A STUDY OF ACRYLONITRILE POISONING IN RELATION TO METHAEMOGLOBIN-CN COMPLEX FORMATION

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Observations are recorded on methaemoglobin-CN complex formation in rats poisoned with acrylonitrile, potassium cyanide, and acetone cyanohydrin. For information on methaemoglobin-CN formation, the methaemoglobin level was increased by sodium nitrite.

The results show that the rate of methaemoglobin-CN formation in rats killed by acrylonitrile is lower than in animals surviving potassium cyanide or acetone cyanohydrin poisoning, and much lower than in animals killed by potassium cyanide. These findings indicate that the toxicity of acrylonitrile cannot be solely due to the liberation of cyanide.

There is a divergence of opinion about the toxic action of acrylonitrile; that the action of acrylonitrile was due to cyanide was accepted by Dudley and his collaborators (Dudley and Neal, 1942; Dudley, Sweeney, and Miller, 1942) on the basis of symptoms, and by Brieger, Rieders, and Hodes (1952), who detected blood cyanide, cyanmethaemoglobin, and thiocyanate excretion. The determination of the rate of cyanmethaemoglobin formation in the range of normal methaemoglobin levels seems, however, to be somewhat problematical.

On the other hand, Ghiringhelli (1956) and Beneš and Černá (1959) found that only 20 to 25% of the cyanide ions were excreted as thiocyanate in acrylonitrile poisoning, and because of this, the latter supposed that acrylonitrile killed by the action of the intact molecule rather than by the detached cyanide ions.

Nevertheless, the relatively low thiocyanate excretion does not disprove the cyanide effect. In Ghiringhelli's (1956) experiment, the guinea-pigs treated with an LD₅₀ of acrylonitrile excreted over five and a half times more thiocyanate than the animals treated with an LD₅₀ of sodium cyanide. Consequently, although the incomplete liberation of cyanide has been demonstrated, the liberation of cyanide in lethal amounts has not been shown to be impossible.

The toxic action of cyanide is characterized by its ability to form complexes with metalloproteins. Therefore, this study has been based on the assumption

that the question of the liberation of cyanide in lethal amounts can be answered by determining the rate of metalloprotein-CN complex formation. Although the most important metalloprotein in this relation is the cytochromoxidase, nevertheless the methaemoglobin offers the easiest way to approach the problem.

It is well known that the formation of both cytochromoxidase-CN and methaemoglobin-CN complexes depends on the cyanide concentration. From the following equation,

$$\frac{K_M}{K_O} \times \frac{\text{Meth-CN}\%}{\text{Meth}\%} = \frac{\text{oxidase-CN}\%}{\text{oxidase}\%},$$

if meth-CN% and meth % are determined, it is possible to calculate the approximate degree of cytochromoxidase inhibition. The cause of uncertainty in this calculation is that K_M and K_O (the dissociation constants of meth-CN and oxidase-CN) are only estimated in *in vitro* experiments. The dissociation constant of meth-CN is 6×10^{-8} (Scheler, Schoffa, and Jung, 1958), and the dissociation constant of oxidase-CN in pre-incubation with cyanide is 3×10^{-8} (Wainio and Greenlees, 1960).

To obtain information on metalloprotein-CN complex formation it was necessary to increase the methaemoglobin level by sodium nitrite treatment. Thus, the study also involved a test of the protective effect of methaemoglobin formation. For purposes of comparison, data on the methaemoglobin-CN

formation caused by potassium cyanide and acetone cyanohydrin are also presented.

Experimental Procedures and Methods

Male albino rats weighing between 160 to 180 g. were used.

The materials were the following: 10×10^{-1} M aqueous acrylonitrile; 5×10^{-2} M aqueous potassium cyanide; 5×10^{-2} M aqueous acetone cyanohydrin; and 1% aqueous sodium nitrite.

