Methaemoglobin formation by aromatic amines

S. MCLEAN, G. A. STARMER* AND J. THOMAS

*The Department of Pharmacy, and *Department of Pharmacology, University of Sydney, Sydney, N.S.W., Australia*

Methaemoglobin formation induced in cats by many substituted anilines has been investigated in an attempt to correlate chemical structure with activity. Although no precise relation emerged, some generalizations could be made. 4-Substituents (except methyl and methoxy) increased the activity of aniline. The activity of aniline was either unaltered or reduced by 2- and 3-substituents. Polyhalo-, polymethyl-, methoxy-, ethoxy- and carboxyester substituents reduced activity, and carboxy groups abolished it. Steric effects around the amino-group were not important. The activities of the compounds are discussed in relation to their absorption, distribution, metabolic transformation and the activities of their metabolites.

Although many aromatic amines have been examined for their methaemoglobin-forming activity over the past 50 years (reviewed by Bodansky, 1951; Kiese, 1966), the results of different workers are not directly comparable since the animal species used and the experimental conditions varied. In an attempt to correlate chemical structure with methaemoglobin-forming activity, many ring substituted derivatives of aniline were administered to the cat and the methaemoglobin responses obtained compared with that observed for aniline given by the same route and at the same molar dose level.

EXPERIMENTAL

Chemical

Some amines were obtained commercially. These were purified and characterized by their physical constants and those of their derivatives. Other amines were prepared by standard methods and purified until acceptable elemental analyses were obtained (McLean, 1968). The acetanilide and isobutyranilide used were described previously (McLean, Murphy & others, 1967).

Biological

Cats were used because of their sensitivity to methaemoglobin formation (Lester, 1943; Spicer, 1950). Only adult cats (older than 24 weeks) were used, since new-born kittens have been shown to reduce methaemoglobin faster than adult cats (Müller-Oerlinghausen & Baethke, 1966). Methaemoglobin determination, administration of compounds and general procedure was as described by McLean & others (1967).

RESULTS

Methaemoglobin in untreated cats. Of the 152 animals examined, nearly all (92%) had 3% or less of their haemoglobin as methaemoglobin; the average value (1·1%) being less than the limit of sensitivity of the assay (1·3%).

Irreversible destruction of haemoglobin. After experiments in which methaemoglobin formation induced by a representative sample of aromatic amines and amides

was followed for several hours, the level of "intact" haemoglobin (oxy- plus methaemoglobin) (Robin & Harley, 1964) was, on average, 95% of the initial value (Table 1).

Table 1. *"Intact" haemoglobin (oxy-plus methaemoglobin) after methaemoglobin formation induced by various drugs expressed as a percentage of initial haemoglobin concentration*

Compound			Dose mmol/kg	Route	No. cats	Mean % methaemoglobin formed	Duration of expt. (h)	Mean % "Intact" Hb
Acetanilide	..	..	1·25	oral	1	60·1	8	70·7
Acetanilide	..	..	1·0	oral	1	66·1	6	111·6
i-Butyranilide		..	1·0	oral	1	78·0	6	117·8
Acetanilide + SKF 525A*		..	1·0 60 mg	oral ⎫ i.p. ⎭	1	82·3	6	110·3
2,4-Dimethylaniline	..		0·25	i.v.	5	6·3	5	91·9
2,4-Difluoroaniline	..		0·25	i.v.	3	62·5	5	95·2
4-Fluoroaniline		..	0·25	i.v.	3	66·0	5	84·4
3-Bromoaniline		..	0·25	oral	5	46·6	5	93·6
Lignocaine	..	..	0·20	i.v.	4	6·0	4	96·1
Prilocaine	..	..	0·20	i.v.	4	50·5	4	99·9

* Administered 45 min before acetanilide.

