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RELEASE OF CHOLINESTERASE FROM RAT LIVER BY NICOTINAMIDE AND CARBON TETRACHLORIDE

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Abstract—Influence of carbon tetrachloride (CCl_4) and nicotinamide (NA) on the concentration of cholinesterase (ChE) in liver and plasma was studied in normal and ethyl-*p*-nitrophenyl thiobenzene phosphonate (EPN)-treated female rats. The enzyme was assayed by the Warburg manometric technique using propionylcholine iodide as the substrate. The ChE content of liver declined by about 80 per cent in 18 hr after oral administration of CCl_4 (1·25 ml/kg body wt.) to normal rats. The plasma ChE levels increased substantially within 8 hr, but then declined rapidly to reach the basal values within a 24-hr period. NA failed to prevent the CCl_4-induced release of ChE from liver at doses (500 mg/kg body wt., i.p. twice at 8-hr intervals) known to be effective against the hepatotoxic action of CCl_4. Instead, it caused about 50 per cent reduction in the ChE content of liver with the consequent elevation of the plasma enzymic levels. These effects were dose related and reversible. The decline in plasma ChE activity subsequent to EPN treatment was significantly less in NA-treated animals and almost negligible in CCl_4-treated ones. NA had no effect *in vitro* on ChE activity, nor did it reactivate the enzyme inhibited *in vivo* as a result of EPN administration. The protective effect of NA was characterized by a 2- to 3-fold increase in the LD_{50} value and some prolongation of the survival time; whereas, CCl_4, while failing to influence the LD_{50} value, exerted greater effect on the survival time.

THE CENTRILOBULAR necrosis and fatty degeneration of the liver produced by CCl_4[1] are known to be preceded by several biochemical changes, prominent among them being the loss of some enzymes[2–9] and inability of liver parenchyma to synthesize proteins[10] and β-lipoproteins.[11] The mechanism by which these changes are brought about is not known but the necrogenic effects can be delayed or partly prevented by a number of agents.[12–19] On the basis of the observation that promethazine[15, 16] and other antioxidants[17–19] greatly diminished CCl_4-induced necrosis, it has been suggested that peroxidative decomposition of the membrane structural lipids is responsible for the hepatotoxic action of CCl_4.[20–24] Gallagher,[18, 19] however, ascribed the protective effect of nicotinic acid and tryptophan to the accelerated rate of formation of oxidized pyridine nucleotides in the liver.

While studying this phenomenon, we noticed that nicotinamide had no influence on the CCl_4-induced depletion of cholinesterase (ChE) from liver. Rather, when given alone, nicotinamide by itself caused a substantial reduction in ChE content of liver. This observation prompted us to study the action of nicotinamide in more detail and the results are presented here. CCl_4, which has been shown to replenish from liver as much as 25 per cent of the circulating enzyme,[9] was used as the reference compound. These agents were also tested in female rats treated with the anti-ChE agent ethyl-*p*-

nitrophenyl thiobenzenephosphonate (EPN), and their plasma ChE levels were determined thereafter. The protective effect of nikethamide against EPN toxicity in atropinized female rats[25] is considered to be exerted through nicotinamide, a metabolite of nikethamide.[26]

MATERIALS AND METHODS

Female albino rats (150–250 g) were maintained on purina chow diet and water *ad lib.* Blood samples were withdrawn under light ether anesthesia by cardiac puncture, using heparinized syringes and centrifuged at 4° for 15 min at 3200 *g* to obtain plasma. Liver samples were removed, rinsed in chilled saline, weighed and homogenized for 2 min at 4° in 0·025 M $NaHCO_3$ buffer (50 mg of tissue/ml) with a Sorvall omnimixer at top speed. The samples of plasma and homogenates were appropriately diluted with the buffer and the ChE activity determined by Ammon's manometric technique[27] as described for red cell ChE.[28] Propionylcholine (iodide), freshly prepared in buffer, was used as the substrate (final concentration, 0·01 M). The activity of ChE is expressed in μl CO_2 evolved in 1 min at N. T. P. per liver per 100 g of body wt., or per g of tissue.

