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ELIMINATION OF *N*-HYDROXY ARYLAMINES FROM THE BLOOD OF GUINEA PIGS

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Abstract—The rate of elimination of *N*-hydroxy-aniline, *N*-hydroxy-2-aminofluorene, and *N*-hydroxy-*p*-aminopropiophenone from the blood of guinea pigs has been studied by determining the concentrations at various times after the i.v. injection and during the i.v. infusion of the *N*-hydroxy arylamines or nitroso analogs. Guinea pigs were found to dispose of the *N*-hydroxy arylamines very rapidly. The rate of elimination of nitrosobenzene exceeded by far that in rabbits, dogs, and cats, and *p*-nitrosopropiophenone was eliminated 3 times more rapidly than from blood of rabbits.

It is concluded that the rapid disposal of *N*-hydroxy derivatives produced by microsomal *N*-hydroxylation of arylamines plays an important role in the refractoriness of guinea pigs to the carcinogenic and ferrihemoglobin forming activity of 2-aminofluorene.

Experiments with red cells *in vitro* showed that 2-nitrosofluorene rapidly disappears in red cells. Only a fraction of it was found to be reduced to the amine.

THE REFRACTORINESS of guinea pigs to the carcinogenic action of 2-aminofluorene has been shown not to be due to incapacity for *N*-hydroxylating arylamines to active *N*-hydroxy derivatives. Guinea pig liver microsomes *N*-hydroxylate arylamines at rates similar to those observed with the microsomes of other animals.[1, 19] Furthermore guinea pigs excrete arylamines, including 2-aminofluorene and 4-aminobiphenyl partly as *N*-hydroxy derivatives in the urine,[13, 19, 21] Therefore, the rate of disposal of *N*-hydroxy arylamines in guinea pigs became of interest.

One path of inactivation of *N*-hydroxy arylamines is reduction to the amines, which has been observed *in vivo* and *in vitro*.[5, 17, 22] The velocities of *N*-hydroxy-2-aminofluorene disappearance and 2-aminofluorene formation in guinea pig red cells were studied and compared with those in red cells of other species.

The *N*-hydroxy derivatives of aniline, 2-aminofluorene, and *p*-aminopropiophenone were used in these experiments. *In vivo* the *N*-hydroxy arylamines are carried in the blood partly as such and partly as nitroso analogs. A rapid interconversion of the two states occurs in red blood cells.[17] Therefore, no large difference in the metabolic behavior is expected when the *N*-hydroxy compound or its nitroso analog is injected into the blood. In describing concentrations in the blood, elimination rates, etc. the name of the *N*-hydroxy arylamine or its nitroso analog is used as the sum of both. *N*-hydroxy-aniline and *N*-hydroxy-2-aminofluorene which were i.v. injected in large doses were applied as nitroso analogs in order to avoid the direct ferrihemoglobin

formation of the full dose. In addition to the i.v. injection of single large doses, continuous infusion of the *N*-hydroxy arylamines were carried out in order to determine elimination velocities at low concentrations in the blood more precisely.

MATERIALS AND METHODS

Nitrosobenzene was prepared by oxidizing phenylhydroxylamine with chromic acid. The phenylhydroxylamine had been synthetized according to Utzinger's[23] method.

N-hydroxy-*p*-aminopropiophenone was prepared as reported in Graffe *et al.*[5]

2-Nitrosofluorene had been prepared by Dr. G. Renner (See Von Jagow *et al.*[13]).

For i.v. injection or infusion, the substances were suspended in 0·9% sodium chloride solution containing 10–30% Tween 80.

N-hydroxy arylamines and their nitroso analogs were determined as nitroso compounds by the method of Herr and Kiese[9] and by the u.v. absorbance of the nitroso compounds. Blood samples were hemolyzed with cold water immediately after being taken. 10 ml hemolyzate were mixed with 3 drops of a 10% potassium hexacyanoferrate(III) solution and extracted with 6 ml carbon tetrachloride. The carbon tetrachloride extracts were washed twice with 10 ml 0·5 N sulfuric acid and in the experiments with 2-nitrosofluorene 7 times.

