J. Pharm. Pharmac., 1967, **19**, 146–154 Received September 5, 1966

Methaemoglobin formation induced by aromatic amines and amides

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Two series of anilides have been examined for their ability to induce the formation of methaemoglobin in cats. The first series is one in which the aniline was acylated by a series of acids and the second is one in which alkyl groups were substituted on the benzene ring and the acyl moiety kept as the acetyl group. In neither series was any correlation found between the methaemoglobin-forming ability and stability of the amide group. In the first series, as the size of the acyl group was increased so the activity rose to a peak and then declined. It is suggested that this phenomenon is related to absorption of the compounds from the gastrointestinal tract. In the second series a parallelism in response was observed between amides given orally and the corresponding amines administered intravenously, suggesting that the nature of the amine liberated on hydrolysis is the major determining factor in the methaemoglobin forming ability of amides.

AROMATIC amines and their derivatives are known to produce methaemoglobinaemia (Bodansky, 1951). Drugs such as phenacetin, paracetamol, acetanilide, some sulphonamides (Harris, 1963) and prilocaine (Scott, Owen & Richmond, 1964), which are all aromatic amides, have been reported to form methaemoglobin. Further oxidation of haemoglobin to irreversible degradation products, including sulphaemoglobin and Heinz bodies, has been observed with certain drugs (Allen & Jandl, 1961). Brodie & Axelrod (1948, 1949) showed that the formation of methaemoglobin by acetanilide and phenacetin in man is related to the concentration of free amine in the blood, but the free amines do not oxidize haemoglobin *in vitro* (Prankerd, 1961). Methaemoglobin is thought to be formed *in vivo* by an oxidation product of the amine (Brodie & Axelrod, 1949; Kiese, 1965). Thus the reactions involved in the formation of methaemoglobin by aromatic amides such as acetanilide are (i) hydrolysis of the amide to the corresponding amine, (ii) metabolism of the amine to the appropriate species, and (iii) oxidation of haemoglobin by the amine metabolite.

The concentration of the active metabolite in the red cell depends on the absorption, distribution and excretion characteristics, both of the amide and of its metabolites. Since erythrocytes possess reductase systems capable of reversing this oxidation reaction (Harris, 1963), the actual concentration of methaemoglobin within the red cell depends on the relative rates of its formation and reduction.

We have examined the effect of retardation of the hydrolysis of aromatic amides on methaemoglobin formation.

It has long been known that the rate of chemical hydrolysis of anilides is slowed by *ortho*-substituents (Semerano, 1931). Thomas & Stoker (1961) have shown that *ortho*-substitution of benzoic esters, which are hydrolysed by a similar mechanism to anilides, retards both hydroxylation

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and esterase catalysed hydrolysis. Lignocaine, a derivative of 2,6-dimethylaniline, has been shown to be extremely refractory to chemical hydrolysis (Bullock & Grundy, 1955). Davis (1909) showed that increasing the length and bulk of the acid group of an anilide slowed the rate of its hydrolysis.

The effects of substitution in both the acid and amine parts of the acetanilide molecule on methaemoglobin formation have been examined.

Experimental

CHEMICAL

All compounds, except paracetamol, phenacetin and lignocaine, were prepared by standard methods and they are listed, together with physical constants, in Table 1.