Protein content of liver was determined by the method of Lowry *et al.*[29] with crystalline bovine albumin as the reference standard. For histological assessment of liver, specimens were fixed in formol-saline and stained with hematoxylin-eosin according to the standard laboratory technique.

The following experiments were done: (a) CCl_4 mixed with an equal volume of peanut oil was given orally to unanesthetized animals in a dose of 1·25 ml/kg body wt. Control animals were given an equivalent volume of peanut oil. The concentration of ChE and the protein content of liver were determined at 0-, 2-, 5- and 18-hr intervals. (b) Nicotinamide, dissolved in distilled water (125 mg/ml), was injected i. p. (500 mg/kg body wt.) and 8 hr later a similar dose of nicotinamide was repeated; the second was immediately preceded by an administration of CCl_4 or oil. Control animals received appropriate amounts of saline and peanut oil. Estimations of ChE and liver protein were done 18 hr after the second injection. (c) In another set of experiments, nicotinamide was injected i. p. at dose levels of 250, 500 and 1000 mg/kg body wt. and the enzymic activity was estimated 18 hr later. In chronic studies, nicotinamide was given in a dose of 500 mg/kg body wt. once daily for 14 days to determine the long-term effect on the enzymic activity and protein content of liver. (d) Oral LD_{50} for EPN, using peanut oil as the vehicle, was determined in nicotinamide-treated and untreated animals by the method of probits.[30] Nicotinamide, 500 mg/kg body wt., was injected twice at 5 hr intervals, the second injection being accompanied with oral administration of EPN. The LD_{50} value was similarly determined in rats which had received a single dose of CCl_4 5 hr earlier. Control animals received peanut oil or saline *in lieu* of CCl_4 or nicotinamide respectively. (e) A group of animals was treated as in (d) and the plasma levels of ChE were determined 2 hr after EPN administration (15 mg/kg body wt.). The degree of ChE inhibition induced by EPN under various treatments was expressed as per cent of enzymic activities determined before the start of the experiments. Blood was obtained from the tail vein for the first determination. (f) To study the effects *in vitro* of nicotinamide on the ChE activity, aliquots (2·0 ml) of liver homogenates prepared as above, or plasma samples, were incubated in duplicate at 37° for 1 hr together with 1·0 ml of saline containing varying amounts of nicotinamide (final concentrations ranging from 0–10 mM). The samples were then appropriately diluted

and the enzymic activity determined as before. In another series of experiments, blood samples from EPN-treated animals were obtained as soon as the toxic symptoms appeared and treated as above in order to determine if the inhibited enzyme could be reactivated by nicotinamide.

The significance between means was determined by the Student's *t*-test.

RESULTS AND DISCUSSION

Preliminary experiments were conducted to determine the degree of alteration induced in the hepatic levels of ChE by intragastric administration of CCl_4 (Table 1).

TABLE 1. EFFECT OF CCl_4 ADMINISTRATION ON THE HEPATIC LEVELS OF CHOLINESTERASE (ChE) IN RATS*

Time (hr)	Liver ChE	Liver weight (g/100 g body wt.)	Protein content (g/100 g wet liver wt.)
0	543 ± 153	3·76 ± 0·32	16·4 ± 0·7
2	446 ± 128	3·55 ± 0·19	16·3 ± 0·3
5	452 ± 134	3·51 ± 0·38	15·6 ± 0·5
18	120 ± 54†	3·56 ± 0·27	16·5 ± 0·5
After 10 days	528 ± 147	3·67 ± 0·39	

* The values are the mean ± S. D. from five rats. The ChE activity is expressed as μl CO_2 per liver per 100 g body wt. in 1 min. Dose of CCl_4:peanut oil mixture (1:1; v/v) was 2·5 ml/kg body wt. given orally by gastric tube.
† $P < 0{\cdot}001$.

