Utilization of tryptophan, nicotinamide and nicotinic acid as precursors for nicotinamide nucleotide synthesis in isolated rat liver cells

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1. Incubation of isolated rat hepatocytes with nicotinamide or nicotinic acid showed that while both vitamers were taken up from the incubation medium, neither was utilized to any significant extent as a precursor of the nicotinamide nucleotide coenzymes, NAD and NADP, and neither was capable of preventing the loss of nucleotides that occurs on incubating the cells.

2. Incubation of hepatocytes with tryptophan showed that *de novo* synthesis from tryptophan permitted replacement of the nucleotides lost during incubation; at high concentrations of tryptophan there was an increase above the initial intracellular concentration of NAD(P). Incubation of hepatocytes with tryptophan also resulted in the formation and release from the cells of a considerable amount of niacin, as well as the two principal metabolites of NAD(P), N^1-methyl nicotinamide and methyl pyridone carboxamide.

3. It is suggested that, in the liver, preformed niacin is not utilized for nucleotide synthesis, and indeed the function of the liver appears to be synthesis of niacin from tryptophan, and its release for use by extrahepatic tissues that lack the pathway for *de novo* synthesis of nicotinamide nucleotides from tryptophan.

Previous studies from this laboratory have suggested that the *de novo* synthesis of nicotinamide nucleotides (NAD and NADP) from tryptophan may be a more important source of these coenzymes than is the utilization of dietary nicotinamide or nicotinic acid. The enzyme kinetic variables are such that the three enzymes involved in the incorporation of preformed niacin into nucleotides, nicotinamide phosphoribosyltransferase (*EC* 2.4.2.12), arylformamidase (nicotinamide deamidase; *EC* 3.5.1.19) and nicotinate phosphoribosyltransferase (*EC* 2.4.2.11) (see Fig. 1), all act at or near their maximum rates at the normal intracellular concentrations of nicotinamide and nicotinic acid (Bender *et al.* 1982). This suggests that there would be little or no utilization of any additional niacin which might be available from the diet or arising from the catabolism of NAD(P).

Feeding rats on diets providing high intakes of either nicotinic acid or nicotinamide leads to a relatively small increase in the concentration of NAD(P) in the liver. A tenfold increase in dietary niacin resulted in only a 50 % increase in liver NAD(P). By contrast, a threefold increase in dietary tryptophan resulted in a 2·6-fold increase in liver NAD(P), as well as a very considerable increase in the urinary excretion of N^1-methyl nicotinamide and methyl pyridone carboxyamide, the two principal metabolites of nicotinamide, resulting from hydrolysis of NAD(P) (McCreanor & Bender, 1986).

The present study was undertaken in order to investigate further the relative importance of utilization of preformed niacin and *de novo* synthesis from tryptophan in the synthesis of nicotinamide nucleotide coenzymes in isolated rat hepatocytes.

METHODS

Cell preparation and incubations

Male Wistar rats, weighing 200 g, fed from weaning on animal house stock diet (Diet 86; A. Dixon & Sons Ltd, Ware, Herts), were used for the present study. Isolated hepatocytes

Tryptophan

↓

↓

Quinolinic acid

↓ a

Nicotinic acid ————— b —————→ Nicotinic acid
mononucleotide

↓ d ↑   ↓

Nicotinamide   Nicotinic acid
adenine dinucleotide

======= c =======→ **Nicotinamide**
←—————— e —————— **adenine dinucleotide**
Nicotinamide   **(NAD)**

↓ f   ↕

                                 Nicotinamide
                                 adenine dinucleotide
                                 phosphate (NADP)