Methaemoglobin formation by aniline

Route of administration. Table 2 shows the formation of methaemoglobin after oral and intravenous administration of aniline at 0·25 and 0·0625 mmol/kg. A Student's t-test showed that the maximum methaemoglobin response after intravenous administration at 0·25 mmol/kg was higher than that after oral administration ($P < 0.02$). At 0·0625 mmol/kg, the difference in maximum response was not significant ($P > 0.5$), although the first hour mean was lower after oral administration ($P = 0.05$).

Table 2. *Formation of methaemoglobin after administration of aniline*

Dose mmol/kg	No. cats.	Route admin.	Methaemoglobin % ± s.e. after time:						
			1 h	2 h	3 h	4 h	5 h	Mean	Mean max.
0·25	9	i.v.	65·1 5·1	67·6 1·3	63·4 3·6	58·2 2·8	53·7	61·6	72·3 4·1
0·0625	19	i.v.	34·5 1·6	33·5 2·3	24·2 2·5	19·5 2·6	12·8 2·3	24·9	36·8 1·7
0·25	5	Oral	45·7 4·1	49·1 3·8	52·5 4·0	49·5 3·2	43·9 2·7	48·1	53·2 3·8
0·0625	5	Oral	25·4 5·8	30·6 5·3	28·0 3·9	24·8 4·6	17·5 2·8	25·2	33·9 5·0

Time to maximum response. To determine when the maximum response occurred, aniline (0·0625 mmol/kg) was given intravenously to 5 cats and the methaemoglobin concentration determined at 10 min intervals for 90 min, and thereafter at 120 and 150 min. The results obtained are shown in Fig. 1. The maximum methaemoglobin level was reached between the first and second hour in every animal and remained fairly constant during this period.

Methaemoglobin formation by substituted anilines

Halogen substituted anilines. The results obtained with the haloanilines are shown in Table 3 from which it can be seen that 3- and 4-fluoroaniline and 2,4-difluoroaniline formed as much methaemoglobin as aniline, while 2-fluoroaniline and 2,5-difluoroaniline were less active. Trifluoromethyl-, tetrafluoro- and pentafluoroaniline were
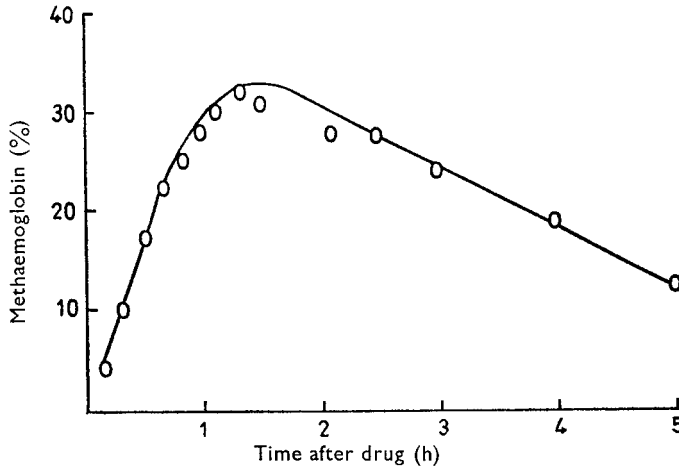
FIG. 1.   The mean methaemoglobin percentage formed in cats after intravenous administration of aniline at 0·0625 mmol/kg.   Each point is the mean of values from 5 cats.

