Biochemical Pharmacology, Vol. 27, pp. 1135–1138.

0006-2952/78/0415-1135$02.00/0

# LIBERATION OF CYANIDE FROM SUCCINONITRILE
## 2. THE EFFECT OF ETHANOL

ANNA RITA CONTESSA, MAURA FLOREANI, ANNA CHIARA BONETTI and RENATO SANTI
Institute of Pharmacology, University of Padua, Largo E., Meneghetti 2, Padua, Italy

(*Received* 13 *May* 1977; *accepted* 10 *October* 1977)

**Abstract**—The influence of drug treatment on $CN^-$ release from DNS both *in vivo* and *in vitro* has been investigated. Treatment of rats with phenobarbital, 3–4 benzopyrene did not modify cyanide liberation from DNS.$CCl_4$ administration almost completely abolished the generation of $CN^-$ and consequently strongly decreased the acute toxicity of DNS. On the contrary, acute or chronic ethanol treatment significantly increased $CN^-$ formation from DNS in rat liver slices, increasing the toxicity of the drug. Thus ethanol administration to mice lowered the $LD_{50}$ i.p. from 63 mg/Kg to 38 mg/Kg. These results suggest that the considerable increase of DNS toxicity is due to a stimulation of cyanide release and a substrate limitation incurred in detoxification.

We have demonstrated previously both *in vivo* and *in vitro* that succinonitrile (DNS), a widely employed antidepressant, released cyanide in the liver of rats and rabbits, and that the cyanide was subsequently converted by rhodanese to thiocyanate [1–3]. About 70 per cent of the DNS administered *in vivo* is recovered in the urine as thiocyanate. Cavanna and Pocchiari obtained similar results upon the administration to mice of $^{14}$C-DNS [4]. Similarly, Lodi *et al.* have presented evidence in humans indicating that the main route of DNS metabolism led to the formation of cyanide, since over 90 per cent of the equivalent DNS given to the subjects appeared as urinary thiocyanate [5, 6]. Recently, Curry has studied the kinetics of excretion for labelled DNS and its metabolic products in the mouse, and has found all of the administered drug modified within 24 hr, yielding thiocyanate in greatest amount [7].

Since all authors concur in that thiocyanate excretion is commeasurate with the amount of DNS administered, the idea is reasonable or even likely that DNS toxicity depends upon the relative rates of two essentially distinct hepatic processes: upon the rate of cyanide liberation relative to the rate of its conjugation with sulfur.

The purpose of this study has been to establish *in vivo* and *in vitro* if these two metabolic processes could be altered and a consequently modified toxicity of the drug found at therapeutic dosages. For this purpose, ethanol, phenobarbital, and $CCl_4$ have been employed due to their well known effects on hepatic drug metabolism.

A preliminary report of this study has been presented at Congress of Società Italiana di Farmacologia, Roma, 5–6 ottobre 1973.

* The animals drank about 15 ml of 20% (v/v) ethanol per diem, equal to 3 ml absolute ethanol.

### MATERIALS AND METHODS

Male albino Wistar rats (body wt 250–300 g) and white male mice (body wt 25–30 g) maintained on laboratory diet have been used. Ethanol has been administered cronically as drinking fluid *ad libitum* (concentration 20% v/v) up to the time of DNS treatment or sacrifice.

The urine was collected from groups of three rats before and after DNS and the thiocyanate excreted was determined by the method of Rosenthal *et al.* [8] with the following modification: 0.5 ml of urine was diluted with 4.5 ml ethanol 95 per cent. After centrifugation, an aliquot of supernatant (generally 0.5–1 ml) was made up to 8 ml with $H_2O$ and 2 ml of ferric nitrate solution was added. The resulting color intensity was measured at 450 nm.

The $LD_{50}$ was determined according to Weil [9] using four dose levels of DNS and five animals each level. Mortality was determined 72 hr after drug administration.

*Assays.* 500 mg of liver slices, prepared as previously described were incubated in 4 ml Krebs–Ringer phosphate buffer pH 7.4 containing: 1.25 mM DNS plus 1.25 mM Na-thiosulfate or 2.5 mM aniline or 2.5 mM aminopyrine plus 2.5 mM semicarbazide [3]. The aerobic incubation was carried out for 2 hr in a Dubnoff shaker at 37°.