N^1-Methyl nicotinamide

↓ g
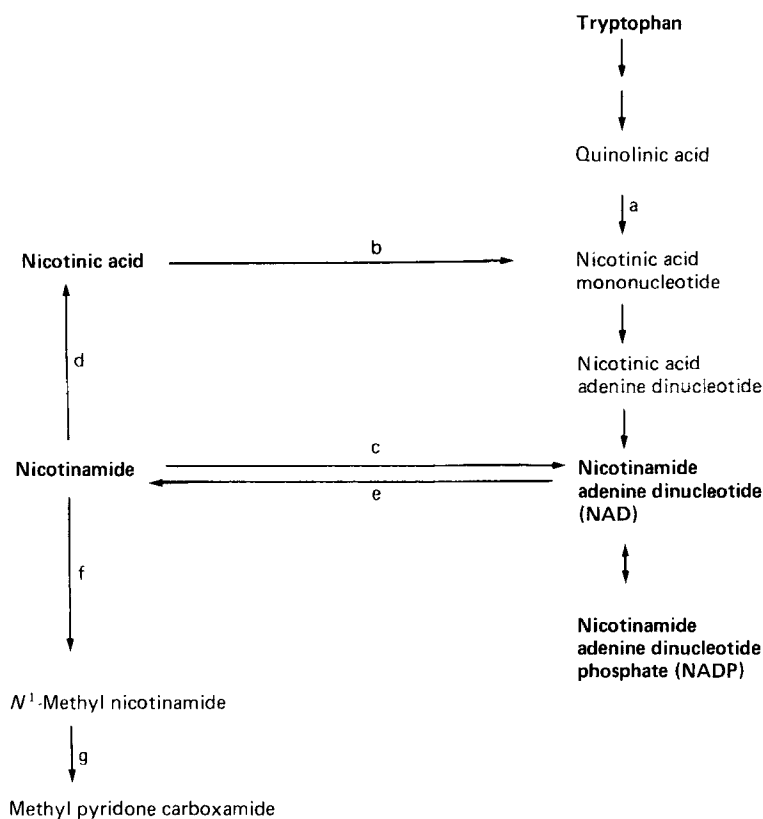
Methyl pyridone carboxamide

Fig. 1. Pathways of nicotinamide nucleotide metabolism. a, Nicotinate–nucleotide pyrophosphorylase (decarboxylating) (quinolinate phosphoribosyltransferase; *EC* 2.4.2.19); b, nicotinate phosphoribosyltransferase (*EC* 2.4.2.11); c, nicotinamide phosphoribosyltransferase (*EC* 2.4.2.12); d, arylformamidase (nicotinamide deamidase; *EC* 3.5.1.19); e, NAD$^+$ nucleosidase (NADase; *EC* 3.2.2.5) and NAD$^+$ ADP-ribosyltransferase (poly(ADP-ribose) transferase; *EC* 2.4.2.30); f, nicotinamide methyltransferase (*EC* 2.1.1.1); g, aldehyde oxidase (*EC* 1.2.3.1).

were prepared by portal–caval perfusion with collagenase (*EC* 3.4.24.3), essentially as described by Elliott *et al.* (1976), except that instead of cannulating the inferior vena cava, this blood vessel was cut, and the perfusion medium was recirculated after draining on to the dissection tray, as described by Romero & Viña (1983) for reverse (caval–portal) perfusion. After washing in ice-cold Krebs-Ringer phosphate–bicarbonate buffer containing 5 mM-glucose, the entire yield of cells from the liver of a 200 g rat was suspended in 50 ml ice-cold Krebs-Ringer phosphate–bicarbonate buffer containing 5 mM-glucose. The dry weight of cells in the preparation was determined by drying replicate 1 ml portions of the cell suspension to constant weight at 105°; typically, such a preparation contained 10–20 mg dry weight of cells/ml.

Incubations were performed in 25-ml conical flasks which had been siliconized by treatment with a solution of dichlorodimethylsilane (20 g/l carbon tetrachloride) in order to prevent cell adhesion to the glass. The cell suspension (1 ml) was incubated with 4 ml Krebs-Ringer phosphate–bicarbonate buffer containing 5 mM-glucose, 5 mM-glutamine, 4·5 mM-lactate and 0·5 mM-pyruvate, and 1 ml of a solution of tryptophan, nicotinamide or nicotinic acid in Krebs-Ringer phosphate–bicarbonate buffer containing 5 mM-glucose.