Table 3.   *Formation of methaemoglobin by halo-anilines*

Compound	Dose mmol/kg	Route	No. cats	Methaemoglobin % ± s.e. after time :						
				1 h	2 h	3 h	4 h	5 h	Mean	Mean max.
2-Fluoroaniline	0·25	i.v.	5	44·2	51·3	48·0	46·4	37·5	45·5	52·9
				2·5	4·3	3·0	4·3	6·1		4·0
3-Fluoroaniline	0·25	i.v.	5	46·5	65·2	65·0	63·4	63·7	60·8	69·1
				4·3	1·9	4·0	4·3	4·4		3·0
4-Fluoroaniline	0·25	i.v.	5	64·3	73·2	66·1	63·0	63·4	66·0	74·9
				3·3	4·7	5·9	5·9	3·4		4·5
2,4-Difluoroaniline	0·25	i.v.	5	69·6	61·7	62·5	62·2	56·6	62·5	71·4
				6·5	4·3	2·4	6·3	5·1		5·2
2,5-Difluoroaniline	0·0625	i.v.	5	16·0	12·1	8·7	5·0	3·5	9·0	16·0
				2·4	1·3	0·9	1·0	0·5		2·4
2-Trifluoromethylaniline	0·0625	Oral	3	5·1	3·1	1·5	0·9	0·5	2·2	5·5
				2·3	0·8	0·2	0·1	0·2		2·1
3-Trifluoromethylaniline	0·0625	Oral	3	8·2	7·3	5·8	5·6	5·3	6·4	8·8
										2·8
Pentafluoroaniline	0·25	Oral	1	0	1·1	3·3	1·8	0·7	1·4	3·3
	0·0625	Oral	1	0	0	0	0	0	0	0
2,3,4,5-Tetrafluoroaniline	0·25	Oral	1	4·0	3·0	2·4	4·9	2·4	3·3	4·9
	0·0625	Oral	1	2·9	2·3	0	1·0	0·6	1·4	2·9
2,3,5,6-Tetrafluoroaniline	0·25	Oral	1	9·0	8·6	10·7	13·4	10·3	10·4	13·4
	0·0625	Oral	1	1·3	1·6	0	0	0	0·6	1·6
2-Chloroaniline	0·25	Oral	5	57·6	62·0	57·1	47·3	41·2	53·1	64·9
				2·2	4·1	3·2	5·3	7·7		2·7
3-Chloroaniline	0·25	Oral	5	24·8	39·9	46·3	53·1	58·0	47·3	60·4*
				2·1	5·1	5·4	5·2	6·8		6·2*
4-Chloroaniline	0·0625	Oral	5	17·1	33·1	46·4	53·8	57·8	45·2	60·7†
				3·1	6·0	7·5	7·6	8·1		8·1†
2,4-Dichloroaniline	0·25	Oral	5	39·3	37·3	31·5	27·6	22·6	31·7	42·0
				9·3	9·4	8·2	8·6	7·6		9·9
2,6-Dichloroaniline	0·25	Oral	5	14·3	22·2	20·4	18·7	13·5	17·0	23·3‡
				2·1	5·4	4·7	6·1	5·0		4·7‡
2,4,6-Trichloroaniline	0·25	Oral	5	35·9	43·7	39·7	37·6	36·8	38·8	44·3
				4·7	5·6	5·2	4·5	6·9		5·5
2-Bromoaniline	0·25	Oral	5	58·8	59·0	57·2	54·9	52·2	56·4	65·3
				8·0	4·8	2·5	3·2	3·7		4·7
3-Bromoaniline	0·25	Oral	5	23·3	40·8	50·9	50·5	59·8	46·6	62·0§
				4·2	7·7	8·5	8·0	7·5		8·2§
4-Bromoaniline	0·0625	Oral	5	14·6	29·7	43·8	49·7	52·6	42·2	56·1‖
				1·8	2·8	4·6	6·2	5·9		4·7‖
2,6-Dibromoaniline	0·25	Oral	5	3·5	5·5	2·7	2·3	0·7	2·6	6·0¶
				0·8	1·7	2·0	2·1	0·5		1·6¶
2,4,6-Tribromoaniline	0·25	Oral	5	18·2	21·7	17·7	20·7	17·5	19·1	23·6
				5·5	5·8	6·3	7·9	5·6		6·5

Some mean results include measurements made over 5 h, these are :
* 56·6 ± 4·5 at 6 h, 52·2 ± 4·3 at 7 h.
† 54·5 ± 8·2 at 6 h, 51·4 ± 9·5 at 7 h, 47·8 ± 9·6 at 8 h.
‡ 12·6 ± 3·9 at 6 h.
§ 54·6 ± 7·0 at 6 h.
‖ 54·4 ± 5·3 at 6 h, 50·8 ± 3·8 at 7 h.
¶ 0·9 ± 0·9 at 6 h.

