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Metabolism of ethylene glycol dinitrate (ethylene dinitrate) in the rat following repeated administration

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Clark, D. G., and Litchfield, M. H. (1969). *Brit. J. industr. Med.*, **26**, 150-155. **Metabolism of ethylene glycol dinitrate (ethylene dinitrate) in the rat following repeated administration.** The *in vivo* metabolism of ethylene glycol dinitrate (EGDN) following repeated administration in the rat, and the *in vitro* metabolism of EGDN and nitroglycerine following repeated administration in the rat and the dog have been studied. No changes were detected in the *in vivo* or *in vitro* metabolism after repeated injection, and the pattern of excretion of EGDN metabolites in the urine did not alter. It is suggested that the tolerance that develops to the cardiovascular effects of these compounds may be due to the changing influence of some physiological compensatory mechanisms rather than to any change in the pattern of metabolism.

It is a well-known clinical observation that tolerance to the cardiovascular effects of nitroglycerine (NG) and other vasodilator organic nitrates can occur after a short period of regular administration, leading to a reduced response to subsequent doses (Goodman and Gilman, 1965). This tolerance is readily lost after cessation of dosing. A similar situation has been reported among workmen employed in the manufacture of dynamite containing ethylene glycol dinitrate (EGDN). Headache and dizziness and a fall in blood pressure often appear during the first few days of employment. Tolerance then develops and the rest of the working week is usually free from effects (Ebright, 1914; Forssman, Masreliez, Johansson, Sundell, Wilander, and Böstrom, 1958). On returning to work on Monday after a weekend free from exposure, however, the first contact with EGDN often leads to headache again. This is known in the dynamite industry as 'Monday head' (McGuinness and Harris, 1961). Tolerance to the cardiovascular effects of EGDN and a return to the original susceptibility following two days free from exposure has also been shown to occur in the rabbit (Gross, Bock, and Hellrung, 1942).

Tolerance could be due to a change in the pattern of metabolism of EGDN, a tolerant animal metabolizing EGDN in a different manner, or at a different rate, from a non-tolerant animal. The work of Crandall (1933) suggests that tolerance to organic nitrates in the dog is due to a reduction in the rate of breakdown in the blood, but the work of Hasegawa and Sato (1963) suggests that, in the rabbit, tolerance to EGDN is due to an increase in the rate of breakdown.

Since the available evidence is conflicting we have studied the *in vivo* metabolism of EGDN following repeated administration to the rat, and the *in vitro* metabolism of EGDN and NG following repeated administration to the rat and the dog. A previous publication (Clark and Litchfield, 1967) has dealt with the metabolism of EGDN in rats that had received only a single injection of EGDN.

Methods

The animals used were specific pathogen-free female rats

of the Alderley Park (albino) strain (body weights 190 to 230 g.), and female beagle dogs (body weights 8 to 12 kg.).

EGDN and NG (supplied by I.C.I. Nobel Division as the 99% pure materials) were used as 10% solutions in corn oil for the *in vivo* studies and as 10% solutions in ethanol for *in vitro* work.

The collection and preparation of rat blood samples, the analysis of EGDN, ethylene glycol mononitrate, inorganic nitrite and inorganic nitrate, and the collection and analysis of urine were as previously described (Clark and Litchfield, 1967).

Venous blood was taken from the dogs into heparinized tubes for the *in vitro* studies. NG was estimated in ether extracts of blood by a technique similar to that used for the determination of EGDN.

Dogs were given subcutaneous injections of 10 mg./kg. EGDN once a day. Rats were given daily subcutaneous injections of 65 mg./kg. EGDN five days a week for several weeks. By not dosing the rats over the weekends it was hoped to simulate more closely the conditions in industry, where exposure occurs during the working week and the weekends are free from exposure. Animals that had not been previously injected with EGDN were used as controls.

Blood pressures in the conscious rat were recorded from a cannula in the dorsal aorta (Weeks and Jones, 1960).

Results

Tolerance to the hypotensive action of EGDN in the conscious rat

The fall in blood pressure in response to a single subcutaneous injection of 65 mg./kg. EGDN was recorded in conscious rats. Subcutaneous injections of 65 mg./kg. EGDN were then given once a day, five days a week, for 10 weeks, the fall in blood pressure being recorded after several of these injections. Although there was some variation in the responses of individual rats to injections of EGDN, the results illustrated in Fig. 1 are from a typical rat. They show that the first injection of EGDN caused a marked fall in blood pressure (Fig. 1a), but with repeated injections tolerance developed (Fig. 1b and c). This tolerance, however, was lost after a period of 60 hours free from injection and there was a return to the original susceptibility (Fig. 1d). Thus, the rat does not differ from the rabbit (Gross *et al.*, 1942) or the dog (Crandall, 1933) in the development of tolerance.

