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The influence of anaesthetic agents on the formation of methaemoglobin induced by aniline in cats

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Administration of anaesthetic agents modifies the methaemoglobin formation induced by aniline in cats. The maximum amount of methaemoglobin is reduced, so too is the rate at which the methaemoglobin disappears. Studies with phenylhydroxylamine indicate that these changes are due to anaesthetics modifying the metabolism of aniline to phenylhydroxylamine rather than modifying the action of phenylhydroxylamine on systems within the red blood cell. The microsomal metabolism inhibitor SKF 525A has no effect on methaemoglobin formation induced by aniline.

IN examining the ability of aromatic amines and amides to induce the formation of methaemoglobin, we have used cats which were not anaesthetized (McLean, Murphy & others, 1967), although most other workers have used anaesthetized animals. Kiese (1963) used chloralose (0·05 g/kg) and urethane (0·5 g/kg) in both cats and dogs. He reported that the maximum methaemoglobin level was attained 4 hr after the intravenous administration of *m*-toluidine and 6 hr after the intravenous administration of *o*-toluidine. We have observed that the maximum methaemoglobin level was reached after 2 hr with *o*-toluidine and after 4 hr with *m*-toluidine in cats when no anaesthetic was used. Because of these differences and others of a similar nature between anaesthetized and conscious animals, we decided to examine the effects of various anaesthetic procedures on the formation of methaemoglobin induced by aniline in cats.

Experimental

CHEMICAL

All the anaesthetic agents were obtained from commercial sources. Aniline hydrochloride was recrystallized until analytically pure. Phenylhydroxylamine was prepared by the method of Vogel (1959) and had m.p. 80–81°.

BIOLOGICAL

The methaemoglobin of the cats was determined by the cyanmethaemoglobin method as previously described (McLean & others, 1967).

PROCEDURE

Food was withheld from the animals for 16 hr before the start of an experiment. For ease of handling, the cats, anaesthetized or conscious, were restrained in jackets made from strong cloth. The anaesthetic agents were given intraperitoneally, except ethyl chloride which was administered by the open drop method. Chloralose was injected as a solution in

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propylene glycol or in aqueous suspension; urethane and sodium pentobarbitone were given as aqueous solutions.

Various degrees of central nervous system (CNS) depression were produced by the procedures adopted. Surgical anaesthesia was induced by chloralose 75 mg/kg, chloralose 50 mg/kg with urethane 500 mg/kg, and pentobarbitone 40 mg/kg. Unconsciousness without loss of corneal reflex was induced by chloralose 50 mg/kg and deep sedation by chloralose 35 mg/kg. Ethyl chloride and oxygen 95%–carbon dioxide 5% mixture were used to produce brief periods of unconsciousness. As soon as muscular relaxation occurred, administration of ethyl chloride was stopped; full consciousness returned after 1–2 min. The cats were given ethyl chloride while the aniline hydrochloride solutions were being injected and then every hour when blood samples were taken. Aniline hydrochloride solutions for intravenous injection were adjusted to pH 5·5 and made isotonic with sodium chloride. Injections were made slowly into the femoral vein with a dose volume of up to 10 ml. A single dose level of aniline (0·0625mmole/kg) was used in all animals. Phenylhydroxylamine solution was administered by intravenous injection into the femoral vein at a dose of 0·5 mg/kg (0·00459 mmole/kg) and an injection volume of 1 ml. The phenylhydroxylamine was dissolved in 0·9% sodium chloride solution adjusted to pH 8 with sodium bicarbonate. Because of the instability of phenylhydroxylamine solution, which became cloudy on standing for 30 sec, the dose for each cat was made up under nitrogen and administered immediately. The saline was previously boiled, and bubbled with nitrogen while cooling. Phenylhydroxylamine was given to five conscious cats and also to five cats which had been anaesthetized with chloralose 50 mg/kg with urethane 500 mg/kg.

