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THE NATURE OF THE TOXICITY OF 2-OXO-OXIMES

BY

BERYL M. ASKEW, D. R. DAVIES, A. L. GREEN, AND R. HOLMES

From the Chemical Defence Experimental Establishment, Ministry of Supply, Porton, Wilts

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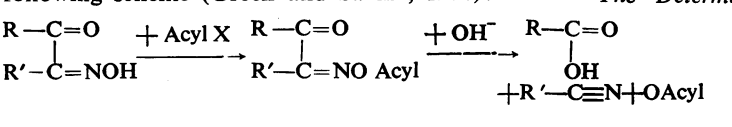
Some oximes have recently been tested as antidotes to organophosphorus anticholinesterase poisoning (Childs, Davies, Green, and Rutland, 1955; Holmes and Robins, 1955; Wilson and Ginsburg, 1955). Many of them are effective, particularly 1:2-dione monoximes of the type R.CO.C(R′):NOH where R is alkyl or aryl and R′ is H or alkyl. Those of greatest interest are mono*iso*nitrosoacetone (MINA; $R=CH_3$, $R'=H$) and diacetyl monoxime (DAM; $R=R'=CH_3$).

When tested therapeutically at equimolar doses, MINA and DAM proved equally effective in saving the lives of rats poisoned with *iso*propyl methylphosphonofluoridate (sarin). MINA, however, rapidly abolished acetylcholine-like effects, whereas, in those animals which had received DAM, convulsive spasms still persisted after several hours (Askew, 1956). However, along with other 2-oxo-aldoximes (R′=H), MINA is appreciably more toxic than either simple oximes or the related 2-oxo-ketoximes (R′=alkyl), so that its effectiveness is limited by the dose which may be given without toxic side effects. Hence it seemed desirable to determine how 2-oxo-aldoximes are more toxic than other oximes.

When oximes are given to animals, the concentration in the blood decreases relatively rapidly, but very little unchanged drug can be recovered from the urine (Davies and Rutland, 1956, unpublished). Breakdown in the body might occur by hydrolysis to hydroxylamine, or by enzymatic reduction to an amine, but neither of these products would explain the high toxicity of 2-oxo-aldoximes.

Many oximes are known to react in neutral solution *in vitro* with acylating agents of the type Acyl X, where Acyl may also be sulphonyl or phosphoryl and X is an acid radical—e.g., acetate or halide. With 1:2-dione monoximes, the intermediate then breaks down according to the following scheme (Green and Saville, 1956):



When R′ is H as in 2-oxo-aldoximes such as MINA, hydrogen cyanide (HCN) would be formed, whereas when R′ is alkyl as in 2-oxo-ketoximes such as DAM, no free HCN would be obtained. Of all normal oximes it is only 2-oxo-aldoximes which could break down directly by the above reaction sequence to give free HCN.

Acylating and phosphorylating agents exist in the body, hence this type of breakdown of 2-oxo-aldoximes to give HCN may occur *in vivo*. This possibility has been examined and it has been shown that sufficient HCN is formed by the metabolism of MINA and DINA (di*iso*nitrosoacetone, HC(:NOH).CO.HC:NOH) to account for their toxicity.

METHODS

Analytical Procedures

The Determination of Cyanide.—The method of Aldridge (1945) was used for the estimation of plasma cyanide and thiocyanate. The method does not differentiate between them when both are present together, thus a determination upon the plasma of an animal poisoned with an oxime which would liberate HCN would include both cyanide and thiocyanate. A part of the latter would be that normally present, whilst the remainder would be derived by detoxication of the HCN arising from the metabolism of the oxime. No attempt has been made analytically to differentiate between the cyanide and thiocyanate, but the amount of HCN derived from an appropriate oxime has been calculated by subtracting the control HCNS level from the total cyanide (i.e., HCN and HCNS) expressed as HCNS, and then converting the difference to HCN.

Blank estimations for cyanide were carried out on all oxime preparations used, and, with the exception of DINA, no free cyanide was detected. This negative result also showed that the oxime itself did not interfere with the test for cyanide. The amount of free cyanide found in DINA preparations was small and was insufficient to account for the increase of cyanide found after the injection of DINA into various animals.

The Determination of Oxime.—The method is essentially that of Blom (1926) for hydroxylamine. It has been modified for use with oximes in small volumes of blood. The principle

depends upon the acid hydrolysis of the oxime to hydroxylamine, which is then oxidized with iodine to nitrous acid. The nitrous acid is used to diazotize sulphanilic acid, the diazonium salt then being coupled with α-naphthylamine to give a pink colour.

