammonium acetate.

replaced by unlabeled nicotinic acid (68 per cent on day 1; 66 per cent on day 5)· Nicotinamide riboside prepared as described in Methods has an elution pattern identical to the endogenous material in bile (Fig. 1b). The neutral and negatively charged derivatives of the pyridine nucleotides are, of course, not retained by the column, nor are the majority of bile pigments and bile salts. 1-Methylnicotinamide, however, elutes in the same region.

When the fractions containing the cationic material from bile are lyophilized, redissolved in a small volume of distilled water, and subjected to paper electrophoresis as described in Methods, it is clearly seen that the radioactivity is not associated with 1-methylnicotinamide but with a faint fluorescent spot (after development over methyl ethyl ketone : ammonia) with an R_f identical to that of nicotinamide riboside. Figure 2a illustrates a run in which internal standards of both nicotinamide riboside and 1-methylnicotinamide were added to the lyophilized sample from a CM-25 Sephadex separation of the cationic material from bile, as well as a similar sample prepared from urine. It can be seen that the label in the cationic fraction of bile is all associated with the nicotinamide riboside, while the label in the cationic fraction of urine is all associated with 1-methylnicotinamide. The biliary material also had an R_f identical to nicotinamide riboside on paper chromatograms using 60% acetone in water or 60% isopropanol as solvents.

Although chromatographic and electrophoretic evidence does not constitute proof of structure, it seems virtually certain that the cationic material in bile is identical with nicotinamide riboside since it behaves identically with nicotinamide riboside in every respect examined (Sephadex chromatography, paper electrophoresis, paper chromatography, emission and excitation spectrum of the methyl ethyl ketone adduct), and since there are no other known intermediates in the synthesis and breakdown of the pyridine nucleotides which have properties consistent with those observed. On the

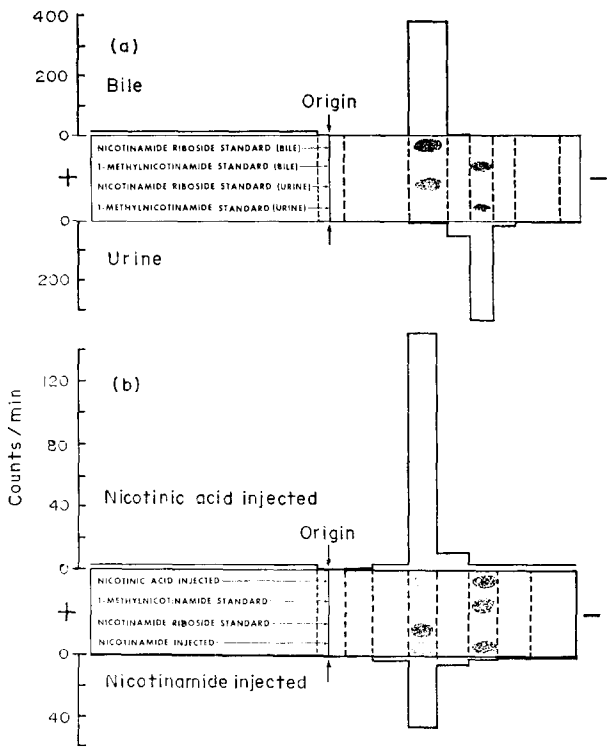

FIG. 2. (a) Paper electrophoresis of cationic nicotinyl derivatives from bile and urine. The cationic fraction obtained from Sephadex CM-25 chromatography was lyophilized, dissolved in a small volume of water and separated by paper electrophoresis as described in Methods. Bile and urine contained added standards of unlabeled nicotinamide riboside or 1-methylnicotinamide as indicated. Dotted lines indicate distance from origin at which strips were cut for counting of radioactivity. (b) Paper electrophoresis of cationic nicotinyl derivatives from the bile of rats administered 150 mg/kg of nicotinic acid or 500 mg/kg of nicotinamide. Samples collected during the period of maximum nicotinamide riboside excretion were fractionated by Sephadex CM-25 chromatography. The cationic fraction was lyophilized, dissolved in a small volume of water containing unlabeled 1-methylnicotinamide, and subjected to paper electrophoresis as described in Methods.