Blood samples were taken from the femoral vein just before administration of aniline and at each hr afterwards until the methaemoglobin level began to fall. Blood samples were also taken in a similar manner just before the administration of phenylhydroxylamine, and then every hr for 5 hr.

Methaemoglobin estimations were made in duplicate on each sample. Dilution of 0·1 ml quantities of heparinized blood in 10 ml of M/60 phosphate buffer pH 6·6 was made immediately after withdrawal of the samples since it has been shown that methaemoglobin is reduced to haemoglobin when whole blood is allowed to stand at room temperature (Climie, McLean & others, 1967). At least five cats were used for each anaesthetic treatment.

In some of the experiments body temperatures of the cats were monitored by means of rectal thermocouples inserted to a depth of 6 cm. All experiments were made in a thermostatically controlled air-conditioned laboratory.

Results and discussion

The results obtained with aniline are given in Table 1 and the statistical treatment is given in the appendix and Tables 3 and 4. A number of conclusions may be drawn from these. The first relates to the effect of the

TABLE 1. TYPICAL RESULTS FOR METHAEMOGLOBIN (% OF TOTAL HAEM PIGMENTS) FORMED IN CATS AFTER INTRAVENOUS INJECTION OF ANILINE. DOSE 0·0625 MM/KG. THE METHODS OF ADMINISTERING THE ANAESTHETICS ARE GIVEN IN THE TEXT. The values for the first 5 hr only were taken for the statistical treatment of the results, data for which were also drawn from results not shown here in the interests of brevity.

Treatment given to cats	Cat No.	Methaemoglobin formed %							
		Time after administration of aniline (hr)							
		1	2	3	4	5	6	7	8
No anaesthetic	1	31·4	17·9	7·8	3·4	1·1	—	—	—
	2	36·9	30·6	19·9	14·1	11·7	—	—	—
	3	25·2	32·3	33·7	32·3	27·6	—	—	—
Chloralose (35 mg/kg)	1	19·1	28·5	25·0	20·0	11·4	7·7	—	—
	2	21·3	25·7	32·4	37·9	26·8	16·2	—	—
	3	13·4	24·6	27·2	23·7	22·0	19·4	—	—
Chloralose (50 mg/kg) + propylene glycol (1 ml/kg)	1	22·7	28·9	34·5	36·0	39·8	37·8	33·9	29·8
	2	36·2	40·0	35·2	31·9	25·2	18·5	—	—
	3	21·8	27·4	25·2	19·1	10·4	5·2	—	—
Chloralose 50 mg/kg in aqueous solution	1	2·3	12·6	12·6	17·7	—	—	—	—
	2	19·7	38·5	32·4	44·5	—	—	—	—
	3	14·2	28·8	25·2	16·4	—	—	—	—
Chloralose (75 mg/kg)	1	19·0	24·7	22·7	26·2	22·7	23·7	—	—
	2	15·9	15·2	15·9	15·2	13·4	8·3	—	—
	3	14·2	19·2	18·2	20·9	15·3	14·9	—	—
Chloralose (50 mg/kg) + urethane (500 mg/kg)	1	13·0	20·1	27·6	25·9	27·2	28·4	25·6	25·9
	2	20·1	16·5	15·8	11·9	10·4	7·2	4·3	—
	3	16·4	17·1	16·8	10·1	11·1	7·4	4·0	—
Ethyl chloride	1	25·6	27·4	16·7	12·6	4·2	—	—	—
	2	15·3	20·1	15·3	14·9	8·7	—	—	—
	3	18·8	18·8	15·6	10·9	—	—	—	—
Sodium pentobarbitone (40 mg/kg)	1	11·8	22·2	13·3	8·8	4·8	4·4	—	—
	2	17·0	21·0	23·2	22·3	18·3	14·3	—	—
	3	0	0	Cat died					