Since no significant change in the protein content of liver was noticed during the first few hours, which is in agreement with earlier studies,[31] the concentration of ChE was calculated in terms of either the total liver activity per 100 g body wt. or as activity per g of tissue. The results show that the enzymic levels declined markedly 18 hr after the administration of CCl_4 but then returned to the normal values within 10 days of the cessation of treatment. Corresponding with these changes, the enzymic activity of plasma increased substantially (about 60 per cent; $P < 0{\cdot}01$) in about 8 hr and then declined rapidly reaching the normal levels within 24 hr. The elevation of plasma per liver ChE ratio (from 0·55 ± 0·19 to 2·05 ± 0·86; $P < 0{\cdot}01$), which was determined 18 hr after the administration of CCl_4, could thus be attributed to the low concentration of ChE in liver rather than to any change in the plasma enzymic levels.

Our results show that pretreatment of animals with nicotinamide did not significantly affect the extent of CCl_4-induced depletion of ChE from liver (Table 2, Nos. 2 and 4). Rather, when given alone at the same dosage level, nicotinamide produced similar changes in the enzymic concentration, although these were less severe (Table 2, No. 3). In agreement with earlier studies,[32] a pronounced fall in the weight of liver was noticed in the nicotinamide-treated animals. The CCl_4-treated rats which did not show any significant change in the liver weight were equally affected when subjected to the joint treatment. None of the treatments produced any significant change in the gross protein content of liver as estimated by the method of Lowry *et al.*[29]

Since nicotinamide is used therapeutically in fairly large doses for prolonged periods,

TABLE 2. EFFECTS OF NICOTINAMIDE ON THE HEPATIC LEVELS OF CHOLINESTERASE (ChE) IN NORMAL AND CCl_4-TREATED RATS*

No.		Treatment	Time of injection (hr)	Liver ChE (μl CO_2/liver/ 100 g body wt. in 1 min.)	Liver weight (g/100 g body wt.)	Protein content (g/100 g wet liver wt.)
1.	I	Saline	0			
	II	Saline + peanut oil	8	510 ± 179	3·76 ± 0·35	16·5 ± 0·3
2.	I	Saline	0			
	II	Saline + CCl_4 in oil	8	116 ± 45†	3·50 ± 0·25	16·3 ± 0·6
3.	I	Nicotinamide	0			
	II	Nicotinamide + oil	8	250 ± 55‡	3·16 ± 0·29‡	16·9 ± 0·7
4.	I	Nicotinamide	0			
	II	Nicotinamide + CCl_4 in oil	8	103 ± 22†	3·04 ± 0·29‡	17·3 ± 0·4

* The values are the mean ± S. D. from 7 rats. The ChE activity was determined 18 hr after treatment II. Dose of nicotinamide, 500 mg/kg body wt., i.p.; dose of CCl_4:peanut oil mixture (1:1, v/v), 2·5 ml/kg body wt. *per os*.
† $P < 0·001$.
‡ $P < 0·01$.

TABLE 3. EFFECT OF CHRONIC ADMINISTRATION OF NICOTINAMIDE ON THE HEPATIC LEVELS OF CHOLINESTERASE (ChE) IN RATS*

Days	ChE activity (μl CO_2 evolved/min/g wet liver wt.) Control	Treated	Per cent decrease
3	86 ± 27	70 ± 21	19
6	104 ± 11	79 ± 15†	24
13	109 ± 24	63 ± 12‡	42

* The values are the mean ± S. D. for five rats. Dose of nicotinamide, 500 mg/kg body wt., i.p. once daily.
† $P < 0·05$.
‡ $P < 0·001$.