basis of one or more of the above criteria nicotinamide, nicotinic acid, nicotinamide-*N*-oxide, 1-methylnicotinamide, the pyridones of 1-methylnicotinamide, nicotinamide and nicotinic acid mononucleotide, NAD^+, $NADP^+$ and nicotinuric acid have been unequivocally shown not to be this major metabolite in bile, leaving only nicotinamide riboside as the likely identity of this metabolite.

The data obtained from the experiment using nicotinic acid-carboxyl-^{14}C are shown in Fig. 3. The results obtained using nicotinic acid-6-^{14}C were similar. Biliary excretion of radioactivity accounted for slightly more than 10 per cent of the dietary intake. The loss of radioactivity from the hepatic pyridine nucleotide pool does not occur via a single exponential decay. A mean of 51 per cent of the label was turned over during the first 2 days, while only 16 per cent was turned over during the second 2 days after replacement of the nicotinic acid-^{14}C with unlabeled nicotinic acid. The observed rate of biliary excretion of the label was sufficient to account for 55 per cent of the hepatic turnover during the initial 2 days and 100 per cent during

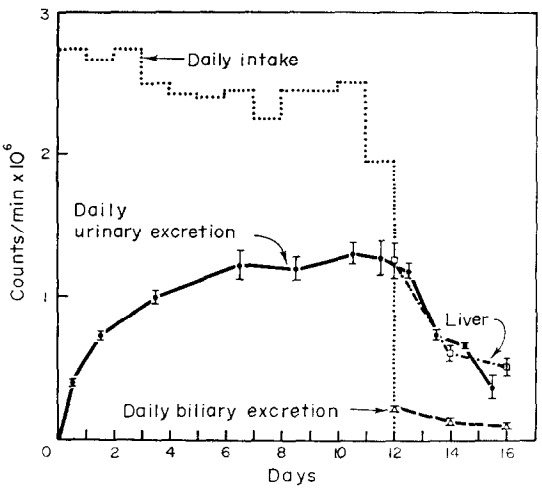

FIG. 3. Excretion of nicotinic acid-^{14}C metabolites in bile and urine. Approximately 5 mg/day/kg of body wt of nicotinic acid-^{14}C ($2{\cdot}7 \times 10^6$ counts/min/mg) was included in the diet for 12 days. The nicotinic acid-^{14}C was then replaced with an equal quantity of unlabeled nicotinic acid. The biliary excretion of radioactivity was determined on four animals at 2-day intervals beginning the day the nicotinic acid-^{14}C was replaced with unlabeled nicotinic acid. After bile collection the animals were sacrificed and the radioactivity in liver was determined. The vertical bar denotes S. E. M. There were six groups of two rats per cage initially. Groups of four animals were sacrificed on alternate days beginning on day 12.

the second 2 days. The nicotinamide riboside excretion determined chemically was about 7 μmoles/day/kg of body wt. If this biliary nicotinamide riboside is derived from the hepatic NAD^+ (approximately 20 μmoles/kg of body wt), half the hepatic NAD^+ would be eliminated via the bile in about 2 days. Experimentally, it was observed above that half the label in liver was eliminated in 2 days after the isotope was removed from the diet. It is therefore apparent that the biliary system could be playing a major role in the regulation of hepatic pyridine nucleotide dynamics.

Test for 1-methylnicotinamide excretion in bile after intravenous administration

Although it was shown above that the major pyridine nucleotide metabolite in rat bile *in vivo* is nicotinamide riboside and that virtually no 1-methylnicotinamide can be detected under conditions of normal dietary nicotinic acid intake, it was still of interest to determine if intravenously administered 1-methylnicotinamide would be transported into bile. The possibility that the liver might be able to accumulate 1-methylnicotinamide and excrete it into bile when the plasma concentration is increased to abnormally high levels is raised by the observation of Schanker and Solomon[4] that 1-methylnicotinamide can decrease the biliary excretion of exogenous quaternary ammonium compounds transported into bile. These authors suggested on this evidence that 1-methylnicotinamide was itself probably secreted into bile.