The flasks were sealed with rubber stoppers (Suba-Seals), and were gassed throughout the incubation with a gentle stream of oxygen–carbon dioxide (95:5, v/v), in a water-bath at 37°, shaking at about 100 rev/min. Unincubated control flasks remained on ice throughout the incubation period.

The incubation was allowed to continue for 30 min, then the contents of each flask were poured into ice-cold tubes and the cells harvested by centrifugation for 10 min at 2000 *g*, 4°. The cell pellet was used immediately for determination of NAD(P) and niacin, as described below; the incubation medium was frozen and stored at −20° until required.

Measurement of nicotinamide nucleotides

The total content of nicotinamide nucleotide coenzymes (NAD and NADP, both oxidized and reduced forms) was determined by the following modification of the fluorimetric method that has been described previously (Bender *et al.* 1982), itself a modification of methods described by Kaplan *et al.* (1951) and Lowry *et al.* (1961). The cell pellet was suspended in 1 ml of an ice-cold solution of 1 vol. hydrogen peroxide using a Polytron tissue emulsifier. To this suspension was then added immediately 1 ml of an ice-cold solution of 0·1 M-sodium sulphate in 0·02 M-sulphuric acid; immediately after mixing the tubes were placed in a boiling water-bath for 5 min. They were then allowed to cool and denatured protein was removed by centrifugation for 10 min at 2000 *g*. The supernatant fraction was neutralized by the addition of 40 μl 5 M-sodium hydroxide and each sample was divided into 0·4 ml portions. To one portion of each sample, 0·4 ml NAD$^+$ nucleosidase (NADase, *EC* 3.2.2.5) from *Neurosopora crassa* (0·1 unit/ml; Sigma Chemical Co., Poole, Dorset) was added, and these samples were incubated at 30° for 30 min, in order to permit correction of subsequent fluorescence for interference from materials other than NAD(P). To another portion was added 0·4 ml 20 μM-NAD, as an internal standard; an equal volume of water was added to two further portions. These samples were kept on ice while the enzymic destruction of NAD continued in the 'blank' tube. To each tube 2 ml 6 M-NaOH were added, then samples were heated in a boiling water-bath for 5 min. After cooling, the fluorescence at 460 nm (activation 360 nm) was measured using an Aminco–Bowman spectrophotofluorimeter.

Measurement of total niacin

Nicotinic acid and nicotinamide were determined colorimetrically after reaction with chloramine-T and potassium cyanide, by the following modification of the method of Carlson (1966).

At the end of the incubation period, 2-ml portions of the incubation mixture were subjected to centrifugation as described previously; the supernatant fraction (incubation medium) was frozen at −20° until required, and the cells were resuspended in 2 ml water, using a Polytron tissue emulsifier, and placed in a boiling water-bath for 2 min. After cooling they were centrifuged for 10 min at 2000 *g* to remove denatured protein, and the supernatant fraction was frozen and stored at −20° until required.

