Biochemical Pharmacology, Vol. 21, pp. 2945–2950. Pergamon Press, 1972. Printed in Great Britain.

ACCELERATED TURNOVER RATE OF NICOTINAMIDE–ADENINE DINUCLEOTIDES IN THE LIVER OF RATS INTOXICATED WITH CARBON DISULPHIDE*

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(*Received* 23 *March* 1972; *accepted* 24 *May* 1972)

Abstract—The effects of chronic exposure to CS_2 on turnover rate of nicotinamide–adenine dinucleotides in the liver of the rats were studied. The time course of NAD synthesis was followed using ^{14}C-labelled nicotinic acid and ^{14}C-nicotinamide, as precursors. Chronic exposure to CS_2 was shown to increase the rate of synthesis and degradation of NAD in the liver; indicated clearly with ^{14}C-nicotinic acid as precursor. A similar trend although less evident, has been noticed using ^{14}C-nicotinamide. An increased rate of nicotinamide deamidation observed *in vitro* is compatible with the conclusion of an increased turnover rate of nicotinamide–adenine dinucleotides in the liver of rats chronically exposed to CS_2.

IN PREVIOUS studies[1–3] we have established that chronic exposure to carbon disulphide (CS_2) brings about an increased excretion of the total amount of nicotinamide metabolites in the urine. The hypothesis according to which an increased excretion of these metabolites would result in depletion of tissue nicotinamide–adenine dinucleotides could not be proven experimentally. As previously discovered,[4] chronic exposure to CS_2 does not provoke any significant changes in the levels of these nucleotides in rat tissues.

The aim of the present study was to verify the alternative assumption of an increased metabolic turnover of nicotinamide in the tissues of rats exposed to CS_2. We performed studies on the dynamics of nicotinamide–adenine dinucleotides synthesis in liver using ^{14}C-labelled nicotinamide and nicotinic acid as precursors, and on the rate of nicotinamide deamidation, an intermediate reaction of metabolic conversion of nicotinamide to NAD.

MATERIALS AND METHODS

Animals. The experiments were performed on white female rats of the Wistar strain, 3 months old, of an average weight of 185 ± 15 g at the beginning of experiment. The animals were exposed in an inhalation chamber to CS_2 at a concentration 1·7/1·45–2·05/mg/l of air, 5 hr daily, 6 days per week over a total period of 6 months. The control animals were maintained under the same conditions without exposure to CS_2.

* This investigation has been carried out under the Polish–American Agreement 05-003-3 with the Occupational Health Program, PHS, U.S.A.

After the last daily exposure each rat was injected intraperitoneally with 2 μmoles of nicotinamide-7-C^{14}* or nicotinic acid-7-C^{14}, specific activity 10 mc/mmole.

Extraction and assay of NAD from liver. The first stage of the procedure involving isolation of oxidized nicotinamide–adenine dinucleotides from the liver of rats was carried out according to the method described by Gordon.[5] Immediately after sacrifice, the livers were homogenized in cold 5 %(w/v) trichloroacetic acid and homogenates were subsequently centrifuged. The supernatant was mixed with 10 vol. of cold acetone and the mixture was left overnight at −20° in order to precipitate the nicotinamide dinucleotides. This acetone precipitate was collected by centrifugation, washed with ether and redissolved in water, and the extract containing NAD and NADP was placed on a Dowex 1 × 8 (200–400 mesh) column in the formate form. The nucleotides were eluted from the column with a formic acid gradient as described by Hurlbert *et al.*[6] The eluate was continuously monitored spectrophotometrically for ultraviolet absorption and the fractions corresponding to NAD were combined. The content of NAD in the fractions collected was determined fluorimetrically using the method of Sokal *et al.*[7] For determination of radioactive NAD in the eluate, an aliquots of the same fractions were placed on aluminum plates, evaporated to dryness and the radioactivity was measured in a gas flow counter at infinite thinness. The specific radioactivity of NAD has been expressed in cpm/nmole of NAD.

Assay of nicotinamide amidohydrolase activity. Activity of nicotinamide amidohydrolase was determined in rat liver homogenates *in vitro* by measuring formation of nicotinic acid from nicotinamide according to the isotopic method of Kirchner *et al.*[8] Tissue homogenate was also prepared according to the same authors. The reaction mixture contained in 1 ml 0·13 M = 1 μc nicotinamide-7-C^{14}; 0·05 M potassium phosphate buffer pH 7·4; 5 mg/ml bovine serum albumin and liver homogenate (20 mg of liver protein/ml). Samples were incubated for 2 hr at 38°. The reaction was stopped by addition of 0·04 ml 1 N acetic acid. The mixture was heated for 2 min in boiling water and 4 mM unlabelled nicotinic acid was added as carrier. The blank samples were treated similarly without incubation. The protein precipitate was removed by centrifugation at 3000 rev/min, for 5 min and labelled nicotinic acid was separated from the radioactive nicotinamide using paper electrophoresis according to Sundaram *et al.*[9] The electrophoresis was carried out at room temperature in 0·05 M veronal buffer, pH 8·6, applying a potential difference of 400 V for 1 hr. Nicotinic acid was detected directly by exposing the paper strip to cyanogen bromide vapours. The radioactivity of the spot was measured using a Nuclear Chicago gas flow counter.