TABLE 1. ANALYSIS AND MELTING POINTS OF ANILIDES AND AMINE HYDROCHLORIDES

Compound	M.p. °C	Found			Required		
		C	H	N	C	H	N
Acetanilide	114	71·3	6·7	10·4	71·1	6·7	10·4
2-Methylacetanilide	110	72·5	7·4	9·5	72·5	7·4	9·4
3-Methylacetanilide	65–6	72·8	7·6	9·4	72·5	7·4	9·4
4-Methylacetanilide	153	72·6	7·2	9·3	72·5	7·4	9·4
2,6-Dimethylacetanilide	177	73·4	7·9	8·5	73·6	8·0	8·6
2,6-Dimethylpropionanilide	122	74·9	8·5	8·1	74·6	8·5	7·9
2,6-Dimethylbutyranilide	135–7	75·7	8·8	7·6	75·4	8·9	7·3
2,6-Dimethylvaleranilide	72–3	76·1	9·3	7·4	76·1	9·3	7·0
2,6-Dimethylbenzanilide	168	80·0	6·6	6·1	80·0	6·7	6·2
Propionanilide	105	72·3	7·4	9·4	72·5	7·4	9·4
Butyranilide	96	73·5	7·8	8·8	73·6	8·0	8·6
Isobutyranilide	105	74·0	8·0	9·0	73·6	8·0	8·6
Valeranilide	62	75·0	8·8	8·2	74·6	8·5	7·9
Hexanilide	93–5	75·2	8·8	7·6	75·4	8·9	7·3
Octanilide	53	76·4	9·8	6·6	76·7	9·6	6·4
Decanilide	66–7	77·7	10·2	6·0	77·7	10·1	5·7
Dodecanilide	76–7	78·5	10·4	5·1	78·5	10·5	5·1
Aniline hydrochloride	198	55·9	6·6	10·6	55·6	6·2	10·8
2-Methylaniline hydrochloride	215–6	58·7	7·0	10·0	58·5	7·0	9·8
3-Methylaniline hydrochloride	208	58·6	7·2	9·6	58·5	7·0	9·8
4-Methylaniline hydrochloride	243	58·2	7·0	9·8	58·5	7·0	9·8
2,6-Dimethylaniline hydrochloride	179	61·0	7·6	8·7	61·0	7·6	8·9

Determination of water solubility. An amount of the compound, much in excess of its expected water solubility, was mixed with fresh glass-distilled water and the mixture slowly rotated in a water bath at 25° ± 0·1° for 21 days. The mixture was then filtered and duplicate samples of the filtrate measured into tared flasks. Water was removed from the samples by freeze drying and the flasks were dried in an oven at 80° for 30 min, allowed to cool and weighed. The method was checked by applying it to solutions of known concentration when quantitative recovery of the amides was obtained. A plot of log solubility of the amides against the number of carbon atoms in the acyl group is a straight line. The values obtained are given in Table 2.

BIOLOGICAL

Selection of species. It has been shown that different species form various amounts of methaemoglobin after administration of aromatic

amines and amides. For both acetanilide and phenacetin the order has been found to be cat>man>dog>rat (Lester, 1943; Spicer, 1950). We used cats and dogs.

TABLE 2. MAXIMUM METHAEMOGLOBIN (%) FORMED IN CATS AFTER ORAL ADMINISTRATION OF A HOMOLOGOUS SERIES OF ANILIDES. DOSE 1 M-MOLE/KG. EACH VALUE IS THE MEAN OF THE VALUES OBTAINED FROM 6 CATS. HYDROLYSIS RATES GIVEN AS TIME IN HR FOR 50% HYDROLYSIS. SOLUBILITY IN WATER MOLE/LITRE AT 25°

Compound	Mean max. % MetHb formed	s.d.	Base catalysed hydrolysis rate*	Solubility in water mole/litre 25°
Acetanilide	71·8	57·7	3·8	0·0464
Propionanilide	84·2	164·0	6·3	0·0132
Isobutyranilide	87·0	127·3	30·7	0·0090
n-Butyranilide	88·0	49·9	13·0	0·0057
Valeranilide	75·6	11·4	18·5	0·0021
Hexanilide	75·7	34·9		0·00055
Octanilide	69·3	72·3		
Decanilide	46·1	184·9		
Dodecanilide	18·5	489·5		

* Davis, 1909.

