The Effect of Sodium Nitrite and Para-Aminopropriophenone Administration on Blood Methemoglobin Levels and Red Blood Cell Survival

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It is generally recognized that the administration of certain drugs causes the appearance of methemoglobin (hemoglobin) in the blood of animals and man. We have had occasion to administer methemoglobin-forming drugs to volunteer subjects, both in connection with investigations of the relationship of methemoglobin formation to drug-induced hemolysis and the effect of methemoglobin formation on the course of sickle cell anemia. It was necessary, therefore, to define more precisely than was possible from the existing literature, the effect of methemoglobin-forming agents in animals and human subjects.

Methemoglobin-forming agents may be divided into two general classes. First, there are direct oxidants of hemoglobin which apparently react stoichiometrically with hemoglobin to form the oxidation product, methemoglobin. Secondly, there are those compounds which will result in the oxidation of more molecules of hemoglobin than there are molecules of drug. Such substances apparently act by mediating the oxidation of hemoglobin by oxygen and are themselves re-utilized many times. Sodium nitrite, a direct oxidant, and para-aminopropriophenone (PAPP), one of the most potent of the indirect oxidants, have been studied in the present investigation. When it seemed likely that one of the agents studied (PAPP) was hemolytic, a finding which has not been reported previously, this aspect was also investigated.

Materials and Methods

Experimental animals: Female rats of the Wistar strain were used. The weights of the animals used are specified in the individual experiments.

Human subjects: Hospitalized subjects with non-hematologic diseases, including a glioma and healing tuberculosis were studied. In addition, investigations were carried out on patients with sickle cell disease.

Drug administration: Sodium nitrite for administration to human subjects was obtained in the form of 65 mg. tablets (Eli Lilly & Company). Rats were given solutions of 10 mg. of sodium nitrite per ml. of water, prepared from sodium nitrite (J. T. Baker Chemical Co.) just prior to use. Para-aminopropriophenone (Eastman Organic Chemicals) was dissolved in propylene glycol at a concentration of 10 mg./ml. unless otherwise specified. Propylene glycol was used as a solvent because Tepperman et al. had shown that in aqueous solution or suspension this compound was a very weak methemoglobin-forming agent. We have confirmed this observation. Solutions of PAPP were used within one week of time of preparation. Preliminary studies indicated that there was no significant change in the methemoglobin-forming properties of this compound in this period of time. Further, it was found that autoclaving did not appreciably affect the methemoglobin-forming proper-

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ties of the compound. However, unautoclaved preparations were used in the studies reported here. Injections of sodium nitrite and PAPP were given subcutaneously into the backs of the rats. Intragastric feedings were carried out using a syringe attached to a #10 French catheter.

Methemoglobin determinations: Methemoglobin determinations were made by the method of Evelyn and Malloy, modified by the addition of 0.2 per cent saponin to the M/60 phosphate buffer. In the case of the differential hemolysis experiments, methemoglobin determinations were carried out at a pH of 7.4, the conversion factors being modified appropriately.

Differential osmotic hemolysis studies: Ten ml. of blood were centrifuged at 375 g for 10 minutes. The plasma was discarded and ice-cold 0.9 per cent sodium chloride was added to the red cells to give a total volume of 30 cc. The suspension was centrifuged at 2 C. at 170 g for 15 minutes and the supernatant discarded. No significant reduction of methemoglobin takes place at this temperature. Buffered saline, equivalent to 0.65 per cent sodium chloride, prepared by the method of Dacie, was added to make a total volume of 30 cc. and the re-suspended red cells were incubated at 0 C. for 10 minutes. The suspension was then centrifuged at 2 C. for 15 minutes at 170 g. The supernatant was removed for hemoglobin and methemoglobin determinations. Buffered saline, equivalent to 0.60 per cent sodium chloride, was then added and the procedure repeated. Subsequently, buffered saline equivalent to 0.55 per cent, 0.50 per cent, 0.45 per cent, 0.40 per cent, and 0.30 per cent were added. In each case, the hemolysate representing the cells hemolyzed by the salt strength used, but not by the previous salt strength, was collected and assayed for hemoglobin and methemoglobin content. It was noted that a few unhemolyzed red cells remained in the supernatant after centrifugation at 170 g. Therefore, the studies were repeated in another patient, using a higher centrifuge speed, giving rise to 680 g, to remove all suspended erythrocytes. Otherwise, the same procedure was followed.

