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Role of inorganic nitrite in methaemoglobin formation after nitrate ester administration to the rat

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Summary

1. Methaemoglobin is rapidly formed during the incubation of rat red cells with inorganic nitrite, whereas ethylene glycol dinitrate at a higher concentration has little effect.

2. The concentrations of blood inorganic nitrite in the rat after ethylene glycol dinitrate or glyceryl trinitrate injection can be reproduced by the infusion of nitrite. The methaemoglobin formed after ethylene glycol dinitrate or glyceryl trinitrate injection is equivalent to that produced from nitrite infusions simulating its formation from the nitrate esters.

3. It is concluded that methaemoglobin arising from nitrate ester administration is formed principally by the action of the metabolite inorganic nitrite.

Introduction

The formation of methaemoglobin *in vitro* and *in vivo* by nitrate esters was noted by Hay (1883) and Haldane, Makgill & Mavrogordato (1897). Wilhelmi (1942) concluded from the ratio of ester given to cats and the amount of methaemoglobin formed that the reaction was molecular and not catalytic as with aromatic nitro-compounds. She suggested that the reaction might be mediated through the action of the metabolite inorganic nitrite, a long established producer of methaemoglobin *in vitro* and *in vivo* (Gamgee, 1868 ; Smith, Alkaitis & Shafer, 1967).

Marshall (1945) demonstrated differences between the initial rates of methaemoglobin formation from the incubation of sodium nitrite or glyceryl trinitrate with blood the former acting after a lag phase, the latter reacting immediately, and concluded that the nitrate ester formed methaemoglobin directly. Hasegawa & Sato (1963a) noted differences in the spectral changes in blood brought about by the *in vitro* reaction of inorganic nitrite in comparison with ethylene glycol dinitrate and used this as the basis for the claim that all the methaemoglobin formed after ethylene glycol dinitrate injection in rabbits was the result of the action of the intact nitrate ester molecule (Hasegawa & Sato, 1963b).

However, in animals given nitrate esters we have noted a correlation between the concentration of blood nitrite and the amount of methaemoglobin produced. This paper describes the results of a study to elucidate the parts played by nitrate esters and inorganic nitrite in forming methaemoglobin after administration of the esters to rats.

Methods

Heparinized rabbit and rat blood or rat erythrocytes washed three times with 1·0% w/v NaCl solution were used and haemolysates prepared by the addition of 0·05 ml whole blood or 0·02 ml washed erythrocytes to 4·0 ml 0·02 M disodium hydrogen phosphate buffer at pH 6·6. To the haemolysates 0·04 ml 0·25% sodium nitrite solution or 0·2 ml 1% ethylene glycol dinitrate solution in alcohol was added and incubated at 25° C. The absorbance from 700–450 nm was measured in 1 cm cells on a Unicam SP 800 recording spectrophotometer at timed intervals. Control determinations were also carried out on haemolysates to which 0·04 ml water or 0·2 ml alcohol was added.

Ethylene glycol dinitrate or glyceryl trinitrate dissolved in corn oil was given by subcutaneous injection at doses of 60 and 120 mg/kg respectively to specific pathogen-free female rats of the Alderley Park (albino) strain (body weights 200–250 g). Sodium nitrite in physiological saline was infused via the femoral vein from a slow injector pump into the animals in restraining cages. The rates of infusion necessary to attain various blood nitrite concentrations were established and the final infusion schedules used to match the formation of nitrite from ethylene glycol dinitrate and glyceryl trinitrate are given in Table 1. At intervals rats were anaesthetized with ether, and the nitrite and methaemoglobin content of blood from the dorsal aorta determined immediately by the methods of Litchfield (1967) and Evelyn & Malloy (1938) respectively.