At doses of 1-methylnicotinamide equivalent to those used by Schanker and Solomon[4] (5 mg/kg = 19 μmoles/kg) no increase in the fluorescence yielding material in bile was detected. At 38 μmoles/kg (approximately the molar equivalent of the total hepatic content of pyridine nucleotides), the excretion was transiently increased to about 50 per cent over the endogenous background during the hour after administra-

tion, and 230 μmoles/kg was required to produce a 4-fold increase in the endogenous fluorescence, and this increase only persisted for about 3 hr. The maximum observed rate of 1-methylnicotinamide excretion was less than 0·68 μmoles/hr/kg of body wt or 0·3 per cent of the injected dose per hr, which is very slow indeed compared with other organic cations known to be secreted into bile which may be eliminated to the extent of 20–50 per cent of the injected dose in 1 hr.[4,11,12]

Effect of increased liver NAD⁺ on nicotinamide riboside execretion in bile

If the biliary excretion of nicotinamide riboside plays an active role in the regulation of pyridine nucleotide dynamics, its rate of excretion should be correlated with the hepatic pyridine nucleotide turnover. Petrack *et al.*[13] have shown that i.p. injections of 150 mg/kg of nicotinic acid or 500 mg/kg of nicotinamide produce a several-fold increase in hepatic NAD^+ content and that this newly formed NAD^+ is rapidly eliminated, so the NAD^+ content of liver returns to normal in from several hr to about a day. This is a much more rapid turnover of this NAD^+ pool than normally occurs. Pyridine nucleotide intermediates are normally conserved through extensive reutilization.[14] If an increased biliary excretion served to remove nicotinamide riboside from the reutilization cycle during periods of elevated NAD^+, one ought certainly to see a dramatic increase under the condition of this marked increase in hepatic NAD^+. It is important that, after the above doses of nicotinic acid or nicotinamide, significant formation of NAD^+ occurred only in liver and kidney.[13] After similar doses in the mouse, it has been demonstrated that the newly formed NAD^+ is present in the soluble fraction of liver, and that there is little change in NADH, $NADP^+$ and NADPH.[10]

Figure 4 shows that the biliary excretion of nicotinamide riboside increases about 5-fold and 10-fold, respectively, after i.p. administration of 150 mg/kg of nicotinic

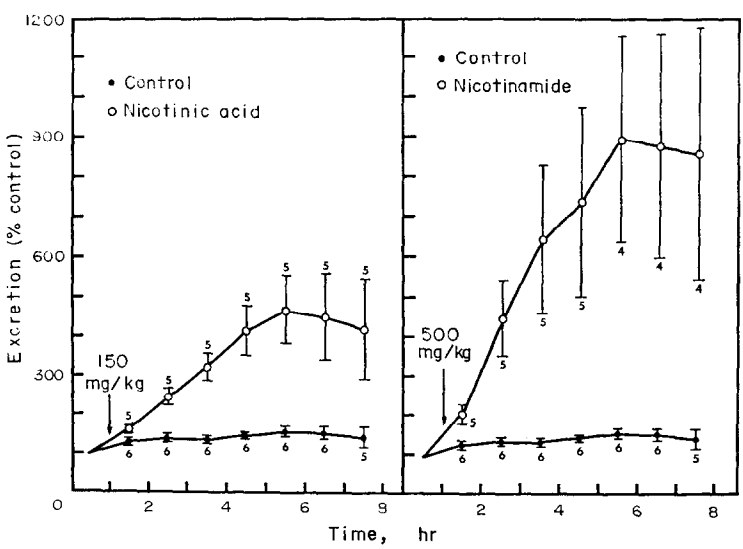

FIG. 4. Biliary excretion of nicotinamide riboside after i.p. administration of 150 mg/kg of nicotinic acid or 500 mg/kg of nicotinamide. Arrows denote administration of nicotinic acid or nicotinamide. The vertical bar denotes S. E. M.

