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In vitro degradation of nitroprusside in relation to in vivo decomposition and mechanism of action

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Summary

The in vitro degradation of the vasodilator nitroprusside (200 ng/ml) was systematically studied in various media, viz. phosphate buffer (pH 7.4), albumin solution, cysteine and glutathione solutions, plasma, methaemoglobin and haemoglobin solutions, erythrocyte suspensions, whole blood and in 100,000 *g* crude aortic soluble fraction. Only in the aortic soluble fraction a fast decay of the nitroprusside concentration was observed. The extrapolated half-life time of about 2.6 min for the undiluted fraction approaches the in vivo half-life time as deduced from infusion experiments by other authors. Cyanide in a 100-fold excess over nitroprusside did not exert any effect on the rate of degradation in aortic soluble fraction. Probably the antagonizing effect of cyanide on the relaxation can be ascribed to a less efficient formation of nitrosyl-haeme from the prosthetic haeme-protein associated with guanylate cyclase and/or a direct effect on guanylate cyclase.

Introduction

The cardiovascular drug, nitroprusside, is characterized by a rapid restoration of the pre-infusion blood-pressure levels, i.e. within 2–5 min after termination of therapeutic infusions (Palmer and Lasseter, 1975; Kreye and Marquard, 1979). This might be caused by a relatively fast in vivo degradation process. However, the exact mechanism of action and the site of the rapid in vivo degradation of nitroprusside (Kreye, 1980) are not known.

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On incubation with homogenates originating from several types of tissue, nitroprusside appeared to decompose in a non-enzymatic way (Casinelli, 1956; Hill, 1942; Smith and Kruszyna, 1974). It was found that nitroprusside disappears in solutions of cysteine and methionine (Page et al., 1955), while a small loss was observed on incubation with glutathione and ascorbic acid (Nakamura et al., 1977). However, also haemoglobin might be involved in the rapid in vivo metabolism (Smith and Kruszyna, 1974). An in vitro half-life time of about 40 min on the incubation of 20 μg/ml nitroprusside with rat blood was observed (Kreye and Reske, 1982). In these cited studies the obtained in vitro values greatly exceed the in vivo half-life times, derived from infusion

experiments (Palmer and Lasseter, 1975; Kreye and Marquard, 1979). This discrepancy can, at least partly, be explained by the unrealistic, high nitroprusside concentrations used in these studies because of the limited sensitivity of the employed (indirect) analytical methods. On the other hand, a more recent study points at the involvement of peripheral vascular beds in the in vivo degradation process (Kreye and Reske, 1982).

In order to identify factors governing the in vivo degradation of the drug, the in vitro decomposition was studied systematically, i.e. in phosphate buffer (pH 7.4), in (solutions of) blood components, in whole blood and in crude aortic soluble fractions at the relevant level of 200 ng/ml. This concentration was calculated by applying the equation for the steady-state plasma levels during an infusion, assuming a distribution volume of 2.5 litres (Rodkey and Collison, 1977) being the plasma volume, and a biological half-life time of 2 min derived from infusion experiments. Furthermore, the infusion rate was set equal to the average infusion rate for adults of 200 μg/min (Goodman Gilman et al., 1980). In addition it is noteworthy that the relevance of the calculated value is indicated by incubation studies with pre-contracted bovine coronary arterial strips (Gruetter et al., 1979). A dose-dependent relaxation was observed with the largest increase in the extent of relaxation occurring in the concentration range 20–200 ng/ml. For the determination of nitroprusside a sensitive pulse polarographic method was used (Leeuwenkamp et al., 1984; 1985). The estimated detection limit in plasma and serum is 20 ng/ml, permitting determination of nitroprusside down to 10% of its initial concentration in the in vitro experiments.

Experimental

For details on the polarographic method and the used apparatus one is referred to foregoing publications (Leeuwenkamp et al., 1984, 1985). All potentials are given with respect to the saturated sodium chloride calomel electrode.

Reagents and materials

Sodium nitroprusside, potassium hexacyanoferrate(II), potassium hexacyanoferrate(III), L-cysteine hydrochloride, reduced glutathione, perchloric acid, sodium dithionite, sodium dihydrogenphosphate monohydrate, disodium monohydrogen phosphate dihydrate, triethanolamine hydrochloride, ascorbic acid and sodium cyanide were of analytical grade (Merck, F.R.G.) and used as received. Crystallized human albumin fraction V, twice recrystallized human haemoglobin type IV and Sephadex G-25-300 were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. For the preparation of all solutions demineralized water was additionally purified with a Milli-Q Water Purification System (Millipore, Bedford, MA, U.S.A.).

Procedures

For the preparation of the buffered solutions an iso-osmotic phosphate buffer (pH 7.4) consisting of 3.23 g sodium dihydrogenphosphate monohydrate and 16.91 g disodium monohydrogen phosphate dihydrate in 1 litre water was used. 3.0 ml of the media were spiked with 50 μl of an aqueous standard solution of nitroprusside (12 μg/ml). When stored in the dark this spiking solution is indefinitely stable. Prior to the actual incubations, the solutions were equilibrated for 10 min. All incubations were carried out in the dark at 37 ± 0.2°C to avoid photodecomposition of nitroprusside. At preselected time intervals the incubations were stopped by adding to the incubated media 5.0 ml cold phosphate buffer (0°C, pH 7.4). Subsequently, the samples were treated according to the methods below. The percentage degraded nitroprusside was calculated by comparing the measured peak-current with the peak-current at $t = 0$ min (zero-time control). All the measured currents were corrected for the background current, which was evaluated by conducting the whole procedure in nitroprusside-free medium.