Determination of methaemoglobin. Methaemoglobin was estimated by a modification of the photometric cyanomethaemoglobin method of Evelyn & Malloy (1938). Solutions were prepared as described by Hawke, Oser & Summerson (1954). Light absorption measurements were made on a Hilger and Watts Uvispek equipped with a quartz prism using matched glass cuvettes of 1 cm optical path. The absolute amounts of haem pigments in the samples were not determined and the results are expressed as percentage haemoglobin converted to methaemoglobin. Where irreversible oxidation occurs the concentration of both haemoglobin and methaemoglobin may be lowered and the values obtained by this method are suspect. Examination for irreversible oxidation products of haemoglobin was made using the method of Harley, J. D. & Robin, H. (personal communication). For aniline, at a dose level of 0·25 m-mole/kg in cats, the extent of oxidative degradation was found to be insignificant.

Procedure. Food was withheld from the animals for 16 hr before the start of an experiment. The animals were unanaesthetized, the cats being restrained in jackets made from strong cloth.

Compounds, which were administered orally, were reduced to fine powder and suspended in a 1% methylcellulose mucilage. Solutions of the amine hydrochlorides, which were given intravenously, 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 approximately 10 ml.

Blood samples were taken from the femoral vein just before administration of a drug and at each hour afterwards for 5 hr with the amines and 6 hr with the amides.

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 was made immediately after withdrawal of the samples.

The lysis of the cells which occurs on dilution prevents reduction of methaemoglobin by cellular enzymes (Onji & Tyuma, 1965).

At least 5 cats were used for each compound at each dose level. Two dogs were used for each compound and a crossover procedure was adopted.

Results and discussion

Twenty cats were given aniline intravenously at a single dose level (0·0625 m-mole/kg) and the amount of methaemoglobin present in the blood determined hourly for 5 hr, by which time the response had passed its peak. The maximum amounts of methaemoglobin formed follow an approximately normal distribution (Fig. 1). The experiment was repeated one week later on 5 of the cats. The maximum responses for each cat

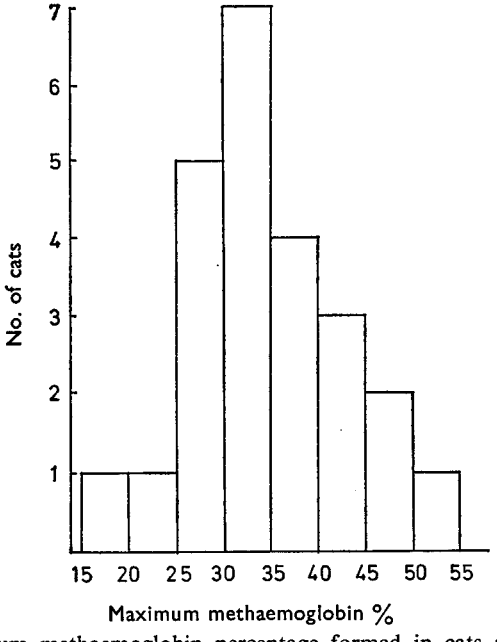

FIG. 1. Maximum methaemoglobin percentage formed in cats after intravenous administration of aniline solution at pH 5·5. Dose 0·0625 m-mole/kg.

on the two occasions were in good agreement. For both aniline and acetanilide a linear dependence of mean response upon log dose was shown to exist (Fig. 2). The slopes and positions of the regression lines were calculated by standard statistical methods (Burn, Finney & Goodwin, 1950). Similar relationships were shown to exist for the methylanilines and the methylanilides.

Intraperitoneal injection of acetanilide in a 30% ethanolic solution resulted in the formation of less methaemoglobin than when the same dose was given orally. Because of this the intraperitoneal route was not used subsequently.