Both nicotinic acid and nicotinamide yield a cyanide adduct with the same absorption spectrum, but that from nicotinic acid has a considerably greater molar extinction coefficient than that from nicotinamide. Therefore samples were treated with a preparation of arylformamidase to convert nicotinamide to nicotinic acid; 30 mg freeze-dried *Micrococcus lysodeikticus* powder (Sigma Chemical Co.) was suspended in 10 ml Krebs–Ringer phosphate–bicarbonate buffer, and 0·4 ml of this suspension was added to 2 ml of the sample of incubation medium or cell lysate. After incubation at 37° for 20 min, protein was denatured by placing the tubes in a boiling water-bath for 2 min. After cooling,
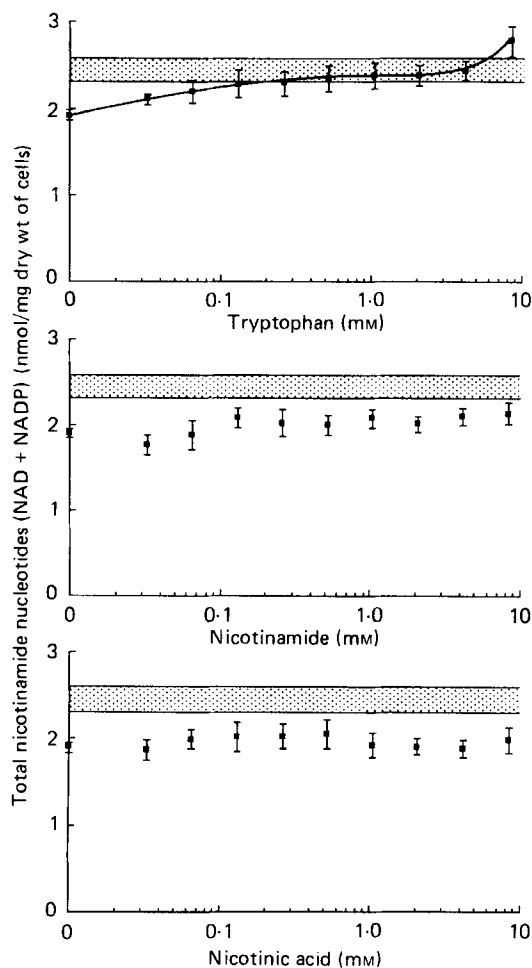
Fig. 2. Hepatocyte total nicotinamide nucleotides (NAD and NADP) after incubation with tryptophan, nicotinamide or nicotinic acid. ▨, Nicotinamide nucleotide content of unincubated cells; points show mean values, and standard deviations represented by vertical bars, for duplicate incubations with five different preparations of hepatocytes (for details of incubations see p. 280).

3 vol. acetone were added and denatured protein and insoluble buffer salts were removed by centrifugation at 2000 g for 10 min. Acetone was then removed by the addition of 1 vol. chloroform, and 1·5 ml portions of the aqueous phase were mixed with 0·5 ml chloramine-T (50 g/l) and 0·5 ml KCN (10 mg/ml in 60 mg Tris and 175 μl 1 M-hydrochloric acid/ml water). After standing at room temperature for 15 min the absorbance was measured at 410 nm.

Measurement of N^1-methyl nicotinamide

N^1-Methyl nicotinamide in the incubation medium was determined by the small-scale modification of the alkali–ketone fluorimetric method of Carpenter & Kodicek (1950) that has been described previously (Bender, 1980).
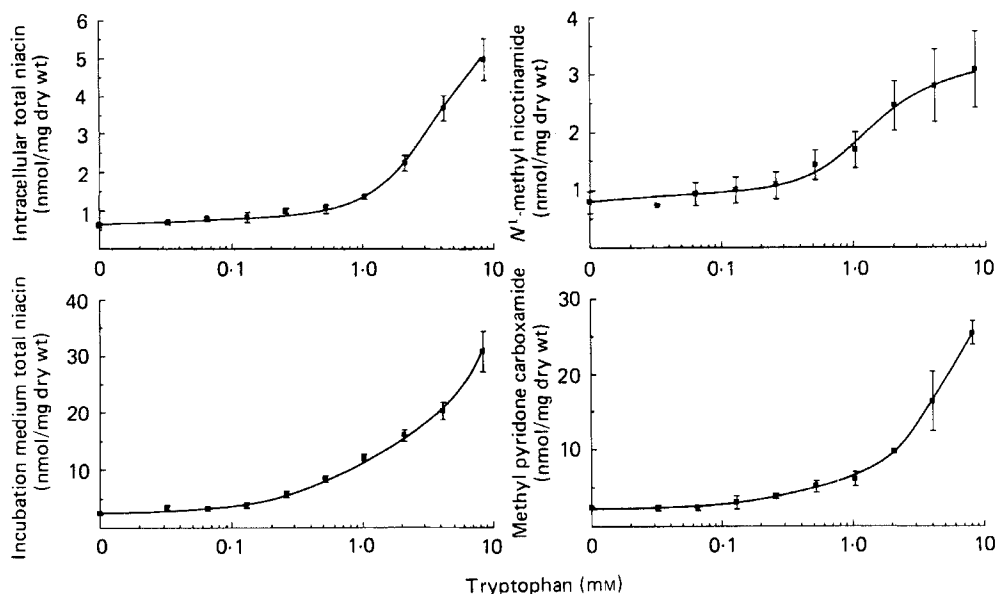
Fig. 3. Formation of niacin (nicotinamide and nicotinic acid, measured together after enzymic deamination), N^1-methyl nicotinamide and methyl pyridone carboxamide, by hepatocytes incubated with tryptophan. Points show mean values, and standard deviations represented by vertical bars, for duplicate incubations with five different preparations of hepatocytes (for details of incubations see p. 280).