its long-term influence on the hepatic levels of ChE was determined (Table 3). As a continued increase in the activity of several enzymes occurs in postnatal rat extending into adulthood,[33] each nicotinamide-treated group was accompanied with a control group receiving saline for the same duration. A progressive decline in the concentration of ChE (expressed per g of tissue) occurred as a result of the treatment; control animals, on the other hand, showed an approximate 25 per cent increase in the enzymic activity. The dose–response relationship, both in respect to the enzymic concentration and liver weight was revealed in acute experiments where nicotinamide was injected twice (d.i.) at 8-hr intervals (Table 4). Single injections (s.i.), however, were effective only at 1 g/kg dose level. Upon cessation of treatment, the ChE content of liver returned to the normal levels within a week. Histological examination of livers obtained from animals treated acutely or chronically with nicotinamide did not reveal any cellular damage.

As observed in earlier studies,[26] rats could be protected against EPN-poisoning by

pretreatment with nicotinamide (Table 5). The protective effect was characterized by a 2-fold increase in the LD_{50} value for EPN and a marked prolongation of the median death time. CCl_4 had no influence on the mortality rate, though the survival time was markedly prolonged.

In view of the earlier observation that nicotinamide and CCl_4 release substantial amounts of ChE from liver, the plasma enzymic levels were determined in these animals 2 hr after the administration of EPN (15 mg/kg body wt.). The results (Fig. 1) showed

TABLE 4. EFFECTS OF VARYING DOSES OF NICOTINAMIDE ON WEIGHT AND CHOLINESTERASE (ChE) ACTIVITY OF RAT LIVER*

Dose (mg/kg)	Activity/g of liver		Wet liver wt./100 g body wt.	
	s.i.	d.i.	s.i.	d.i.
0	93 ± 29	106 ± 24	3·78 ± 0·46	4·31 ± 0·16
250	95 ± 25	71 ± 19	3·66 ± 0·29	4·17 ± 0·33
500	86 ± 29	61 ± 21†	3·56 ± 0·35	3·91 ± 0·23†
1000	51 ± 17†	37 ± 13‡	3·05 ± 0·35†	3·71 ± 0·16‡

* The values are the mean ± S. D. from five rats. Nicotinamide was injected i.p. once (s.i.) or twice (d.i.) at 8-hr intervals, and the ChE activity, expressed in μl CO_2 evolved in 1 min, was determined 18 hr later.
† $P < 0{\cdot}05$.
‡ $P < 0{\cdot}001$.

TABLE 5. EFFECTS OF NICOTINAMIDE AND CCl_4 AGAINST ETHYL-*p*-NITROPHENYL THIOBENZENE PHOSPHONATE (EPN)-POISONING IN FEMALE RATS*

Treatments	LD_{50} (mg/kg)	Approx. survival time (hr)
Saline + EPN (control)	11·5 (8·2–14·1)	4½
Nicotinamide + EPN	27·5 (22·4–33·5)	7
Peanut oil + EPN (control)	12·0 (8·4–16·0)	4½
CCl_4 + EPN	13·0 (9·1–17·3)	37

* For the experimental details see Methods under (d). No. of animals: thirty in each test. Ninety-five per cent confidence limits in parentheses.

that the decline in plasma ChE activity subsequent to EPN-treatment was significantly less in nicotinamide-treated animals and almost negligible when CCl_4 was used as the test substance. Nicotinamide and CCl_4 given alone caused some 15 to 20 per cent increase in the plasma ChE activity. It may be mentioned that the withdrawal of blood (1·5 to 2·0 ml) for determining the pretreatment values of ChE resulted in an 8 to 9 per cent decline in the enzymic levels (probably because of the subsequent dilution of blood) and the results presented in Fig. 1 are corrected for this change. Experiments *in vitro* showed that nicotinamide, in concentrations ranging from 0·1 to 1 mM, had no influence on the activity of ChE nor did it reactivate the enzyme inhibited *in vivo* as a result of EPN administration.

