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CHEMICALLY INDUCED METHEMOGLOBINEMIAS IN THE MOUSE

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Abstract—Nitrobenzene, aniline, *p*-aminotoluene (*p*-AT), *p*-aminoacetophenone (*p*-AAP) and *p*-aminopropiophenone (*p*-APP) have widely different activities as methemoglobin-forming agents in mice. As assessed by circulating levels after intraperitoneal administration, *p*-AT was the least potent compound of the series, but nitrobenzene and aniline were also only weakly active even at lethal doses. Three hydroxylamine analogues from the above series were all about equipotent as methemoglobin-forming agents in mice. Moreover, at a dose of 0·1 m-mole/kg each produced a transient methemoglobinemia that was remarkably similar to that resulting from *p*-APP. A methemoglobinemia with similar temporal characteristics was also produced by *o*-aminophenol, but this compound is about ten times less potent. *p*-Aminophenol was even weaker, being about as active as aniline. When aniline or nitrobenzene was given in combination with sodium nitrite, circulating methemoglobin levels were prolonged. Such a synergism was not seen when either was given in combination with *p*-APP. Methylene blue *in vivo* attenuated the methemoglobinemic response to both *p*-APP and nitrite. It was more effective, however, against the latter, as evaluated at doses that produced equivalent peak circulating levels. In its time pattern the effect of nitrite was similar *in vivo* (intact mouse) and *in vitro* (mouse red cell suspension). In contrast, methemoglobinemia produced by phenylhydroxylamine appears to persist longer in red cells than in the intact animal. Apparently, factors external to the red cell are important in terminating some kinds of induced methemoglobinemias.

IN HIS classic review of methemoglobinemia and the chemicals that produce it, Bodansky[1] drew attention to the circumstance that "the cat has been used almost entirely for the valuation of methemoglobin-forming compounds because of the ease with which this pigment is formed in this species." While such a statement may hold for chemicals that require biotransformation to active methemoglobin-forming metabolites, the response of cat erythrocytes to sodium nitrite is similar to that of canine or human red cells.[2] On the other hand, the apparent resistance of the mouse to the methemoglobin-forming properties of nitrite is probably due to higher rates of methemoglobin reductase activity and not a hemoglobin that is dramatically more resistant to oxidation. Besides an unusually brisk NADH-dependent reductase system, the mouse red cell appears to contain also a NADPH-dependent reductase that can be coupled with methylene blue to further accelerate the reduction of methemoglobin. Such circumstances make it possible to characterize the temporal features of the entire methemoglobinemic response to many chemicals within the span of a few hours.

Coupled with an obvious economic advantage, this species may be particularly valuable for the study of chemically induced methemoglobinemias. Surprisingly, the mouse as well as the cat is recognized as a good experimental subject for what may be a related process, Heinz-body anemia.[3]

Our findings with a variety of chemical methemoglobin-forming agents in mice are reported here. They have led to the formulation of certain generalities about chemically induced methemoglobinemia in this species. In these investigations we have drawn heavily from the numerous published experiences of Kampffmeyer and Kiese and their associates,[4–9] who have previously reported some findings which also occur in other species.

METHODS

Charles River CD_1 30-g female mice and Sprague–Dawley female rats were employed in these experiments. Methods for following the methemoglobinemia in mice after intraperitoneal administration of various chemicals, including techniques for blood sampling and analyses, have been previously described.[10] The techniques for the experiments *in vitro* with washed mouse and rat erythrocytes are also as previously described.[2] Blood from these pentobarbital–ether-anesthetized rodents was taken by open-chest cardiac puncture in heparinized syringes. Heinz-body formation in peripheral blood was demonstrated by the method of Beutler *et al.*[11] Chemicals injected in propylene glycol or dimethyl sulfoxide were prepared in concentrations of 33·6 μmoles/ml for doses of 0·1 m-mole/kg and appropriate multiples of that concentration for higher doses. Statistical comparisons were by Student's *t*-test.

p-Aminopropiophenone (*p*-APP) and *p*-aminoacetophenone (*p*-AAP) were purchased from Eastman. Phenylhydroxylamine (PHA) was obtained from K & K Laboratories. *p*-Hydroxylaminoacetophenone (*p*-HAAP) and *p*-hydroxylaminotoluene (*p*-HAT) were synthesized. Repeated attempts to synthesize *p*-hydroxylaminopropiophenone (*p*-HAPP) were unsuccessful.

p-HAAP. *p*-Nitroacetophenone was synthesized by the reaction of the magnesium salt of diethylmalonate with *p*-nitrobenzoyl chloride, followed by decarboxylation.[12] The *p*-nitroacetophenone was reduced to *p*-HAAP with zinc in an alcoholic ammonium chloride solution, by a modification of Bamberger's method.[13] The crude hydroxylamine was purified by elution chromatography on silicic acid. The purity of the product was determined by thin-layer chromatography (silica gel, developed by 2:1 benzene:ether). Melting point: 121·5–122·0°. Analysis: calc. 63·56%, C; 6·00%, H; 0·26%, N; found 63·72%, C; 6·06%, H; 0·31%, N.