Cysteine solutions

The cysteine solutions were prepared by dissolving cysteine hydrochloride in the iso-osmotic phosphate buffer, previously deoxygenated by purging with nitrogen. In order to prevent the oxidation of cystine to cysteine during incubation as indicated by the appearance of a polarographic

peak at about −450 mV, the incubations were conducted under a nitrogen atmosphere. The polarographic measurements were carried out after addition of 5.0 ml cold phosphate buffer (0°C, pH 7.4) and of 7.0 ml 2.1 M perchloric acid to 3.0 ml of the incubated solutions.

Glutathione solutions

The employed procedure was analogous to that for cysteine solutions as described in the Section *Cysteine solutions*.

Albumin solutions

For these incubation experiments the physiological albumin concentration of 4 g/100 ml in the iso-osmotic phosphate buffer was employed. After the incubations 5.0 ml cold phosphate buffer (0°C, pH 7.4) and subsequently 7.0 ml of a 2.1 M perchloric acid solution containing 6.4 mg/ml potassium hexacyanoferrate(II) were added. The precipitated proteins were removed by centrifugation (5 min, 2000 *g*) at ambient temperature. The resulting opalescent supernatant was filtered twice through an acid-resistant membrane filter (0.45 μm; Sartorius, The Netherlands), in order to remove remaining small particles which appeared to cause much noise in the polarographic measurements.

Human plasma

Fresh blood obtained from apparently healthy volunteers was collected in sterilized and siliconized capped bottles containing 75 I.U./ml heparin (Heparin Novo). The plasma was separated by centrifugation (10 min, 2000 *g*) and the collected plasma layer was centrifuged a second time (5 min, 5000 *g*) to remove rigorously the remaining erythrocytes. The obtained plasma was divided in 3.0-ml portions. These were frozen and stored at −20°C.

The plasma samples were slowly thawed before use. After incubation the degradation was stopped by the immediate addition of 5.0 ml cold phosphate buffer (0°C, pH 7.4) followed by 7.0 ml 2.1 M perchloric acid, containing 2 mg/ml potassium hexacyanoferrate(II), to precipitate the plasma proteins which were removed by centrifugation. For the measurements 10.0 ml of the resulting supernatant were used. At the level of 200 ng/ml nitroprusside a relative standard deviation of 1.5% was observed (n = 4).

Erythrocyte suspensions

Fresh heparinized blood obtained from apparently healthy volunteers was used for preparation of the suspensions. A total amount of 200 ml was centrifuged (10 min, 2000 *g*) and the plasma layer was carefully removed. Subsequently, 200 ml cold iso-osmotic phosphate buffer (0°C, pH 7.4) were gently mixed with the packed red blood cells. This procedure was repeated 3 times. Portions of 3.0 ml were stored in capped vials at about 4°C. The suspensions are stable for 2 days as revealed by microscopic examination. After 3 days a considerable disintegration of the red blood cells was observed. Again 5.0 ml phosphate buffer (0°C, pH 7.4) and 7.0 ml perchloric acid containing 4 mg/ml potassium hexacyanoferrate(II) were added to the incubated samples and the precipitated material was removed by centrifugation (5 min, 5000 *g*). Finally the clear, yellowish supernatant was used for the measurements. Standard addition experiments showed that the recovery from the suspensions is quantitative (100 ± 2%; n = 4).

Human blood

10.0 ml of fresh heparinized human blood were – prior to the incubation – equilibrated for 10 min at 37°C. After spiking of these samples with 200 ng/ml nitroprusside, the portions were incubated in duplicate with frequent shaking. At preselected time intervals the incubations were terminated and the samples were rapidly cooled, while swirling, in an ice bath (0°C). Subsequently, the plasma was separated by centrifugation (10 min, 1000 *g*) and 3.0 ml of the separated plasma layer were pipetted. The thus obtained plasma samples were stored overnight at −20°C. In a previous study (Leeuwenkamp et al., 1985) it was shown, that nitroprusside is essentially stable in blood and thus in plasma when stored at this temperature. The samples were analyzed the day after according to the method outlined in the Section *Human plasma*.