Metabolism of EGDN *in vitro*

A group of six rats and three dogs was injected with EGDN for five weeks and five days respectively. Twenty-four hours after the last injections blood was taken from the animals. The rat blood was pooled and incubated at 37°C. with 50 μg./ml. EGDN; the three samples of dog blood were each incubated at 37°C. with 50 μg./ml. EGDN. Samples were withdrawn at intervals for the analysis of EGDN, inorganic nitrite, and inorganic nitrate. Blood from control animals was also incubated with EGDN under the same conditions. The results summarized in Table 1 (rat) and Table 2 (dog) show that the *in vitro* metabolism of EGDN in blood from repeatedly injected and control animals did not differ. However, a marked species difference is apparent in that blood from both groups of dogs metabolized EGDN much more rapidly than blood from the rats.

Metabolism of NG *in vitro*

Since Crandall (1933) has shown that dogs given EGDN repeatedly metabolize NG *in vitro* more

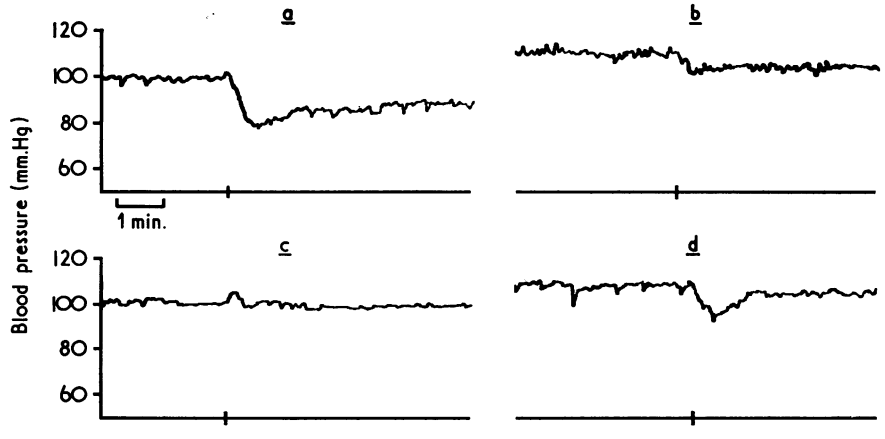

FIG. 1. Mean arterial blood pressure in the conscious rat in response to subcutaneous injections of 65 mg./kg EGDN once a day, five days a week, for 10 weeks. The moment of injection is marked on each trace. The traces show the fall in blood pressure due to (*a*) the first injection, (*b*) the second injection, and (*c*) the 50th injection of EGDN. Trace (*d*) shows the response to an injection 60 hours after the 50th injection.

TABLE 1

THE *in vitro* BREAKDOWN OF 50 μG./ML. EGDN IN WHOLE BLOOD TAKEN FROM CONTROL RATS AND FROM RATS REPEATEDLY INJECTED WITH EGDN

Time (*min.*)	*EGDN remaining* (*μg./ml.*)		*Inorganic nitrite* (*μg./ml.*)		*Inorganic nitrate* (*μg./ml.*)	
	Test	*Control*	*Test*	*Control*	*Test*	*Control*
15	37·5 (3·1)	39·0 (2·2)	1·4 (0·3)	1·6 (0·2)	3·0 (0·8)	2·0 (0·8)
30	25·0 (3·4)	26·0 (2·7)	1·9 (0·1)	2·0 (0·4)	7·5 (1·4)	7·0 (1·8)
60	8·5 (1·1)	10·0 (0·6)	1·2 (0·2)	1·3 (0·2)	13·0 (2·0)	12·5 (1·8)
120	0·5 (0·3)	1·0 (0·5)	0·4 (0·1)	0·4 (0·1)	20·0 (1·8)	20·0 (1·6)

The results are the means of four experiments. Standard deviations are given in parentheses.