p-HAT. Commercial *p*-nitrotoluene (recrystallized from ligrion) was reduced to *p*-HAT under the same conditions as above. Melting point 90–91°.

p-HAPP. The malonic ester synthesis described above was attempted with methyldiethylmalonate. This route was not successful; perhaps steric hindrance resulted from the presence of the extra methyl group. The reaction of *p*-nitrobenzoyl chloride with either diethyl cadmium or ethyl lithium failed to yield *p*-nitropropiophenone. Bromination of *p*-nitro-*n*-propylbenzene synthesized by nitration of *n*-propylbenzene with subsequent hydrolysis to the ketone in 90% formic acid showed some promise. However, it was not possible to separate the *meta*- and *para*-isomers resulting from the nitration step.

RESULTS

Lethal effects. In preliminary experiments to fix appropriate dose ranges it was noted that unmolested mice in large cages given a lethal dose of nitrobenzene survived for 7 to 24 hr, and animals fatally poisoned by aniline often survived for 10 hr or longer. Considerable variations in survival time, however, were encountered with both chemicals. In general, animals given larger doses of either substance survived for shorter periods of time.

The behavior of both nitrobenzene- and aniline-poisoned mice suggested central nervous system involvement. Animals given lethal doses of nitrobenzene rapidly lost the righting reflex (10 to 15 min) and became comatose, with shallow respirations. Often, however, the mice regained the righting reflex after several hours, only to lose it again prior to death, apparently in respiratory arrest. A few animals showed tremor and some had running movements while in coma. In contrast, aniline-poisoned mice presented primarily signs of central stimulation. Loss of righting reflex and running movements were observed less frequently than with nitrobenzene. Most exhibited tremor, Straub tail reaction, hypersensitivity to sensory stimuli, exophthalmos, chromodacryorrhea, and terminal convulsions. It was our impression that aniline was more toxic to animals confined in restraint cages, where such phenomena as hyperkinesis, salivation, and aggressive behavior were especially pronounced. Because the signs of aniline poisoning were reminiscent of *d*-amphetamine intoxication, repeated attempts were made to demonstrate a significant difference in mortality between isolated and aggregated groups of animals.[14] These attempts were unsuccessful.

TABLE 1. INFLUENCE OF ARGININE ON THE LETHALITY OF ANILINE AND NITROBENZENE IN FEMALE MICE WITH METHEMOGLOBIN LEVELS AT TIME OF DEATH*

Regimen	No. dead/No. tested	Mean per cent methemoglobin	N
Aniline	14/16	8·2	1
Aniline + arginine	12/12	4·2	3
Nitrobenzene	12/12	4·0	4
Nitrobenzene + arginine	12/12	2·7	4

* Aniline and nitrobenzene injected i.p. without diluent via a microliter syringe, 6·4 and 8·1 m-moles/kg respectively. Arginine, 6·0 m-moles/kg, was given s.c. at the same time. Recorded mortalities are for 48 hr. N = number of animals on which methemoglobin analyses were performed.

The results of another experiment to gain insight into the central mechanism of action of aniline or nitrobenzene are summarized in Table 1. These data show that arginine, which in human hepatic insufficiency[15] is reputed to relieve some of the symptoms of ammoniemia, particularly the coma, had no influence on the lethality of either chemical when given in doses which are said to suppress the central excitatory actions of hydrazine.[16] Table 1 also contains the results of methemoglobin analyses performed immediately after death on a few animals on each regimen. Whereas these data may indicate some slight suppression of methemoglobin formation by arginine, they confirm that methemoglobin *per se* could not have been the cause of death in either aniline or nitrobenzene poisoning. Irrespective of the time of death, only very low levels of methemoglobin were found on analysis. Although the values in Table 1

TABLE 2. PER CENT CIRCULATING METHEMOGLOBIN AT VARIOUS TIMES AFTER THE INJECTION OF AROMATIC AMINES OR NITROBENZENE IN FEMALE MICE*