Haemoglobin solutions

The obtained haemoglobin preparation con-

tains at least 75% methaemoglobin, as specified by Sigma, while the remainder is haemoglobin in the Fe(II) state. Haemoglobin was quantitatively converted into methaemoglobin by adding a 2-fold M excess of potassium hexacyanoferrate(III) to a 1.5 $\times 10^{-2}$ M haemoglobin solution. Complete conversion was verified spectrophotometrically using the extinction coefficients of methaemoglobin ($E^{542nm} = 7.14\ mM^{-1} \cdot cm^{-1}$, $E^{630nm} = 3.7\ mM^{-1} \cdot cm^{-1}$) and of haemoglobin ($E^{542nm} = 14.37\ mM^{-1} \cdot cm^{-1}$, $E^{630nm} = 0.17\ mM^{-1} \cdot cm^{-1}$) (Craven and DeRubertis, 1978). Hexacyanoferrate(III) was removed by passing the solution over a small column (1 × 5 cm) Sephadex G-25-300, previously equilibrated with the employed buffer, and the red methaemoglobin fraction was collected. The concentration of methaemoglobin in the fraction was determined spectrophotometrically using calibration curves at the wavelengths of 542 nm and 630 nm. As found, the methaemoglobin concentration was approximately a factor of two lower than in the solution applied to the column. Oxyhaemoglobin was obtained in a similar way. However, instead of potassium hexacyanoferrate(III), sodium dithionite in a 10-fold M excess was used (Smith and Kruszyna, 1974; Craven and DeRubertis, 1978). The incubations concerning oxyhaemoglobin ($E^{542nm} = 14.37\ mM^{-1} \cdot cm^{-1}$, $E^{630nm} = 3.7\ mM^{-1} \cdot cm^{-1}$) (Craven and DeRubertis, 1978) were conducted in a nitrogen atmosphere. Incubation was followed by immediate addition of 5.0 ml cold phosphate buffer (0°C, pH 7.4) and 7.0 ml 2.1 M perchloric acid containing 10 mg/ml potassium hexacyanoferrate(II). Finally the measurements were performed in 10.0 ml of the yellowish brown, clear supernatant obtained by centrifugation (10 min, 5000 *g*). Deoxyhaemoglobin ($E^{555nm} = 13.04\ mM^{-1} \cdot cm^{-1}$, $E^{542nm} = 11\ mM^{-1} \cdot cm^{-1}$) (Craven and DeRubertis, 1978) was prepared by repeated evacuation of oxyhaemoglobin solutions and equilibration with nitrogen. Prior to the incubations the solutions were spiked with 50 µl of the nitroprusside standard solution (12 µg/ml), freed from oxygen, in a nitrogen filled home-made glove bag.

Crude aortic soluble fractions

Bovine aortic material was obtained from the local slaughter house and was stored on dry-ice. The aorta was carefully washed with cold and deoxygenated iso-osmotic phosphate buffer. Connective tissue and fat were removed and subsequently the cold aortic tissue was minced with a razor blade. Homogenate was prepared from 41 g aortic tissue in 160 ml cold buffer (0°C, pH 7.4) (Ystral, X1021, 25,000 rpm). The cold homogenate was centrifuged for 30 min at 5000 *g* at 0–4°C (Sorvall RC-5B Refrigerated Superspeed Centrifuge DuPont Instruments) and the resulting supernatant at 100,000 *g* for 60 min at 0–4°C (Sorvall OTD-65 Ultracentrifuge DuPont Instruments). The resulting supernatant was finally purged with nitrogen and stored at −20°C under nitrogen in order to prevent auto-oxidation of in particular guanylate cyclase present in this fraction (Axelsson and Andersson, 1983). After the incubation – performed in a nitrogen atmosphere – and the prompt addition of 5.0 ml cold phosphate buffer (0°C, pH 7.4), the samples were treated with 7.0 ml 2.1 M perchloric acid containing 3.4 mg/ml potassium hexacyanoferrate(II). The clear and colourless supernatant was used for the measurements.

Additional experiments were done in fractions obtained by dilution of the original crude fraction in order to evaluate the rate of degradation in undiluted aortic material. The dilutions were prepared by adding the appropriate amount of cold, deoxygenated phosphate buffer (0°C, pH 7.4) to the originally obtained soluble fraction. Due to the procedure used for the preparation of the soluble fraction, dilution of the aortic material occurs. A dilution factor of 4 was estimated, taking into account the volumes of the aortic material and the sedimented material.

The protein content of the prepared soluble fractions was determined by the Bio-Rad, Coomassie brilliant blue G-250 procedure, as described by the Bio-Rad Laboratories. Lyophilized bovine plasma albumin was used as a standard. The prepared crude aortic soluble fraction appeared to contain 7.0 mg/ml protein.

Results and Discussion

Prior to the actual incubation experiments, the applicability of two buffers was investigated, viz. phosphate buffer (pH 7.4) and Tris buffer (pH 7.4). It was intended to use the selected buffer for the preparation of, for instance, the cysteine solutions as well as for quenching the degradation process in the incubated media by immediate addition of cold buffer to these solutions. In the iso-osmotic phosphate buffer essentially no degradation could be observed on incubation at 37°C over a time period of 60 min in contrast to the Tris buffer in which little degradation was found (about 5% in 60 min). Consequently, the phosphate buffer was preferred. Since albumin (4 g/100 ml) is the major protein present in plasma, decomposition of 200 ng/ml nitroprusside in the presence of the indicated albumin concentration was investigated. A slow decrease of the nitroprusside concentration was observed (Fig. 1). Obviously, albumin will play only a minor role in the in vivo degradation process despite its relatively high concentration in plasma (6.7×10^{-4} M).

On incubation with sulfhydryl compounds (e.g. cysteine), the formation of S-nitroso products was recently reported (Ignarro et al., 1981). It is clear that formation of these products will be accompanied by decomposition of nitroprusside. The concentration of cysteine in plasma is relatively high (1.2×10^{-4} M) (Diem and Lentner, 1973) and therefore a rather rapid degradation of nitroprusside in plasma was anticipated. On incubation in solutions containing 1.2×10^{-4} M cysteine a rapid linear decrease of the nitroprusside concentration was observed with a half-life time of about 50 min (Fig. 1). However, as already indicated, the degradation process in the albumin solutions proceeds comparatively slowly, despite the fact that albumin is a sulfhydryl-rich protein. The sulfhydryl moieties of albumin are, however, located in hydrophobic clefts. Consequently, these groups are relatively inaccessible for the hydrophilic nitroprusside anion. The lack of reactivity of nitroprusside towards albumin and other proteins was ascribed to this inaccessibility and/or a negatively charged environment of the sulfur sites (Johnson and Wilkins, 1984). As in cysteine solutions, in human plasma (individual A) the loss of nitroprusside is almost linear up to 60 min (Fig. 1) and proceeds at a rate comparable to that in solutions containing physiological cysteine concentrations. In the plasma of a second individual (not shown) a somewhat slower, linear decline in the nitroprusside concentration was observed with a half-life time of about 35 min. Representative polarograms for plasma (individual A) on incubation are given in Fig. 2. Apparently, degradation of nitroprusside in plasma is largely due to interaction with cysteine present in the plasma. Employing a lower concentration of cysteine (1.2×10^{-5} M), a non-linear and much slower decomposition is observed (Fig. 3). Thus the approximately linear loss of nitroprusside as found in plasma and cysteine solutions represents the initial linear part of a non-linear decay curve. In the presence of 200 ng/ml cysteine essentially no degradation of nitroprusside was detectable (Fig. 3).