Procedures.—*a.* Before the experiment it was necessary to determine the LD_{50} of the cyanide compounds in question. The substances were injected subcutaneously, the dosage series calculated in millimole alternated in accordance with the scheme of Deichmann and LeBlanc (1943), *i.e.* the larger exceeded the smaller by 50%.

b. Subsequently, 10 animals were treated with a dose $1.5 \times LD_{50}$. Five animals from each group were given 30 mg. sodium nitrite per kg. of body weight intraperitoneally 30 minutes before the treatment with the cyanide compounds. An additional four animals treated with sodium nitrite were given potassium cyanide in a dose $2.25 \times LD_{50}$.

c. Blood samples for methaemoglobin-CN estimation were taken from the heart immediately after death or from the tail vein one hour after the injection of the cyanide compounds.

d. Methaemoglobin and Methaemoglobin-CN Determination.—To 10 ml. of phosphate buffer, pH 6.6, 0.1 ml. blood was transferred, and haemolysed with a trace of saponin. To remove turbidity, the solution was centrifuged at 1,900 G for 20 minutes.

The optical density was read at 630 $m\mu$ in a Beckman spectrophotometer model DU, with tungsten lamp and 10 mm. glass cuvettes (slit 0.035 mm.) before (E_1) and after (E_2) adding a drop of 20% potassium ferricyanide to the solution. The increase of optical density is inversely proportional to the total methaemoglobin concentration. After this, one drop of 10% acetone cyanohydrin was added to the sample to convert the methaemoglobin to methaemoglobin-CN, and the optical density was read again at 630 $m\mu$ (E_3). The decrease of density is inversely proportional to the original methaemoglobin-CN concentration. After the potassium ferricyanide and acetone cyanohydrin treatment, the samples were allowed to stand three minutes for complete conversion.

The total haemoglobin was determined as methaemoglobin-CN at 540 $m\mu$ (slit: 0.02) after the sample was diluted with buffer to a double volume and treated with a drop of concentrated ammonium hydroxide (E_4).

The calculation was as follows.

Total haemoglobin g.% = $E_4 \times 30.34$.

Total methaemoglobin g.% = $(E_4 \times 30.34) - (E_2 - E_1) \times 45.8$.

Methaemoglobin-CN g.% = $(E_4 \times 30.34) - (E_2 - E_3) \times 50.7$.

Methaemoglobin g.% = (total methaemoglobin g.%) - (methaemoglobin-CN g.%).

The maximal error is 0.30 g.% methaemoglobin-CN. The rate of methaemoglobin-CN formation is expressed

in percentages as a ratio of methaemoglobin-CN to total methaemoglobin.

The factor for calculating total haemoglobin was based on the extinction coefficient given by Zijlstra and Kampen (1960).

Results

Median lethal doses (Table 1) were exceeded at a similar rate in the investigation of both the preventive effect of sodium nitrite and the methaemoglobin-CN formation.

TABLE 1
APPROXIMATE MEDIAN LETHAL DOSES OF
ACRYLONITRILE, POTASSIUM CYANIDE, AND ACETONE
CYANOHYDRIN ADMINISTERED SUBCUTANEOUSLY
TO ALBINO RATS

Compound	Millimole/kg.	mg./kg.
Acrylonitrile	1.8	95.8
Potassium cyanide	1.6×10^{-1}	10.4
Acetone cyanohydrin	1.0×10^{-1}	8.5

Table 2 shows the proportion of animals dying to the number that were given one of the three cyanide compounds with or without sodium nitrite treatment.

TABLE 2
EFFECT OF METHAEMOGLOBINAEMIA ON
MORTALITY RATIOS IN ALBINO RATS POISONED
WITH ACRYLONITRILE, POTASSIUM CYANIDE,
AND ACETONE CYANOHYDRIN

Compound	Dose (millimole/kg.)	No. That Died/No. Given Cyanide Compound	
		Without Sodium Nitrite	With Sodium Nitrite
Acrylonitrile	2.8	5/5	5/5
Potassium cyanide	2.4×10^{-1}	5/5	1/5
Potassium cyanide	3.7×10^{-1}	—	4/4
Acetone cyanohydrin	1.6×10^{-1}	5/5	0/5

On this experimental condition the sodium nitrite which was effective in the case of both potassium cyanide and acetone cyanohydrin, changed neither the mortality ratio nor the period between the injection of acrylonitrile and death. The survivals in minutes are given in Table 3.