Dose (m-mole/kg)	Compound	0 time	10 min	20 min	40 min	60 min	120 min	180 min
0·1	*p*-APP	1·3 ± 0·6	35·7 ± 7·1	34·0 ± 3·8	26·0 ± 2·8	10·9 ± 2·5	2·4 ± 0·8	
	p-APP†		33·8 ± 10·5	25·3	20·2	12·5	5·9	
	p-AAP		3·6	3·2	0·6	0·9		
	Aniline		2·8	5·0	2·1	2·9		
	p-AT		1·5	1·6	3·0	0		
	Nitrobenzene		4·2	3·9	2·0	0·4		
0·5	*p*-APP		48·0	49·0 ± 8·0	52·0	46·6	25·0	
	p-AAP		18·2	16·1	10·2	4·8		
	Aniline		7·0	8·4	5·8	3·5		
	p-AT		6·4	4·9	4·9	2·6		
	Nitrobenzene		3·9	4·1	3·6	2·8		
1·0	*p*-APP†		51·4	52·5 ± 13·0	49·6	45·0	29·9	3·2
	Aniline		8·8	8·2	6·6	5·8 ± 1·3		
	p-AT		4·5	6·0	4·2	3·1		
	Nitrobenzene		3·0	1·5	1·6	3·3 ± 1·9		
5·0	Aniline		14·6	15·4	14·5	(2/3 dead)		
	p-AT		6·0	6·3	5·2	3·7		
	Nitrobenzene		8·3	9·8	10·2	9·0		
10·0	Nitrobenzene		5·5	7·2	(2/3 dead)			

* Values are either mean ± S.D. for six animals or the simple average for three. All compounds were given intraperitoneally in propylene glycol except for those indicated (†) which were dissolved in dimethyl sulfoxide. The highest dose of nitrobenzene was given without diluent.

are so low that they are not established as significantly different from normal, they may represent an unusual persistence when compared with the effects of most other compounds reported on here (cf. Tables 2, 3, and 7).

An attempt was then made to define methemoglobin levels at the LD_{50} of *p*-APP. Given intraperitoneally in dimethyl sulfoxide (50 mg/ml), the LD_{50} in female mice was 2·3 m-mole/kg. A value of about half this, however, has been reported previously for similar conditions.[17] Death occurred in less than 30 min at doses above the LD_{50} and usually in less than 2 hr at or below the LD_{50}. Loss of righting reflex, coma, tremors, and terminal convulsions, perhaps of anoxic origin, were noted. The heart continued to beat after the cessation of respiration. Blood samples were taken by cardiac puncture immediately after death. It was not possible, however, accurately to assay these samples for methemoglobin. Red cells from such animals were resistant to hemolysis by saponin, and blood specimens appeared to contain considerable cellular debris. On microscopic examination many cells were crenated. Spectrophotometric analyses suggested the presence of "sulfhemoglobin"-like pigments. Evidence for the presence of gross hemolysis and Heinz-body formation is seen in Fig. 1, where a blood specimen from an animal given only dimethyl sulfoxide can be compared with a sample from an animal given a lethal dose of *p*-APP in dimethyl sulfoxide.

Methemoglobin-forming properties. Table 2 summarizes the results obtained with four aromatic amines and nitrobenzene, evaluated as methemoglobin-forming agents in mice. These findings are consistent with those of many investigators using different species.[1] *p*-APP is clearly the most potent of the chemicals tested in that it produces the highest circulating level of methemoglobin at the lowest dose. At a dose of 0·1 m-mole/kg, only *p*-APP produced a definite methemoglobinemia. Its time course was characterized by a rapid rise to peak circulating levels within 10 to 20 min after injection and a relatively abrupt decline toward normal values over the succeeding 2 hr. Dimethyl sulfoxide, a more satisfactory solvent for *p*-APP, can apparently be substituted for propylene glycol as a vehicle, since the methemoglobinemias produced by both preparations were not significantly different 10 min after injection, and they had a similar time course.

With a fivefold increase in dose of *p*-APP (0·5 m-mole/kg in Table 2), significantly more methemoglobin was present, and the time course of the methemoglobinemia seemed to be more prolonged. At this dose, *p*-APP also showed evidence of producing a distinct methemoglobinemia, and its time course was similar to that resulting from the lower dose of *p*-APP. A further increase in the dose of *p*-APP to 1·0 m-mole/kg did not result in a significant elevation of the peak methemoglobin concentration and did not dramatically prolong the duration of the methemoglobinemia. As evidenced by the results obtained at a dose of 5·0 m-mole/kg, aniline is perhaps more potent as a methemoglobin-former in mice than *p*-aminotoluene. Again, even at doses in the lethal range, aniline produced only low levels of methemoglobin when compared with *p*-APP (cf. Tables 1 and 2).

The results in Table 2 also confirm that nitrobenzene is a poor methemoglobin-forming agent in mice. In over a hundredfold range in doses, including a supralethal dose, nitrobenzene failed to produce any indication of a graded response in terms of methemoglobin levels assayed in the first hour after injection. Confinement in a warm restraint cage and repeated blood sampling probably constituted significant

additional stress, since both aniline and nitrobenzene under these conditions produced death in less than an hour.