anaesthetic treatments on the methaemoglobin formed after the administration of aniline. From a consideration of the "mean effect" column in Table 4 it may be seen that there was no significant difference in the methaemoglobin produced when aniline was given to cats which were either unanaesthetized, or which had received chloralose at doses of 35 or 50 mg/kg. However, the methaemoglobin produced was significantly reduced after the administration of chloralose 75 mg/kg, chloralose 50 mg/kg with urethane 500 mg/kg, ethyl chloride or sodium pentobarbitone 40 mg/kg. There was no significant difference among these four anaesthetic procedures in their effectiveness in depressing methaemoglobin formation and the effect appears to be related to CNS depression itself. Perhaps the most surprising finding is that ethyl chloride had such a marked effect, since the animals were only rendered unconscious for short periods (less than 2 min) at the time of administration of aniline hydrochloride solution and at each hr when blood samples were taken. The second conclusion about the effect of anaesthetic treatments on methaemoglobin formation induced by aniline can be obtained from the "linear effect" column in Table 4. This effect relates to the rate at which methaemoglobin levels fell from hr 1 to hr 5 after the administration of aniline. For the unanaesthetized animals it can be seen that the level of methaemoglobin fell relatively rapidly with time, but with all the

anaesthetic treatments the rate of fall was significantly reduced. Again it is evident that this effect is a function of the animal being under CNS depression rather than the chemical nature of any anaesthetic agent. With the exception of the ethyl chloride treatment, there was no significant difference in the response obtained with any of the anaesthetic procedures used. This effect is more sensitive to anaesthetics than the reduction in the amount of methaemoglobin formed since even the low doses of chloralose (35 mg/kg and 50 mg/kg) have the same effect as the other anaesthetics. The ethyl chloride treatment was the only one which produced a less pronounced change in the time course of methaemoglobin removal than any of the other anaesthetics, but even this treatment produced a response which was significantly different from the results with conscious animals.

Under anaesthesia with chloralose 50 mg/kg with urethane 500 mg/kg the rectal temperature fell by approximately 1°/hr. However, the effects of anaesthesia on methaemoglobin formation and removal cannot be explained simply as a result of hypothermia since after chloralose 35 mg/kg and ethyl chloride there was no measurable fall in body temperature during the course of the experiment.

TABLE 2. METHAEMOGLOBIN (% TOTAL HAEM PIGMENTS) FORMED IN CATS AFTER INTRAVENOUS INJECTION OF PHENYLHYDROXYLAMINE (0·00459 MMOLE/KG)

Time after phenylhydroxylamine administered (hr)	Methaemoglobin formed %									
	Unanaesthetized cats					Cats treated with chloralose 50 mg/kg + urethane 500 mg/kg				
	1	2	3	4	5	1	2	3	4	5
1	32·3	38·5	32·2	47·8	33·9	37·9	25·9	40·9	32·5	47·6
2	28·5	29·5	9·8	42·9	19·4	23·9	18·9	30·2	21·4	34·9
3	23·8	16·6	8·3	28·2	12·7	18·3	13·6	22·9	20·1	33·1
4	20·3	10·6	3·8	26·9	5·7	—	10·4	13·1	22·2	24·8
5	18·0	9·3	4·2	23·3	5·7	—	6·3	6·8	10·7	17·9

The appearance of methaemoglobin following the administration of aniline to cats is the result of at least three processes. One is the metabolism of aniline to the active methaemoglobin-forming species and a second is the action of this metabolite on the haemoglobin in the intact erythrocytes. The third process involved is the erythrocyte reductase systems of the red blood cell which reduce methaemoglobin back to haemoglobin. Kiese (1963) considered that the active metabolite of aniline is phenylhydroxylamine and there is much evidence to support this view. The effects observed after administration of anaesthetics can therefore be due to the anaesthetic either reducing the rate of metabolism of aniline to phenylhydroxylamine, reducing its effect on haemoglobin, or affecting the methaemoglobin reductase systems in the erythrocyte.