The detailed procedure was as follows: *Reagents*—(1) iodine: 1.3 g. was dissolved in 100 ml. glacial acetic acid; (2) sulphanilic acid: 1 g. was dissolved in 75 ml. of distilled H_2O followed by the addition of 25 ml. glacial acetic acid; (3) α-naphthylamine: 0.3 g. was boiled with 100 ml. of distilled H_2O, the solution was filtered and 25 ml. glacial acetic acid was added to 75 ml. filtrate.

Procedure.—10% Trichloroacetic acid (TCA) (0.4 ml.) was added to a mixture of blood (0.2 ml.) and water (0.2 ml.), which was then centrifuged. The supernatant fluid was separated and a sample (0.2 ml.) was added to 10% sulphuric acid (0.8 ml.) and water (2.0 ml.). After being heated on a steam bath for 30 min. the solution was cooled to below 30° C. and was treated with iodine (0.1 ml.) to convert the free hydroxylamine into nitrous acid. This solution was then used to diazotize sulphanilic acid (0.1 ml.) in aqueous 40% sodium acetate (1.5 ml.). After removal of excess iodine with decinormal sodium thiosulphate (0.1 ml.), α-naphthylamine (0.1 ml.) was added. The total volume was made up to 5 ml., and after 10 min. the colour was read on an EEL absorptiometer (604 filter).

Calibration curves were obtained by adding known quantities of oxime to blood and subjecting these to the above procedure at the same time as the unknowns. The method has been used to determine oxime concentrations over the range 20–800 μg./ml. whole blood.

In vivo *Experimental Procedures*

In experiments upon rats, albinos (400 g.) in groups of 6 were used. After treatment with oxime or cyanide they were killed by a blow on the head and drained of blood, which was collected in heparinized tubes. Control animals, to which no oxime or cyanide had been given, were sacrificed at the same time and in a similar manner.

Rabbits and dogs (anaesthetized with urethane and pentobarbitone respectively) were used in order to make simultaneous serial estimations of cyanide and oxime in the blood. Blood samples from a T-cannula inserted into the femoral artery were obtained before, and at intervals after, the injection of the oxime. Samples of 3 ml. were taken from the rabbits and 4 ml. from the dogs, each sample being divided into two for the separate estimation of cyanide and oxime.

The cyanide plus thiocyanate was estimated in plasma, since Aldridge (1945) showed that thiocyanate could not be estimated in whole blood. The oxime was usually estimated in whole blood, for it was found to be uniformly distributed between cells and plasma.

Results

The signs of MINA or DINA poisoning are hyperventilation and muscular tremors, followed by prostration and loss of the righting reflex; respiration and heart beats then become progressively slower, culminating in death. These effects are similar to those observed in slow poisoning with cyanide, and in fact control groups of rats poisoned with 12 mg./kg. KCN s.c. could not be distinguished by direct observation from a group which had received a lethal dose of MINA (150 mg./kg. s.c.).

Qualitative confirmation of the formation of free HCN can be very easily obtained in MINA- or DINA-poisoned rats, for, when the gastro-intestinal tract is exposed, a very strong smell of HCN is at once apparent; it is also quite marked in the blood collected for analysis.

In some of the experiments on dogs, the expired air was passed through 10% caustic soda solution to absorb any HCN. The solution was analysed and approximately 8 μg. of HCN obtained in each of three successive 5-min. collection periods starting 15 min. after the intraperitoneal injection of MINA, 60 mg./kg.

The presence of free HCN in the plasma of rats poisoned with MINA (150 mg./kg. s.c.) was further demonstrated by the inhibitory effect of such plasma upon the oxidation of ascorbic acid

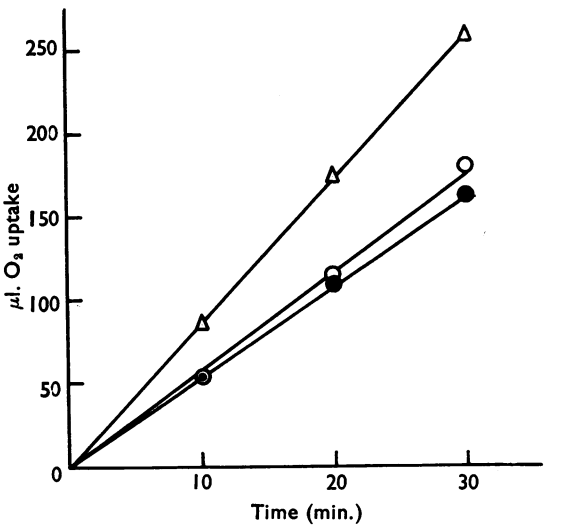


FIG. 1.—The inhibitory effect of plasma from rats poisoned with KCN (open circles) and MINA (filled circles) upon the oxidation of ascorbic acid by the cytochrome oxidase of rat heart. Control with normal plasma, triangles.

by a cytochrome oxidase preparation from normal rat heart (Schneider and Potter, 1943). This is shown in Fig. 1, which also includes for comparison the effect of plasma from KCN-poisoned rats (12 mg./kg. s.c.).

