Biochemical Pharmacology, Vol. 34, No. 22, pp. 4081–4084, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

POSSIBLE ROLE OF HYDROXYL RADICALS IN THE METABOLISM OF SUCCINONITRILE

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(Received 29 July 1985; accepted 22 August 1985)

Succinonitrile is a dinitrile which has been utilized as an antidepressant (1). It is metabolized to cyanide (CN^-) upon incubation with mouse liver microsomes and the acute toxic actions of this drug are amenable to treatment with CN^- antidotes (2). The toxicity of succinonitrile in mice is also prevented by pretreatment with carbon tetrachloride (2) and delayed by pretreatment with ethanol (3).

Except for acrylonitrile, the mechanisms of metabolism of nitriles to CN^- have not been well studied. Doherty *et al.* (3) reported that ethanol and 4-methylpyrazole altered the toxicity of succinonitrile in mice. These agents are scavengers of hydroxyl radicals ($OH^\bullet$, 4,5). The above report suggested to us that $OH^\bullet$ could play a role in the metabolism of succinonitrile, and perhaps other nitriles, to CN^-. Figure 1 shows a possible mechanism by which $OH^\bullet$ might attack succinonitrile and other saturated nitriles. The scheme presented here is a simplified model and it is conceivable that some of the postulated intermediates could also be attacked by $OH^\bullet$. Cyanohydrins are unstable under alkaline conditions and will spontaneously decompose to release CN^-. The present studies were conducted to investigate the conversion of succinonitrile to CN^- in a model $OH^\bullet$ generating system and to investigate the hypothesis that $OH^\bullet$ may be involved in the metabolism of succinonitrile in a mouse microsomal system.

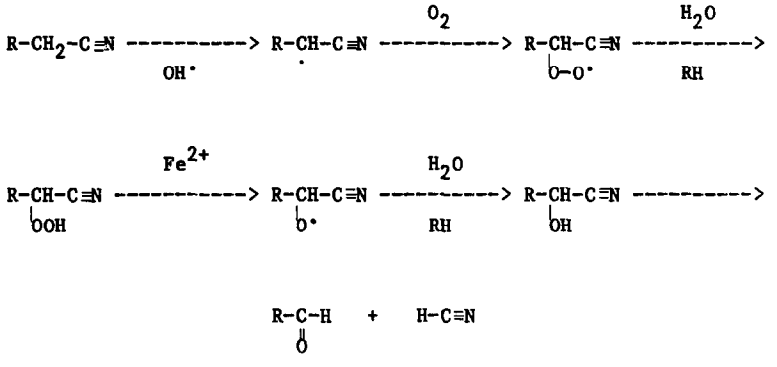

Fig. 1. Postulated mechanism for the production of cyanide from saturated nitriles by reaction with hydroxyl radicals. In the steps involving the peroxy and the alkoxy radicals hydrogen may be abstracted either from H_2O or from some other donor (RH) depending on the system (iron-ascorbate, microsomes, etc.).

METHODS

An iron-ascorbic acid system was used as a model $OH^\bullet$ generating system (5). This system contained 2 mM ascorbic acid, 0.167 mM ferrous ammonium sulfate, and 0.33 mM EDTA (final concentrations) in 0.1 M phosphate buffer, pH 7.4. This buffer and others used in these experiments were passed through a column containing Chelex-100 resin to decrease the amount of contaminating trace metals. Succinonitrile (5 or 10 mM) was incubated in this model system for 5 min at 37°. In some experiments, the $OH^\bullet$ scavengers dimethyl sulfoxide (DMSO,

1-10 mM) or mannitol (5-40 mM) were added to the reaction. All reactions were carried out in stoppered tubes and 0.4 ml of 100% trichloroacetic acid (TCA) was added after 5 min. The reaction mixture was transferred to the outer well of a Conway diffusion cell and CN^- was trapped in 0.4 ml NaOH (0.1 M) during a 2-hr period. CN^- was analyzed colorimetrically (6) with a detection limit of 0.04 nmole. CN^- recovery was determined in each experiment and zero-time control values were subtracted from all data points.