Thiocyanate formation was estimated as previously described [3]. The *p*-hydroxylation of aniline was determined according to Imai *et al.* [10] and the *N*-demethylation of aminopyrine was measured by the method of Nash [11] as modified by McLean and Day [12].

### RESULTS

*In vivo.* Pretreatment of mice with either $CCl_4$ or ethanol powerfully affected the acute toxicity of DNS. Table 1 shows that $CCl_4$ increased the $LD_{50}$ from 63 to over 303 mg DNS per Kg, and conversely, prolonged pretreatment with ethanol decreased

Table 1. The effect of ethanol and $CCl_4$ on acute toxicity of DNS in mice

| Drug | $LD_{50}$ mg/Kg i.p. | Confidence interval mg/Kg |
|---|---|---|
| — | 63.11 | 49.17 to 80.97 |
| 1. Ethanol | 38.12 | 29.4 to 48.78 |
| 2. $CCl_4$ | >303.00 | — — |

1. Drinking fluid 25% (v/v) for 2 months.
2. $CCl_4$ 2 ml/Kg in corn oil s.c. 24 hr before DNS administration.

it by 40 per cent. These results suggest that the toxicity of the drug is linked to the appearance of cyanide, its major metabolic product, rather than to the presence of DNS *per se*. That is, DNS toxicity is increased by ethanol because of increased metabolism, while $CCl_4$ treatment decreased metabolism and toxicity of DNS.

Figure 1a shows the effect of $CCl_4$ on the urinary excretion of $SCN^-$ subsequent to DNS administration. It is well known that cyanide is liberated from precursor compounds in small part in the pulmonary route, most of it being conjugated with sulfur and then excreted in the urine. Therefore, the lack of $SCN^-$ excretion after DNS administration to $CCl_4$ pretreated animals, in conjunction with the diminished DNS toxicity is consistent with the idea that $CCl_4$ blocks cyanide formation from DNS. However, the latter inhibition clearly can be ascribed to a blockage of $CN^-$ formation from DNS, because rhodanese activity tested in the animals pretreated with $CCl_4$ have consistently yielded normal values.

The DNS linked elevation of urinary thiocyanate excretion was not significantly affected by ethanol pretreatment. Figure 1b shows that the ethanol pretreated animals excreted less thiocyanate than did controls, and that augmented $SCN^-$ excretion occurred (as in controls) upon the simultaneous administration of ethanol + sodium thiosulfate. (The latter has no effect on the urinary $SCN^-$ excretion of control animals). These results appear not to be in line with the toxicity elevated upon ethanol

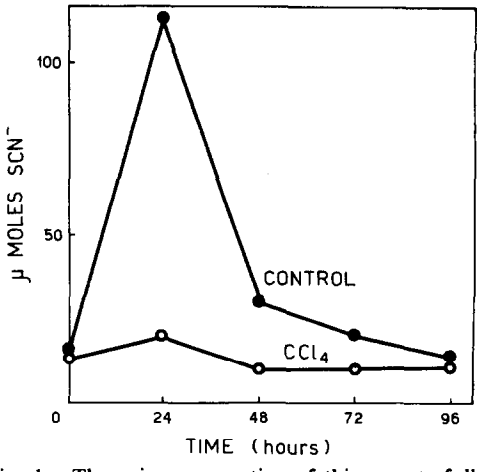


Fig. 1a. The urinary excretion of thiocyanate following i.p. administration of 50 mg/Kg DNS to the rat. Effect of $CCl_4$ 2 ml/Kg s.c. treatment 24 hr before DNS.

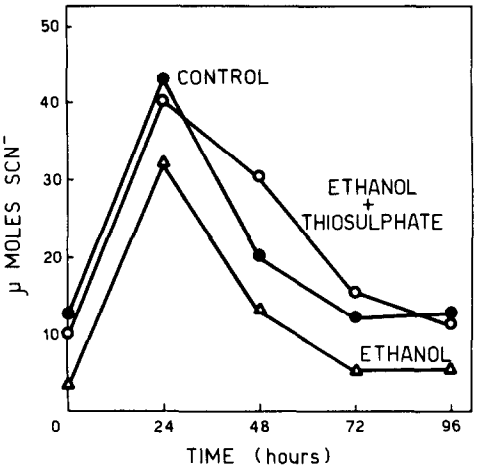


Fig. 1b. The urinary excretion of thiocyanate following i.p. administration of DNS 20 mg/Kg to the rat. Effect of ethanol drinking fluid 25% (v/v) for 8 days up to the time of DNS administration. Effect of thiosulfate 80 mg/Kg i.p. twice daily for 2 days before and 3 days after DNS administration.

pretreatment, however, they are attributable to an impaired conversion of cyanide to thiocyanate due to lack of sulfur. This hypothesis gains support from the observation that ethanol is also conjugated with sulfur, yielding ethylsulfate [15]. Even if this process is not conspicuous under normal conditions, it may lead to a deficiency in sulfur donors in the presence of a continuously abundant ethanol supply.