TABLE 2

THE *in vitro* BREAKDOWN OF 50 μG./ML. EGDN IN WHOLE BLOOD TAKEN FROM CONTROL DOGS AND FROM DOGS REPEATEDLY INJECTED WITH EGDN

Time (*min.*)	*EGDN remaining* (*μg./ml.*)		*Inorganic nitrite* (*μg./ml.*)		*Inorganic nitrate* (*μg./ml.*)	
	Test	*Control*	*Test*	*Control*	*Test*	*Control*
15	16·4	14·3	2·8	2·1	11·2	12·0
30	5·7	4·5	2·0	1·4	14·6	15·6
60	0·6	0·0	0·9	0·4	18·6	20·0
120	0·0	0·0	0·4	0·3	19·2	20·0

The results are the means of three experiments.

slowly than control dogs, we carried out further experiments to see if the metabolism of NG *in vitro* was in any way different from that of EGDN.

Rats and dogs were injected with EGDN in the manner described in the previous section. Samples of blood were incubated at 37°C. with 50 μg./ml. NG, and aliquots were withdrawn at intervals for the estimation of NG and inorganic nitrite. The results summarized in Tables 3 and 4 show that repeated administration of EGDN did not lead to any change in the *in vitro* metabolism of NG. In these experiments no marked species difference in the rate of breakdown of NG in dog and rat blood was observed.

Metabolism of EGDN *in vivo*

Seventy-five rats were given daily injections of EGDN for 10 weeks. Groups of five rats were taken at standard times after the final injection of EGDN, and the blood EGDN, inorganic nitrite, and

TABLE 3

THE *in vitro* BREAKDOWN OF 50μG./ML. NG IN WHOLE BLOOD TAKEN FROM CONTROL RATS AND FROM RATS REPEATEDLY INJECTED WITH EGDN

Time (*min.*)	*NG remaining* (*μg./ml.*)		*Inorganic nitrite* (*μg./ml.*)	
	Test	*Control*	*Test*	*Control*
15	22·3	23·1	3·4	3·8
30	9·2	9·4	2·8	3·3
60	2·0	2·1	1·0	1·1
120	0·0	0·0	0·4	0·4

The results are the means of two experiments.

TABLE 4

THE *in vitro* BREAKDOWN OF 50 μg./ML. NG IN WHOLE BLOOD TAKEN FROM CONTROL DOGS AND FROM DOGS REPEATEDLY INJECTED WITH EGDN

Time (*min.*)	*NG remaining* (*μg./ml.*)		*Inorganic nitrite* (*μg./ml.*)	
	Test	*Control*	*Test*	*Control*
15	27·5	28·7	1·1	0·8
30	14·7	15·7	1·0	0·7
60	4·2	3·7	0·7	0·5
120	0·0	0·0	0·4	0·1

The results are the means of three experiments.

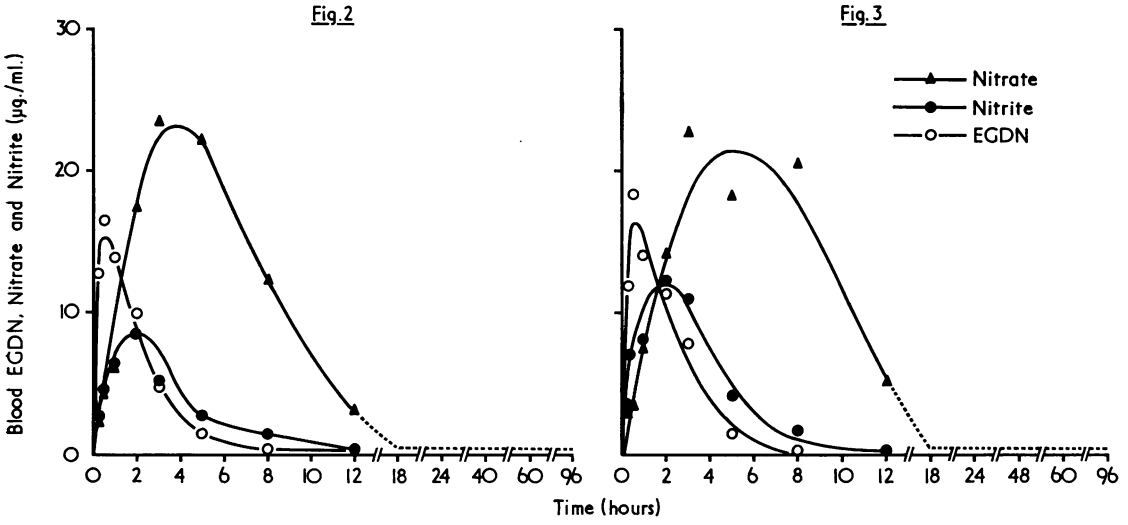

FIG. 2. Blood levels of EGDN, inorganic nitrate, and inorganic nitrite after the final subcutaneous injection of 65 mg./kg. EGDN in rats repeatedly injected with EGDN for 10 weeks. Each point is the mean of five estimations. The mean coefficients of variation of the points on the curves are 25% for EGDN, 23% for nitrate, and 31% for nitrite.