The results obtained with hydroxylamine analogues of three compounds listed in Table 2 are shown in Table 3. Despite wide differences in the activity of the free amines,

TABLE 3. PER CENT CIRCULATING METHEMOGLOBIN AT VARIOUS TIMES AFTER THE INJECTION OF AROMATIC HYDROXYLAMINES IN FEMALE MICE*

Compound	10 min	20 min	40 min	60 min
p-HAAP	38·3 ± 5·8	32·8	6·2	0·7
PHA	42·1 ± 4·3	28·9	8·4	3·6
p-HAT	33·4 ± 5·7	18·5	5·1	2·3

* All chemicals given i.p. in propylene glycol, 0·1 m-mole/kg. Values are either mean ± S.D. for six animals or the simple average for three. Ten-minute values for PHA and *p*-HAT are significantly different ($P < 0.01$).

the hydroxylamine derivatives all form roughly equivalent amounts of methemoglobin 10 min after administration. Moreover, they all produce a methemoglobinemia with a time course similar to that following an identical dose of *p*-APP (Table 2). Such results suggest that potency differences observed in the free amine series may reflect differences in the rates of metabolism to active methemoglobin-forming compounds, or differences in the rates of detoxication of active intermediates.

Several chemicals were tested for an ability to modify *p*-APP metabolism in mice. It was anticipated that an alteration in the rate of metabolism would manifest itself as an alteration in the intensity or time course of the methemoglobinemia. The selection of iproniazid and SKF 525-A as test compounds was suggested by the results *in vitro* of Kampffmeyer and Kiese.[4] Table 4 shows that pretreatment with a large

TABLE 4. PER CENT CIRCULATING METHEMOGLOBIN AT VARIOUS TIMES AFTER THE INJECTION OF *p*-APP IN FEMALE MICE*

Pretreatment	10 min	20 min	40 min	60 min	120 min
None	35·7 ± 7·1	34·0 ± 3·8	26·0 ± 2·8	10·9 ± 2·5	2·4 ± 0·8
SKF 525-A		30·6 ± 8·2 ($0.3 < P < 0.4$)			
Iproniazid	28·6	22·8 ± 4·2 ($P < 0.01$)	16·8	10·0	0
Sodium azide	25·3	27·0 ± 3·3 ($P < 0.01$)	22·8	20·2 ± 3·5 ($P < 0.01$)	4·3

* *p*-APP given i.p. in propylene glycol, 0·1 m-mole/kg. Values are either mean ± S.D. for six animals or the simple average for three. All pretreatments were given by the i.p. route: SKF 525-A, 25 mg/kg 60 min prior to *p*-APP; iproniazid, 100 mg/kg 12 hr prior to *p*-APP; and sodium azide 25 mg/kg 10 min prior to *p*-APP.

dose of SKF 525-A had no significant effect on *p*-APP-induced methemoglobinemia as judged by levels measured 20 min after injection. As would be predicted from the work of Kampffmeyer and Kiese, however, iproniazid pretreatment produced a significant depression in circulating methemoglobin levels 20 min after *p*-APP.