acid and 500 mg/kg of nicotinamide. The excretion begins to rise at about the same time the hepatic NAD^+ is reported to begin increasing,[13] but in the case of nicotinic acid administration remains high up to 7 hr after the injection, while Petrack *et al.*[13] report the NAD^+ to be back to normal by this time. Analysis of the hepatic NAD^+ in our rats showed that the NAD^+ was still elevated 7 hr after the nicotinic acid injection (see Fig. 5). The increase in NAD^+ therefore persists longer in our experiments, indicating that the turnover is probably slower than that observed in the experiments of Petrack *et al.* This may be due to the fact that our rats were anesthetized throughout the experiment, while their rats remained conscious and were sacrificed in groups.

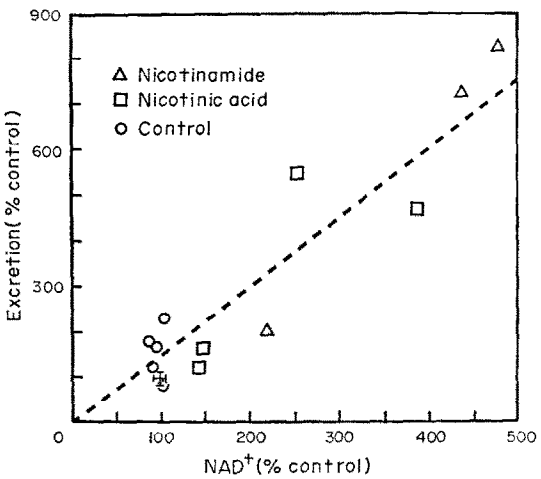

Fig. 5. Biliary excretion of nicotinamide riboside as a function of increased hepatic NAD^+. The horizontal bar denotes S. E. M. of NAD^+ content of liver from six sham-operated control rats (content = 0·575 ± 0·036 S. E. M. μmole/g of liver). The vertical bar denotes S. E. M. of biliary nicotinamide riboside excretion of the sixteen rats from Fig. 4 during the 1-hr control period of bile collection (0·306 ± 0·041 S. E. M. μmole/hr/kg of body wt).

Figure 5 shows the nicotinamide riboside excretion during the period prior to sacrifice (expressed as per cent of the excretion during the control period) vs the NAD^+ content of liver at the time of sacrifice (expressed as per cent of the average NAD^+ content of a group of sham-operated control animals). It appears the increased nicotinamide riboside excretion is correlated with the increased hepatic NAD^+ and is independent of the precursor injected. It also appears that much of the variability observed in Fig. 4 is due to a variable increase in NAD^+ rather than to variable excretion of nicotinamide riboside in response to the elevated NAD^+, since Fig. 5 shows that every case of increased NAD^+ is associated with a corresponding increase in nicotinamide ruboside excretion.

Bile from two animals in each of the groups injected with ^{14}C-labeled NAD^+ precursor was fractionated on a column of CM-25 Sephadex to determine the ^{14}C-nicotinamide riboside excretion in bile. Concentration of the cationic fractions obtained from the CM-25 Sephadex columns, and paper electrophoresis of the concentrate showed that the ^{14}C label in the cationic fraction was associated exclusively with nicotinamide riboside (see Fig. 2b). Although nicotinamide is

extensively methylated by liver[15] and Petrack *et al.*[13] observed 1-methylnicotinamide-^{14}C in urine after both nicotinamide-^{14}C and nicotinic acid-^{14}C administration, 1-methylnicotinamide-^{14}C was not observed in bile after injections of either nicotinic acid or nicotinamide, indicating that 1-methylnicotinamide endogenously formed in liver is not excreted via the bile. Table 1 gives the data obtained from the analysis of