Measurement of methyl pyridone carboxamide

Methyl pyridone carboxamide in the incubation medium was determined colorimetrically after removal of interfering substances with MB-5113 mixed-bed ion-exchange resin, hypobromite-catalysed rearrangement to the amine, diazotization, and coupling with napthylethylene diamine, as described previously by Bender (1983), a modification of the methods described by Holman (1954) and Joubert & de Lange (1962).

RESULTS

Incubation of the hepatocytes in the absence of substrate resulted in a fall in the intracellular content of nicotinamide nucleotides from a mean of 2·45 (SD 0·136) nmol/mg dry weight in unincubated cells to 1·92 (SD 0·118) nmol/mg ($P < 0.001$, t test). As can be seen in Fig. 2, incubation in the presence of tryptophan resulted in the preservation of intracellular NAD(P), and at unphysiologically high concentrations of tryptophan there was a slight increase above the unincubated concentration (to 2·78 (SD 0·353) nmol/mg dry weight; $0.02 > P > 0.01$, t test). By contrast, neither nicotinamide nor nicotinic acid, over the same range of concentrations, resulted in any significant preservation of intracellular NAD(P).

As can be seen from Fig. 3, incubation of hepatocytes with tryptophan resulted in significant formation of niacin, with both intracellular accumulation and a considerable amount in the extracellular incubation medium. Relatively large amounts of both N^1-methyl nicotinamide and methyl pyridone carboxamide were also recovered from the incubation medium after incubation with higher concentrations of tryptophan.
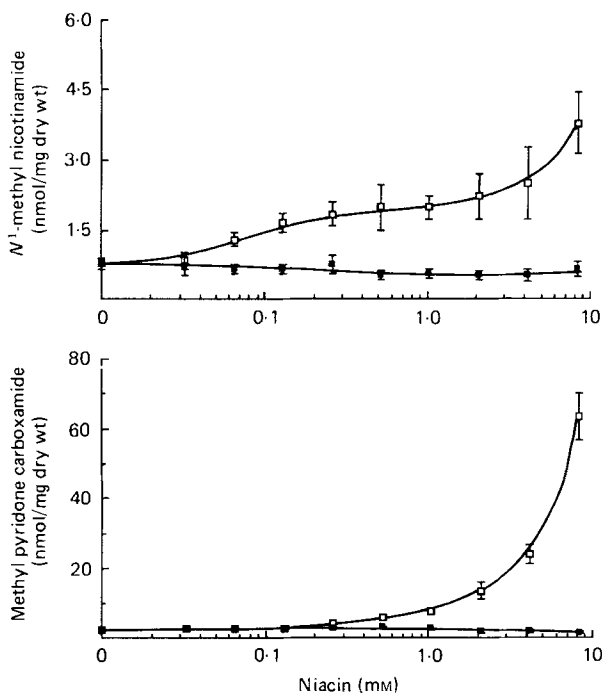
Fig. 4. Formation of N^1-methyl nicotinamide and methyl pyridone carboxamide by hepatocytes incubated with nicotinamide (□), or nicotinic acid (■). Points show mean values, and standard deviations represented by vertical bars, for duplicate incubations with five different preparations of hepatocytes (for details of incubations see p. 280).