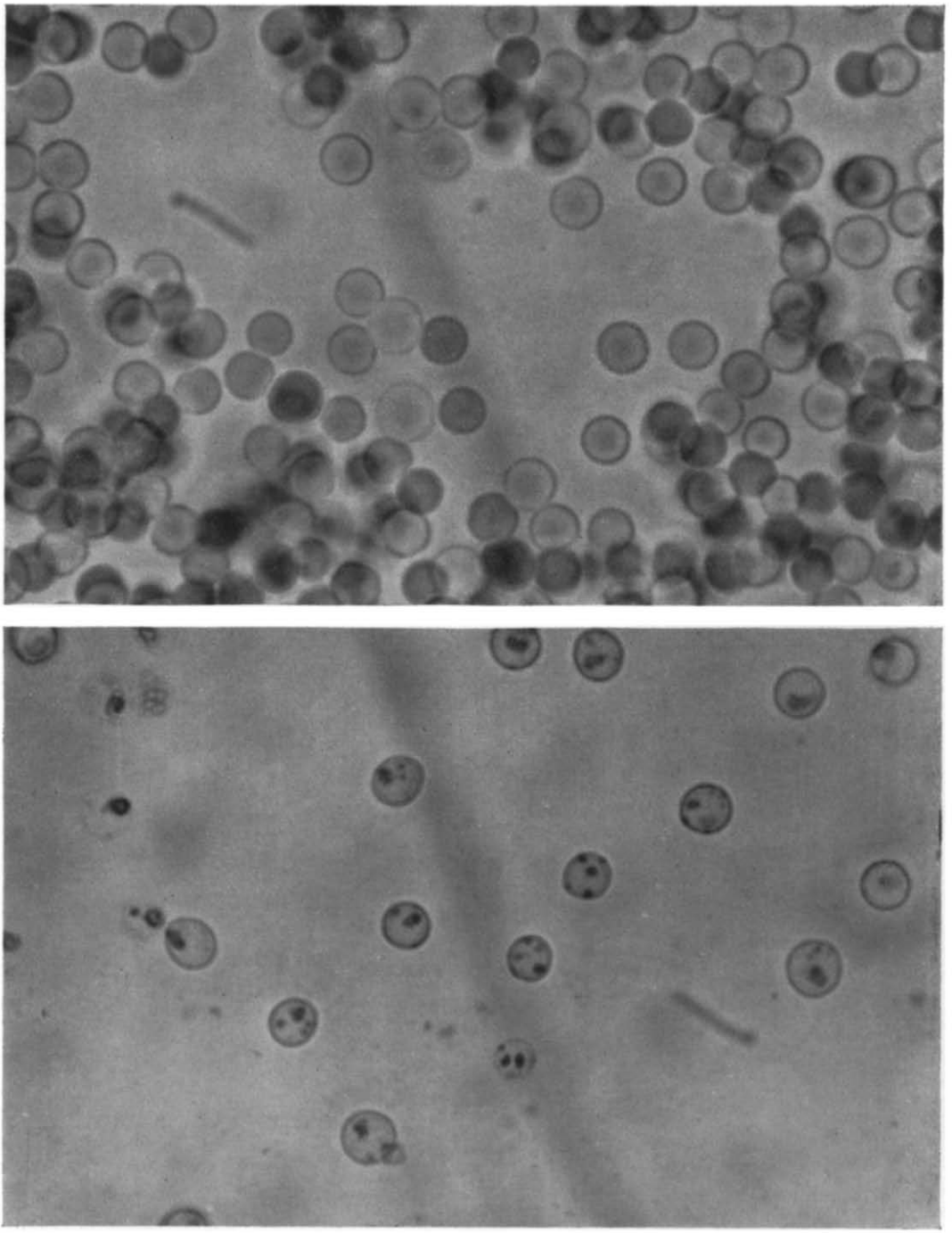

FIG. 1. Mouse erythrocytes (× 970) stained with crystal violet to reveal the presence of Heinz bodies.[11] These identically treated specimens were taken from an animal 15 min after injection with DMSO (above) and from an animal 15 min after injection of a lethal dose of *p*-APP in DMSO (below).

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Sodium azide produced a biphasic modification of the time course. Levels were significantly depressed at 20 min but significantly elevated at 60 min. None of these changes, however, can be interpreted as an influence on *p*-APP metabolism since, as shown in Table 5, iproniazid and sodium azide produced similar effects on the time

TABLE 5. PER CENT CIRCULATING METHEMOGLOBIN AT VARIOUS TIMES AFTER THE INJECTION OF SODIUM NITRITE IN FEMALE MICE*

Pretreatment	20 min	40 min	60 min	120 min	180 min
None	33·4 ± 6·2	34·1 ± 8·4	25·2 ± 7·2	8·1 ± 6·4	1·1
Iproniazid	25·7 ± 3·0 ($P < 0·05$)				
Sodium azide	24·4 ± 2·6 ($P < 0·01$	33·6	39·6 ± 5·6 ($P < 0·01$)	25·2	

* Sodium nitrite given i.p. in water, 1·1 m-moles/kg. Values are either mean ± S.D. for six animals or the simple average for three. Iproniazid and sodium azide given in the doses and sequences indicated in Table 4.

course of nitrite-induced methemoglobinemia. The latter agent is well known not to require biotransformation to active methemoglobin-forming metabolites.

The apparent persistence of circulating methemoglobin after aniline or nitrobenzene, suggested by the data in Table 1, could be explained if these substances or a metabolite common to them inhibit methemoglobin reductase activity. If such were the case,

TABLE 6. PREDICTED VERSUS ACTUAL PER CENT CIRCULATING METHEMOGLOBIN AT VARIOUS TIMES AFTER COMBINED TREATMENTS IN FEMALE MICE*

Treatment	20 min	40 min	60 min	120 min	180 min
Aniline, $NaNO_2$					
Predicted		39·9		8·6	2·7
Actual		44·1 ± 9·8		30·8	7·4
Nitrobenzene, $NaNO_2$					
Predicted		37·4		9·4	1·1
Actual		42·8 ± 13·7		25·3	7·9
Aniline, *p*-APP					
Predicted	31·9		19·2	6·4	
Actual	29·9		12·4	3·9	
Nitrobenzene					
Predicted	26·9		14·9	7·2	
Actual	25·9		10·6	4·0	