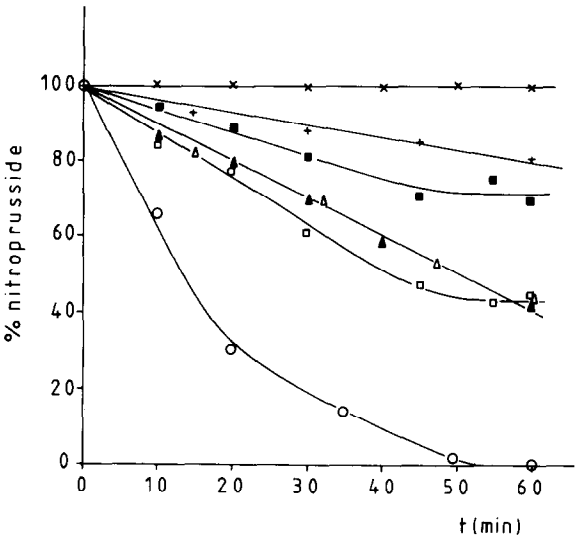

Fig. 1. In vitro degradation of nitroprusside with an initial concentration of 200 ng/ml on incubation at 37°C in blood and blood components: percentage nitroprusside vs time. × phosphate buffer (pH 7.4); ▲ cysteine solution with physiological concentration of cysteine (1.2×10^{-4} M); + albumin solution (4 g/100 ml); △ human plasma (individual A); □ erythrocyte suspension; ■ 2-fold diluted erythrocyte suspension; ○ human blood.

The decay curves for nitroglycerin in a medium

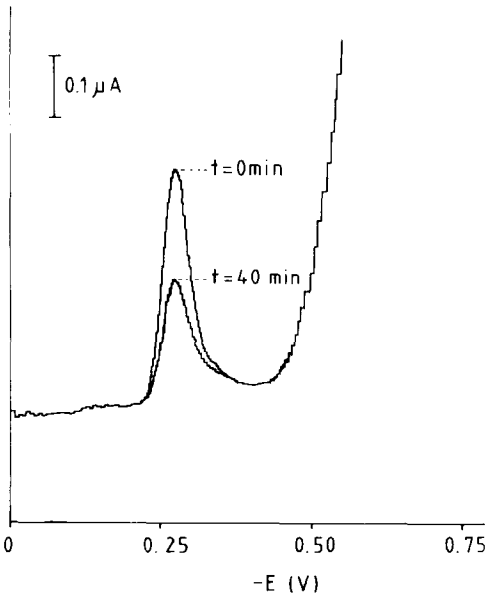

Fig. 2. Typical polarograms (DME, HPDPP) for nitroprusside with an initial concentration of 200 ng/ml on incubation at 37°C in human plasma (individual A), t = 0 min (zero-time control) and t = 40 min, respectively.

of resuspended erythrocytes and in whole blood are very similar, implying that degradation of the substance in intact blood is associated to a large

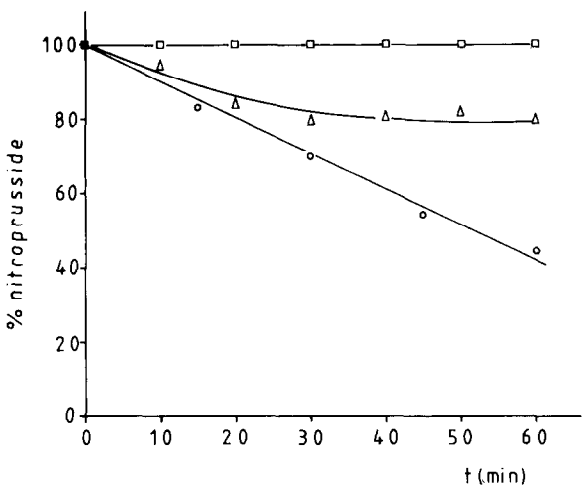

Fig. 3. Plots for nitroprusside with an initial concentration of 200 ng/ml on incubation at 37°C in cysteine solutions with varying cysteine concentrations: ○ 20 μg/ml; △ 2 μg/ml; □ 200 ng/ml.