TABLE 1. INCREASE IN HEPATIC NAD$^+$ AND BILIARY EXCRETION OF NICOTINAMIDE RIBOSIDE AFTER I.P. ADMINISTRATION OF NICOTINIC ACID-^{14}C (150 mg/kg) OR NICOTINAMIDE-^{14}C (500 mg/kg)

	Biliary nicotinamide riboside excretion (μmoles/kg/hr)	Biliary nicotinamide-^{14}C riboside excretion (μmoles/kg/hr)	Biliary ^{14}C excretion (μmoles/kg/hr)	Increase in hepatic NAD$^+$ (μmoles/kg)	Liver ^{14}C (μmoles/kg)	Urinary ^{14}C excretion* (μmoles/kg/hr)
Nicotinic acid	1·8† 2·1‡	1·4†	4·9† 5·0‡	52	109	2·4 as 1-methyl-nicotin-amide
Nicotinic acid	1·5† 1·2‡	0·92†	4·9† 2·3‡	34	68	1·8 as nico-tinamide 4·3 total
Nicotinamide	1·6† 1·4‡	1·3†	17·9† 13·3‡	88	350	
Nicotinamide	1·0† 1·2‡	1·0†	9·6† 8·0‡	84	239	

* Data for urinary excretion of metabolites derived from NAD$^+$ catabolism after nicotinic acid (150 mg/kg) obtained by Petrack *et al.*[13] in conscious rats during the period of maximum hepatic NAD$^+$.

† Period over which bile subjected to chromatography was collected (5–7 hr after precursor administration).

‡ One-hr period immediately before sacrifice.

bile and liver from two nicotinic acid–^{14}C and two nicotinamide-^{14}C-injected rats. Biliary excretion data for fluorometrically determined nicotinamide riboside and total ^{14}C label are given for both the 1-hr period preceding sacrifice and for the period over which the bile subjected to chromatography was collected (5–7 hr after precursor administration). Liver content of NAD$^+$ and of radioactivity was determined 7 hr after the i.p. administration of nicotinamide or nicotinicacid.

DISCUSSION

The present results suggest that an important function of the biliary system in pyridine nucleotide dynamics may have previously been overlooked. Although urinary excretion of nicotinamide derivatives accounts for the major portion of ingested nicotinic acid at levels approximating the normal dietary intake, the biliary excretion of nicotinamide riboside and other unidentified products derived from pyridine nucleotides is large enough to account for most, if not all, of the hepatic pyridine nucleotide turnover.

Under conditions leading to a marked increase in hepatic NAD$^+$ turnover (i.e.

after large doses of nicotinic acid or nicotinamide), the biliary excretion of nicotinamide riboside increases dramatically during the period of NAD^+ breakdown. A comparison of the rate of biliary excretion of nicotinamide riboside observed in our experiments with the rate of urinary excretion of NAD^+ metabolites observed by Petrack *et al.*[13] after the same dose of nicotinic acid reveals that the biliary excretion of nicotinamide riboside in bile is equal to 35–50 per cent of the rate of excretion of total NAD^+ metabolites in urine, even though NAD^+ turnover was more rapid in their experiments. This is remarkable in light of the negligible role previously assigned to the biliary route in removing pyridine nucleotide catabolites.[16] Further, only nicotinamide riboside excretion has been measured in bile under these conditions of elevated NAD^+ in liver. It was shown above that non-cationic metabolites are present in bile under conditions of normal dietary levels of nicotinic acid. Although nicotinamide riboside is the major biliary metabolite under conditions of normal dietary levels of nicotinic acid, this is not necessarily true after large doses of NAD^+ precursors. The other metabolites in bile should be determined, both under normal conditions and under conditions of altered NAD^+ turnover. Experiments are needed in which urinary and biliary excretion are measured simultaneously during the period of NAD^+ buildup and breakdown, preferably with simultaneous determinations of important intermediates in liver, in order to assess the relative importance of the urinary and biliary routes.