* Aniline and nitrobenzene given i.p. in propylene glycol, 1·0 m-mole/kg, 20 min prior to either $NaNO_2$ or *p*-APP in DMSO. The predicted responses are derived from the appropriate $NaNO_2$ control data of Table 5 or the *p*-APP control data (0·1 m-mole/kg in DMSO) of Table 2, to which have been added the appropriate aniline or nitrobenzene data of Table 2; e.g. the mean response 40 min after nitrite has been summed with response 60 min after aniline. Additional controls, each representing the average of three mice, required for this calculation are: 6·7, 0·5, and 1·6 for 80, 140 and 200 min respectively after aniline and 2·4, 1·3, and 0, the corresponding values after nitrobenzene. Actual values are either mean ± S.D. for six animals or the simple average for three.

one might predict an alteration in the time course of nitrite-induced methemoglobinemia in animals pretreated with these two agents. As shown in Table 6, the methemoglobinemic response 40 min after nitrite (60 min after injection of aniline or nitrobenzene) was not different from the sum of the responses when the chemicals were

given separately. Although a threefold difference between predicted and observed circulating methemoglobin levels existed 2 hr after nitrite injection, an inhibition of methemoglobin reductase activity cannot be held responsible, because no such influence occurred with *p*-APP-induced methemoglobinemia (Table 6).

As further confirmation that neither aniline nor nitrobenzene, nor any of their major metabolites, has an influence on methemoglobin reductase activity, they were tested with washed rat erythrocytes. From the "control" reponse of Fig. 2 it can be

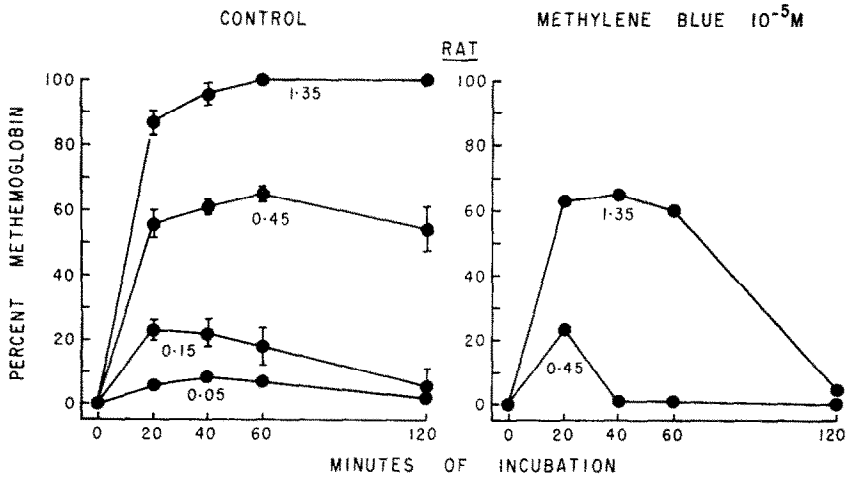

FIG. 2. Methemoglobinemic response to sodium nitrite by washed rat erythrocytes in Krebs–Ringer phosphate glucose, pH 7·4. Numerals refer to nitrite-to-heme mole ratios. Vertical bars show standard deviations. On the right, the cells were preincubated for 15 min with 10^{-5} M methylene blue before the addition of nitrite. See text for an explanation of the "control" response.

seen that, whereas the rat offers an obvious advantage over the mouse in terms of the blood volume that can be collected for experiments *in vitro*, the ongoing methemoglobin reductase activity after nitrite is less brisk than in mouse cells.[2] Net reductions of methemoglobin were nevertheless clearly apparent in the control response within the 2-hr incubation period at nitrite-to-heme mole ratios of 0·45 and lower. Methylene blue elicited an acceleration of reductase activity, as it does with mouse red cells.[2]

The so-called control response of Fig. 2 is in reality a composite presentation of five separate experiments, including a true control and the responses observed when the following additional chemicals were added to the incubating cell suspension 15 min prior to the addition of nitrite: *o*-aminophenol 10^{-5} M, *p*-aminophenol 10^{-4} M, aniline 10^{-3} M, and nitrobenzene 10^{-4} M. As evidenced by the small standard deviations of Fig. 2, the responses to nitrite in the presence and absence of these chemicals were so uniform that all results were pooled to construct the indicated "control" curves.

o-Aminophenol was also tested at 10^{-4} M, at which concentration it proved to be *per se* a fairly potent methemoglobin-producing agent. Levels above 60 per cent were generated in an hour. At 10^{-5} M the methemoglobin production by *o*-aminophenol did not exceed 5 per cent throughout the experiment.

The effects of the two aminophenol isomers were also tested *in vivo*, as shown in Table 7. In mice, *o*-aminophenol was clearly more active than *p*-aminophenol. In terms of peak levels of methemoglobin produced, *o*-aminophenol was about as potent as nitrite (see control response in Table 5) and roughly tenfold less active then phenylhydroxylamine (Table 3), but the time course of *o*-aminophenol-induced methemoglobinemia more closely resembled the latter than the former.