The results presented here show that CCl_4 caused depletion by nearly 80 per cent of

the preformed stores of ChE from liver with the consequent elevation of plasma ChE activity. Similar changes were observed after nicotinamide administration, although these were less severe. The increase in plasma ChE activity in both instances, however, was only transient. Slater and Greenbaum[31] observed similar changes in the serum activity of β-glucuronidase after the administration of CCl_4.

The manner in which nicotinamide affords protection against EPN toxicity is not clear. Rosenberg and Coon[26] demonstrated that prior treatment of rats with nicotinamide resulted in a marked protection of tissue ChE (brain, heart, lung and diaphragm)

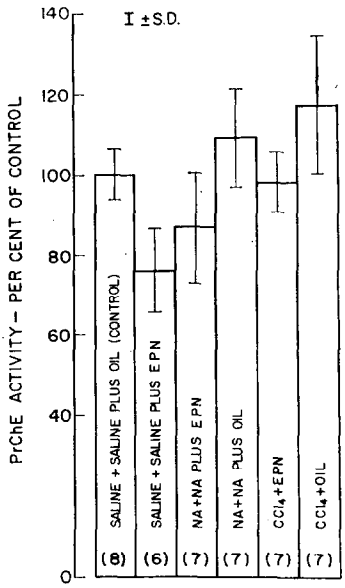

FIG. 1. Influence of nicotinamide and CCl_4 on plasma levels of ChE in EPN-treated and untreated female rats. Dosages: Nicotinamide (NA), 500 mg/kg body wt., i.p.; CCl_4, 2·5 ml of 1:1 (v/v) mixture in peanut oil per kg body wt. *per os.*; EPN, 15 mg per 4 ml peanut oil per kg body wt. *per os.* plus sign denotes an interval of 5 hr. Blood samples were obtained 2 hr after the second treatment. For other experimental details see under Methods (e). Figures in parentheses represent the number of animals. Control vs. EPN, $P<0{\cdot}001$; Control vs. NA + NA plus EPN, $0{\cdot}05>P>0{\cdot}01$; EPN vs. NA + NA plus EPN, $0{\cdot}1>P>0{\cdot}05$; EPN vs. CCl_4 + EPN, $0{\cdot}01>P>0{\cdot}001$; Control vs. CCl_4, $P<0{\cdot}01$.

against inhibition after EPN administration. Their experiments *in vitro* and our own, however, have shown that nicotinamide has no direct action on ChE nor does it reactivate the enzyme inhibited as a result of EPN administration. It is difficult to ascribe the observed protective effect *in vivo* of nicotinamide to the elevated levels of plasma ChE. First, because the extent of the increase in plasma enzyme, which was observed, was so small and transient in nature that it seems very unlikely to be of any pharmacological significance. Second, the important acute toxic actions of anti-ChE agents are caused by the accumulation of acetylcholine in the vicinity of nerve terminals (both central and peripheral) as a result of the inhibition of acetyl-ChE.[34] However, Douglas and Paton[35] have suggested that inhibition of blood ChEs may result in the elevation of the levels of the circulating acetylcholine and thereby contribute to the discrete effects composing the syndrome of anti-ChE poisoning.

The observation that nicotinamide increases both the LD_{50} value of EPN and the survival time, whereas CCl_4 prolongs only the survival time, suggests that their mode of action is different and may be quite unrelated to the release of ChE from liver. It is well established that EPN, which is nontoxic by itself, must undergo conversion to its oxygen analogue before any adverse effects become apparent.[36-39] The enzymes responsible for its conversion and the subsequent breakdown to *p*-nitrophenol reside in the microsomes of the liver[40] which are vulnerable to the disruptive action of the hepatotoxin.[3, 41, 42] The logical explanation for the prolongation of survival time after CCl_4 administration, therefore, seems to be the failure of liver microsomal enzymes to effect conversion of the nontoxic EPN to the toxic form.

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