2-Aminofluorene in suspensions of red cells incubated with 2-nitrosofluorene was determined by 2 methods. That is, in addition to the estimation with Brodie and Axelrod's[4] method designed for aniline the amine was isolated by thin-layer chromatography (TLC) and estimated by its absorbance of u.v. light; the maximum in methanol solution being at 287 mμ. 10 ml of red cell suspension were extracted with 30 ml benzene. 25 Ml benzene extract were evaporated in a rotary evapomix. The residue was applied to a thin-layer of silica gel HF 254 + 366 and chromatographed with a mixture of 1 vol. hexane and 2 vol. ethyl acetate. The 2-aminofluorene was spotted under u.v. light. The spot with the same R_f as authentic 2-aminofluorene, i.e. about 0·48, was eluted with 3 ml methanol. After the u.v. absorbance had been determined, 2 ml methanol solution were evaporated *in vacuo* and the residue dissolved in 5 ml 0·5 N hydrochloric acid for determination of u.v. absorbance in acid aqueous solution.

Ferrihemoglobin was determined by the increase in absorbance of hemolyzed blood at 550 mμ on addition of cyanide.[14]

Red blood cells, which had been washed 3 times with 0·9% sodium chloride solution were suspended in an equal volume of Krebs–Ringer phosphate solution pH 7·4. The resulting hemoglobin concentration was 15–17 g in 100 ml. Glucose was added with a concentration of 0·2%. The suspensions were incubated at 37° under air with 10^{-4} M (19·5 μg/ml) 2-nitrosofluorene, the corresponding amount being dissolved in a volume of methanol less than 1 per cent of the red cell suspension.

Male guinea pigs weighing on the average 720 g were anesthetized by intraperitoneal injection of 1 g urethane/kg. A jugular vein was cannulated for infusions and a carotid artery for taking blood samples. In the infusion experiments 0·04 ml fluid/min was infused.

RESULTS

Intravenous injections of N-*hydroxy arylamines or their nitroso analogs*

Preliminary experiments showed that large doses of the *N*-hydroxy arylamines

may be i.v. injected for accurate determination of elimination rate. An increase in ferrihemoglobin concentration beyond 40 per cent of total hemoglobin, however, was avoided in order to exclude an effect of severe hypoxia on the elimination. Fig. 1 shows the results of these experiments.

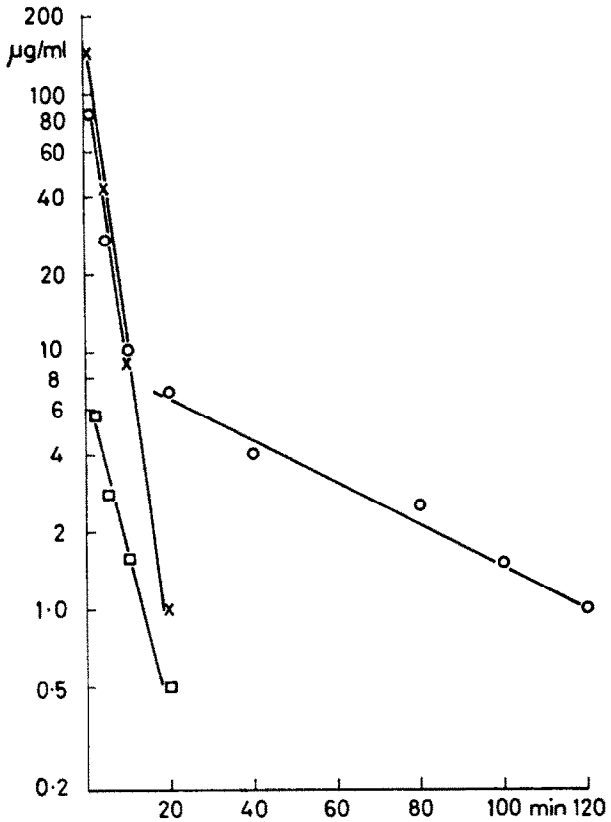

FIG. 1. Decrease in concentration of nitroso compounds observed in the blood of guinea pigs after the i.v. injection of: ×—30 mg nitrosobenzene/kg; ○—40 mg 2-nitrosofluorene/kg;□—5 mg *N*-hydroxy-*p*-aminopropiophenone/kg.
The guinea pigs were anesthetized with 1 g urethane per kg injected i.p.

The i.v. injection of 30 mg nitrosobenzene/kg produced a nitrosobenzene concentration in the blood as high as 200 μg/ml. The rate of nitrobenzene elimination from blood was found to be proportional to the concentration as far down as 1 μg/ml. The half-life of nitrosobenzene in guinea pig blood may be calculated to be about 2 min.

N-hydroxy-*p*-aminopropiophenone, because of its high ferrihemoglobin forming activity, was injected in smaller doses, 5 mg/kg, which produced concentrations less than 10 μg/ml in the blood. The rate of elimination was lower than that of nitrosobenzene, the half-life time being 4 min.