The Level of Cyanide in Oxime-poisoned Rats.—Separate groups of rats were given DINA, MINA, or DAM. Of two further groups, one was given KCN and the remaining served as untreated controls. Rats in each group were killed a few minutes before the previously determined expected time of death from the particular substance administered. Thus with MINA, after 150 mg./kg. s.c. the average time to death was 70 min., and these rats were therefore killed 50 min. after injection. Animals given KCN (12 mg./kg. s.c.), or DINA (25 mg./kg. s.c.), and which were expected to die about 25 min. after poisoning, were killed after 20 min. Since DAM is much less toxic, the time to death was not determined and, after a dose of 500 mg./kg. i.p., which caused acute effects, the animals were killed at an arbitrary interval of 20 min. The blood of each animal was collected separately and the plasma of each was analysed for "total cyanide"—cyanide plus thiocyanate. The results are shown in Table I.

TABLE I

THE CONCENTRATION OF CYANIDE IN THE PLASMA OF RATS POISONED WITH OXIMES OR KCN
(6 rats in each group)

	Dose (mg./kg. s.c.)	Mean HCNS+HCN	Mean HCN
		Expressed as μg. HCNS/ml. Plasma	HCNS+HCN−HCNS (Controls)×0·46 μg. HCN/ml. Plasma
Controls ..		3·25 (2·77–3·76)	0
KCN ..	12	6·42 (6·04–6·75)	1·45
DINA ..	25	7·20 (6·66–7·87)	1·82
MINA ..	150	9·27 (8·50–9·95)	2·77
DAM ..	500 (i.p.)	3·16 (2·80–3·90)	0

If the cyanide in the plasma, following a killing dose of KCN, is taken as a criterion of cyanide lethality it is apparent from Table I that sufficient amounts can build up from these doses of DINA or MINA to account for their toxicity.

As mentioned in the introduction, DAM would not be expected to produce cyanide or thiocyanate directly, but would give rise to acetonitrile if it were broken down in the same way as the 2-oxo-aldoximes. However, aliphatic nitriles are metabolized to thiocyanate (Stoa, 1952), and it was found that a dose of 200 mg./kg. i.p. of acetonitrile (equivalent to 500 mg./kg. of DAM) caused a significant rise in total cyanide of the plasma of rats without producing marked effects. The rats were killed 20 min. after injection and the mean total cyanide (expressed as HCNS) of the plasma of 5 rats was 4.93 μg./ml. compared with the control level of 3.25 μg./ml. As may be seen from Table I, no increase in total cyanide was found 20 min. after the injection of 500 mg./kg. i.p. of DAM.

The Rate of Metabolism of Oximes in Rabbits and Dogs.—Simultaneous values of blood oxime and plasma cyanide were obtained after the intraperitoneal injection of 150 mg./kg. MINA to rabbits. The results showed a steady decrease in the concentration of oxime accompanied by a corresponding rise in plasma cyanide.

Similar experiments were carried out on dogs, not only because more frequent sampling was possible, but also to enable observations to be carried on for a longer period. The results with MINA and DAM are illustrated in Figs. 2 and 3, and show with MINA (Fig. 2) an inverse relationship between oxime and cyanide concentrations similar to that found in the rabbit. In experiments with DAM, despite a fall in oxime concentration and the presence of obvious toxic effects, no free cyanide was detected at any stage during the

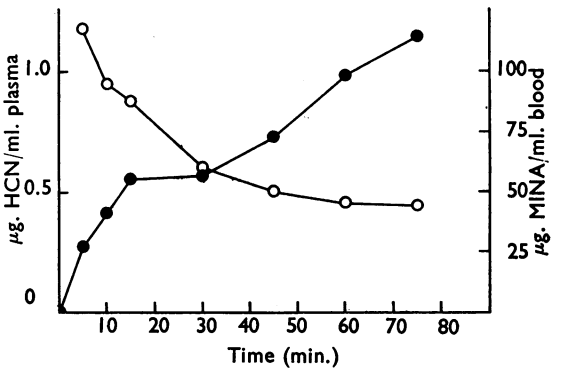


FIG. 2.—Blood concentrations of MINA (open circles) and cyanide (filled circles) in a dog given MINA, 60 mg./kg. i.p., at zero time.