extent with interaction in the interior of the red blood cells (Sokoloski et al., 1983). This is very plausible because of the known lipophilicity of the uncharged nitroglycerin molecule. Intracellular haemoglobin plays an important role in the degradation process of nitroprusside (Smith and Kruszyna, 1974). It is, however, unlikely that the hydrophilic nitroprusside dianion passes rapidly the negatively charged outer membrane by diffusion and subsequently the lipid phase of the red cell membrane (Krysinski et al., 1984). In order to probe the role of the red blood cells in the degradation process, the in vitro degradation of 200 ng/ml nitroprusside was studied in erythrocyte suspensions. In Fig. 1 the obtained decay curves for an undiluted and a 2-fold diluted suspension are shown. Assuming a normal erythrocyte count of 5×10^9 cells/ml (Ganong, 1975) for the undiluted suspension it can be calculated that at about t = 45 min, the time at which the degradation stops completely, 4000 nitroprusside ions per erythrocyte cell are decomposed. One red cell contains the approximate amount of 29 pg haemoglobin (Ganong, 1975). This amount corresponds to 2.7×10^8 haemoglobin molecules. Obviously decomposition in the suspension does not proceed via interaction with haemoglobin present in the interior of the red blood cells. In view of the already supposed inability of nitroprusside to pass rapidly the membrane, the degradation must occur on the outer side of the membranes. Since the loss of nitroprusside occurs only up to about 45 min, it can be concluded that the capacity of the groups – located on the outer membrane and involved in the degradation process – is limited. Besides, these groups are apparently not regenerated after the interaction with nitroprusside. The foregoing calculation shows that about 4000 nitroprusside ions degrade per erythrocyte. As a consequence, 4000 groups – interacting with nitroprusside – are located at the outer membrane, assuming a stoichiometric interaction. The conclusion that intracellular haemoglobin is not involved in the degradation process, is supported by the observed decay of nitroprusside on incubation with a 10-fold M excess of oxyhaemoglobin (Fig. 4). In contrast to erythrocyte suspensions, in oxyhaemoglobin solutions the nitroprusside concentration de-

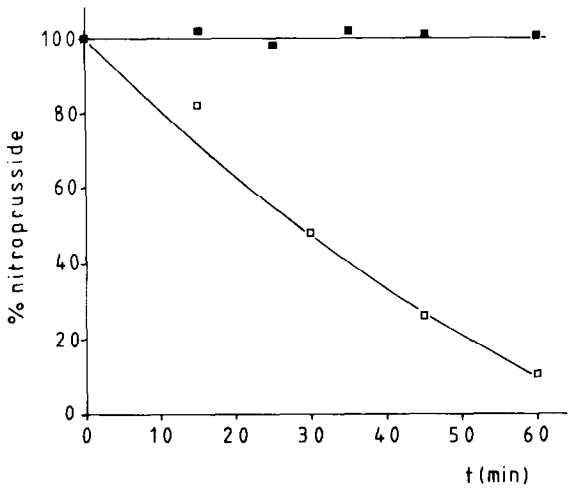

Fig. 4. Percentage remaining nitroprusside vs time for nitroprusside with an initial concentration of 200 ng/ml on incubation at 37°C in oxyhaemoglobin (□) and methaemoglobin (■) solutions, respectively.

creases continuously. On the basis of the decay curves for plasma and erythrocyte suspensions, in blood a non-linear decomposition curve was expected, i.e. an initial rapid loss followed by a much slower degradation phase corresponding with decomposition in plasma. As can be seen in Fig. 1, the anticipated biphasic decay for blood was indeed observed. It must, however, be noted that the degradation curve for human blood is not simply the summation of the decay curves found for plasma and the undiluted erythrocyte suspension. In the first place blood contains other cells in addition to the red blood cells. Secondly, degradation products formed in the plasma can in principle interfere with the decomposition process occurring on the outer membrane of the erythrocytes. Since the initial rapid degradation process is saturable, decomposition according to the slow second phase – related to decomposition in plasma – will be operative after a certain time during an infusion. Therefore, during infusion the actual half-life time will be equal to the half-life time as observed for plasma (about 45 min).

In summary, from the results presented concerning degradation of nitroprusside in blood and blood components it is obvious that the intriguingly rapid in vivo loss – reflected by an exceptional rapid reversal to the pre-infusion blood-pressure values after cessation of a therapeutic infusion – cannot be understood in terms of interaction with blood constituents. This conclusion is in complete accordance with the results of others (Kreye and Reske, 1982). These authors found an in vitro half-life time of about 40 min for 20 μg/ml nitroprusside on incubation with whole rat blood. Because of the high initial nitroprusside concentration employed in that study, degradation according to the slow, plasma-related phase will be observed accounting for the half-life time of about 40 min. Furthermore, the peak in the cyanide concentration in the red blood cells is preceded by a peak in that level in plasma (Vesey et al., 1977). This finding suggests that decomposition occurs outside these cells. However, over 90% of nitroprusside is exclusively present in the plasma (Rodkey and Collison, 1977). Thus, nitroprusside does not enter the erythrocytes and consequently mediation of the degradation by intracellular haemoglobin can be excluded. The quoted half-life time for nitroprusside in rat blood contradicts with the biological half-life times of about 30 s and 5 min, derived from infusion experiments with rats (Kreye et al., 1977; Kreye and Marquard, 1979). Obviously, the erythrocytes do not play a key role in the degradation process.

In vivo rat experiments have suggested that the peripheral vascular beds represent major sites for the in vivo decomposition (Kreye and Reske, 1982). In that study a substantial loss of nitroprusside (30–35%) was observed during one single passage of, for instance, the hind leg vein. Apparently, the blood vessels are important sites for the in vivo decomposition. This is not surprising because nitroprusside and other vasodilators establish their effect, i.e. relaxation, via a direct action on the blood vessels. According to recent studies the enzyme guanylate cyclase is activated by nitro-vasodilators resulting in an enhanced formation of cyclic-GMP, which induces relaxation and thus vasodilatation. The role of cyclic-GMP is clearly demonstrated by two facts. In the first place the lipophilic 8-bromo derivative of cyclic-GMP is a potent relaxant of smooth muscles (Schulz et al., 1979). Secondly, relaxation induced by a nitro-vasodilator such as nitroprusside is