TABLE 1. *Schedule of i.v. nitrite infusion in rats to simulate the release of inorganic nitrite following the s.c. injection of* 60 *mg/kg ethylene glycol dinitrate* (*I*) *or* 120 *mg/kg glyceryl trinitrate* (*II*)

Infusion period (hours)	NO_2-infused (μg/min)
I	
0–1·0	37·5
1–2·5	24·8
2·5–3·5	18·8
3·5–4·5	12·3
4·5–5·0	7·5
II	
0–1·5	37·5
1·5–3·0	26·3
3·0–4·5	22·6
4·5–6·0	18·8

Results

Figures 1 and 2 respectively show the spectra produced after 15 min reaction between rat whole blood or washed erythrocytes and nitrite or ethylene glycol dinitrate. The traces for ethylene glycol dinitrate are the same as for the controls. With nitrite each sample produces a spectrum where the major peaks of haemoglobin at 578 and 542 nm have been virtually eliminated and a significant peak at 630 nm characteristic of methaemoglobin is formed. The spectrum produced by rabbit blood had a less pronounced shoulder at 542 nm than with the rat but is the same in other respects. If ethylene glycol dinitrate remains in contact with haemolysate for one hour a very small peak at 630 nm appears indicating that some reaction has occurred and analysis shows that 1 μg/ml nitrite is present.

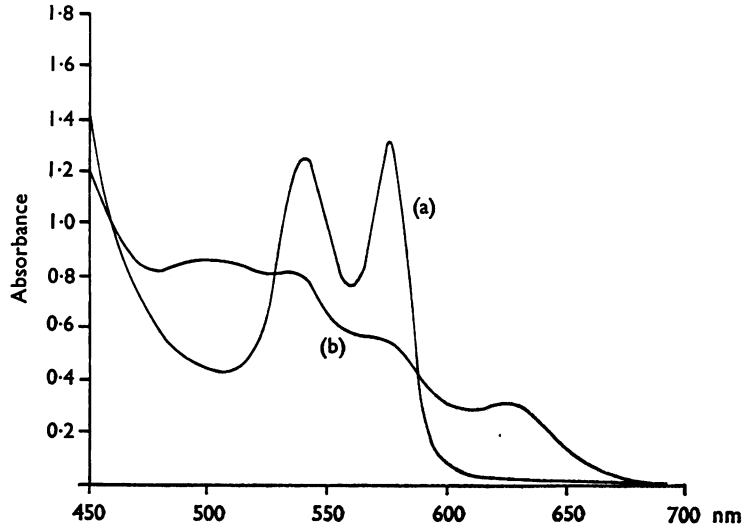

FIG. 1. Spectra produced from the 15 min incubation of 500 μg/ml ethylene glycol dinitrate (a) or 25 μg/ml inorganic nitrite (b) in haemolysate prepared from 0·05 ml rat blood in 4·0 ml 0·02 M disodium hydrogen phosphate buffer. Spectrum for the haemolysate alone coincides exactly with (a).

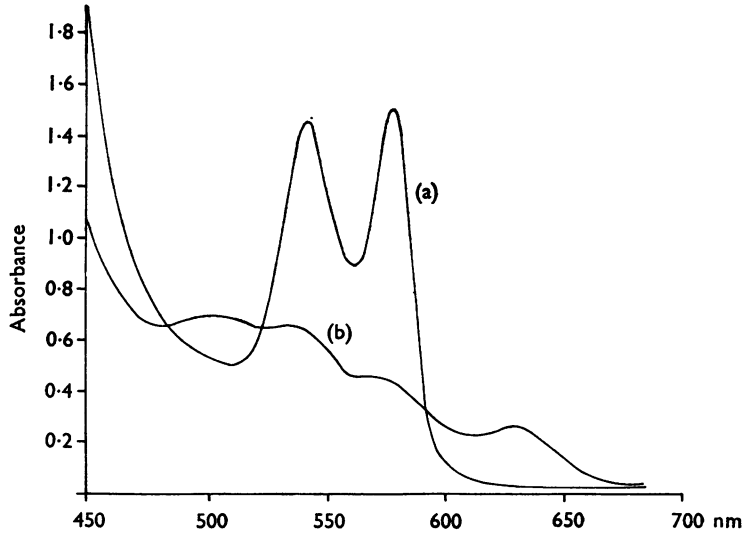

FIG. 2. Spectra produced from the 15 min incubation of 500 μg/ml ethylene glycol dinitrate (a) or 25 μg/ml inorganic nitrite (b) in haemolysate prepared from 0·02 ml rat washed erythrocytes in 4·0 ml 0·02 M disodium hydrogen phosphate buffer. Spectrum for the haemolysate alone coincides exactly with (a).