Activity of amidohydrolase was expressed as nmoles of nicotinic acid formed from nicotinamide in 60 min under the above conditions, per milligram of liver protein.

Protein was determined by the method of Lowry *et al.*[10] using bovine serum albumin, fraction V, as a standard.

Statistical calculations. The differences in specific radioactivities of NAD between

* Nicotinamide-7-C^{14} sp. act. 59·6 mc/mM and nicotinic acid-7-C^{14} sp. act. 59·1 mc/mM produced by Radiochemical Centre, Amersham, England.

the groups were ascertained by a statistical analysis based on the model of double classification with equal number of observations within subclasses.[11] The Student's *t*-test of significance has been used in statistical analysis of differences in activity of nicotinamide amidohydrolase.

RESULTS

After 6 months of exposure to CS_2 rats displayed symptoms of chronic intoxication such as disturbances in equilibrium, muscular weakness and paresis of the hind limbs.

Nicotinic acid is the precursor most rapidly incorporated into liver NAD.[12] The time course of its incorporation found experimentally is presented in Fig. 1. In both groups of animals (control and exposed to CS_2) maximal incorporation has occurred already at 30 min after injection of the precursor. At the later periods of observations the specific radioactivity of NAD was much lower.

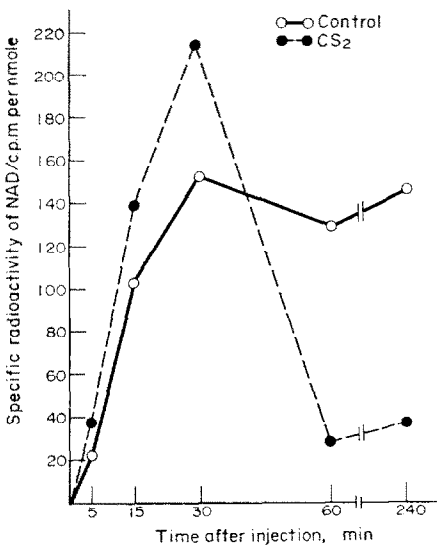

FIG. 1. Time course of incorporation of nicotinic acid into NAD in the liver of rats after intraperitoneal injection of 2 μmoles of ^{14}C-nicotinic acid (total activity 20 μc). Each point represents mean value of three separate determinations.

Within 4 hr of observation of both groups of animals no differences in the concentration of NAD in the liver were noticed. There were distinct differences in the rate of incorporation of nicotinic acid into NAD. Exposure to CS_2 resulted in a markedly higher incorporation of nicotinic acid during the first 30 min after injection of the precursor. The difference between control and exposed animals is statistically highly significant ($P < 0{\cdot}01$).

Also the decay of the specific radioactivity of NAD in the later periods (30–240 min) was faster in rats intoxicated with CS_2.

^{14}C-nicotinamide is less effectively incorporated into NAD than nicotinic acid. Maximal activity of NAD in the liver observed 2 hr after administration of this precursor was three times less than that after nicotinic acid (Fig. 2). Similarly to the

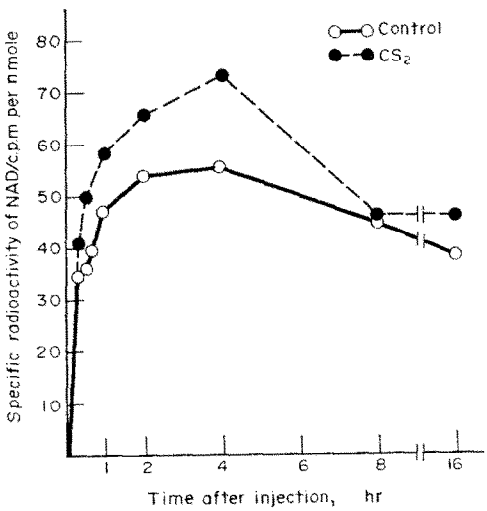

FIG. 2. Time course of incorporation of nicotinamide into NAD in the liver of rats after intraperitoneal injection of 2 μmoles of ^{14}C-nicotinamide (total activity 20 μC). Each point represents mean value of three separate determinations.

experiment with nicotinic acid, there were no changes in the concentration of NAD in both experimental groups and nicotinamide was incorporated more extensively into NAD liver of rats exposed to CS_2. The difference in the time course of incorporation noticed within the first 2 hr is statistically significant. A tendency for a more rapid decrease can be also seen in exposed animals within the later period (2–8 hr) of observations.