In other studies, female CD-1 mice (25-30 g), obtained from Charles River Breeding Laboratories, were used. Livers were removed, pooled, and homogenized in 0.1 M phosphate buffer, pH 7.4, containing 0.15 M KCl. Microsomes were obtained following differential centrifugation and, after washing, were resuspended in buffer and stored at -60°. Succinonitrile (5-50 mM) was incubated at 37° with 1 mg microsomal protein and NADPH (2.5 μmoles) in a final volume of 2 ml phosphate or Tris buffer (0.1 M, pH 7.4) for periods ranging from 5 to 30 min. The reaction was terminated by addition of 0.4 ml of 100% TCA and the reaction mixture was transferred to a diffusion cell for analysis of CN^-, as described above. In some experiments, DMSO (2.5-20 mM), mannitol (10-80 mM), sodium benzoate (25-100 mM), desferrioxamine (50-800 μM), or a solution of ferrous ammonium sulfate:EDTA, 1:2 (Fe:EDTA, 5-40 μM) was added to the incubations. Data for CN^- production from succinonitrile in microsomes were corrected based on CN^- recovery experiments. All experiments were conducted at least twice.

RESULTS AND DISCUSSION

The initial studies utilized a model $OH^\bullet$ generating system to investigate whether $OH^\bullet$ could react with nitriles to yield CN^-. CN^- production from succinonitrile was concentration dependent, with CN^- production approximately doubling as the concentration of succinonitrile was increased from 5 to 10 mM. Compared to the complete system (control), omission of one or more of the components of the model system resulted in alterations in the amount of CN^- generated from succinonitrile. In the absence of EDTA, CN^- production from succinonitrile increased slightly (120% of control) whereas in the absence of ascorbic acid or iron CN^- production decreased to 8 and 23% of control, respectively. When only ascorbic acid was present, CN^- production was 26% of that found when succinonitrile was incubated with the complete system. These results suggest that ascorbic acid and iron are critical components for the production of CN^- from succinonitrile. It is conceivable that Chelex-100 treatment of the buffer did not remove all the contaminating iron, and that the remaining iron contributed to the production of CN^- when only ascorbic acid was added to the incubation mixture containing buffer and succinonitrile.

Addition of DMSO or mannitol to the model system with succinonitrile resulted in marked inhibition of CN^- generation (Fig. 2). The IC_{50} values for inhibition by DMSO of CN^- generation were 2.28 and 2.17 mM, at succinonitrile concentrations of 5 and 10 mM