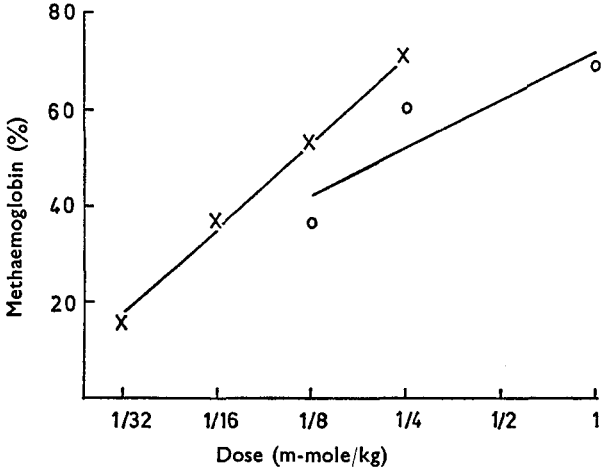

FIG. 2. The relationship between the log of the dose and the mean maximum percentage of methaemoglobin formed in cats after intravenous administration of aniline solution and oral administration of acetanilide suspension. Each point is the mean of the values obtained from 5 cats. Aniline ×——×, acetanilide ○——○.

ANILIDES

The mean maximum methaemoglobin responses obtained for a series of anilides administered orally to cats at the same molar dose level are listed in Table 2. Six cats were used for each compound. The time course for methaemoglobin production by acetanilide, *n*-butyranilide and decanilide is shown in Fig. 3.

For the series from acetanilide to dodecanilide, analysis of variance showed that the response differs ($F_{8,45} = 23{\cdot}18$; $P < 0{\cdot}1$) with a peak response for the compounds propionanilide, *n*-butyranilide and isobutyranilide. Though the variances cannot be considered equal, the conclusion that the means differ with a peak response around butyranilide seems fairly sound. There is no apparent correlation between the stability of the anilides to chemical hydrolysis (Table 2) and the methaemoglobin response. Hydrolysis is therefore unlikely to be the rate-limiting step in methaemoglobin formation by these compounds. Since the same amine, aniline, is liberated on hydrolysis of anilides, differences in the methaemoglobin forming ability of the individual members of the series is presumably due to variation in the concentration of aniline available for further metabolism to the active species.

The compounds become less water soluble as the homologous series is ascended (Table 2) and so the oil-water partition coefficients would be expected to rise (Albert, 1960). It is also suggested by Albert that the lipid-water solubility characteristics of drugs influence their rates of dissolution, absorption and distribution within the tissues, both to sites of metabolism and sites of loss.

With this series it would appear that as the series is ascended a more favourable lipid-water solubility ratio is achieved with an optimum value

for methaemoglobin production around butyranilide. Thereafter, as water solubility decreases so does the methaemoglobin forming ability. At a limiting water solubility the dissolution rate of a compound will become significant in the regulation of absorption. In this series of experiments the particle size of the material was not controlled in any way and further work is being done on this aspect of the problem.

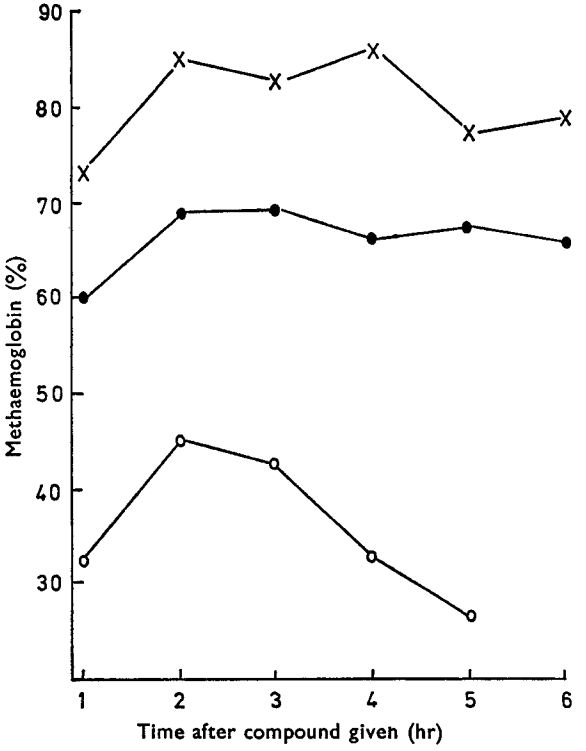

FIG. 3. The mean methaemoglobin percentage produced in cats at hourly intervals after administration of acetanilide ×——×, n-butyranilide ●——● and decanilide ○——○. Each point is the mean of the values obtained from 5 cats. Dose 1 m-mole/kg.

