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BIOCHEMICAL EFFECTS OF ACRYLONITRILE ON THE RAT LIVER, AS INFLUENCED BY VARIOUS PRETREATMENTS OF THE ANIMALS

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SUMMARY : Acrylonitrile is extensively used in the synthesis of plastics, some of which are used for the packaging of food and beverage. A single dose of AN causes an increase in serum lactate and sorbitol dehydrogenase levels, as well as a decrease of liver cytochrome P-450 content and microsomal aldrin oxide synthetase activity. Those effects are prevented by pretreatments of the animals with either inducers of the mixed function oxidases or L-cysteine ; diethylmaleate pretreatment enhances the increase of the soluble enzyme levels.

Acrylonitrile (CH_2=CH-CN, vinyl cyanide) (AN) is extensively used in the manufacture of synthetic fibers and resins, as an intermediate in the synthesis of pharmaceuticals, dyes and antioxidants and as a grain and tobacco fumigant.
Total world production of acrylonitrile is estimated to have been about 2,400 million kg in 1976.
Despite the large-scale use of AN, very few data exist on its toxicologic properties as well as on its metabolism.
Acute experiments have shown that high doses of AN produce adrenocortical insufficiency, toxicity to the central nervous system and congestive lung oedema [1-4], and a rapid decrease in reduced glutathione in the liver, lung, kidney and adrenal [5,6].
Those toxic symptoms can be prevented by pretreatment of the animals with either phenobarbital [7] or L-cysteine [8].
AN is partially metabolized in the organism to cyanide, which is then transformed to thiocyanate and eliminated in the urine ; however, the fate of the remaining fraction of AN is not yet clear and it is generally considered that the toxicity of AN

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Abbreviations
AN, acrylonitrile ; PB, phenobarbital ; DEM, diethylmaleate ; 3-MC, methyl-3-cholanthrene ; MFO, mixed function oxidase.

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is due not only to the hydrogen cyanide liberated during its metabolism but also either to acrylonitrile itself or to some unknown metabolite [9]. Moreover, evidence has been obtained that AN was mutagenic towards several Salmonella typhimurium strains in the presence of a 9,000 g supernatant of liver from mice and rats [10-11] as well as towards some Escherichia coli strains, but without metabolic activation system [12].

An interim report concerning ongoing 2 years study incorporating AN in drinking water of rats indicates that a proportion of the animals administered AN developed squamous-cell papillomas of the forestomach, Zymbal gland carcinomas and microgliomas of the CNS [13].

More recently, O'Berg [14] reported preliminary results of an epidemiologic study indicating a significantly increased incidence of lung and large intestine cancer among AN exposed workers.

In order to get further insight into the possible mechanisms of biological action of AN, we investigated the effects of the chemical on some relevant liver enzymatic systems and analysed the influence on those parameters, of pretreatment of the animals with agents able to modify its biochemical effects.

MATERIALS AND METHODS

Chemicals : AN (purity 99.5 %) and propionitrile (purity 99.0 %) were obtained from Aldrich, Belgium. Aldrin, Dieldrin and Endrin were generously provided by the Shell Co, the Netherlands. All other commercial products were of the purest grade available.

Animals and pretreatments : Adult male Wistar rats (230-270 g) were fed ad libitum and fasted 24hrs before sacrifice. They were treated with intraperitoneal doses of 3, 10 and 30 mg/kg AN in 0.9 % NaCl and killed by decapitation after various times. PB (2 x 75 mg/kg, i.p.) and 3-MC (2 x 40 mg/kg, i.p.) were administered 48 and 24hrs before treatment. Arochlor 1254 (60 mg/kg, i.p.) was injected 5 days before treatment.
L-cysteine (500 mg/kg, per os) and DEM (0.6 ml/kg, i.p.) were administered 5 min. and 30 min.respectively before AN administration.

Microsomal fractions : Liver microsomes were prepared as described by de Duve et al [15]. Protein concentrations were determined by the method of Lowry et al., using bovine serum albumin as a standard [15].

Enzyme assays
Serum glutamic-oxalacetic transaminase (SGOT), glutamic pyruvic transaminase (SGPT), lactate dehydrogenase (LDH) and sorbitol dehydrogenase (SDH) activities were assayed with Boehringer reagent kits.
The content of cytochrome P-450 was evaluated using the procedure of Raj and Estabrook [17] ; glucose-6-phosphatase was assayed by the method of de Duve et al [15], N-acetyl-β-glucosaminidase

TABLE 1. Effects of AN (10 mg/kg, i.p.) on marker enzymes of the endoplasmic reticulum, at different times after injection.