only weakly active. The time course of the methaemoglobin response to the fluoro-anilines was similar to that found for aniline (Table 2). 3-Chloroaniline was as active as aniline, while 4-chloroaniline was much more active. A Student's t-test showed that 2-chloroaniline produced a higher maximum level of methaemoglobin than aniline ($P < 0.05$). The mean maximum response to 2,4-dichloroaniline did not differ significantly from that to aniline ($P > 0.3$) or 2,6-dichloroaniline ($P > 0.1$), due partly to the high variance of the response to 2,4-dichloroaniline. The maximum methaemoglobin response to 2,4,6-trichloroaniline was not significantly different from that to 2,4-dichloroaniline and aniline ($P > 0.2$) but was greater than that to 2,6-dichloroaniline ($P = 0.02$). Aniline was more active than 2,6-dichloroaniline ($P < 0.01$).

The pattern of the responses to the various bromoanilines was similar to that observed for the corresponding chloroanilines; 2- and 3-bromoaniline were as active as aniline, and 4-bromoaniline was much more active. 2,4,6-Tribromoaniline formed less methaemoglobin than aniline and 2,6-dibromoaniline formed less than 2,4,6-tribromoaniline ($P < 0.05$). Although the monobromoanilines formed the same amount of methaemoglobin as the corresponding chloroanilines, 2,6-dibromoaniline and 2,4,6-tribromoaniline formed less methaemoglobin than the corresponding chloroanilines. All the chloro- and bromoanilines tended to produce a long-lasting methaemoglobin response, especially the 3- and 4-haloanilines which produced a maximum response in the fifth or sixth hour.

Alkyl substituted anilines. Methyl substituents tended, if anything, to lower the methaemoglobin forming activity of aniline (Table 4). Although the rate of formation of methaemoglobin after 3-methylaniline was slower than after aniline, 2- or 4-methylaniline, the log dose-maximum response curves for 3-methylaniline and aniline

Table 4. *Formation of methaemoglobin after intravenous administration of some alkyl-anilines*

Compound		No. of cats	Dose mmol /kg	Methaemoglobin % ± s.e. after time:					Mean	Mean max.
				1 h	2 h	3 h	4 h	5 h		
2-Methylaniline	..	9	0.25	57.4	63.9	64.5	63.3	57.1	61.3	70.1
				3.8	3.4	3.6	4.9	3.9		3.9
3-Methylaniline	..	8	0.25	28.8	46.8	57.5	60.8	58.6	50.5	60.2
				2.9	5.2	6.1	6.5	6.6		6.5
4-Methylaniline	..	9	0.25	28.1	34.3	32.7	33.2	32.1	32.1	39.6
				5.3	5.1	4.7	4.6	5.0		4.8
2,3-Dimethylaniline	..	5	0.25	16.9	16.3	15.2	13.4	9.5	14.3	20.2
				4.8	5.5	6.0	6.1	4.0		5.4
2,4-Dimethylaniline	..	15	0.25	6.6	6.5	7.6	6.9	3.7	6.3	10.4
				1.8	2.2	2.9	2.8	2.1		3.7
2,5-Dimethylaniline	..	5	0.25	30.7	35.2	30.0	28.6	21.1	29.1	36.3
				5.0	7.7	4.6	2.6	4.3		7.1
2,6-Dimethylaniline	..	5	0.25	8.7	8.6	7.6	6.3	4.8	7.2	10.3
				2.6	2.1	1.8	1.8	1.2		1.9
3,4-Dimethylaniline	..	5	0.25	11.8	14.1	15.6	16.6	12.2	14.1	18.0
				2.1	2.5	3.1	3.7	3.3		3.4
3,5-Dimethylaniline	..	5	0.25	44.4	41.9	38.4	35.9	31.5	38.3	46.5
				3.0	5.3	3.5	4.0	4.9		3.9
2-Ethylaniline ..	..	5	0.0625	26.0	21.3	13.4	9.2	9.8	15.9	27.1
				4.4	2.9	2.8	1.5	1.6		4.1
3-Ethylaniline ..	..	5	0.0625	20.6	23.2	25.0	23.9	22.1	23.0	28.3
				2.8	3.7	5.1	4.2	4.4		4.3
4-Ethylaniline ..	..	5	0.0625	38.0	61.0	64.8	61.2	56.9	56.4	66.7
				7.4	3.6	5.2	5.0	4.6		4.5