Results

1. The time-course of methemoglobin-formation by sodium nitrite and PAPP in rats: Eight rats weighing from 150 Gm. to 190 Gm. were studied. Four rats were given PAPP, 10 mg./Kg. body weight, two by the subcutaneous route, two by the intragastric route. The other four animals were given sodium nitrite, 50 mg./Kg. body weight, two by the subcutaneous route, two by the intragastric route. In figure 1, the time-course of the administration of PAPP by the intragastric and subcutaneous routes are shown. The results of intragastric and subcutaneous administration of sodium nitrite follow a similar time-course. When both the sodium nitrite and PAPP were injected subcutaneously at different sites, an additive effect was observed: higher methemoglobin levels resulted than were observed after injection with either compound alone (fig. 2).

2. The effect of concentration of PAPP on methemoglobin formation: It was found that the concentration of PAPP in propylene glycol injected exerted a distinct influence on the levels of methemoglobin achieved. Twelve rats weighing from 250-350 Gm. each were studied. A total dose of PAPP amounting to 5 mg./Kg. body weight was given to all animals. However, six animals were injected with a solution containing 1 mg./ml., while in the other six rats a solution of 10 mg./ml. was used. Figure 3 demonstrates that higher levels of methemoglobinemia were achieved when more concentrated material was administered, although the total amount of PAPP given was the same in
Fig. 1.—The time course of methemoglobinemia following the administration of 10 mgs. PAPP/Kg. body weight to four rats.

both groups. The difference in methemoglobin level is significant statistically at a confidence level of greater than .01 at one-half and one hour after drug administration.

3. The dosage response curve of rats given PAPP and sodium nitrite: Sixty-six rats weighing from 100–250 Gm. each were studied, following intragastric or subcutaneous administration of sodium nitrite or PAPP. Methemoglobin levels were determined in each case at one-half hour, one hour, and two hours. The maximum level achieved has been recorded in figures 4 and 5. These data indicate that there is no significant difference between the maximum levels of methemoglobin achieved by administration of either of these compounds when the intragastric route is compared with the subcutaneous route. Further, it is evident that PAPP is, mg. for mg., a more potent methemoglobin-forming agent than sodium nitrite. A threshold effect is observed with PAPP: maximum levels of methemoglobin are achieved at dosages of approximately 10 or 15 mg./Kg. Several-fold increases in the quantity of drug administered elicits no greater maximum levels of methemoglobinemia. However, there is a tendency for high levels of methemoglobinemia to be sustained for longer periods of time at the higher dosages of PAPP. A similar effect has previously been described in the case of acetanilid and aniline.
Fig. 2.—The time course of methemoglobinemia following the subcutaneous injection of 30 mg. sodium nitrite/Kg. body weight, 15 mg. PAPP/Kg./body weight, and both compounds. An additive effect is clearly present.

4. The time-course of methemoglobinemia induced in humans with PAPP and sodium nitrite: Three patients without hematologic disease were given single doses of 50 mg., 100 mg., and 150 mg. of PAPP. One patient was also given 130 mg. and 260 mg. of sodium nitrite in single doses. No untoward symptoms were noted in any of the subjects. The change in methemoglobin levels following drug administration is presented in figure 6.

5. The effect of red cell age on methemoglobin content of erythrocytes: The effect of red cell age on the methemoglobin content of red cells after administration of PAPP was investigated in two normal subjects. These data are presented in figures 7 and 8. It is evident that in normal subjects the older, more easily lysed, red cells could not be demonstrated to contain significantly greater quantities of methemoglobin than younger cells. Similar results were observed in two rabbits. These data are not in agreement with those published earlier by Jung. However, since details of his method of separation are not given, no comparison of our data with his is possible. This is in contrast to our findings in subjects with sickle cell disease who show a marked decrease
in the amount of methemoglobin in the younger, more osmotically resistant, red cells. 8 9

6. The effect of chronic PAPP and sodium nitrite administration on methemoglobin levels: While, as shown in figures 1–3 and 6, a single dose of PAPP or sodium nitrite elicits a peak level of methemoglobinemia at one-half to one hour after the administration of drug, very constant levels of methemoglobin are achieved in the blood of subjects with sickle cell disease when PAPP is administered regularly at four-hour intervals. The relatively broad oscillations in methemoglobin levels become damped with the passage of time (fig. 9). Similar results have been observed when sodium nitrite is administered to patients with sickle cell disease over prolonged periods of time. Thus, it is possible to produce very stable levels of methemoglobinemia in human subjects by administering these compounds at four-hour intervals.