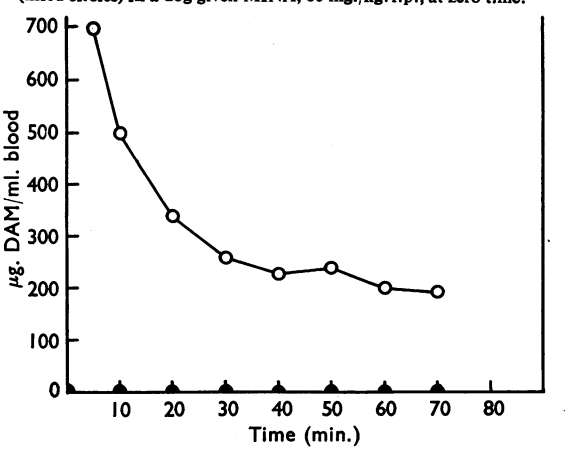


FIG. 3.—Blood concentrations of DAM (open circles) and cyanide (filled circles) in a dog given DAM, 500 mg./kg. i.p., at zero time. No free cyanide found.

experiment (Fig. 3). In one of the experiments on dogs, plasma was analysed for cyanide for up to 5 hr. after the administration of DAM (500 mg./kg. i.p.) and again no increase in total cyanide was found.

The Protection of MINA-poisoned Rats with Cyanide Prophylaxis.—If cyanide is actually produced in MINA poisoning and is responsible for the toxicity of this compound, known procedures which protect the animal against cyanide should be equally effective with MINA.

This was tested by giving MINA to groups of 4 rats, both alone or 10 min. after prophylaxis against cyanide. Prophylaxis consisted of either 22.5 mg./kg. sodium nitrite plus 1 g./kg. sodium thiosulphate, or 1 mg./kg. *p*-aminopropiophenone plus 1 g./kg. sodium thiosulphate, all given i.p. The MINA (150 mg./kg.) was generally given intraperitoneally, but in a few experiments was administered subcutaneously (200 mg./kg.) so that there was no possibility of direct reaction with prophylactic agent in the peritoneal cavity. With MINA alone, all rats showed severe effects and 7/8 died between 1–4 hr. With both forms of prophylaxis no toxic effects were seen and there were no deaths. In comparative prophylactic experiments with KCN (12 mg./kg. s.c.) no deaths or signs of cyanide poisoning occurred, although the KCN alone was always lethal.

Discussion

In the introduction, it has been suggested that the breakdown *in vivo* of DINA or MINA could lead to the formation of hydrogen cyanide, whereas by the same process DAM could not. Thus the relative toxicities of these substances should be related to the rate and extent of the formation of hydrogen cyanide. The toxicities of DINA, MINA, and DAM are compatible with this idea, the order is DINA>MINA≫DAM. This is explicable since its structure suggests that 1 mole of DINA gives rise to 2 moles of HCN, whereas 1 mole of MINA produces only 1 mole of HCN, and DAM yields no free HCN. In addition, DINA is more unstable than MINA and appears to break down more quickly; thus with a dose of DINA equivalent on a molar basis to one-quarter that of MINA used, two-thirds of the amount of cyanide produced by MINA was obtained from DINA (Table I).

The confirmation of this hypothesis depends upon the identification of cyanide in the tissues of animals poisoned with DINA or MINA. Quantitative estimations of total cyanide in the plasma of rats given lethal doses of DINA and MINA and for comparison KCN showed that more total cyanide was present following oxime poisoning than after KCN. As defined previously, estimations of total cyanide did not differentiate between thiocyanate and cyanide. However, the presence of free HCN has been unequivocally demonstrated. Thus in the experiments with cytochrome oxidase, it was shown that comparable amounts of HCN were present in the plasma from rats killed with MINA or KCN. Again, the existence of HCN, which can be detected by its odour in MINA poisoning, has been confirmed by its estimation in the expired air of MINA poisoned dogs. That cyanide has arisen directly from the breakdown of MINA is suggested by the inverse relationship of plasma cyanide and whole blood MINA concentrations found in rabbits and dogs.

Further evidence confirming the proposed mechanism of MINA toxicity has been obtained by the complete protection against lethal doses of MINA afforded by standard cyanide prophylaxis.

If DAM were broken down by the same process as MINA, acetonitrile would be produced, but no evidence of this mechanism has been obtained. The acute toxic effects of DAM appear to be due to the compound itself; for example, an intravenous injection of DAM (150 mg./kg.) caused immediate cardiac arrest in rats and rabbits.

While it has been shown that the blood level of DAM falls fairly rapidly after injection, the fate of the substance is not known. However, from the experiments described, it does not appear to be metabolized by the same pathway as DINA and MINA.

Summary

1. The nature of the toxicity of the oximes mono*iso*nitrosoacetone (MINA), di*iso*nitrosoacetone (DINA), and diacetyl monoxime (DAM) has been investigated.

2. It has been shown that this is due, with MINA or DINA, to the formation of hydrogen cyanide.

3. There is no evidence to suggest that a similar mechanism will explain the toxicity of DAM.

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