FIG. 3. Blood levels of EGDN, inorganic nitrate, and inorganic nitrite after a subcutaneous injection of 65 mg./kg. EGDN given 60 hours after the last injection in rats repeatedly injected with EGDN for 10 weeks. Each point is the mean of five estimations. The mean coefficients of variation of the points on the curves are 23% for EGDN, 26% for nitrate, and 33% for nitrite.

inorganic nitrate were determined for each animal. The results are presented in Figure 2.

A further group of 75 rats was injected with EGDN daily for 10 weeks, but in this group the rats were given the final injection 60 hours after the penultimate injection. Groups of five rats were then taken at the standard times after the final injection, and blood EGDN, inorganic nitrite, and inorganic nitrate were determined. In this way the metabolism of EGDN after a weekend free from exposure was studied. Figure 3 summarizes these results. A comparison of the two graphs shows no marked differences in the time-course of the various metabolites in the blood. Free EGDN reached a peak within 30 to 60 minutes of injection and fell to zero in eight hours; inorganic nitrite concentration was maximal within two hours and zero within 12 hours. Inorganic nitrate slowly reached a peak between three and five hours after injection and returned to the preinjection level of 1 μg./ml. at 18 hours.

Although the metabolism of EGDN in these two groups of rats was similar, the peak concentrations of inorganic nitrate and nitrite in the blood were greater than those in rats that had not previously been injected with EGDN (Clark and Litchfield, 1967). Since this could indicate a change in metabolism following repeated injections of EGDN, further experiments were undertaken to clarify this point.

Groups of 20 rats were given daily injections of EGDN for periods of zero, two, four, and eight weeks. Blood was taken at intervals after the last injection and analysed for inorganic nitrate and nitrite. The results (Table 5) show a random variation in nitrate and nitrite levels and indicate that the increase, noted above, in nitrate and nitrite in rats injected for 10 weeks could have been due to experimental variation. Further evidence to support this view was furnished by a detailed study of the metabolism of EGDN in rats injected daily for three weeks (unpublished observations). In this case the pattern of metabolism was very close to that of control rats.

In addition to inorganic nitrate and nitrite, ethylene glycol mononitrate (EGMN) is released during the metabolism of EGDN. The production of EGMN in rats repeatedly injected with EGDN has, therefore, been measured. Each rat in a group of 28 was injected with EGDN daily for 10 weeks, and blood was taken at intervals after the last injection. Another group of rats was injected for a similar period but the animals in this group were given a further injection of EGDN 60 hours after the penultimate injection. Blood was taken at intervals after the final injection and analysed for EGMN. The results in Table 6 show that there is no overall difference in the production of EGMN by rats before and after a weekend free from exposure

TABLE 5

CONCENTRATIONS OF INORGANIC NITRITE AND NITRATE IN THE BLOOD OF RATS FOLLOWING A FINAL INJECTION OF 65 MG./KG. EGDN AFTER DOSING WITH EGDN FOR VARYING PERIODS

Time after last injection of EGDN (hrs)	*Nitrite concentration (μg./ml.)*				*Nitrate concentration (μg./ml.)*			
	Period of dosing (wks)				*Period of dosing (wks)*			
	0	*2*	*4*	*8*	*0*	*2*	*4*	*8*
1	8·0 (1·5)	11·5 (1·9)	10·0 (2·3)	8·3 (1·3)	9·4 (3·9)	9·2 (3·4)	14·9 (7·3)	14·5 (6·0)
2	8·3 (2·0)	12·5 (2·8)	10·4 (3·6)	8·9 (2·2)	23·7 (5·5)	14·7 (6·8)	16·0 (7·7)	14·0 (1·8)
5	2·6 (1·0)	4·6 (0·8)	3·2 (1·3)	3·8 (1·8)	23·1 (4·3)	22·2 (5·6)	17·9 (5·2)	23·9 (4·7)
8	1·2 (0·2)	1·2 (0·4)	0·7 (0·2)	1·2 (0·2)	16·2 (4·3)	15·6 (4·3)	12·4 (3·4)	20·2 (7·3)

Groups of five rats were used for each determination. Standard deviations are given in parentheses.