7. The effect of PAPP and sodium nitrite on red blood survival: Blood from a normal rat was labeled with Cr\textsuperscript{51} and aliquots were injected into six rats weighing 147 to 182 Gm. The baseline Cr\textsuperscript{51} survival was observed for a period of nine days. Two rats were then given two daily injections of 5 mg.

of PAPP solution in propylene glycol per Kg. of body weight, two were given
The relationship between dosage of sodium nitrite and maximum methemoglobin levels in rats.

50 mgs. sodium nitrite/Kg. body weight twice daily, and two were given .87 cc. propylene glycol twice daily and served as controls. The average Cr\textsuperscript{51} survival curves, hemoglobin levels and methemoglobin levels one hour after drug administration are presented in figure 10. It is noted that the administration of PAPP resulted in rapid destruction of labeled erythrocytes, while the administration of sodium nitrite in doses which caused an equivalent degree of methemoglobinemia, resulted in no demonstrable shortening of erythrocyte survival. In these studies no significant fall in the hemoglobin level of any of the rats was observed; the hemolytic anemia of the PAPP-treated rats was well compensated. In another experiment in which 20 mgs. PAPP/Kg. was administered, marked shortening of Cr\textsuperscript{51} survival was accompanied by substantial degrees of anemia in the experimental animals.

**Discussion**

Relatively little information is available on the time-course of drug-induced methemoglobinemia. While some data have been published relating to dosage of drug to level of methemoglobinemia in various experimental animals,\textsuperscript{1,5,6,10,11} in man, drug-induced methemoglobinemia has been reported chiefly as a sporadic toxic side effect of ingestion of unknown quantities of various chemicals, particularly aniline derivatives and nitrite, or as an emergency treatment for intoxication with cyanide. In the case of two drugs, acetanilid and phen-
Fig. 5.—The relationship between dosage of PAPP and maximum methemoglobin level in rats.

acetin, data relating formation of methemoglobin to dosage has been published for man, as well as for experimental animals, but we are not aware of data relating the dose of nitrite or para-aminopropriophenone to methemoglobin-formation in man. In the present investigations we have defined more precisely the effect of PAPP and sodium nitrite in inducing methemoglobinemia in the Wistar rat and have presented for the first time quantitative studies of methemoglobin formation in hematologically normal human subjects. As in earlier
Fig. 6.—The effect of oral administration of PAPP and sodium nitrite on the methemoglobin concentration of the blood of three hematologically normal subjects.

Fig. 7.—The distribution of methemoglobin in the fractionally osmotically lysed red cells of a hematologically normal subject. In this study, a centrifugal force of 170 g was used to sediment red cells (see Methods).
Fig. 8.—The distribution of methemoglobin in the fractionally osmotically lysed red cells of a hematologically normal subject. In this study, a centrifugal force of 680 g was used to sediment red cells (see Methods).

Fig. 9.—The effect of repeated administration of PAPP at four-hour intervals to a subject with sickle cell disease. Each arrow represents a single dose of 100 mgs. PAPP. It is noted that the broad oscillations in the methemoglobin levels which are present on the initiation of intermittent PAPP therapy become markedly damped and that after several days the methemoglobin level is essentially independent of its time relationship to any single dose.

studies, it has been demonstrated that humans are much more susceptible to the methemoglobin-forming effect of drugs than are rats. These studies indicate that the induction of significant degrees of methemoglobinemia by the administration of sodium nitrite or PAPP may be carried out relatively safely in man. It must be recognized that considerable variations in methemoglobin levels may exist from person to person. However, the limited studies reported may serve to provide the clinical investigator with some information regarding to level to which methemoglobin may be expected to rise following the administration of varying doses of these drugs.

The studies of others have suggested that young erythrocytes have a greater capacity for the reduction of methemoglobin than do older red cells. In sickle cell disease, we have previously demonstrated that young cells contain
Fig. 10.—The effect of subcutaneous injection of PAPP, sodium nitrite, and propylene glycol (PG) on the methemoglobin level, hematocrit, and survival curve of rats. Each curve represents the mean value obtained from two animals. Although the level of methemoglobinemia achieved by the administration of sodium nitrite was slightly higher than that achieved by the administration of PAPP, sodium nitrite administration resulted in no shortening of red cell survival, while PAPP administration caused red cell survival to be shortened.

less methemoglobin after the administration of a methemoglobin-forming agent than do older cells. In the present study, however, investigation of the red cells of hematologically normal human subjects and rabbits failed to
demonstrate such difference when the cells were fractionated by differential osmotic hemolysis. This suggests that in a normal red cell population the difference in the quantity of methemoglobin formed in older and younger red cells must be quite small.