After an injection of 40 mg/kg, 2-nitrosofluorene disappeared at the same rate as nitrosobenzene until the concentration had dropped to about 10 μg/ml. Then the elimination continued at a much lower rate. In this phase, the elimination rate was

again proportional to the concentration of 2-nitrosofluorene in the blood, the half-life being 36 min.

The concentration of ferrihemoglobin increased rapidly after the i.v. injection of the nitroso compounds. The maximum concentrations attained are given in Table 1.

TABLE 1. MAXIMUM CONCENTRATIONS OF FERRIHEMOGLOBIN FOUND IN THE BLOOD OF GUINEA PIGS AFTER THE I.V. INJECTION OF NITROSOBENZENES

Substance	Dose mg/kg	Ferrihemoglobin / Hemoglobin	min after injection
Nitrosobenzene	30	0·4	10
2-Nitrosofluorene	40	0·3	20
N-Hydroxy-*p*-amino-propiophenone	5	0·3	5

Intravenous infusions of nitrosobenzene

The i.v. infusion of nitrososobenzene at a rate of 0·5 mg/kg and min caused a quick increase in nitrobenzene concentration in the blood. In 5 min, concentrations between 4 and 5 μg/ml were attained which remained in this range for an hour while the infusion went on, as may be seen in Fig. 2. The ferrihemoglobin concentration rose more slowly than the nitrosobenzene concentration. After 40 min it leveled off to a concentration equal to about 30 per cent of the hemoglobin.

During the 60 min infusion a dose of 30 mg nitrosobenzene/kg had been infused.

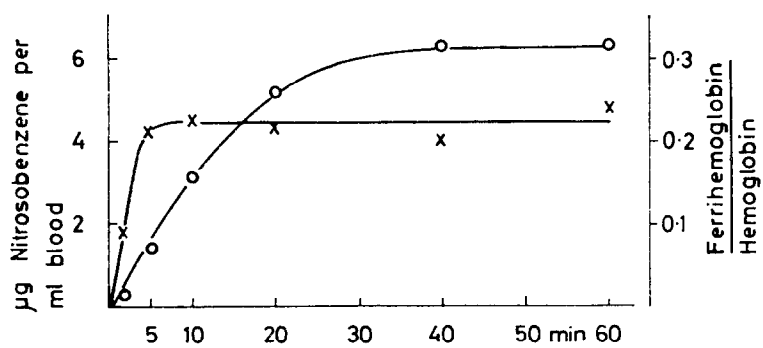

FIG. 2. Nitrosobenzene and ferrihemoglobin concentrations in the blood of guinea pigs during the i.v. infusion of 0·5 mg nitrosobenzene/kg and min.
The symbols indicate the means of 6 experiments.
×—Nitrosobenzene; ○—Ferrihemoglobin.

Intravenous infusions of 2-nitrosofluorene

2-Nitrosofluorene was infused at the rates of 0·25, 0·5, 1·0 and 1·5 mg/kg and min. The results of the nitrosofluorene determinations in the blood during the infusions are summarized in Fig. 3. At all infusion velocities, no tendency of the 2-nitroso-fluorene concentration to level off was observed.

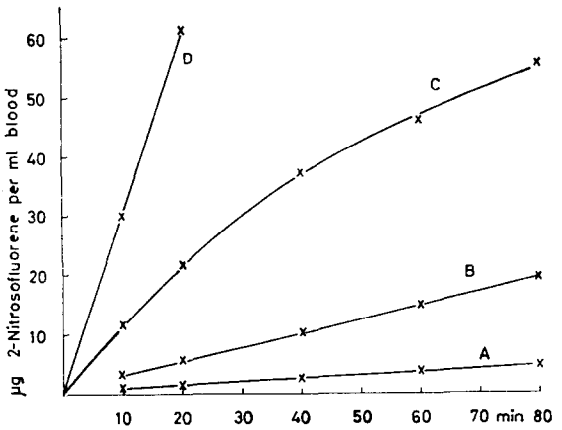

FIG. 3. 2-Nitrosofluorene concentrations in the blood of guinea pigs during the i.v. infusion of 2-nitrosofluorene at various velocities.

	Mg 2-Nitrosofluorene/kg and min	No. of experiments
A	0·25	2
B	0·5	7
C	1·0	3
D	1·4	1

Ferrihemoglobin concentrations were determined in experiments with 0·5 mg and 1·0 mg 2-nitrosofluorene/kg and min. In 60 min the ferrihemoglobin concentration had increased to 17 and 33 per cent of the hemoglobin.