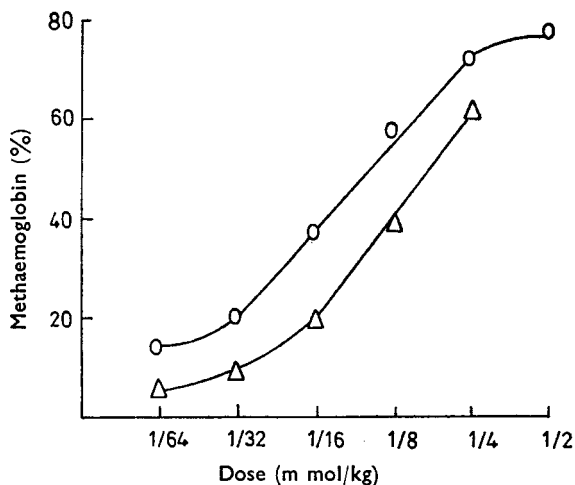FIG. 2. The relationship between log dose of 3-methylaniline (△), aniline (○) and the maximum methaemoglobin response. 3-Methylaniline was given to 8 cats at 0·25 mmole/kg and to 2 cats at each other dose. Aniline was given to at least 5 cats at each dose level.

were approximately parallel (Fig. 2). Pretreatment with SKF 525A (60 mg/kg i.p. 45 min before administration of the amine) had little effect on methaemoglobin formation by 2-, 3- and 4-methylaniline. All the dimethylanilines formed less methaemoglobin than aniline (Table 4). The most active were 2,5- and 3,5-dimethylaniline. A Student's *t*-test showed no significant difference in the maximum methaemoglobin responses to 2,3-, 2,4- 2,5- and 3,4-dimethylaniline. 2,4,6-Trimethylaniline formed no methaemoglobin. The level of methaemoglobin formed by the dimethylanilines remained relatively constant over 5 h. 2,4-Dimethylaniline (0·25 mmol/kg i.v.) was given to 15 cats and the frequency distribution of the maximum responses was found to be skewed, with more than half the animals forming less than 5% methaemoglobin (Fig. 3).
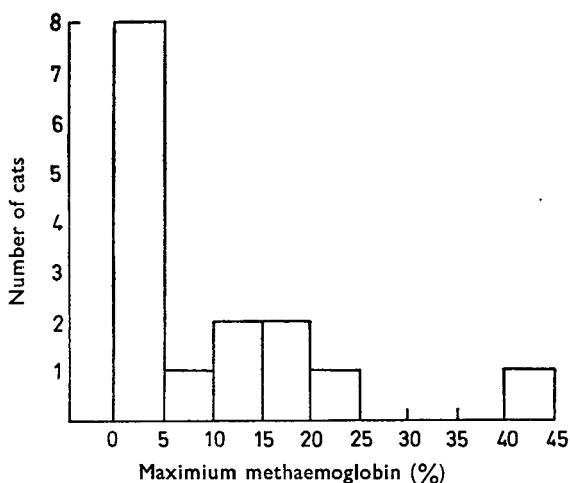

FIG. 3. Frequency distribution of the maximum methaemoglobin response in cats after intravenous administration of 2,4-dimethylaniline at 0·25 mmol/kg.

Ethyl groups in the 2- and 3-positions slightly reduced the methaemoglobin-forming activity of aniline ($P < 0.05$) while 4-ethylaniline was much more active than aniline (Table 4). The response to 4-ethylaniline was maximal at the third hour.