The individual variation in methaemoglobin response to dodecanilide was much greater than with the other anilides. This is possibly due to irregular absorption.

ANILINE AND MONOMETHYL SUBSTITUTED ANILINES

The mean maximum methaemoglobin responses obtained in cats for aniline and the methylanilines administered intravenously at the same dose level are given in Table 3. For treatment differences it was found $F = 9{\cdot}8$, indicating that the treatments differ. A Least Significant Difference test showed that it is due to 4-methylaniline forming much less methaemoglobin than the other amines.

TABLE 3. FORMATION OF METHAEMOGLOBIN AFTER INTRAVENOUS ADMINISTRATION OF ANILINE AND METHYLANILINES. DOSE 0·25 M-MOLE/KG

Compound	No. of cats used	Mean max. % MetHb formed	s.d.
Aniline	9	72·3	104·5
2-Methylaniline ..	9	70·1	136·1
3-Methylaniline ..	9	60·2	325·3
4-Methylaniline ..	8	39·6	187·1

It can be seen (Fig. 4) that the time course of the response to 3-methylaniline differs from that to aniline and 2-methylaniline. Although the maximum amounts of methaemoglobin formed do not differ significantly for the three compounds, the rate of methaemoglobin formation after 3-methylaniline is much slower. This difference in rate was also observed with the corresponding acetylated amines when they were administered orally.

The same order of response (Table 5) occurred when these compounds were administered to the dog.

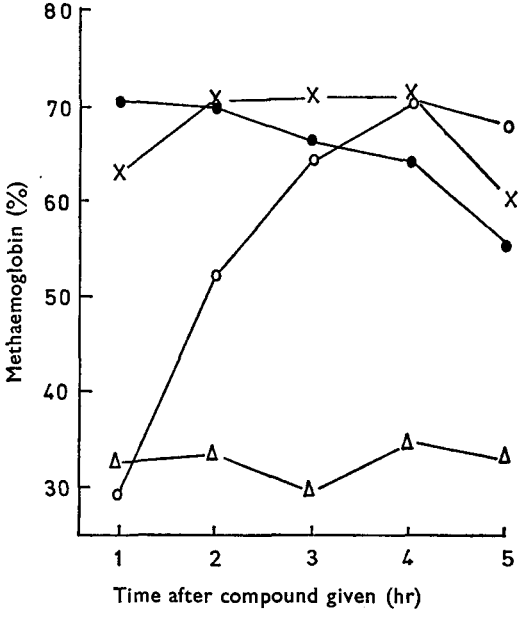

FIG. 4. The mean methaemoglobin percentage produced in cats at hourly intervals after intravenous administration of solutions of aniline ●——●, 2-methylaniline ×——×, 3-methylaniline ○——○, 4-methylaniline △——△. Each point is the mean of the values obtained from 5 cats. Dose 0·25 m-mole/kg.

SUBSTITUTED ANILIDES

The results obtained for this series in the cat are shown in Table 4. The variances for the treatments were not found to be homogeneous, therefore reservations must be made in interpreting the F value of 106.

It does seem large enough to admit the possibility of real differences in treatments. Further, when the treatment sum of squares was broken down for orthogonal components the major contribution was found to come from 4-methylacetanilide versus the rest. Thus it was concluded that 4-methylacetanilide forms less methaemoglobin than the rest. There was also an indication that 2- and 3-methylacetanilide form more methaemoglobin than acetanilide, phenacetin and paracetamol. Other comparisons showed that acetanilide, phenacetin and paracetamol formed the same amount of methaemoglobin.