TABLE 7. PER CENT CIRCULATING METHEMOGLOBIN AT VARIOUS TIMES AFTER THE INJECTION OF AMINOPHENOLS IN FEMALE MICE*

Isomer	10 min	20 min	40 min
p-Aminophenol	7·2	4·9	2·3
o-Aminophenol	32·6 ± 5·2	11·9	1·4

* Compounds given i.p. in aqueous solution, 1·0 m-moles/kg. Values shown are either mean ± S.D. for six animals or the simple average for three.

TABLE 8. PEAK PER CENT CIRCULATING METHEMOGLOBIN PRODUCED IN FEMALE MICE AND THE EFFECT OF METHYLENE BLUE*

Methemoglobin-former	Control	After methylene blue
$NaNO_2$	34·1 ± 8·4	3·1 ± 0·2
p-APP	35·7 ± 7·1	10·5 ± 2·6

* Methylene blue, 50 mg/kg, was given i.p. 20 min prior to $NaNO_2$ or *p*-APP. Each nitrite response was measured 40 min after its injection, as previously reported.[2] The *p*-APP responses (0·1 m-mole/kg in propylene glycol as in Table 1) were measured 10 min after injection. Values are means ± S.D. for six animals.

A further distinction among chemicals that induce methemoglobinemia is illustrated in Table 8. Here it can be seen that methylene blue was less effective in attenuating the methemoglobinemic response to *p*-APP than it was in reversing nitrite-induced methemoglobinemia. A dose of methylene blue that completely blocked the peak nitrite response was only partially effective against the peak *p*-APP response.

Methylene blue was also less effective against the methemoglobinemic response of washed mouse erythrocytes to phenylhydroxylamine (Fig. 3) than against the nitrite response previously demonstrated.[2] Whereas the nitrite response has roughly a 1:1 stoichiometry in terms of moles of nitrite required to oxidize moles of heme,[2] Fig. 3 shows that phenylhydroxylamine can produce methemoglobin in an approximately 1:10 stoichiometry. Such a disparity suggests that either arylhydroxylamines react in a cyclic manner with hemoglobin or they influence cellular metabolism in such a way as to predipose to methemoglobin formation. Others[18] have suggested that peroxide formation may be involved in the genesis of some methemoglobinemias. No support for this hypothesis was obtained when phenylhydroxylamine was incubated with mouse cells in the presence of 25 mM aminotriazole (Fig. 3). This concentration of AT has been shown to inhibit human red cell catalase if peroxide is present.[19] This possibility, however, cannot be said to be eliminated, since catalase may

be relatively unimportant in red cells as a means of inactivating peroxide.[20] Probably the most significant feature of the experiments shown in Fig. 3 is the disparity in the time course of the methemoglobinemia induced by phenylhydroxylamine *in vitro* and *in vivo* (Table 3). *In vitro* the response to phenylhydroxylamine resembles that to nitrite. Only in the presence of methylene blue does the *in vitro* response resemble the

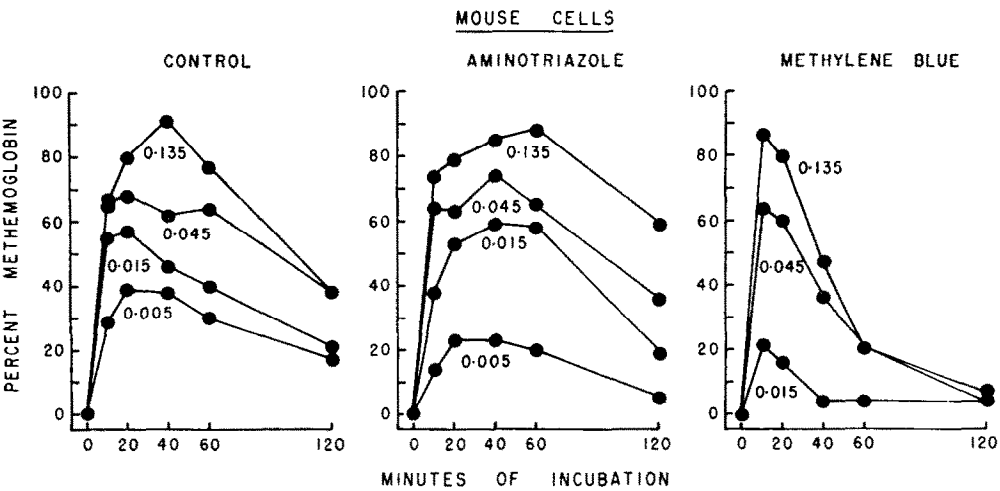

FIG. 3. Methemoglobinemic response to phenylhydroxylamine by washed mouse erythrocytes in Krebs–Ringer phosphate glucose, pH 7·4. Numerals refer to PHA-to-heme mole ratios. In the center, cells were preincubated with 25 mM aminotriazole, on the right with 10^{-5} M methylene blue, both for 15 min prior to nitrite addition.

in vivo response. No such disparity occurs with nitrite.[2] These findings suggest that factors external to the red cell are important in terminating the methemoglobinemia induced by some chemicals.