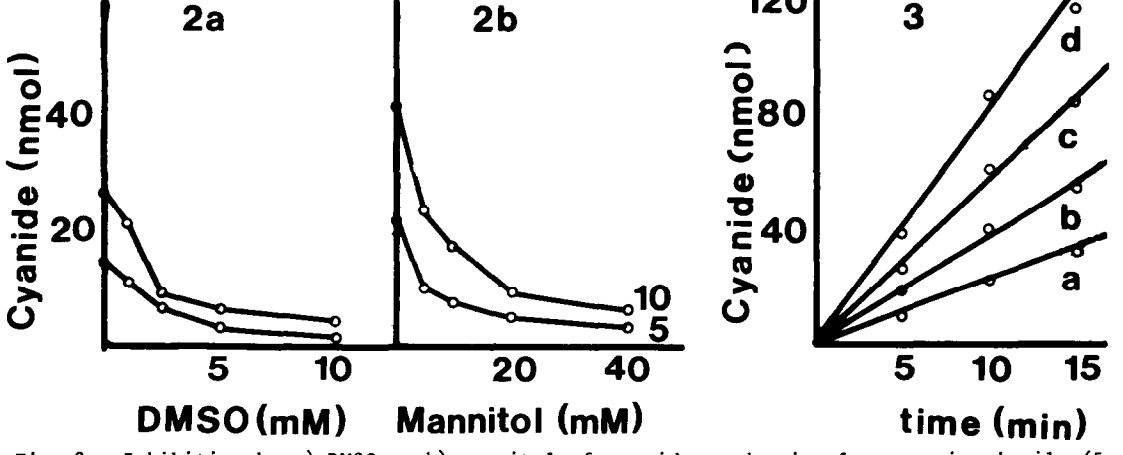

Fig. 2. Inhibition by a) DMSO or b) mannitol of cyanide production from succinonitrile (5 to 10 mM) in an iron-ascorbic acid hydroxyl radical generating system.

Fig. 3. Time and concentration dependence of succinonitrile (a, 5 mM; b, 10 mM; c, 20 mM; and d, 50 mM) metabolism to cyanide by mouse microsomes.

respectively. The IC_{50} values for inhibition by mannitol of CN^- production were 3.8 and 6.5 mM, at succinonitrile concentrations of 5 and 10 mM respectively. These data suggest that, in this model system, DMSO is approximately twice as potent as mannitol, as an inhibitor of succinonitrile conversion to CN^-. The results also suggest that mannitol is acting as a competitive inhibitor of the oxidation of succinonitrile to CN^-. DMSO should also be a competitive inhibitor of the oxidation of succinonitrile to CN^- in this system, yet similar IC_{50} values were obtained when the nitrile concentration was doubled. The reason for this finding is unclear.

Succinonitrile was also metabolized to CN^- by the mouse liver microsomal system. As shown in Fig. 3, this metabolism was concentration-dependent and linear with time for at least 15 min. Lineweaver-Burk and Eadie-Hofstee plots were both linear and yielded similar values for K_m (20.6 mM) and V_{max} (5.09 nmoles CN^-/mg protein/min). The metabolism of succinonitrile to CN^- was dependent on the presence of NADPH and did not occur when a boiled microsomal suspension was utilized. These data suggest that this metabolism is an enzymatic process. Succinonitrile was also metabolized to CN^- when mouse microsomes were incubated in Tris buffer. Under these conditions, the V_{max} was increased slightly and the K_m was increased approximately 2.3-fold (data not shown) compared to values calculated for these parameters in a phosphate buffer system. Systems containing H_2O_2 and metal ions, which presumably would produce $OH^\bullet$, will oxidize Tris to free radical products (7). It is plausible that Tris could serve as an $OH^\bullet$ scavenger. Competition of Tris with succinonitrile for $OH^\bullet$ could account for the change in K_m when Tris was used as the buffer system.

Addition of DMSO to microsomes during incubation with succinonitrile resulted in a marked decrease in CN^- generation. As shown in Fig. 4, this inhibition appeared to be competitive. The IC_{50} values for inhibition by DMSO of CN^- production were 3.3, 6.3, and 11.8 mM when the succinonitrile concentrations were 5, 10, or 50 mM, respectively. Recovery of $NaCN^-$ incubated with microsomal suspensions was not altered by DMSO. At the concentrations used in these studies, mannitol did not inhibit succinonitrile metabolism to CN^-. Sodium benzoate, also an $OH^\bullet$ scavenger, inhibited the metabolism of succinonitrile approximately 33% only at the highest concentration (100 mM) utilized in these studies. Desferrioxamine did not affect the metabolism of succinonitrile to CN^- (data not shown). Addition of Fe:EDTA to the microsomal system resulted in a concentration-dependent increase in the metabolism of succinonitrile to CN^- (Table 1). DMSO, mannitol, and desferrioxamine inhibited the metabolism of succinonitrile in this system (Table 1).

The data presented here indicate that $OH^\bullet$ may react with succinonitrile to cause release of CN^-. It is possible that other saturated nitriles may also participate in $OH^\bullet$ mediated oxidation reactions. When Fe:EDTA was used, a stimulation of the microsomal metabolism of

Table 1

Effect of Iron:EDTA (1:2) and hydroxyl radical scavengers on succinonitrile metabolism to cyanide in microsomal systems[a].