TABLE 3
SURVIVAL IN MINUTES

With Sodium Nitrite		Without Sodium Nitrite	
	93		104
	111		115
	116		118
	130		121
	141		122
Average	118		116

TABLE 4
COMPARISON OF METALLOPROTEIN-CN COMPLEX-FORMING CAPACITY OF CYANIDE
COMPOUNDS AFTER SODIUM NITRITE ADMINISTRATION

Compound	No. of Rats	Time of Taking Blood Samples	Ratio of Meth-CN to Total Meth		Approximate Inhibition of Cytochromoxidase (%)
			Mean	S.D.	
Acrylonitrile	5	After death	55.8	14.8	71.5
Potassium cyanide	5	After death	85.6	8.7	92.2
Potassium cyanide	4	One hour after			
Acetone cyanohydrin	5	cyanide administra- tion	72.4	12.7	84.0

Blood samples taken from rats killed by acrylonitrile or potassium cyanide revealed a significant difference ($t = 4.06$, $0.01 > p > 0.001$) in the rate of methaemoglobin-CN formation expressed in percentage as a ratio of methaemoglobin-CN to total methaemoglobin. Blood samples from surviving animals taken one hour after the injection of potassium cyanide or acetone cyanohydrin also showed a higher rate of methaemoglobin-CN formation than those of animals killed by acrylonitrile.

Table 4 shows in addition to the methaemoglobin-CN values, the approximate inhibition of cytochromoxidase calculated from the ratio of methaemoglobin-CN: methaemoglobin and based on the assumption that $K_M:K_O = 2$.

Discussion

One of the most important features of the mechanism of intoxication by cyanide ions is the metalloprotein-CN complex formation. There have been no studies on record in which this fundamental phenomenon has been analysed in poisoning with acrylonitrile. Approaching the problem from this point of view, this work has revealed clear differences between acrylonitrile and potassium cyanide or acetone cyanohydrin.

In contrast to low thiocyanate excretion found by Ghiringhelli (1956) and Beneš and Černá (1959), more than half of the methaemoglobin binds cyanide ions in animals killed by acrylonitrile. Notwithstanding, this diversion of CN does not seem to play a prominent role in the lethal effect of acrylonitrile.

This hypothesis is supported by two facts.

a. Although the rate of methaemoglobin-CN formation is high, it is lower than in animals surviving potassium cyanide or acetone cyanohydrin poisoning, and much lower than in animals killed by potassium cyanide.

b. The considerable quantity of cyanide trapped by methaemoglobin had no effect either on the mortality or the time of survival.

However, the hypothesis may not be generally

applicable. Dudley and Neal (1942), Dudley *et al.* (1942) and Brieger *et al.* (1952) showed that there was a discernible difference between species in their susceptibility to acrylonitrile. In the experiments of Brieger and co-workers (1952) the cyanide concentration in the blood of dogs was always higher than in rats, whereas the reverse applied to the urine thiocyanate. In spite of rapid cyanide detoxication in rats, a very remarkable quantity of metalloprotein-CN complex formation was detected in the present study. In species with slower conversion of cyanide to thiocyanate, therefore, the possibility of lethal cyanide liberation cannot be excluded.

Nevertheless, the facts mentioned above show that the toxicity of acrylonitrile is not due to the metabolic formation of cyanide ions in every case, and the hypothesis of Beneš and Černá (1959), which is accepted by Paulet and Desnos (1961), seems to be justified. Thus, the toxicity of acrylonitrile cannot be due solely to cyanide liberation as Williams (1959) has implied.

It is well known that acrylonitrile is an exceptionally reactive compound, and it is entirely reasonable to assume that important constituents of biochemical mechanisms can be damaged, *e.g.* by cyanoethylation. There are very many compounds having the structure HX, where H is a reactive hydrogen atom which adds readily to the carbon-carbon double bond of acrylonitrile (American Cyanamid Company, 1951).

There is a possibility that after acrylonitrile administration the cyanide formed *in statu nascendi* in the tissues might act at a lower concentration than after administering potassium cyanide. In this case, on the basis of both the difference in methaemoglobin-CN complex formation caused by potassium cyanide and acrylonitrile and the negative effect of sodium nitrite treatment, we must suppose that the cyanide formation after acrylonitrile administration is a result of a reaction including cytochromoxidase and acrylonitrile. If this hypothesis is true, the importance of the direct effect of acrylonitrile should be again stressed.

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