DISCUSSION

Results presented here indicate that *p*-aminopropiophenone in large doses has profound effects on mouse erythrocytes above and beyond methemoglobin formation. Perhaps these additional effects are responsible for the somewhat different time courses of the methemoglobinemia induced by different doses of *p*-APP (0·1 vs. 0·5 m-mole/kg, Table 2). With several other chemicals, however, the responses of mice were so weak that one would be hard pressed to demonstrate a significant elevation of methemoglobin levels above normal (Tables 1 and 2). It is probably useful to limit the discussion to those agents capable of converting at least a third of the circulating pigment to methemoglobin at their peak activity. This level can be generated by several substances at doses which do not elicit gross signs of toxicity.

Differences between the methemoglobinemias induced by *p*-APP and sodium nitrite in mice have been previously reported.[10] Peak levels of methemoglobin were attained within 10 to 20 min after an intraperitoneal injection of *p*-APP and had declined to near normal after 2 hr. In contrast, peak levels build up more slowly after sodium nitrite and decline more slowly so that the methemoglobinemia is more prolonged. All additional agents studied in this report which produced a "one-third" methemoglobinemia resembled *p*-APP rather than sodium nitrite; i.e. compare control

response of Table 5 with the low dose responses to *p*-APP (Table 1), arylhydroxylamines (Table 3), and aminophenols (Table 7). Hydroxylamine hydrochloride also belongs in the latter group.[21] Although the reason is not known, it is apparent that the methemoglobinemic response of mice to sodium nitrite is atypically slow and persistent. Surprisingly, the fast-reacting group includes at least one agent (*p*-APP) that requires biotransformation to an active methemoglobin-forming substance.

A second generality that can be drawn about the one-third methemoglobinemias deals with the potencies of the various agents. It seems that again there are two general classes. One class includes sodium nitrite, hydroxylamine,[21] and *o*-aminophenol, which are active at doses of 1 m-mole/kg. The other class is about ten times more potent (0·1 m-mole/kg) and includes the arylhydroxylamines. Whereas *p*-APP seems to belong in the latter class, no meaningful statements can be made about other methemoglobin-forming agents which require metabolism to active forms. Again the explanation for this generality is not clear, but the potency distinction carries over to the situation *in vitro*; e.g. compare mole ratio for nitrite to heme in Fig. 2, with those for PHA to heme in Fig. 3.

The hypothesis that hydroxylamines are the active methemoglobin-forming metabolites in arylamine-induced methemoglobinemia has recently been revived.[6] No direct evidence on this question is contributed by the results presented here, but of the various compounds tested, the arylhydroxylamines did most closely match the methemoglobin-forming properties of *p*-APP. On the other hand, the aminophenols (Table 7) which are established metabolites of aniline would appear to account for its metheoglombin-forming properties without evoking the existence of phenylhydroxylamine *in vivo*. The N-hydroxylation of aromatic amines is said to be a NADPH-mediated, oxygen-dependent reaction of liver microsomes. Unhappily, agents reported[4] to influence the rate of this reaction *in vitro* appear to have no influence *in vivo*. It is particularly surprising that the reaction is not inhibited by SKF 525-A (as shown here) or Lilly 18947 as reported by Bonner.[17]

The influence of iproniazid seems best interpreted as an acceleration of methemoglobin reductase activity (Tables 4 and 5) since it has a similar effect with either nitrite or *p*-APP. It might for example couple with the NADPH-dependent reductase as does methylene blue. Whereas this effect is not dramatic, it is surprising that it is seen as long as 12 hr after treatment. Apparently isoniazid has a similar effect.[17] The influence of sodium azide is more difficult to interpret, but one may rule out an effect on *p*-APP metabolism, since a similar effect is seen with nitrite. The difficulty of interpretation lies in the fact that azide interferes in the determination of methemoglobin by forming an azide–methemoglobin complex.[10] The presence of azide in such a system, however, would lead one to underestimate the amount of methemoglobin present. Therefore, one may safely interpret the second part of the biphasic response as a true potentiation of the methemoglobinemia produced by either nitrite or *p*-APP (cf. results at 60 min in Tables 4 and 5). Such an effect could conceivably be produced by an inhibition of methemoglobin reductase activity or of catalase activity, provided that peroxide generation is common to both nitrite and *p*-APP and is responsible for methemoglobin production by these agents. Aminotriazole, however, is without effect on PHA-induced methemoglobinemia *in vitro*, as shown in Fig. 3. Clearly, additional experimental work is needed to clarify these and other phenomena associated with chemically induced methemoglobinemia.

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