*In vitro*. These tests have employed rat liver slices, as the splitting of DNS could only be demonstrated in such preparations [3]. Table 2 demonstrates markedly elevated cyanide liberation (measured as $SCN^-$) from DNS in the liver of ethanol pretreated rats relative to the controls.* This elevation was already evident (+70 per cent) and significant after a single treatment with a high dose of ethanol. It became much more obvious upon chronic ethanol administration (20% v/v in water *ad*

Table 2. The effect of ethanol treatment and ethanol withdrawal on the $SCN^-$ formation from 1.25 mM DNS by rat liver slices

| Ethanol treatment | No. of animals | nMole $SCN^-$ g tissue wet wt/2 hr | % Stimulation |
|---|---|---|---|
| — | 51 | 330 ± 13 | — |
| single dose | 19 | 559 ± 50 | +70 |
| 4 days | 8 | 975 ± 133 | +196 |
| 8 days | 14 | 935 ± 36 | +184 |
| 15 days | 10 | 863 ± 91 | +160 |
| withdrawal | 4 | 301 ± 46 | — |

Single dose. 8 hr fasted rats were given 47% (v/v) ethanol 15 ml/Kg by gastric intubation and killed 16 hr later. Drinking fluid ethanol 20% (v/v) up to the time of sacrifice. Withdrawal: ethanol as drinking fluid 20% (v/v) for 8 days and withdrawn 15 hr prior to sacrifice. Experimental conditions as described in Methods. The data represent the difference in $SCN^-$ formation in the presence and absence of succinonitrile. All assays contained Na-thiosulphate. The values are means ±S.E.

Table 3. The effect of drug treatment on the $SCN^-$ formation from DNS, on aminopyrine-demethylase and aniline-hydroxylase by rat liver slices

| Drug | nMole $SCN^-$ g tissue wet wt/2 hr | nMole Formaldehyde*/g tissue wet wt/2 hr | nMole *p*-aminophenol/g tissue wet wt/2 hr |
|---|---|---|---|
| — | (11) 298 ± 20 | (6) 250 ± 28 | (13) 53 ± 4 |
| Ethanol | (14) 935 ± 36 | (5) 491 ± 42 | (7) 191 ± 20 |
| Phenobarbital | (8) 250 ± 24 | (5) 819 ± 100 | (7) 162 ± 22 |
| 3-4 Benzopyrene | (8) 234 ± 23 | — | (8) 80 ± 7.5 |
| $CCl_4$ | (4) 12 ± 1 | — | (3) 0 |

Ethanol as drinking fluid 20% (v/v) for 8 days up to the time of sacrifice.
Phenobarbital. 37 mg/Kg i.p. twice daily for 4 days.
3-4 benzopyrene. 2.5 mg/rat i.p. twice daily for 7 days.
$CCl_4$ 1 ml/kg per os 24 hr before sacrifice.
In parenthesis the number of animals. The values are means ± S.E. Experimental conditions as described in Methods.
* These data represent the difference in formaldehyde production in the presence and absence of aminopyrine. All assays contained semicarbazide.

*lib.*), having reached a maximum level (+196 per cent) after 4 days. Table 2 shows that omission of the alcohol from the drink 15 hr prior to sacrifice restored the rate of $SCN^-$ formation from DNS close to normal. Other authors found that withdrawal of alcoholic drink 24 hr prior to sacrifice abolished the stimulating effect on aminopyrine demethylase and on aniline hydroxylase [6].

Table 3 reports the quantities of hepatic $SCN^-$ formation from DNS in rats pretreated with various pharmaceutical agents. In order to test for the metabolic efficiency of the preparations, also the well established activities of the aminopyrine demethylase and aniline hydroxylase have been followed, providing additional criteria for the functional state of the system. As illustrated, the well known enzyme inducers phenobarbital and 3-4 benzopyrene did not modify the metabolic transformation of DNS, whereas pretreatment of the animals with $CCl_4$ completely inhibited $SCN^-$ formation.