As can be seen from Fig. 4, incubation with nicotinamide resulted in the formation of significant amounts of both N^1-methyl nicotinamide and methyl pyridone carboxamide, whereas incubation with nicotinic acid did not result in any signficant increase in the concentration of either of these metabolites in the incubation medium.

DISCUSSION

The loss of nicotinamide nucleotide coenzymes on incubation of isolated hepatocytes in the presence of glucose, lactate, pyruvate and glutamine as energy-yielding substrates, but without any added precursor for nicotinamide nucleotide synthesis, suggests that under normal conditions there may be a considerable turnover of NAD(P). McCreanor & Bender (1983) showed that exposure of rats to moderately severe hypoxia for 24 h increased the proportion of liver nicotinamide nucleotides in the reduced forms (NADH and NADPH), which are not substrates for either NAD^+ ADP-ribosyltransferase (poly(ADP-ribose) transferase; EC 2.4.2.30) or NAD^+ nucleosidase (NADase, EC 3.2.2.5). This was accompanied by a 68% increase in the total liver content of nicotinamide nucleotides. However, the loss of nucleotides from isolated hepatocytes on incubation reported here may represent a metabolic response to cold shock rather than reflecting the normal turnover of the coenzymes. Cantoni et $al.$ (1986) have reported a similar loss of nicotinamide nucleotides from cultured Chinese hamster ($Cricetulus$ $griseus$) ovary cells which had been cooled to 4° and then incubated at 37°, but not during incubation without

previous cold shock. This loss was apparently due to activation of NAD^+ ADP-ribosyltransferase, since it could be prevented by incubation in the presence of 3-amino-benzamide, a specific inhibitor of NAD^+ ADP-ribosyltransferase which has no effect on NAD^+ nucleosidase (Hunting *et al.* 1985).

The activities of the two enzymes that hydrolyse NAD(P), NAD^+ nucleosidase and NAD^+ ADP-ribosyltransferase, are such that were there no regulatory factors or membrane-permeability barriers, the entire intracellular content of NAD(P) could be hydrolysed within a few minutes (Clark & Pinder, 1969; McCreanor & Bender, 1983). One obvious regulatory factor is the binding of nucleotides to enzymes that use them as co-substrates. Bernofsky & Pankow (1973) have shown that protein-bound NAD is not a substrate for NAD^+ nucleosidase and the affinities of the two enzymes for $NAD(P)^+$ are of the same order of magnitude as the affinities of a number of enzymes that use the coenzymes (McCreanor & Bender, 1983). This means that the catabolic enzymes will have to compete for their substrate with metabolic utilization of NAD(P); this would be expected to limit catabolism under normal conditions. Furthermore, NAD^+ ADP-ribosyltransferase is active in response to DNA damage (Benjamin & Gill, 1980*a*, *b*) and possibly other metabolic stresses (Cantoni *et al.* 1986), and NAD^+ nucleosidase may be mainly an extracellular enzyme rather than truly microsomal (Müller *et al.* 1983; Amar-Costesec *et al.* 1985), in which case it is unlikely to make a significant contribution to NAD turnover in the cell.

The nicotinamide that is released by the hydrolysis of NAD(P) seems not to be used for resynthesis of the coenzymes, but to be lost from the liver cells, both as nicotinamide and nicotinic acid, which can be used by other tissues, and as the excretory products N^1-methyl nicotinamide and methyl pyridone carboxamide.