Intravenous infusions of N-*hydroxy*-p-*aminopropiophenone*

N-Hydroxy-*p*-aminopropiophenone was infused at rates of 0·1, 0·15, and 0·2 mg/kg and min. The lowest infusion velocity produced a barely detectable increase in *p*-nitrosopropiophenone concentration. It remained below 1 µg/ml all the time.

With infusions of 0·15 mg and 0·2 mg/kg and min the concentration of *p*-nitroso-propiophenone rose in 80 min to 2·5 and 4·3 µg/ml blood. As may be seen in Fig. 4, the *p*-nitrosopropiophenone concentration increased as long as the infusion went on.

After 80 min infusion of 0·1, 0·15, and 0·2 mg *N*-hydroxy-*p*-aminopropiophenone/kg and min the ferrihemoglobin concentration was found to have increased to 25, 40 and 45 per cent of the hemoglobin.

2-Nitrosofluorene in the blood of guinea pigs injected with 2-aminofluorene

A quarter to half an hour after doses of 25–500 mg 2-aminofluorene had been injected i.p., guinea pigs were anesthetized by i.p. injection of 1 g urethane/kg. Blood samples for determining 2-nitrosofluorene and ferrihemoglobin were taken from the carotid artery at various times after the injection of 2-aminofluorene.

Traces of 2-nitrosofluorene were found only in the blood of animals dosed with 500 mg 2-aminofluorene/kg. None of the blood samples taken from 4 guinea pigs had a 2-nitrosofluorene content higher than 0·2 µg/ml. The average calculated from

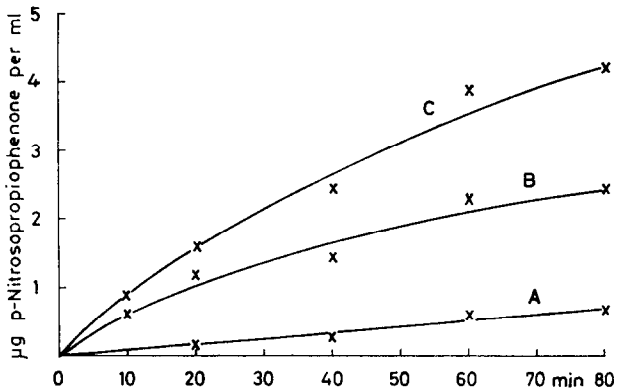

FIG. 4. *p*-Nitrosopropiophenone concentrations in the blood of guinea pigs during intravenous infusions of *N*-hydroxy-*p*-aminopropiophenone.

	Mg *N*-Hydroxy-*p*-aminopropiophenone/kg and min	No. of experiments
A	0·1	5
B	0·15	3
C	0·2	5

the highest concentrations found in the 4 guinea pigs dosed with 500 mg 2-aminofluorene/kg amounted to 0·1 µg 2-nitrosofluorene per ml blood. Because of the small amounts of 2-nitrosofluorene found, these figures may not be very accurate. It is certain, however, that the nitrosofluorene concentration was far below 1 µg/ml.

No increase in ferrihemoglobin concentration was observed after the injection of 500 mg 2-aminofluorene/kg. This confirms the finding of very low concentrations of 2-nitrosofluorene in the blood.

Reduction of 2-nitrosofluorene in red blood cells in vitro

2-Nitrosofluorene disappeared very rapidly from suspensions of guinea pig red cells. Five min after 10^{-4} M (19·5 µg/ml) 2-nitrosofluorene had been added, only 3 µg/ml were recovered. Less than a quarter of the added 2-nitrofluorene was reduced to 2-aminofluorene. The results are summarized in Fig. 5 (A).

In view of the rapid disposal of 2-nitrosofluorene in guinea pig red cells by reactions other than reduction to 2-aminofluorene, the behaviour of 2-nitrosofluorene in the red cells of dogs, rabbits, and cattle was studied. In beef red cells the initial rate of 2-nitrosofluorene reaction was much lower than in the other red cells tested. More than half of the 2-nitrosofluorene added was still present after 5 min incubation. The data presented in Fig. 5 (B) also demonstrate that more than 60% of the 2-nitrosofluorene was recovered as 2-aminofluorene

In red cells from dogs and rabbits the concentration of 2-nitrosofluorene decreased in the first 5 min not quite as rapidly as in red cells from guinea pigs. Dog cells produced a little less 2-aminofluorene and rabbit cells 50 per cent more than guinea pig cells. The ferrihemoglobin concentration increased to about 40 per cent of the hemoglobin in the red cells from dogs and rabbits.