TABLE 6

PRODUCTION OF EGMN *in vivo* IN RATS INJECTED WITH 65 MG./KG. EGDN FOR 10 WEEKS

Time after last injection of EGDN (hrs)	*Blood concentrations of EGMN (μg./ml.)*	
	Group A rats[1]	*Group B rats*[1]
0·5	15·1 (3·9)	12·0 (0·3)
1·0	22·1 (0·7)	18·5 (0·4)
2·0	31·0 (7·7)	28·8 (5·7)
3·0	31·2 (3·2)	27·5 (4·8)
5·0	15·3 (4·0)	16·8 (3·2)
8·0	7·1 (2·3)	7·7 (0·4)
12·0	0·3	0·25

[1]The rats were given their final injection of EGDN 24 hours (group A rats) or 60 hours (group B rats) after the penultimate injection.
The results are the means of four experiments. Standard deviations are given in parentheses.

to EGDN. In both cases EGMN was produced rapidly, reaching a maximum concentration at two to three hours, and then falling almost to zero by 12 hours. These figures do not differ from those previously reported for control rats.

Excretion

A group of rats was injected with EGDN for 10 weeks and the urine was collected over the 24 hours following the last injection. Analysis of the urine (Table 7) showed that inorganic nitrate was the major metabolite, together with small amounts of EGMN. The results did not differ from those obtained on urine from control rats. Thus there was no change in the pattern of excretion following repeated administration of EGDN.

TABLE 7

ANALYSIS OF URINE EXCRETED OVER THE 24 HOURS FOLLOWING AN INJECTION OF EGDN IN A GROUP OF EIGHT RATS INJECTED WITH 65 MG./KG. EGDN FOR 10 WEEKS

Compound	*24-hour excretion (mg.)*	*% of original injection excreted*
EGDN	< 0·1	< 0·1
EGMN	0·5	0·6
Inorganic nitrate ..	56·5	57·5
Inorganic nitrite ..	< 0·1	< 0·1
Total recovery ..	57·1	58·2

The results are the means of two experiments.

Discussion

The *in vitro* results reported here show that the repeated administration of EGDN to rats or dogs did not lead to any change in the metabolism of EGDN or NG in blood taken from these animals. The metabolism was the same as in blood from controls. These results disagree with those obtained by Crandall (1933), who reported that blood taken from EGDN-tolerant dogs metabolized NG at only half the rate of blood taken from control dogs. We are unable to explain this disagreement. It cannot be due to a difference in experimental design, since the species, dose-level, and duration of dosing were similar in both cases.

When the *in vivo* metabolism of EGDN in repeatedly injected rats, both before and after a weekend free from exposure, is compared with that in control rats, it can be seen that the breakdown of EGDN and the liberation of inorganic nitrate, inorganic nitrite, and EGMN did not differ in the three groups of animals. The pattern of excretion of EGDN metabolites also did not differ. Thus the results of the *in vivo* and *in vitro* studies support the conclusion that repeated exposure to EGDN does not lead to any alteration in its metabolism.

This conclusion differs from that of Hasegawa and Sato (1963), who found that when rabbits were injected daily with EGDN, the rate of inorganic nitrate formation increased day by day. They suggested that this could be due to an increase in the amount of the enzyme that metabolized EGDN. A species difference between the rat and the rabbit may explain these conflicting conclusions, but other factors may be involved. If an increased breakdown of EGDN were responsible for the increased formation of inorganic nitrate, the concentration of EGDN in the blood should fall. In fact, Hasagawa and Sato found that it increased. In view of our observation that blood inorganic nitrate levels following EGDN injection can show some variation, the results of Hasegawa and Sato may reflect such a variation rather than a true metabolic change.

Since the metabolism of EGDN does not alter even in rats exposed to EGDN for long periods, it is most probable that metabolic changes do not account for the tolerance that develops to the hypotensive actions of EGDN. Although it is possible that small undetected changes in metabolism occurring at the cellular level could be sufficient to cause tolerance, it is more likely that tolerance is due to the changing influence of some physiological compensatory mechanisms. The speed with which the rat develops tolerance to EGDN following subcutaneous injection suggests that this view may be correct. It is unlikely that metabolic adaptation could develop rapidly but physiological mechanisms could readily be mobilized in a short period. Various physiological mechanisms that could be implicated in tolerance are therefore at present under study in these laboratories.

If the metabolism of EGDN in workmen exposed to EGDN during the manufacture of dynamite is similar to that in the rat, it is possible that the tolerance that develops with repeated exposure, and the partial loss of tolerance leading to 'Monday head' after a weekend free from exposure, is also due to the changing influence of physiological compensatory mechanisms.

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