The observation reported here that incubation of hepatocytes with nicotinamide or nicotinic acid does not prevent or reverse the loss of NAD(P) on incubation is in agreement with previous observations that the kinetic variables of the enzymes involved in utilization of nicotinamide and nicotinic acid in the liver are such that they are all acting at or near their maximum rates under normal conditions, and therefore are unable to utilize any additional substrate which is made available (Bender *et al.* 1982). It is, however, at variance with studies of isolated perfused whole liver (Keller *et al.* 1971; Grünicke *et al.* 1974), in which there was significant incorporation of radioactivity from [^{14}C]nicotinamide into NAD(P), and studies in mice which showed the transient incorporation of radioactivity from [^{14}C]nicotinic acid into liver nucleotides, followed by significant labelling of the liver pool of nicotinamide (Collins & Chaykin, 1972). In all three of these studies, what was observed was the incorporation of radioactivity from [^{14}C]niacin, which may be the result of an exchange reaction catalysed by NAD^+ nucleosidase (Zatman *et al.* 1954), rather than net synthesis of NAD(P). The results reported here clearly show that neither nicotinamide nor nicotinic acid is a significant precursor for net synthesis of the nucleotides.

An alternative explanation would be that neither nicotinamide nor nicotinic acid is taken up into the isolated hepatocyte. However, incubation of hepatocytes with increasing concentrations of nicotinamide together with a constant amount of [^{14}C]nicotinamide showed a concentration-dependent inhibition of uptake of radioactivity. From the results, a value for the transport coefficient (K_T, the substrate concentration resulting in half-maximum rate of transport) for nicotinamide was determined as 0·29 (SD 0·036) μM. Similarly, nicotinic acid inhibited the accumulation of radioactivity from [^{14}C]nicotinic acid, with a K_T of 0·16 (SD 0·012) μM (means of three determinations on separate hepatocyte preparations). It is thus apparent that isolated hepatocytes are indeed capable of taking up niacin from the incubation medium. As shown in Fig. 4, the nicotinamide that was taken up into the cells was methylated, and some of the resultant N^1-methyl nicotinamide was

oxidized to methyl pyridone carboxamide. It is noteworthy that incubation of isolated hepatocytes with nicotinic acid did not result in any increased formation of either methylated metabolite. This is further evidence that nicotinic acid is not a significant net precursor of the nucleotides, since were nicotinic acid metabolized by way of NAD(P), hydrolysis would be expected to result in the release of nicotinamide, a proportion of which would presumably be methylated. These results are in agreement with previous studies of the metabolism of single doses and high dietary intakes of nicotinamide and nicotinic acid in rats, in which the two vitamers resulted in the excretion of different patterns of metabolites (McCreanor & Bender, 1986).

The marked increase in both intracellular and extracellular niacin, and in extracellular N^1-methyl nicotinamide and methyl pyridone carboxamide, after incubation with tryptophan is also in agreement with previous reports which suggested that there is little limitation on the incorporation into nicotinamide nucleotides of quinolinic acid arising from tryptophan, and that a major factor in controlling the intracellular content of NAD(P) is hydrolysis catalysed by NAD^+ nucleosidase or NAD^+ ADP-ribosyltransferase (Bender et al. 1982; McCreanor & Bender, 1983, 1986).

Although studies in vivo (Collins & Chaykin, 1972) and with isolated perfused liver (Keller et al. 1971, Grünicke et al. 1974) have demonstrated the incorporation of radioactivity from [^{14}C]niacin into NAD(P), it is apparent from the results reported here that neither nicotinamide nor nicotinic acid is a significant net precursor of nicotinamide nucleotide coenzymes in the liver. Indeed, it seems more likely that under normal conditions the liver oxidizes tryptophan, forming NAD(P) which is then hydrolysed, resulting in release from the liver of significant amounts of niacin. Extrahepatic tissues can and do utilize both nicotinamide and nicotinic acid from the bloodstream as precursors of the coenzymes (Deguchi et al. 1968; Gerbner & Dervo, 1970; Collins & Chaykin, 1972; Lin & Henderson, 1972; Spector, 1979). Indeed, since most tissues lack one or more of the enzymes of the tryptophan oxidative pathway, they are reliant on a source of preformed niacin, which may be either dietary or the result of hepatic tryptophan metabolism.

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