There are several studies of hepatic pyridine nucleotide metabolism in isolated, perfused rat liver which have followed the synthesis of pyridine nucleotides from nicotinic acid and nicotinamide independently of other body tissues,[15, 17–19] but all have been carried out over relatively short time periods when the biliary nicotinamide riboside excretion would be very small. Although labeled precursors are incorporated into NAD^+ within a few min, only insignificant amounts of label disappear from the NAD^+ pool within 3 hr[18] because catabolites arising from NAD^+ degradation are efficiently reutilized.[14] Since measurements of nicotinyl derivatives of the pyridine nucleotide derivatives in bile from perfused liver are generally made during experiments lasting 3 hr or less,[15, 17, 18] it is not surprising that significant nicotinamide riboside excretion has not been observed. Further, the formation of 1-methylnicotinamide and its pyridones, as well as nicotinamide-*N*-oxide, is most rapid during the first hr after the addition of radioactive nicotinic acid to the perfusate.[18] The rate of formation of these metabolites has fallen to very low levels by 3 hr. Investigation of the biliary nicotinamide riboside excretion from isolated, perfused liver over longer time periods during which the labeled NAD^+ is being eliminated and after the initial rapid formation of 1-methylnicotinamide and its pyridones should help to determine if nicotinamide riboside transport into bile could be an important mechanism for removing intermediates from the pyridine nucleotide cycle.

It is interesting to note that high phosphatase activities, expecially 5′-nucleotidase, are associated with the bile cannaliculi.[20] The presence of a phosphatase in the bile cannaliculi which is an integral part of the excretion of nicotinyl ribosides into bile could provide an efficient and direct control on the removal of intermediates from the pyridine nucleotide cycle. This hypothesis is especially attractive in light of the demonstration by Keller *et al.*[17] that the primary reutilization pathway for nicotinamide derived from NAD^+ degradation is via nicotinamide mononucleotide, which is, of course, phosphorylated nicotinamide riboside. This idea would be even more

appealing if nicotinic acid riboside accounted for the unidentified fraction of label in bile. Hagino *et al.*[15] have reported qualitative identification of nicotinic acid, nicotinamide, nicotinamide riboside, nicotinic acid riboside, and 1-methylnicotinamide in bile from isolated, perfused rat liver. We do not find 1-methylnicotinamide in bile *in vivo* either under conditions of normal dietary nicotinic acid intake or after very large doses of nicotinic acid or nicotinamide. Since nicotinic acid-AD has been shown to accumulate in liver after large doses of both nicotinic acid and nicotinamide,[10] the excretion of nicotinic acid riboside in bile should certainly be determined under these circumstances. Gerber and Deroo[19] have demonstrated that the formation of nicotinic acid riboside in isolated, perfused rat liver parallels that of 1-methylnicotinamide after a pulse label of nicotinic acid-^{14}C.

The observed excretion of nicotinamide riboside into bile also provides the first demonstration of the secretion of an endogenous quaternary amine into bile. It has been known for some time that many organic cations are efficiently transported into bile,[1-3] and it has been proposed that an "active", metabolically dependent transport process exists for this type of compound.[2,4] Such transport systems for the secretion of both anions and cations into the renal proximal tubule[21,22] and for the secretion of organic anions into bile[23] are also well known. In each of these transport systems, excepting organic cations into bile, there are endogenous metabolic products which are secreted by the transport process. Since one likes to think that energy-requiring processes have some inherent physiologic function, it is tempting to postulate that nicotinamide riboside is the physiologic substrate for the biliary organic cation transport system. Certainly the regulation of pyridine nucleotide dynamics in liver would be a valid "reason" for the organism to expend energy on such a transport process. Whether nicotinamide riboside is in fact transported by the same transport system responsible for the biliary secretion of exogenous organic cations remains to be seen.

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