To see if the effect of anaesthetics on methaemoglobin formation is due to effects on metabolism or due to effects in the erythrocytes, experiments were made using phenylhydroxylamine in place of aniline. The results of these are in Table 2 and the statistical treatment is given in the appendix (Table 5), from which it can be seen that there was no

significant difference in the amount of methaemoglobin formed and the rate at which it is removed in unanaesthetized cats or in cats treated with chloralose 50 mg/kg with urethane 500 mg/kg. This result indicates that the anaesthetic agents are exerting their effects on the metabolism of aniline to phenylhydroxylamine rather than on the events which occur in the erythrocytes.

Since the effects of anaesthetics seemed to be on the metabolism of aniline, the effect of SKF 525A, a potent inhibitor of microsomal metabolism, on the formation of methaemoglobin by aniline was examined. Cats were pretreated with SKF 525A at doses of 30 and 60 mg/kg intraperitoneally and then given aniline hydrochloride (0·0625 mmole/kg) intravenously after 45 min. The amount of methaemoglobin formed in the cats treated with SKF 525A was not significantly different from that in untreated cats. The metabolic step of aniline which is essential for methaemoglobin formation was not blocked by SKF 525A.

The effect of anaesthetics on cats has been investigated by Berglund, Nylen & Wallentin (1965), who found that chloralose 50 mg/kg with urethane 100 mg/kg caused metabolic acidosis, whereas sodium pentobarbitone 30 mg/kg, chloralose 50 mg/kg and urethane 1 g/kg did not. Propylene glycol, when used as a solvent, has been shown to reduce the amount of methaemoglobin produced following the administration of *p*-chloracetanilide in rats and guinea-pigs compared with the amount produced when the same dose of *p*-chloracetanilide was given in aqueous solution (Glocklin, 1954). It has now been found that there is no significant difference in the amount of methaemoglobin produced by aniline in animals pretreated with chloralose 50 mg/kg whether the chloralose was given as an aqueous suspension or in propylene glycol. However, the dose of propylene glycol (mg/kg) used by Glocklin (1954) was four times greater than the dose used in the present work.

The fact that anaesthetic procedures have such significant effects on methaemoglobin formation induced by aniline suggests that structure-action studies on methaemoglobin formation induced by aromatic amines should preferably be made on unanaesthetized animals.

The effect of the various anaesthetic procedures on the formation of methaemoglobin induced by other aromatic amines has been examined. Amines used included *o*, *m* and *p*-toluidine as well as 2,3-, 2,4- and 3,4-dimethylaniline, with and without chloralose 50 mg/kg and ethyl chloride. In all instances the effects of the anaesthetic procedures were essentially the same as their effects with aniline. However, in one experiment, sodium

TABLE 3. ANALYSIS OF DISPERSION FOR MEAN AND LINEAR EFFECTS (ANILINE)

Source of variation	d.f.	s.s. mean	s.s. linear	s. products
Between treatments	6	4,948·7	3,749·7	−2,438·0
Within treatments	41	12,944·4	2,341·8	2,587·0
Total	47	17,893·1	6,091·5	149·0

$$\Lambda = 0{\cdot}217$$

$$\frac{1 - \sqrt{\Lambda}}{\sqrt{\Lambda}} \cdot \frac{40}{6} = 7{\cdot}65^{**}$$

TABLE 4. TREATMENT MEANS FOR THE MEAN EFFECT AND LINEAR EFFECT WITH S.E.'S OF DIFFERENCES FOR ANILINE

Treatment	Mean effect	Linear effect
1. No anaesthetic	24·89	−5·74
2. Chloralose 35 mg/kg	22·10	0·23
3. Chloralose 50 mg/kg	26·66	0·49
4. Chloralose 75 mg/kg	14·77	−0·06
5. Chloralose 50 mg/kg + urethane 500 mg/kg	15·40	0·05
6. Ethyl chloride	17·46	−2·78
7. Sodium pentobarbitone 40 mg/kg	15·42	0·31
s.e. of difference for treatments—		
(1,2); . . . ; (1,6)	3·99	1·20
(1,7)	4·37	1·32
(2,3); . . . ; (5,6)	5·03	1·51
(2,7); . . . ; (6,7)	5·33	1·60

