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Species Differences in Methemoglobin Levels Produced by Administration of Monomethylhydrazine.* (32238)

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Previous studies of the effects of monomethylhydrazine (MMH) demonstrated that significant levels of methemoglobin were found in the blood of anesthetized dogs receiving I.V. injections of MMH(1,2). Additional experiments demonstrated that methemoglobin were also produced *in vitro* during incubation of MMH with either canine or human blood(2).

Further studies have been conducted with rats, rabbits, and guinea pigs. The results reported here show that in blood of these 3 species methemoglobin levels found *in vivo* after injection of MMH are markedly lower than previously found in dogs. This species difference was demonstrable also during *in vitro* incubations, when peak methemoglobin levels in rat, rabbit, or guinea pig blood incubated with MMH were much lower than in human or canine blood similarly incubated with MMH.

Procedures. In vivo *studies.* To determine the effects of MMH on methemoglobin levels *in vivo,* methemoglobin content was measured in blood samples taken just before and at intervals after injection of either MMH solution or physiological saline solution.

All studies utilized male animals. Rats were of Sprague-Dawley descent, guinea pigs were albinos of the Hartley strain, and rabbits were New Zealand whites. Monomethylhydrazine (MMH) was obtained from the Eastman Kodak Co. Appropriate dilutions were prepared fresh daily from liquid MMH using physiological saline solution as a diluent. Control animals received injections of corresponding volumes of physiological saline solution. Guinea pigs were injected *via* cardiac puncture during CO_2 anesthesia; rats were injected intravenously *via* the tail vein, and rabbits *via* the marginal vein of the ear. Blood samples were obtained from rats by bleeding from the tail, from guinea pigs by cardiac puncture during CO_2 anesthesia, and from rabbits by bleeding from the marginal vein of an ear.

Blood samples were taken at zero time just before injection of either MMH or saline solution and at selected times thereafter.

* The research reported in this paper was conducted by personnel of the USAF School of Aerospace Medicine, Aerospace Medical Division, AFSC, U.S. Air Force, Brooks AFB, Texas. Further reproduction is authorized to satisfy the needs of the U.S. Government.

TABLE I. Peak Methemoglobin Concentration in Blood of Various Species After Injection of MMH.

	Dose MMH, mmoles/kg	No. of animals	Peak levels* Hb, μmoles/ml	MHb, μmoles/ml	100 MHb / Hb
Guinea pig	.54	2	8.44 (.52)	.24 (.03)†	2.8 (.2)†
	.47	1	6.97	.37	5.3
	.40	2	8.80 (.73)	.30 (.00)	3.4 (.3)
	.22	2	8.63 (.99)	.37 (.00)†	4.3 (.5)†
	.11	1	7.14	.22	3.1
	Controls	9	8.59 ± 1.05	.01 ± .02	.1 ± .3
Rabbit	.54	2	9.99 (.87)	.17 (.11)†	1.6 (1.0)†
	.44	1	8.13	.07	.9
	.33	1	8.77	.18	2.1
	.22	2	7.77 (1.58)	.25 (.11)	3.1 (.7)
	.11	2	9.08 (1.03)	.17 (.11)	1.8 (1.4)
	Controls	8	9.54 ± 1.40	.02 ± .04	.2 ± .4
Rat	.54	2	8.40 (.24)	.26 (.00)	3.1 (.0)
	.43	1	8.26	.18	2.5
	.33	1	8.95	.22	2.4
	.22	3	9.02 (2.64)	.16 (.03)	1.8 (.9)
	.11	2	7.37 (.77)	.15 (.08)	2.1 (1.3)
	Controls	9	9.32 ± 1.14	.05 ± .06	.4 ± .5

* Where data were obtained from more than 1 animal at any dose level, the mean is recorded and the difference between values is shown in parentheses. For controls, the values listed are the mean ± standard deviation.

† One or more animals died before peak level of methemoglobin was reached.

Methemoglobin content was measured by the method of Evelyn and Malloy(3).