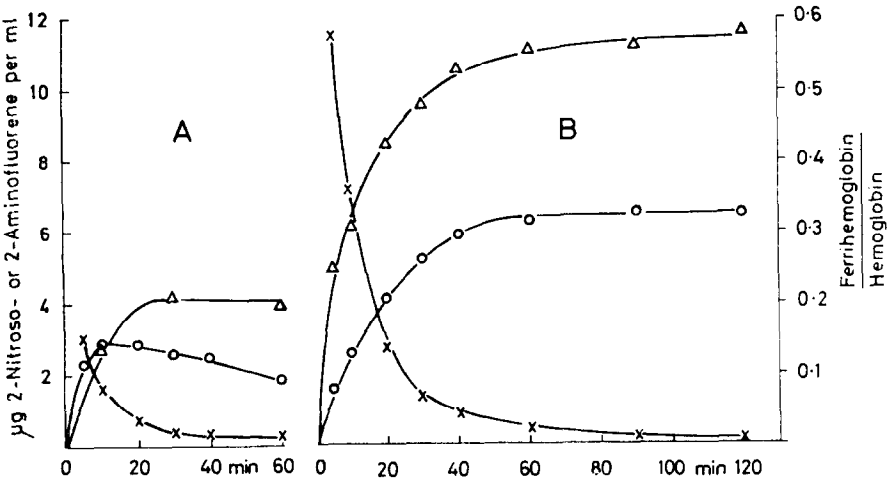

FIG. 5. Reduction of 2-nitrosofluorene (10^{-4} M) to 2-aminofluorene and formation of ferrihemoglobin in red cells from guinea pigs (A) and cattle (B).
The symbols indicate the means of 3(A) and 6(B) experiments
× 2-Nitrosofluorene; △—2-Aminofluorene; ○—Ferrihemoglobin.

DISCUSSION

The experiments demonstrate a rapid disappearance of *N*-hydroxy arylamines from the blood of guinea pigs. The half-life of nitrosobenzene in the blood of cats[8,10] is 3 times longer than in guinea pigs. From infusion experiments on dogs[14] it may be calculated that guinea pigs eliminate nitrosobenzene from the blood 5 times more rapidly than dogs. *p*-Nitrosopropiophenone disappears 4 times more rapidly from the blood of guinea pigs than from the blood of rabbits.[13]

No data are available for the rate of 2-nitrosofluorene elimination from the blood of other animals.

Guinea pigs dispose of a production of *N*-hydroxy-2-aminofluorene as high as 0·25 mg/kg and min at very low concentration in the blood and other tissues. Therefore, only very low concentrations of *N*-hydroxy arylamines can appear in the blood even after large doses of arylamines, although the capacity of guinea pig liver microsomes to *N*-hydroxylate arylamines is not lower than that of microsomes from other species.[1,19] This appears to be an important factor in the resistance of guinea pigs to the carcinogenic action of 2-aminofluorene.

Our infusion experiments reveal two patterns of *N*-hydroxy arylamine elimination. With nitrosobenzene a steady state developed within 5 min after the beginning of the infusion. 2-Nitrosofluorene and *p*-nitrosopropiophenone, however, accumulated in the blood as long as the infusions went on. The accumulation rate was not proportional to the infusion velocity. Doubling of the velocity of 2-nitrosofluorene infusion increased the concentration found 20 or 40 min later by a factor of 4; see Fig. 3. The higher infusion rates seem to exhaust capacities which can rapidly dispose of small amounts of 2-nitrosofluorene. The results of experiments in which a large dose of 2-nitrosofluorene was injected support this supposition.

The experiments with red cells in vitro demonstrate that in addition to the reduction to the amine *N*-hydroxy-2-aminofluorene like other *N*-hydroxy arylamines[7,11,12]

undergoes yet unknown reactions. These reactions are very rapid in red cells from guinea pigs and slow in beef red cells. They transform the *N*-hydroxy arylamines or their nitroso analogs into derivatives which are lacking in the specific actions of *N*-hydroxy arylamines. The part played by these reactions in the rapid disposal of *N*-hydroxy-2-aminofluorene *in vivo* remains to be elucidated.

Previous experiments with other *N*-hydroxy arylamines *in vivo* and in red cells *in vitro* point to large species differences in their half-life and in the ratio of half life *in vivo* to half-life *in vitro*.[2,3,7,8,15,16,20]

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