Enzyme	Control	1/2hr	2hr	4hr	6hr	12hr
Glucose-6-phosphatase (U/g liver)	10.5±2.6	10.8±0.5	7.8.±0.8	7.5.±2	7.9.±0.9	7.3.±0.7
NADPH:cyt c reductase (U/g/liver)	2.3±0.8	2.9±0.4	1.8±0.3	1.9±0.4	3.4±0.4	3.1±1.2

All values are the means of 9 animals ± s.d.

according to Sellinger et al [18], sulfite:cytochrome c reductase according to Wattiaux-Deconinck and Wattiaux [19] and NADPH:cytochrome c reductase by the method of Hogeboom and Schneider [20]. Aldrin oxide synthetase activity was evaluated by the determination of the quantity of dieldrin formed when microsomal preparations were incubated with aldrin, according to the method described by Dubois-Krack et al.[21].
The quantitative estimation of the lipid peroxidation in vivo has been determined on the microsomal fractions, as described by Klaassen and Plaa [22].

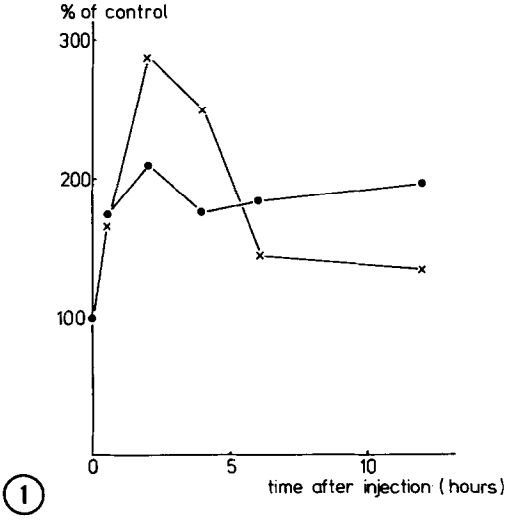
Results and discussion

Results presented in Table 1 demonstrate that the activities of two marker enzymes of the endoplasmic reticulum : glucose-6-phosphatase and NADPH:cyt c reductase are somewhat lowered by treatment of the animals with 10 mg/kg AN ; however, these differences are statistically not significant.
Moreover, as indicated by the absence of effect on the ratio of free to total activities of sulfite:cyt c reductase (marker enzyme of the intermembranar space of mitochondria) and N-acetyl-β-glucosaminidase (marker enzyme of lysosomes) respectively, the treatment does not affect the integrity of these subcellular organites (Table 2).
Animals administered AN show no change in SGOT and SGPT levels as well as no significant lipid peroxidation phenomenon as demonstrated by the very low amount of conjugated dienes formed in the microsomal fraction (not shown) ; however, LDH and SDH levels are increased, and reach the highest values 2 hrs after treatment (Fig.1).

TABLE 2 : Effects of AN (10 mg/kg, i.p.) on the integrity of subcellular structures, at different times after injection*.

Enzyme	Control	1/2hr	2hr	4hr	6hr	12hr
N-acetyl-β-glucosaminidase (% of free activity)	11.3±3.7	16.7±1.9	15±3.6	12.3±3.6	13±2.3	13.7±2.5
Sulfite:cyt c reductase (% of free activity)	17.7±4.5	16.9±1.4	17.4±5.5	18.7±6.1	22.1±6.2	19.2±3.7

Values are the means of 9 animals ± s.d. and are determined by calculating the ratio of the free enzymatic activity (in isotonic medium) over the total enzymatic activity (in the presence of 0.1 % Triton X 100).



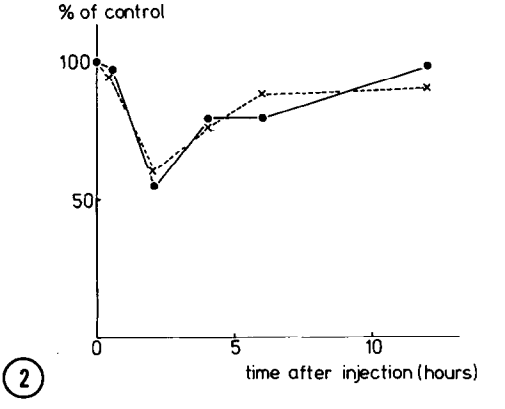


FIG 1. : Time-dependent effect of AN (10 mg/kg) on SDH (×——) and LDH (●——) levels. Expressed as % of control ; each point represents the mean of 9 animals.

FIG 2. : Time-dependent effect of AN (10 mg/kg) on cyt-P450 amount (X-----) and aldrin oxide synthetase activity (●——) Expressed as % of control ; each point represents the mean of 9 animals.

TABLE III : Effects of different doses of AN on LDH and SDH activities, 2 hours after treatment.

Dose of AN (mg/kg)	LDH (U/ml)	SDH (U/ml)
-	0.14 ± 0.03	0.48 ± 0.13
3	0.20 ± 0.05	0.85 ± 0.27
10	0.29 ± 0.08(a)	1.39 ± 0.63(a)
30	0.43 ± 0.08(a)	2.32 ± 0.80(a)

Values are the means of at least 3 animals ± S.D.
(a) Significantly different from control rats (P = 0.05).

The elevation of LDH and SDH levels is dose-dependent (Table 3) and suppressed by pretreatments of the animals with either inducers of the cyt P450 dependent MFO (PB, 3-MC and Arochlor 1254) or L-cysteine (Table 4). Surprisingly however, treatment with AN inhibits the LDH and SDH increases observed when rats are pretreated with 3-MC.