In vitro *studies.* To determine the effects of MMH on methemoglobin levels *in vitro,* methemoglobin content was measured in blood samples before, and at intervals after, the start of incubations with various concentrations of MMH in buffer. Blood for the incubations was obtained from normal rats, rabbits, guinea pigs, dogs, and human subjects.

Incubations were performed with 2 ml heparinized blood in 10 ml volumetric flasks to which were added either 0.1 ml of 0.1 M phosphate buffer, pH 7.4 (control flasks), or 0.1 ml of this buffer containing the desired amount of MMH (experimental flasks). Incubations were carried out for 4 hours at 37°C in a water bath with constant shaking. Aliquots were withdrawn just before addition of buffer or MMH solution, and at selected times thereafter for determination of methemoglobin content.

Results and discussion. In vivo *studies.* The results of administration of MMH to rats, rabbits, and guinea pigs are summarized in Table I. It was previously noted in anesthetized dogs that the methemoglobin level reached a peak approximately an hour after injection of MMH and declined thereafter. A similar pattern in time was found after MMH injection in rats, rabbits, and guinea pigs, except that the maximum levels of methemoglobin were less and were usually reached sooner than one hour after MMH injection. Among all the animals of the 3 species tested in these experiments, the peak methemoglobin level reached 5% of the total hemoglobin content of the blood in only one instance.

It is evident from the data of Table I that the response of the methemoglobin level to increasing doses of MMH was erratic. This lack of correlation between dose and response may reflect animal variability in methemoglobin levels and in response to the toxicity of MMH. Among the 4 rabbits (the fourth rabbit was omitted from Table I because methemoglobin levels were not measured) and 5 guinea pigs receiving doses of 0.40 to 0.54 mmoles/kg, survival times ranged between 19 and 65 minutes. Rats were less sensitive; survival times exceeded 4 hours in all rats at all dose levels of MMH listed.

The fact that some rabbits and guinea pigs did not survive long enough after MMH injection to develop maximum methemoglobinemia would tend to obscure dose-response relationships. This uncertainty is not too great, however, since 3 of 4 rabbits receiving doses larger than 0.3 mM/kg survived past the peak of methemoglobinemia. Since methemoglobin levels were still rising in the last blood samples obtained before death in both guinea pigs injected with 0.54 mmole/kg, the maximum levels at that dose in that species were not measured. However, the peak methemoglobin level with a slightly smaller dose, 0.47 mmole/kg, was measured and was about 5%.

The data obtained with rats, rabbits, and guinea pigs indicate, in general, that the maximum methemoglobin level produced by MMH in these species did not exceed 5% of total hemoglobin with doses as high as 0.54 mmoles/kg. In contrast, it was previously observed that such a dose of MMH in anesthetized dogs resulted in the conversion of more than 30% of the hemoglobin to methemoglobin(2). It follows, therefore, that in anesthetized dogs methemoglobinemia after MMH injection is 5 to 8 times greater than in non-anesthetized rats, rabbits, or guinea pigs.

In terms of methemoglobin formation *in vivo*, the response of a given species to MMH is generally similar to the response of that species to other methemoglobin-producing chemicals. Lester(4) measured methemoglobin *in vivo* after oral administration of acetanilid or acetophenetidin and found rabbits to be unresponsive as methemoglobin formers. Rats formed small amounts of methemoglobin; dogs and man about 6 and 12 times as much, respectively. Francis(5) reported that the drug Dapsone (4,4′-diaminodiphenyl-sulfone) produced methemoglobinemia in various species including man, dog, guinea pig, and mouse, but not in certain other species. The studies cited plus the studies of MMH have shown that dogs developed significant methemoglobinemia in response to MMH as well as to the drugs used by Lester and by Francis, whereas rats similarly treated formed only small amounts of methemoglobin. Rabbits were rather unresponsive to either acetanilid(4) or MMH. Guinea pigs apparently developed significant methemoglobinemia after exposure to Dapsone(5) but only small amounts of methemoglobin after MMH, although the toxicity of MMH discussed above may be a factor in this instance.