The role of methemoglobin formation in drug-induced hemolysis must as yet be considered uncertain. It has been suggested that methemoglobin may be a precursor of further hemoglobin degradation products. On the other hand, the fact that considerable quantities of methemoglobin may exist in red cells of patients with congenital methemoglobinemia without clinically significant anemia occurring, suggests that methemoglobinemia alone does not suffice to cause red cell destruction. In the present studies, we have been able to demonstrate by measuring red cell survival in an experimental situation, that equal degrees of methemoglobinemia are associated with markedly unequal hemolytic effect. It is possible there may be species differences, since it has been demonstrated that the addition of 1 per cent or 4 per cent sodium nitrite to the diet of mice results in anemia and the appearance of Heinz bodies in most of the mature erythrocytes. On the other hand, Clark and Morrissey observed no anemia when sodium nitrite was administered chronically to dogs. The effect of nitrite on red cells has not previously been studied using modern red cell survival technics.

**Summary**

1. The administration of sodium nitrite or para-aminopropiophenone (PAPP) to rats caused a rise in the methemoglobin level with maximum concentrations of methemoglobin occurring generally after one-half to one hour. The level of methemoglobinemia gradually declined thereafter. No significant difference was observed between intragastric and subcutaneous administration either in the time-course or in the maximum level of methemoglobinemia achieved. When administered at separate sites by the subcutaneous route, sodium nitrite and para-aminopropiophenone exert an additive effect in forming methemoglobin.

2. The concentration of PAPP in propylene glycol affected appreciably the course of methemoglobinemia. More concentrated solutions of PAPP caused greater methemoglobin formation, even when the total amount given was identical.

3. The dosage response curve for PAPP and sodium nitrite in rats has been defined.

4. Limited data have been obtained on the dosage response curves of human subjects without hematologic disease after the administration of sodium nitrite and PAPP.

5. No effect of red cell age on methemoglobin content of erythrocytes could be discerned using the method of differential osmotic hemolysis.

6. Stable concentrations of methemoglobin in the blood of human subjects could be achieved by chronic administration of drug every four hours.

7. The administration of 5 mgs. PAPP/Kg. body weight to rats resulted in accelerated destruction of red cells. In contrast, the administration of propylene glycol alone or the administration of 50 mgs. sodium nitrite/Kg. body weight,
a quantity inducing the same degree of methemoglobinemia, failed to accelerate red cell destruction.

**SUMMARIO IN INTERLINGUA**

1. Le administration de nitrito de natrium o de para-aminopropiophenon (PAPP) a rattos causava un augmento in le nivello del methemoglobina. Le concentration maximal de methemoglobina occurreva generalmente post inter un medie hora e un hora. Subsequentemente le nivello de methemoglobina declinava gradualmente. Nulle significative differentia esseva observate inter le administration intragastric e le administration subcutanee, tanto con respecto al curva de responsa e tempore como etiam con respecto al nivello maximal de methemoglobina attingite. Quando administrate in siti separate per via subcutanee, nitrito de natrium e PAPP exerce effectos mutualmente additive super le formation de methemoglobina.

2. Le concentration de PAPP in glycol de propyleno afficeva appreciabilemente le curso del methemoglobinemia. Plus concentrate solutiones de PAPP causava un plus intense formation de methemoglobina, mesmo quando le total quantitates administrate esseva identic.

3. Le curva de dose e responsa pro PAPP e nitrito de natrium in rattos es definte.

4. Esseva obtenite datos incomplete in re le curvas de dose e responsa in s"jectos human sin morbo hematologic post le administration de nitrito de natrium e PAPP.

5. Nulle effecto del etate del erythrocytos super lor contenuto de methemoglobina poteva esser detegite per le methodo del hemolyse osmotic differential.

6. Stabile concentrationes de methemoglobina in le sanguine de subjectos human poteva esser effectuate per le administration chronic del drogas a intervallos de quatro horas.

7. Le administration de 5 mg de PAPP per kg de peso corporee resultava in rattos in un accelerate destruction de erythrocytos. Per contrasto con isto, le administration de glycol de propyleno sol o le administration de 50 mg de nitrito de natrium per kg de peso corporee (un dose capace a effectuar le mesme grado de methemoglobinemia como 5 mg de PAPP) non resultava in un accelerate destruction de erythrocytos.

**REFERENCES**


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