Pretreatment with DEM, which depletes glutathione levels, reinforced the effect of AN on both enzymes.

As indicated in Fig.2, AN treatment significantly diminished cytochrome P-450 contents as well as the activity of aldrin oxide synthetase ; the effect was most pronounced after 2 hours and disappeared rapidly ; it was not dose-dependent and was prevented by pretreatment with PB, 3-MC, Arochlor 1254 or L-cysteine ; however, DEM did not reinforce these decreases.

Contrarily to what we observed with several chemical carcinogens such as benzo(a)pyrene and 3-MC, as well as with styrene, treatment with AN did not significantly decrease the K_M of aldrin oxide synthetase [23].

Additional experiments have shown that the changes in SDH and LDH levels produced by 10 mg/kg AN could equally been observed when the animals were treated with 10 mg/kg propionitrile (CH_3-CH_2-CN) but that this chemical had no effect either on cytochrome P-450 content or on aldrin oxide synthetase activity ;

TABLE 4 : Influence of several pretreatments on the effects of AN (10 mg/kg, i.p., 2hr) on LDH and SDH levels.

Pretreatment of rats	LDH (U/ml)	SDH (U/L)
AN	0.29 ± 0.08	1.39 ± 0.63
PB	0.17 ± 0.03	0.88 ± 0.13
PB + AN	0.19 ± 0.40	0.72 ± 0.06
3MC	0.46 ± 0.10	1.15 ± 0.13
3MC + AN	0.19 ± 0.01	0.46 ± 0.20
Arochlor 1254	0.13 ± 0.03	0.50 ± 0.07
Arochlor 1254	0.18 ± 0.06	0.50 ± 0.10
L.Cystéine	0.13 ± 0.02	0.45 ± 0.09
L.Cystéine + AN	0.16 ± 0.04	0.47 ± 0.15
DEM	0.14 ± 0.03	0.51 ± 0.14
DEM + AN	0.48 ± 0.20(a)	5.9 ± 0.90(a)

Values are the means of 3 animals ± S.D.
(a) Significantly different from control (p = 0.05)

those data indicate the importance of the vinylic double bond of AN in the mechanism of its reactivity towards cytochrome P-450 ; this phenomenon has previously been observed with several other vinylic compounds, such as vinyl chloride, which has also been shown to deplete the cytochrome P-450 content in the liver [24]., but the effect was more persistent
The protective role exerced by the inducers of the MFO on the hepatic effects induced by AN have to be correlated with the observations [7] that adrenal lesions and mortality induced by AN can be prevented by the previous administration of phenobarbital to rats. Further studies will be needed to verify whether the prevention of AN-induced toxic effects are caused by an increased biotransformation of AN itself or one of its toxic intermediates, e.g. an epoxide, into inactive metabolites.

Lack of effect of phenobarbital on the AN-thiocyanate balance has been shown by Gut et al [25].
AN has been shown to deplete glutathione stores in the liver, lung and kidney [5,6] ; therefore, the reduction of the effects of AN by L-cysteine and the enhancement on the SDH and LDH levels by DEM can be easily understood, if one considers the protective effect exerced by this endogenous nucleophilic thiol compound against chemical injury by electrophilic species.
The absence of influence of DEM towards the cytochrome P-450 and aldrine oxide synthetase changes induced by AN remains to be explained.

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TABLE III

Effect of cycloheximide and trypsin (pretreatment) on the synthesis of the collagenase inhibitor by smooth muscle cell cultures

Conditions	Units per dish#
Control	74
plus 2μM cycloheximide	11
trypsin treated control*	76

1 unit of collagenase inhibitor is the amount of dialyzed medium required for 50 percent inhibition of fibroblast collagenase (2.8μg protein). This presumptive unit is determined by titration with fibroblast collagenase.

* Cell sheet was pretreated with 0.05% trypsin in 0.02% EDTA for 1 min. The cell sheet was then rinsed with phosphate buffered saline and serum-free medium was added.

thesized, de novo, by smooth muscle cells, replicate dense cultures were exposed to serum-free medium containing 2μM cycloheximide. After 16 hours, the media from control and cycloheximide treated cells were harvested, concentrated by ultrafiltration and dialyzed against 50mM cacodylate buffer, pH 7.0, containing 5mM $CaCl_2$. The media were analyzed for their inhibitory activity. Table III indicates that in the presence of cycloheximide, the amount (units) of inhibitor in the medium was greatly reduced as compared to control media. In separate experiments, the cell sheet of dense smooth muscle cell cultures was pretreated briefly with 0.05 percent trypsin in 0.02 percent EDTA to remove serum proteins bound non-specifically to the cell surface. The trypsin was removed and the cell sheet was rinsed with phosphate buffered saline. The dishes were supplemented with serum-free medium. After 16 hours, the medium was harvested and assayed for the inhibitor (Table III). The amount of inhibitor in the medium of trypsin treated cultures was similar to that of control. All of the above obser-