TABLE 4. MAXIMUM METHAEMOGLOBIN (%) FORMED IN CATS AFTER ORAL ADMINISTRATION OF SOME ACETANILIDE DERIVATIVES. DOSE 1 M-MOLE/KG. EACH VALUE IS THE MEAN OF VALUES OBTAINED FROM 5 CATS. SOLUBILITY IN WATER MOLE/LITRE AT 25° AND RELATIVE RATES OF HYDROLYSIS ARE GIVEN

Compound	Mean max. % MetHb formed	s.d.	Solubility in water mole/litre 25°	Hydrolysis rate Acetanilide = 1*
Acetanilide	70·5	59·6	0·0464	1·00
2-Methylacetanilide	79·7	168·1	0·0695	0·42
3-Methylacetanilide	83·8	41·3	0·0403	0·89
4-Methylacetanilide	34·5	526·3	0·0069	0·98
Paracetamol	64·5	55·3		
Phenacetin	72·5	19·3		

* Semerano (1931).

Since the water solubility of the simple anilides was considered to be the most important factor influencing their methaemoglobin-forming ability, it is interesting to note that 4-methylacetanilide has a water solubility comparable with that of butyranilide, the most active of the anilides. In fact, neither the water solubility nor hydrolysis rate (Table 4) appears to be important with these compounds. The activities of the acetylated methylanilines correlate with those of the corresponding free amines in both the cat and the dog (Table 5).

TABLE 5. MAXIMUM METHAEMOGLOBIN (%) FORMED IN CAT AND DOG AFTER ADMINISTRATION OF SOME SUBSTITUTED ANILINE COMPOUNDS AND THE CORRESPONDING *N*-ACETYL DERIVATIVES

Compound	Mean max. % MetHb formed			
	Cat		Dog*	
	Amine 0·25 m-mole/kg i.v.	*N*-Acetyl 1 m-mole/kg oral	Amine 0·25 m-mole/kg i.v.	*N*-Acetyl 1 m-mole/kg oral
Aniline	72·3	70·5	30·3	34·1
2-Methylaniline	70·1	79·7	30·5	25·9
3-Methylaniline	60·2	83·8		
4-Methylaniline	39·6	34·5	2·6	11·1
2,6-Dimethylaniline	10·4	4·5	0	0

* Each value is the mean of two experiments in different dogs.

2,6-DIMETHYL SUBSTITUTED ANILIDES

Less than 5% methaemoglobin is formed in the cat after oral administration of 2,6-dimethylacetanilide and the corresponding amine produces the same order of response (Table 5). Neither 2,6-dimethylacetanilide nor 2,6-dimethylaniline produces a response in the dog.

2,6-Dimethylacetanilide is stable towards both acidic and basic catalysed hydrolysis due to the "*ortho* effect", although an enzyme has been isolated from hog liver which is capable of hydrolysing 2,6-dimethylaniline amides (Krisch, 1963).

The stability of the amide bond is probably not of primary importance here. It may be noted that increasing chain length in this series has no significant effect on methaemoglobin production (Table 6). The methaemoglobin response to lignocaine, which is a 2,6-dimethylaniline amide, is shown in the same table.

TABLE 6. MAXIMUM METHAEMOGLOBIN (%) FORMED IN CATS AFTER THE ADMINISTRATION OF A SERIES OF 2,6-DIMETHYLANILINE AMIDES. DOSE 1 M-MOLE/KG ORALLY. FIVE CATS WERE USED FOR EACH COMPOUND EXCEPT 2,6-DIMETHYLBENZANILIDE WHEN ONLY ONE CAT WAS USED.

Compound	Mean max. % MetHb formed
2,6-Dimethylacetanilide	4·5
2,6-Dimethylpropionanilide ..	9·2
2,6-Dimethylbutyranilide ..	5·4
2,6-Dimethylvaleranilide	8·6
2,6-Dimethylbenzanilide ..	7·1
Lignocaine	8·0*

* Dose 0·2 m-mole/kg intravenous.

Thus changes in structure which produce profound retardation of the hydrolysis of aromatic amides do not have a commensurate effect on the methaemoglobin formation which follows their administration. The nature of the amine appears to be of prime importance in determining the amount of methaemoglobin formed by these compounds.

Acknowledgements. One of us (S.M.) wishes to thank the N.S.W. Pharmacy Research Trust for a research scholarship.

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