Iron:EDTA (μM)	Scavenger	Cyanide[b]	% of control
5	none	19.55 + 0.51	100
10	"	18.24 + 0.61	93.3
20	"	20.32 + 0.17	103.9
40	"	23.53 + 0.42	120.4
20	DMSO 10 mM	5.10 + 0.12	25.1[c]
20	Mannitol 80 mM	19.34 + 0.20	95.2[c]
20	Desferrioxamine 400 μM	16.66 + 0.45	82.0[c]

[a]The microsomal system consisted of 1 mg microsomal protein, 2.5 μmoles NADPH, and 5 mM succinonitrile in phosphate buffer.

[b]Cyanide values are nmoles/mg protein/10 min (mean ± S.D.).

[c]This group was compared to the group treated with 20 μM iron:EDTA.

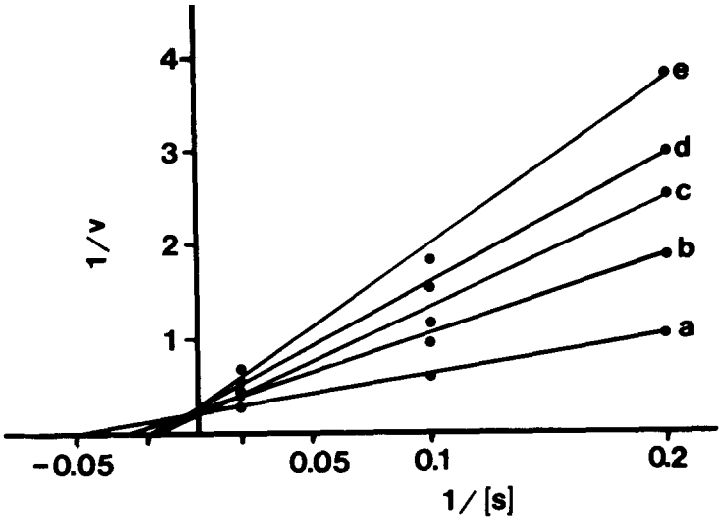

Fig. 4. Lineweaver-Burk plot of DMSO inhibition of succinonitrile metabolism to cyanide by mouse microsomes: (a) succinonitrile alone; (b) thru (e) Succinonitrile plus DMSO 2.5, 5, 10, and 20 mM respectively.

succinonitrile occurred and this metabolism was susceptible to inhibition by mannitol and desferrioxamine, as well as by DMSO. Under these conditions, DMSO was the most effective inhibitor of succinonitrile metabolism. Thus in this system OH• may be involved in the metabolism of the nitrile to cyanide.

DMSO was the only OH• scavenger used in this study that was an effective inhibitor of succinonitrile metabolism to CN^- in the microsomal system (without added Fe:EDTA). The scavengers, mannitol and benzoate, and the chelating agent desferrioxamine did not inhibit succinonitrile metabolism. Several possible explanations may be suggested. These other scavengers may not penetrate the microsomal membrane and therefore would be unable to inhibit OH• mediated reactions occurring within the membrane environment. Alternatively, it is possible that catalase, present in the microsomal preparations, may have destroyed H_2O_2 necessary for production of OH•. However, when azide was added to the microsomal suspension to inhibit catalase the metabolism of succinonitrile was not enhanced (data not shown). It is also possible that succinonitrile is metabolized by an enzyme system, perhaps a specific isoenzyme of cytochrome P-450, which is particularly susceptible to inhibition by DMSO but not inhibited by the other scavengers. In this regard, DMSO has been identified as a competitive inhibitor of the ethanol inducible cytochrome P-450 isoenzyme LM3a (8). Additional experimental investigation will be required in order to distinguish between these possible explanations.

ACKNOWLEDGEMENT This work was supported, in part, by a General Research Support Grant from UMDNJ-Rutgers Medical School.

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