Alkoxy, carbinol and carboxy substituted anilines. The anisidines (2-, 3- and 4-) were all less active than aniline (Table 5). The maximum responses to 3- and 4-phenetidine were not significantly different from that to aniline, possibly because the standard errors for these phenetidines were large. Aniline was more active than 2-phenetidine. The methaemoglobin responses to the anisidines and phenetidines declined relatively rapidly. 4-Aminobenzylalcohol was slightly less active than aniline, while 2-aminobenzylalcohol was very much less active and 3-aminobenzylalcohol was virtually inactive. Benzocaine and procaine, two esters of 4-aminobenzoic acid, were much less active than aniline. The response to the aminobenzylalcohols, benzocaine and procaine fell rapidly from 1 h to 5 h. The aminobenzoic acids (2-, 3-, and 4-) did not form detectable amounts of methaemoglobin.

Table 5. *Formation of methaemoglobin after intravenous administration of some substituted anilines.* Five cats were used in each experiment, except for benzocaine where one animal was used.

| Compound | Dose mmol /kg | Methaemoglobin % ± s.e. after time: | | | | | | Mean max. |
		1 h	2 h	3 h	4 h	5 h	Mean	
2-Anisidine	0·0625	11·5	6·5	3·9	3·2	1·0	5·2	11·5
		3·4	2·8	2·1	1·6	0·6		3·4
3-Anisidine	0·0625	20·1	16·1	12·6	8·5	6·9	12·8	20·1
		5·5	5·3	4·2	4·1	4·0		5·5
4-Anisidine	0·0625	13·4	15·3	7·6	3·5	1·6	8·3	15·3
		2·3	2·6	1·7	1·2	0·7		2·6
2-Phenetidine ..	0·0625	7·3	5·0	2·7	1·7	0·7	3·9	7·8
		3·1	3·2	0·8	0·6	0·2		2·9
3-Phenetidine	0·0625	21·7	25·0	15·6	12·1	7·2	16·3	27·5
		4·6	8·2	4·9	3·6	2·7		7·9
4-Phenetidine	0·0625	37·6	46·7	39·0	27·6	20·1	34·2	46·7
		9·3	9·9	8·8	8·4	6·7		9·9
2-Aminobenzylalcohol	0·25	10·7	10·0	6·9	4·3	2·4	6·9	11·0
		2·9	2·9	2·4	1·7	1·1		2·8
3-Aminobenzylalcohol	0·25	1·2	0·8	0·3	0·6	0·3	0·6	1·4
		0·6	0·2	0·2	0·2	0·2		0·5
4-Aminobenzylalcohol	0·0625	26·8	17·4	12·9	7·8	6·8	14·4	26·8
		3·7	3·9	3·2	1·7	2·0		3·7
Benzocaine	0·25	21·7	13·4	9·8	3·5	2·0	10·1	21·7
Procaine	0·20	6·7	10·4	9·5	7·1		8·4	13·1
		1·6	2·1	3·8	3·0			3·3

DISCUSSION

Methaemoglobin in untreated cats. As the normal level of methaemoglobin in a population of 152 cats was 1·1%, less than the limit of sensitivity of the assay, it was taken as being equal to zero in subsequent experiments.

Irreversible destruction of haemoglobin. Methaemoglobin formation has frequently been found to be associated with the oxidative destruction of haemoglobin and the appearance of irreversible degradation products including sulphaemoglobin and Heinz bodies (Allen & Jandl, 1961; Harley & Mauer, 1960). There is also doubt, however, (Beutler, 1962; Rentsch, 1968) about whether a causal relation exists between methaemoglobin formation and haemoglobin destruction. Cat haemoglobin is known to be resistant to oxidative denaturation (Robin & Harley, 1966). Nevertheless it was