In vivo the infusion rates of inorganic nitrite (Table 1) were successfully adjusted to produce a similar time-course of blood nitrite concentrations as those produced after ethylene glycol dinitrate or glyceryl trinitrate injection (Figs. 3b and 4b). The corresponding amounts of methaemoglobin arising from each pair of experiments were also very similar (Figs. 3a and 4a) with the peak maxima occurring about one hour after the nitrite maxima.

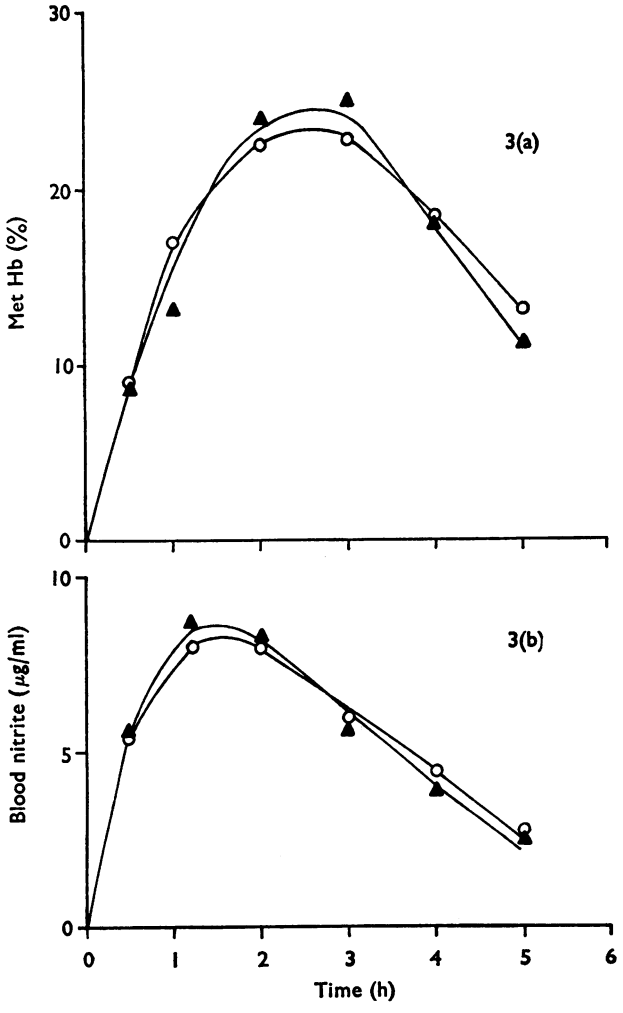

FIG. 3. The amounts of methaemoglobin (a) and inorganic nitrite (b) arising in blood from the injection of 60 mg/kg ethylene glycol dinitrate (○), or the infusion of nitrite (▲), to rats. Each point represents the mean value from five rats.