At the present state it is not clear why cyanide formation from DNS has not been affected by the above enzyme inducers. This problem may be elucidated when the sites of DNS metabolism and of their controls will be clearly established.

## CONCLUSIONS

The experimental study here described indicates that the toxicity of DNS is due essentially to enzymatic release of $CN^-$ in the liver. This release can be influenced by the simultaneous administration of other pharmacological agents, and DNS toxicity can be modified accordingly.

As far as the *in vivo* effect of $CCl_4$ pretreatment is concerned, the *in vitro* tests provide clarification for them. According to the latter results, the diminution of *in vivo* DNS toxicity and the depletion of the urinary $SCN^-$ under the effect of $CCl_4$ is due to the inhibited release of $CN^-$ from DNS.

Pretreatment of the animals with ethanol yielded noteworthy results of scientific as well as of practical implications. Stimulation of the metabolism of various drugs is known to be among the effect of chronic alcohol consumption in large [17-19]. In view of the foregoing, also the metabolic disposal of DNS can be categorized among the drug metabolisms thus affected by ethanol. The *in vitro* tests clearly demonstrate an accelerated $CN^-$ release from DNS under these conditions that also bring about a halving of the $LD_{50}$ for DNS *in vivo*. The high rhodanese activity [20] has been found unaltered under the conditions of ethanol treatment (unpublished observations). Therefore, the greatly increased toxicity of DNS under such conditions must be ascribed to a limiting sulfur availability rather than to a low detoxifying capacity *per se*. The urinary excretion of $SCN^-$ by rats pretreated with ethanol provides evidence for the scarcity of available sulfur in such animals. Indeed, a depletion of the normal sulfur reserves obtains when the organism is confronted with a massive sulfur requirement for detoxification as in the case of DNS.

Therefore, it is reasonable to infer that the augmented DNS toxicity in animals pretreated with ethanol is due to two factors, to the stimulation of $CN^-$ release and to the impaired rate of detoxification resulting from the lack of sulfur.

## REFERENCES

1. H. Hyden and H. Hartelius, *Acta psychiat. neurol. Scand. suppl.* 48 (1948).
2. V. Martini and J. Pattay, *Archs. int. Pharmac.* **99**, 314 (1954).
3. A. R. Contessa and R. Santi, *Biochem. Pharmac.* **22**, 827 (1973).
4. R. Cavanna and F. Pocchiari, *Biochem. Pharmac.* **21**, 2529 (1972).
5. E. Lodi, E. Marozzi, G. Barbi and L. Ventura, *Riv. Patol. Clin.* **27**, 101 (1972).
6. F. Lodi, E. Marozzi, G. Barbi and C. A. Maggi, *Il Farmaco, Ed. Prat.* **28**, 105 (1973).
7. S. H. Curry, *Biochem. Pharmac.* **24**, 351 (1975).
8. O. Rosenthal, C. S. Rogers, H. M. Vars and C. C. Ferguson, *J. biol. Chem.* **185**, 669 (1950).
9. C. S. Weil, *Biometrics* **8**, 249 (1952).
10. Y. Imai, A. Ito and R. Sato, *J. Biochem.* **60**, 417 (1966).
11. T. Nash, *Biochem. J.* **55**, 416 (1953).
12. A. E. M. McLean and P. A. Day, *Biochem. Pharmac.* **23**, 1173 (1974).
13. T. Dambrauskas and H. H. Cornish, *Toxic. appl. Pharmac.* **17**, 83 (1970).

14. G. Ugazio, R. R. Koch and R. O. Recknagel, *Gastroenterology* **60**, 188 (1971).
15. H. Bostroem and A. Vestermark, *Acta physiol. Scand.* **48**, 88 (1960).
16. S. Nakanishi, G. Kinoshita, E. Shiohara and M. Tsukada, *Jap. J. Pharmac.* **22**, 809 (1972).
17. E. Rubin and C. S. Lieber, *Science NY* **162**, 690 (1968).
18. P. S. Misra, A. Lefevre, E. Rubin and C. S. Lieber, *Gastroenterology* **58**, 308 (1970).
19. E. Rubin, P. Bacchin, M. Gang and C. S. Lieber, *Lab. Invest.* **22**, 569 (1970).
20. K. Lang, *Biochem. Z.* **259**, 243 (1933).