accompanied by a marked elevation of the cyclic-GMP levels, inferring a coupling of these events (Schulz et al., 1977; Katsuki and Murad, 1977; Katsuki et al., 1977; Arnold et al., 1977; DeRubertis and Craven, 1976). Recently, it was unequivocally shown that guanylate cyclase deprived from a prosthetic haeme-protein, due to the method of purification employed, is considerably less responsive towards nitro-vasodilators (Craven and DeRubertis, 1978; Craven et al., 1979; Ignarro et al., 1981; 1982b; Ohlstein et al., 1982). Contrarily, intact enzyme obtained via modified purification methods (Gerzer et al., 1981; Ignarro et al., 1982a; Wolin et al., 1982) or enzyme restored by addition of haeme are fully responsive (Craven and DeRubertis, 1978; Ignarro et al., 1984; Ohlstein et al., 1982; Wolin et al., 1982). In addition it is noteworthy, that the intact enzyme contains a stoichiometric quantity of, presumably, haeme-iron (Gerzer et al., 1981; Wolin et al., 1982; Ignarro et al., 1982a). The haeme-protein is essential, because formation of a nitrosyl haeme-protein is an obligatory step in the sequences of events leading to stimulated formation of cyclic-GMP and subsequently to relaxation (Craven and DeRubertis, 1978; Craven et al., 1979; Ignarro et al., 1981, 1982b; Ohlstein et al., 1982). This decisive role of nitrosyl-haeme formation was demonstrated by the minimal effective concentrations for preformed nitrosyl-haeme (0.1 μM) and for nitroprusside (50 μM).

Furthermore, the haeme-protein must be present in the reduced state explaining the fact, that the enzyme is markedly inhibited by the oxidants methylene blue and hexacyanoferrate(III) (Gruetter et al., 1979). These oxidants convert the haeme-protein to the oxidized state. The oxidized haeme-protein is unable to interact with nitroprusside. Consequently, no nitrosyl-haeme will be formed. Both substances also have a pronounced effect on the relaxation of precontracted bovine arterial coronary strips, induced by nitroprusside (Katsuki et al., 1977: Arnold et al., 1977). The observation that in methaemoglobin solutions essentially no decomposition of nitroprusside can be detected is consistent with the above delineated concept. In order to obtain information on the conditions required for the formation of nitrosyl-haemoglobin from haemoglobin, used as a model compound for the prosthetic haeme-protein of guanylate cyclase, degradation of nitroprusside in the presence of oxyhaemoglobin and deoxyhaemoglobin was studied. Spectrophotometric analysis of the incubated solutions revealed that in both cases cyanomethaemoglobin is the predominant species ($\lambda_{max} = 545$ nm). In contrast, in the presence of deoxyhaemoglobin and a reductor (ascorbic acid) in a large excess with respect to nitroprusside, nitrosyl-haemoglobin ($E^{418nm} = 130$ $mM^{-1} \cdot cm^{-1}$, $E^{545nm} = 12.6$ $mM^{-1} \cdot cm^{-1}$, $E^{575nm} = 13.0$ $mM^{-1} \cdot cm^{-1}$) is formed (Craven and DeRubertis, 1978). In conclusion, the haeme-protein must be present in the deoxygenated state and in addition a reductor in a large excess is required. Apparently, the reductor and cyanide released from unstable, reduced nitroprusside (Schmidt et al., 1974) both compete for intermediate haemoglobin in the Fe(III) state. The occurrence of a redox reaction between nitroprusside and haemoglobin in the Fe(II) state is confirmed by the finding that in a methaemoglobin solution essentially no decomposition could be detected (Fig. 4). Therefore, the reaction does not proceed via the β-93 cysteine moiety in haemoglobin.

As already noted, formation of a nitrosyl haeme-protein is a prerequisite for the induction of the biological effect. Since nitrosyl-haeme formation implies that the nitroprusside ion must release the nitrosyl moiety, degradation and induction of the biological effect are closely related (Gruetter et al., 1979).

In order to investigate this supposition and the role of blood vessels in the in vivo decomposition, in vitro degradation of nitroprusside in crude bovine aortic soluble fractions (100,000 *g*) was studied. These fractions contain in particular the enzyme of interest, viz. guanylate cyclase. Typical polarograms are shown in Fig. 5. The obtained decay curve for 200 ng/ml nitroprusside is shown in Fig. 6. In comparison with the other media, a relatively rapid decline in the nitroprusside concentration is observed, while the soluble fraction used is diluted 4 times because of the method employed for the preparation of the fraction. In order to calculate the rate of disappearance in an undiluted soluble fraction, the decomposition was

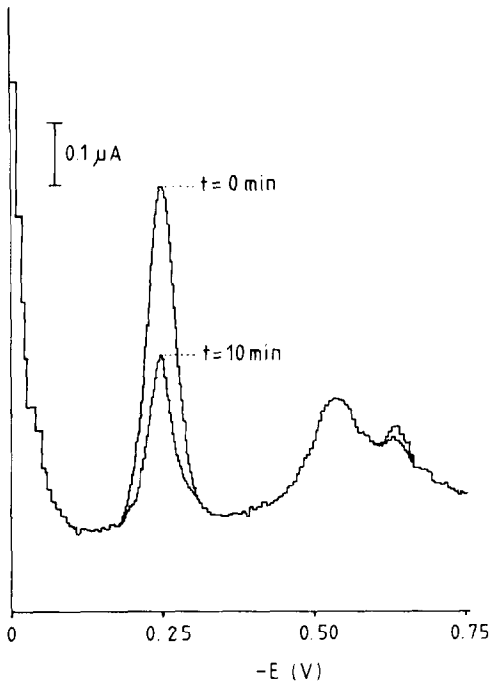

Fig. 5. Typical polarograms (DME, HPDPP) for nitroprusside with an initial concentration of 200 ng/ml on incubation at 37°C in 100,000 *g* crude aortic soluble fraction, t = 0 min (zero-time control) and t = 10 min, respectively.