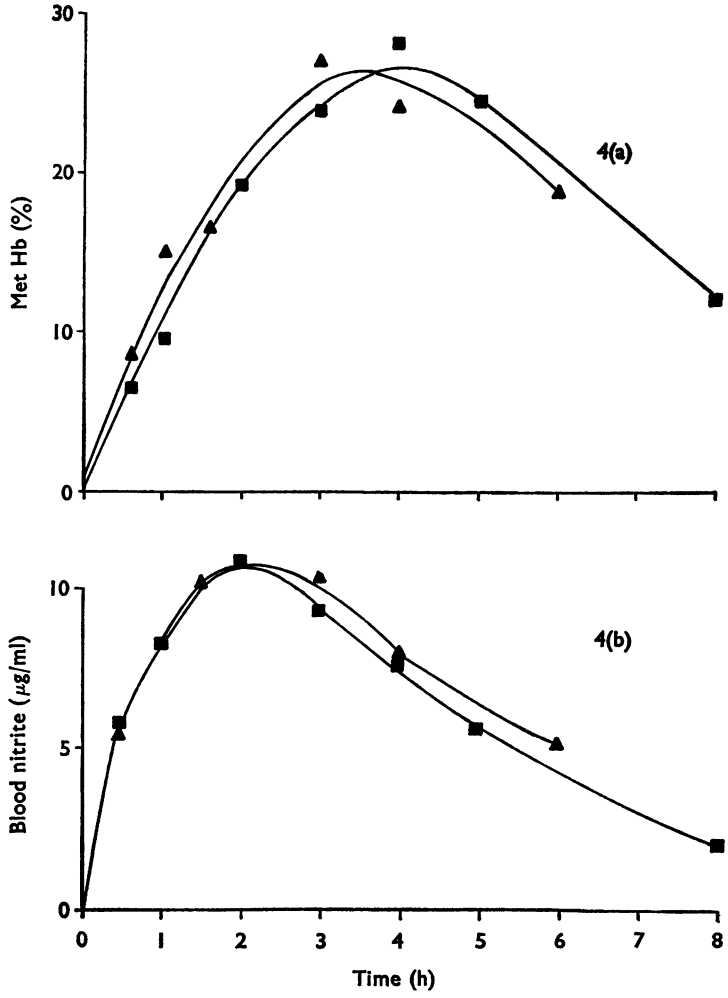

FIG. 4. The amounts of methaemoglobin (a) and inorganic nitrite (b) arising in blood from the injection of 120 mg/kg glyceryl trinitrate (■), or the infusion of nitrite (▲), to rats. Each point represents the mean value from five rats.

Discussion

The *in vitro* experiments showed that up to 500 μg/ml ethylene glycol dinitrate did not form methaemoglobin with rat red cells, whereas much smaller amounts of nitrite effected almost 100% conversion within the same reaction time of 15 minutes. These observations are at variance with the claims of Marshall (1945) and Hasegawa & Sato (1963a) that nitrate esters form methaemoglobin directly from haemoglobin. Their evidence was based upon the observation that spectra of blood from rabbits administered ethylene glycol dinitrate showed an absorption maximum at 500 nm whereas methaemoglobin produced by nitrite *in vitro* did not show this peak. However, the present studies show that rabbit or rat blood react with nitrite to produce spectra with an absorption maximum at 500 nm.

The metabolism of nitrate esters to nitrite has been demonstrated by many studies (Crandall, 1933 ; Von Oettingen, Donahue, Lawton, Monaco, Yogada & Valaer, 1944 ; Needleman & Hunter, 1965 ; Clark & Litchfield, 1967, 1969) and has been questioned only by Hasegawa & Sato (1963b). The concentration of blood nitrite so formed represents a dynamic equilibrium between the breakdown of the nitrate ester and the oxidation of nitrite to inorganic nitrate (Clark & Litchfield, 1967 ; Litchfield, 1971). Since after injection nitrite rapidly disappeared due to oxidation, infusion was used to reproduce nitrite levels found at varying times after an injection of ethylene glycol dinitrate or glyceryl trinitrate in rats. The course of methaemoglobin formation is the same from nitrate ester injection or nitrite infusion and the decay, which is dependent upon reductase action (Stolk & Smith, 1966), is also similar. If ethylene glycol dinitrate or glyceryl trinitrate were to contribute directly to methaemoglobin formation then significantly higher levels of the latter would have been expected from nitrate ester injection and the time-course of reaction would probably have differed from that of the nitrite infusion experiments. It is apparent that nitrite, liberated during metabolism after the injection of nitrate esters, is responsible for the major part, if not the whole, of the methaemoglobin so formed.

The results from the *in vitro* and *in vivo* experiments are complementary, the former showing no direct formation of methaemoglobin by the nitrate ester molecule, the latter demonstrating good correlation between blood nitrite concentrations and methaemoglobin formation. These results can explain the general observation that nitrate esters, such as ethylene glycol mononitrate which produce low concentrations of blood nitrite by metabolism (Clark & Litchfield, 1967) give rise to much smaller amounts of methaemoglobin than glyceryl trinitrate and ethylene glycol dinitrate (Wilhelmi, 1942 ; Gross, Bock & Hellrung, 1942) which produce relatively more nitrite during metabolism.

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