TABLE 1. NICOTINAMIDE AMIDOHYDROLASE ACTIVITY IN THE LIVER OF RATS INTOXICATED WITH CS_2

Groups of animals	No. animals	nmole/mg protein/hr	%
CS_2	10	27·0 ± 1·7*	163
Control	9	16·6 ± 2·1	100

* Difference statistically significant in relation to control: $P < 0{\cdot}01$.

Since certain authors[12–15] suggest that nicotinamide is incorporated into NAD after previous deamidation to nicotinate, we have also studied the activity of nicotinamide amidohydrolase in the liver. As seen from the results presented in the Table 1, activity of this enzyme has been found markedly higher (about 60 per cent) in the liver of exposed rats; this difference being statistically significant ($P < 0{\cdot}01$).

DISCUSSION

The increased excretion of N^1-methylnicotinamide is an early and persisting symptom of metabolic disorders in the course of chronic poisoning with carbon disulphide.[1–3] The mechanism which would bring about such disorders has not been known. If one considers the scheme of metabolism of nicotinamide in mammals actually accepted (Fig. 3) one may conclude that disorders in nicotinamide and its catabolites

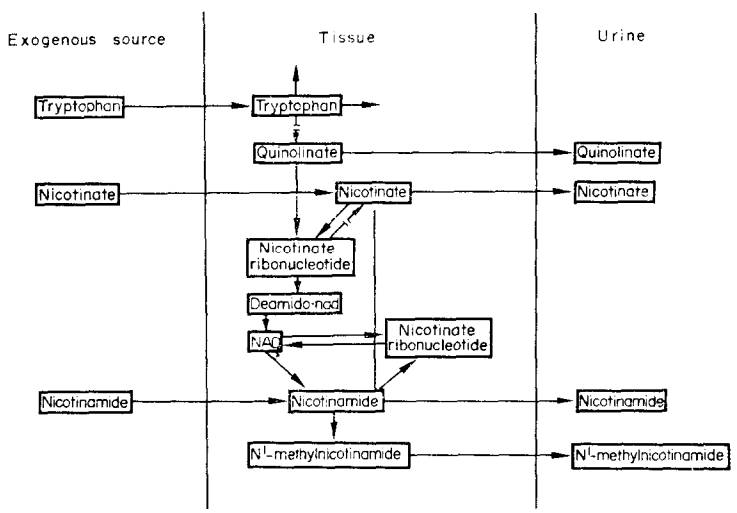

FIG. 3. Metabolism of nicotinamide in the liver according to literature data.[12,13,17–19]

must involve changes in the metabolism of nicotinamide–adenine dinucleotides since this is the only known metabolic pathway for nicotinamide synthesis in the body, and since endogenous formation of nicotinamide from tryptophan must also pass via NAD.[16] However, we have shown in our previous studies that the tissue content of these nucleotides does not change significantly in the course of the chronic CS_2 poisoning.[4] Thus, the changes observed in excretion of nicotinamide metabolites can not be interpreted in terms of depletion of the body stores of nicotinamide dinucleotides.

The results of the present studies have substantiated the alternative assumption of an accelerated turnover of nicotinamide–adenine dinucleotides in animals exposed to CS_2. This conclusion is based on the analysis of the time course of nicotinic acid

incorporation into liver NAD (Fig. 1) where higher rates of both synthesis and degradation of NAD have been found. A similar trend of changes has been noticed when ^{14}C-nicotinamide used as a precursor of NAD, although these data are not as convincing particularly with respect to the rate of NAD degradation.

An increased deamidation of nicotinamide found in the present studies *in vitro* could be considered as another evidence for an accelerated metabolism of nicotinamide dinucleotides synthetized from this precursor. However, because opinions diverge as to the significance of this reaction in NAD synthesis these results must be interpreted cautiously. This reaction has been considered by some authors as an essential step in the process of NAD synthesis in liver[13–15] or in the intestinal wall,[12] while others state that it plays no role *in vivo*.[17]

According to the results of the present investigation, the increased excretion of the nicotinamide metabolites by rats exposed to CS_2[1–3] may result from an accelerated turnover of nicotinamide dinucleotides. However, the question arises as to the endogenous source of the substrate necessary to compensate for the increased rate of this metabolic process, since the uptake of nicotinic acid and nicotinamide from the diet is known to be unchanged in rats exposed to CS_2.[1]

It can be concluded from the Fig. 3 that the only possible explanation seems to involve an increased utilization of metabolites of tryptophan in the direction to NAD. This thesis remains to be experimentally verified.

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