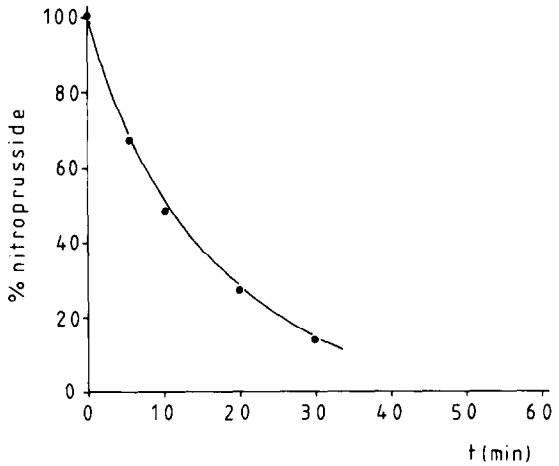

Fig. 6. Decay curve for nitroprusside with an initial concentration of 200 ng/ml on incubation at 37°C in crude aortic soluble fraction (100,000 *g*).

also studied in 6-, 8- and 12-fold diluted soluble fractions, respectively. All the fractions yield linear semilogarithmic plots, indicating first-order decay in all soluble fractions. From these plots the elimination rate constants were evaluated. From the (kinetic) equation $k_{el} = 0.693 \cdot t^{-1/2}$ the corresponding half-life times ($t_{1/2}$) were calculated. In Table 1 the data concerning the aortic soluble fractions are summarized. A linear plot ($r = 0.992$) was obtained for these half-life times vs the dilution factors (Fig. 7). On extrapolation to a dilution factor of 1, i.e. for the undiluted fraction, a half-life time of about 2.6 min was found. Thus degradation of nitroprusside in aortic tissue occurs at an appreciably higher rate than in blood components. Moreover, the calculated value is in reasonable agreement with the observation that after immersion of a saphenous vein strip – relaxed by nitroprusside – in a buffer medium the extent of relaxation diminishes rapidly in a non-linear way, i.e. within 14 min with a half-life time of 4.4 min (Verhaeghe and Shepherd, 1976). In addition, the value of 2.6 min is consistent with the in vivo half-life times derived from infusion experiments in man and animals (Palmer and Lasseter, 1975; Kreye and Marquard, 1979). When decomposition and induction of relaxation are coupled events, the rapid degradation in the aortic material is consistent with the rapid onset of action after the start of a therapeutic infusion.

TABLE 1

EVALUATED FIRST-ORDER ELIMINATION RATE CONSTANTS (k_{el}) AND HALF-LIFE TIMES ($t_{1/2}$) FOR THE INVESTIGATED 100,000 g CRUDE AORTIC SOLUBLE FRACTIONS

Dilution factor	$10^2 \times k_{el}$ (min^{-1}) [b]	$t_{1/2}$ (min)	Corr. coeff. [c]
4 [a]	7.48 (±0.02)	9.27	0.997 (6)
6	6.08 (±0.05)	11.42	0.988 (6)
8	3.99 (±0.01)	17.37	0.999 (6)
12	2.81 (±0.01)	24.69	0.995 (4)

[a] Estimated extent of dilution (vide experimental for the originally obtained crude aortic soluble fraction).

[b] The calculated standard deviation is given in parentheses.

[c] Number of points used in the semilogarithmic plots are given in parentheses.

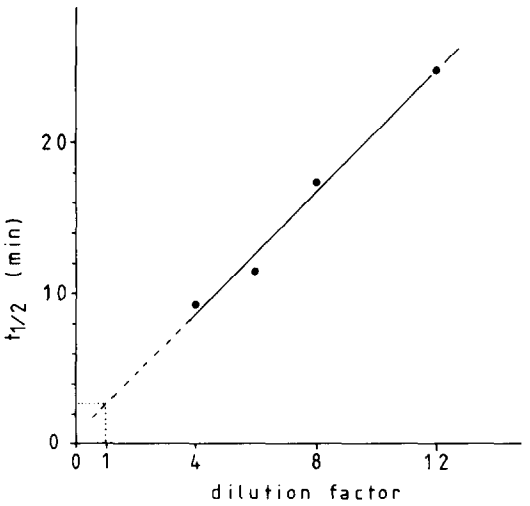

Fig. 7. Plot of the calculated first-order half-life times ($t_{1/2}$) vs the estimated dilution factor.

A retardation of the decomposition was expected in presence of a 100-fold excess of cyanide, if the hypothetical mechanism is correct. In the presence of a relatively high concentration of cyanide, cyanomethaeme might be produced after reduction of nitroprusside and formation of methaeme, which possesses a very high affinity for cyanide. Since methaeme and most probably also cyanomethaeme are unable to interact with nitroprusside, the effective haeme-protein concentration will diminish gradually in the course of the degradation process, leading to a lower decomposition rate. Identical decay curves were obtained in cyanide-free aortic soluble fraction and in media to which a 100-fold excess of cyanide was added over the initial nitroprusside concentration. This result was also obtained when the aortic soluble fractions were preincubated with cyanide for 10 min at 0°C prior to incubation with nitroprusside at 37°C. From the foregoing it can be concluded that nitroprusside decomposes due to interaction with a potent reductor other than the haeme-protein attached to guanylate cyclase. On the other hand, extensive loss of cyanide from the aortic soluble fractions during incubation at 37°C and pH 7.4 must be taken into account. A recent paper points at the same complication arising in incubation studies with cyanide (Rapoport and Murad, 1984).