TABLE II. Peak Methemoglobin Levels During *in vitro* Incubations of MMH with Blood of Various Species.

	MMH conc, μM/ml	Hb, μM/ml	MHb, μM/ml	100 MHb / Hb
Dog	5.4	9.38	6.30	67.1
	2.0	9.42	2.37	25.1
	1.0	9.63	1.22	12.6
	.5	8.64	.78	8.9
Man	5.4	7.53	3.74	49.7
	2.2	7.74	2.04	26.3
	1.1	7.27	1.11	15.2
	.5	8.77	.82	9.2
Guinea pig	5.4	7.01	.93	13.2
	1.1	7.10	.37	5.2
	.5	7.78	.22	2.8
Rabbit	5.4	9.19	1.38	15.0
	2.0	9.25	.48	5.2
	1.1	9.91	.22	2.2
	.5	8.13	.26	3.1
Rat	5.4	8.52	.82	9.5
	2.2	10.24	.22	2.1
	1.1	9.16	.18	2.0
	.5	8.69	.22	2.5

In vitro *studies. In vitro* incubation of blood of various species with MMH concentrations up to 5.4 μmoles/ml for periods as long as 4 hours showed that in blood of all species tested some MMH was formed in the incubation (Table II). As in the *in vivo* studies, the levels of methemoglobin found reached a peak within an hour after addition of MMH and declined thereafter. When levels of MMH less than 2 μmoles/ml were incubated with blood of rats, rabbits, or guinea pigs, the amounts of methemoglobin present after 4 hours of incubation had decreased to levels found in control samples incubated without addition of MMH. Furthermore, with all concentrations of MMH incubated with blood of these species, the peak level of methemoglobin was uniformly much lower than levels reached during incubation of canine blood with corresponding concentrations of MMH. Methemoglobin levels dur-

ing incubation of human blood with MMH were comparable to those found with canine blood, at least up to MMH concentrations of 2 μmoles/ml.

A 2-way analysis of variance(6) was performed using the data in the last column of Table II. This analysis showed that *in vitro* methemoglobin formation was significantly greater with blood of man and dog than with blood of rat, rabbit, and guinea pig ($P<0.01$). The species difference in methemoglobin formation in response to MMH is therefore observed both *in vivo* and *in vitro*. However, the results of *in vitro* studies with MMH differ from the results of *in vivo* studies with acetanilid in that with MMH *in vitro*, canine blood produced at least as much methemoglobin (cf Table II) as did human blood, while with acetanilid *in vivo* the dog produced approximately ½ as much methemoglobin as did man(4).

As was pointed out in previous studies(2), the fact that methemoglobin is formed by incubation of MMH with either red blood cells or crystalline hemoglobin shows that the formation of methemoglobin does not require, nor does it rule out, the intervention of body tissues or of red blood cells.

The reverse process—conversion of methemoglobin back to hemoglobin—is carried out by erythrocytes(7). This process is mediated by the enzymes methemoglobin reductase and NADH diaphorase, appropriately supported by a supply of reduced cofactors generated by erythrocyte metabolism. The latter process can be affected by the rate of carbohydrate metabolism(8). Levels of both glutathione and ascorbic acid also affect the reduction of methemoglobin. The precise interrelationships among the factors responsible for the species differences in methemoglobin reducing capacity are not elucidated, for the differences do not appear to correlate in a simple manner with species differences in levels of glutathione or ascorbic acid, at least as indicated by published data(9).

The observed species differences in methemoglobin levels after exposure to MMH, either *in vivo* or *in vitro*, must reflect differences in the balance between rates of formation and reduction of methemoglobin. Shifting of this balance presumably reflects the capacity of MMH to alter the interplay of carbohydrate metabolism, levels of ascorbic acid and glutathione, and activities of the methemoglobin reductases in the erythrocytes of the species studied.