TABLE 5. TREATMENT MEANS FOR THE TOTAL EFFECT AND LINEAR EFFECT WITH S.E.'S OF DIFFERENCES USING PHENYLHYDROXYLAMINE

Treatment	Total effect	Linear effect
Phenylhydroxylamine	21·25	−6·22
Phenylhydroxylamine + chloralose and urethane	22·70	−6·13
s.e. of difference	5·26	1·17

pentobarbitone was given intravenously at a dose level of 50 mg/kg to five cats which then received aniline (0·0625 mmole/kg i.v.). The methaemoglobin percentages produced were significantly lower than control values for the first 2 hr and all the animals died before the 3rd hr.

Amount of aniline converted to phenylhydroxylamine. The metabolism of aniline is a complex process producing a series of metabolites, of which ring hydroxylation and *N*-hydroxylation are considered to be important. If it is assumed that phenylhydroxylamine is overridingly the most important methaemoglobin forming metabolite of aniline, as is suggested by Kiese (1963), then from the results now obtained with aniline and phenylhydroxylamine it is possible to estimate approximately the proportion of aniline metabolized to phenylhydroxylamine. It can be seen from Tables 4 and 5 that aniline at a dose of 0·0625 mmole/kg and phenylhydroxylamine at a dose of 0·00459 mmole/kg produce the same effects, both with regard to the amount of methaemoglobin produced (total or mean effect) and to the rate at which the methaemoglobin disappears (linear effect) 1 hr after the administration of aniline and phenylhydroxylamine. This indicates that about 7% of aniline is metabolized to phenylhydroxylamine. The fact that the linear effects of aniline and phenylhydroxylamine are close supports the view that phenylhydroxylamine is the methaemoglobin-forming compound derived from aniline. Since if the methaemoglobin-forming metabolite from aniline is not phenylhydroxylamine, it might be expected that the rate of removal of methaemoglobin would be different from the rate with phenylhydroxylamine.

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Appendix

The five hourly observations on each cat make up a set of correlated observations which present a problem of interpretation. A transformation was made to new variates on which meaningful comparisons can be made. The new variates chosen were an average effect, a linear effect and quadratic, cubic and quartic effects. If h_1, h_2, h_3, h_4, h_5 are the observations then the new variates are

$$\begin{aligned} y_1 &= h_1 + h_2 + h_3 + h_4 + h_5, \\ y_2 &= -2h_1 - h_2 + h_4 + 2h_5, \\ y_3 &= 2h_1 - h_2 - 2h_3 - h_4 + 2h_5, \\ y_4 &= -h_1 + 2h_2 - 2h_4 + h_5, \\ y_5 &= h_1 - 4h_2 + 6h_3 - 4h_4 + h_5, \end{aligned}$$

where y_1, y_2, y_3, y_4, y_5 are the mean, linear, quadratic, cubic and quartic effects respectively.

Standard analysis of dispersion techniques may be used on the new variables to test hypotheses concerning treatment differences (Rao, 1965). It is desirable to reduce the number of variables considered as much as possible to simplify the interpretation, so a test of significance was made to determine the additional information on treatment differences supplied by the quadratic, cubic and quartic effects. This was performed by the method of Rao (1965) and gave non-significant results. Consequently, further analysis was carried out using only mean and linear effects.

The analysis of dispersion for these effects is given in Table 3 where Λ is the test statistic for a test of the hypothesis of equality of treatments and $\frac{40}{6}\frac{1-\sqrt{\Lambda}}{\sqrt{\Lambda}}$ may be tested as an F variate with 12 and 80 degrees of freedom (Rao, 1965). This test showed that significant differences between the treatments existed. A detailed comparison of the treatments may be made from Table 4 which sets out the means of the mean effects and the linear effects for each treatment and the standard errors of comparisons to be considered.

It may be noted that a quadratic effect existed in the treatments but no differences between the treatments were evident in this effect.

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