An antagonistic effect of cyanide, at a large cyanide-nitroprusside ratio, on nitroprusside-induced relaxation in experiments with isolated rabbit aortic strips and acutely denervated gracilis muscle was observed (Kruszyna et al., 1982). Very recently cyanide appeared to have an inhibitory effect on both cyclic-GMP formation as well as on the induction of the relaxation (Rapoport and Murad, 1984). Furthermore, cyanide antagonizes in guanylate cyclase preparations the formation of cyclic-GMP (Wolin et al., 1982; Ohlstein et al., 1982). In view of cyanide antagonism, other observations can be explained completely (Krapez et al., 1981). Accordingly, aquocobalamin enhances the blood-pressure lowering effect elicited by nitroprusside because of its known potency to bind cyanide resulting in the formation of cyanocobalamin.

Additionally, degradation of nitroprusside in the presence of 1.16×10^{-4} M glutathione – set equal to the physiological cysteine concentration – was studied. It was shown that glutathione plays an important role in the metabolism of nitroglycerin by perfused rat liver (Needleman and Harkey, 1971). In their perfusion experiments a substantial decrease of the endogenous glutathione reserve of the liver was observed as well as a rapid decomposition of nitroglycerin. A key role of the liver in the metabolism of (hydrophilic) nitroprusside is, however, not very likely. Pretreatments with phenobarbital and SKF 525-A appeared to have no effect on mortality after nitroprusside in rats (Smith and Kruszyna, 1974). Evidently, hepatic microsomal enzymes do not interact with nitroprusside leading to degradation and cyanide formation. It was suggested that glutathione is involved in the in vitro decomposition occurring in liver homogenate (Smith and Kruszyna, 1974). In the presence of 1.16×10^{-4} M glutathione we have observed a decline in the nitroprusside concentration of about 5% over a period of 60 min. Thus, decomposition of nitroprusside in the liver – mediated by glutathione – is unlikely. However, liver perfusion experiments are required to establish firmly the role of the liver in the in vivo degradation process.

In summary, the present in vitro study presents strong (direct) evidence, obtained via aortic soluble fractions, for the conclusion already drawn by others, that the blood vessels represent major sites for the in vivo decomposition (Kreye and Reske, 1982). More recently, it was shown that degradation of nitroglycerin in the vasculature plays an important role in the in vivo metabolism, providing an explanation for unusual pharmacokinetic features such as sample-site dependence in the venous system (Fung et al., 1984). This phenomenon must be taken into account in the set-up of pharmacokinetic studies concerning nitroprusside. The formation of nitrosyl-haeme is preceded by reduction of the nitroprusside ion due to interaction with a reductor other than the haeme-protein associated with guanylate cyclase. However, direct involvement of the prosthetic haeme-protein in the reduction of nitroprusside cannot be ruled out entirely. In Fig. 8 the events, leading to decomposition and induction of the biological effect, are presented schematically.

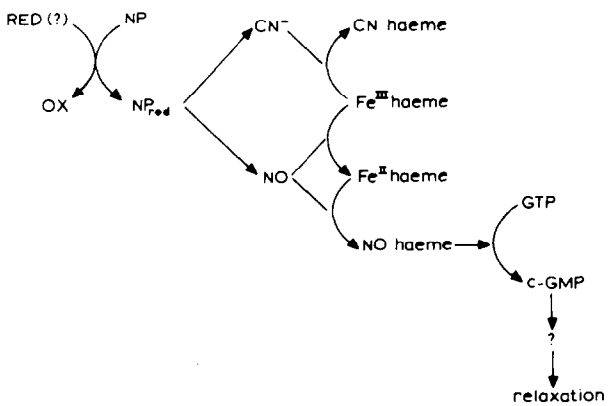

Fig. 8. Sequence of events leading to induction of the biological effect (relaxation). A not identified reductor (RED) reduces the nitroprusside (NP) ion resulting in unstable reduced NP_{red}. Alternatively NP may interact directly with reduced prosthetic haeme-protein (FE^{II}-haeme) of guanylate cyclase (GC). Released NO reacts with (FE^{II}-haeme) under formation of the GC activator nitrosyl-haeme protein (NO-haeme). Activation leads to stimulated formation of cyclic guanosine monophosphate (c-GMP) from guanosine triphosphate (GTP). Unknown steps lead eventually to relaxation. Furthermore, the haeme-protein in the oxidized form (Fe^{III}-haeme) is converted by cyanide, generated from NP_{red}, into the stable cyanometheme (CN-haeme).

Conclusions

Nitroprusside degrades slowly at the relevant initial concentration of 200 ng/ml in whole blood and (solutions of) blood components. The observed half-life times do not account for the rapid in vivo decomposition as found in infusion experiments. However, on incubation in aortic soluble fraction nitroprusside degrades rapidly. For undiluted aortic soluble fraction an (extrapolated) half-life time of about 2.6 min was calculated. This value is comparable to the in vivo half-life time. Evidently, the major site of the extremely rapid in vivo decomposition process is located in the blood vessels. The prosthetic haeme-protein in the reduced state might be involved in the decomposition.

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