Summary. Monomethylhydrazine (MMH) was injected into rats, rabbits, and guinea pigs, and subsequent levels of methemoglobin in the blood were measured. Peak levels of methemoglobin occurred within an hour, but were less than 5% of blood hemoglobin with doses of MMH up to 0.54 mmoles/kg. In contrast, 5 to 8-fold higher levels have been previously observed in anesthetized dogs treated with comparable doses of MMH. *In vitro* incubations of MMH with blood from rats, rabbits, guinea pigs, dogs, and human subjects produced peak levels of methemoglobin within an hour. Peak levels were 4 to 8-fold higher when human or canine blood was incubated with MMH than when rat, rabbit, or guinea pig blood was similarly incubated. These results demonstrate that there is a marked difference among species in the levels of methemoglobin found either *in vivo* after MMH injection or *in vitro* during incubation of blood with MMH.

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Effect of Indomethacin in Endotoxin Shock in the Dog.* (32239)

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Previous publications from these laboratories and elsewhere(1,2,3) indicated that acetylsalicylic acid and phenylbutazone can block the hemodynamic effects of endotoxin in dog. Since the pharmacological activities of these agents overlap with another anti-inflammatory drug, indomethacin, (1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid)(4,5) we became interested to determine whether indomethacin can interfere with the typical cardiovascular response to endotoxin in the dog.

Materials and methods. The early responses of animals that received endotoxin were studied. Experiments were performed on 42 adult mongrel dogs, anesthetized with sodium pentobarbital, 30 mg/kg i.v. The femoral artery and vein were cannulated in all experiments, portal vein pressure was recorded in 10 animals. Arterial pressure was monitored by means of a catheter introduced through the femoral artery. Pressure in the portal vein was assayed by means of a catheter advanced from the splenic vein. Pressures were recorded with Statham pressure transducers connected to a Sanborn direct writing recorder, or to a Grass polygraph. Hematocrit and pH determinations were carried out with blood drawn through a catheter inserted in the femoral vein. Rectal temperature was measured with a thermometer.

Drugs were injected into the femoral vein. Indomethacin was dissolved in a 0.1 M phosphate buffer of pH 8 and was given i.v. in a volume of 5 ml/kg within 2 minutes. Dogs received 20 mg/kg indomethacin 20-30 minutes prior to the i.v. administration of 0.4 mg/kg endotoxin (*E. coli,* Difco Labs). This dose of endotoxin represented the LD_{80}, which was established in separate experiments done with 45 dogs by one of us (Hinshaw). In control studies, dogs received endotoxin only or phosphate buffer and endotoxin. Four dogs were pretreated with 100 mg/kg phenylbutazone HCl instead of indomethacin.

The animals used in the experiments were divided into 5 groups. Group I: 10 animals received 0.4 mg/kg endotoxin only. Group II: 14 dogs were given indomethacin as pretreatment 20-30 minutes before administration of endotoxin; the hematocrit, blood pH and rectal temperature were recorded in 10 animals. Group III: 4 dogs were pretreated with phosphate buffer only. Group IV: In 5 dogs the abdominal wall was opened and portal venous pressure was measured simultaneously with mean systemic arterial pressure. The animals in this group received phosphate buffer only as pretreatment. Group V: 5 dogs were treated similarly to group IV, but indomethacin was given 20 minutes before endotoxin. Group VI: 4 dogs were pretreated with phenylbutazone.

The hydrolysis of benzoyl-L-arginine ethyl ester (BAEe) by highly purified hog pancreatic kallikrein was assayed in a Radiometer pH-Stat. The concentration of BAEe was 3×10^{-3} M in 0.15 M NaCl. The kallikrein used was purified by Dr. E. Werle; it contained 1,000 FU/mg. The reactions were followed at room temperature and the pH was kept constant at 8 by the addition of 0.001 M NaOH. The initial steady rates of hydrolysis were registered.

Results. In the first series of experiments, 10 dogs in Group I were given the LD_{80} dose of endotoxin, while the 14 dogs in

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