considered necessary to determine the degree of haemoglobin destruction in these experiments since the accuracy of the methaemoglobin estimations would be materially affected if such destruction was extensive. A simple Heinz body count in red cells would not have been satisfactory since similar inclusions have been reported in the red cells of normal cats (Schmauch, 1899) with extreme frequency variations (Beritic, 1965). More meaningful information was expected from measurements of "intact haemoglobin" remaining after the formation of methaemoglobin by drugs. After several experiments in which the methaemoglobin responses to a number of aromatic amines were followed for several hours the average "intact haemoglobin" concentration had fallen by only 5% from the initial value. There may have been considerable turnover of red cells with damaged cells being sequestered and new cells released by the reticulo-endothelial system (Kiese & Kaeske, 1942; Rothberg, Corallo & Crosberg, 1959), but this could not be detected by the techniques used. It was concluded that the estimation of methaemoglobin under these conditions was not greatly affected by irreversible destruction of haemoglobin.

Formation of methaemoglobin by aniline. It has been shown (McLean & others, 1967) that the methaemoglobin response to aniline has an approximately normal distribution and that the response is reproducible. A linear dependence of the methaemoglobin response to aniline on log dose was also found. It can be seen from Table 2 that at a high dose level (0·25 mmol/kg) the response was greater after intravenous than after oral administration, but at a lower dose (0·0625 mmol/kg) there was no significant difference except that the initial rate of formation of methaemoglobin was slower when the oral route was used.

After intravenous administration of aniline (0·0625 mmol/kg) the level of methaemoglobin rose steeply almost at once (Fig. 1). The response levelled out at a maximum between 1 and 2 h after the dose had been given, and then declined fairly steadily until at 5 h it was less than half the maximum value. The formation of methaemoglobin could therefore be followed by estimating methaemoglobin levels hourly for 5 h after administration of aniline.

Methaemoglobin formation by substituted anilines

Most aromatic amines induce methaemoglobin formation in the intact animal but not on incubation with blood or red cell suspensions (Bodansky, 1951). Metabolism to the molecular species capable of reacting directly with haemoglobin and oxygen, such as aminophenols and *N*-oxidation products, occurs mainly in the liver (Kiese, 1966). Phenylhydroxylamine (von Issekutz, 1939) and nitrosobenzene (Kiese & Soetbeer, 1950) have been shown to be the most effective metabolites of aniline in producing methaemoglobin *in vivo*. Aminophenols are generally considered to be of minor importance (Kiese, 1966) although some arylamines form highly active aminophenols (Kiese & Rachor, 1964).

Aniline substituted with -Et, -OEt, -CH$_2$OH, -Cl, -Br, or -F was most active if the substituents were in the 4-position and less active if 3-substituted. The activity of the 2-isomer was either less than or equal to that of the 3-isomer. Exceptions were the -Me and -OMe groups, which produced less activity in the 4-position than in the 3-position. These findings are supported by the observations of Kiese (1963) that 4-substitution of aniline by -Cl, alkoxy or acyl residues increased methaemoglobin formation in dogs while the same substituents in the 2- or 3-positions decreased activity. The exception, as in the present study, was 4-methylaniline which formed less

methaemoglobin than either its 2- and 3-isomers, or aniline. Anaesthesia reduces the formation of methaemoglobin by aromatic amines to a marked extent (McLean & others, 1967). Although Kiese (1963) used anaesthetized dogs (50 mg chloralose + 500 mg urethane/kg, i.p.) the order of activities of the substituted anilines was the same as in the present study, where the cats were unanaesthetized.

Kiese (1966) found that 4-substitution of aniline frequently increased the rate of microsomal N-hydroxylation, and von Jagow, Kiese & Renner (1966) observed that 4-substitution of aniline favoured the urinary excretion of N-hydroxy derivatives. It was suggested that the substituents were blocking 4-hydroxylation, a major metabolic reaction of aromatic amines which presumably competes with N-hydroxylation. It has been shown, however, that cats and dogs hydroxylated aniline mainly in the 2-position (Parke, 1960) and therefore any increase in N-hydroxylation due to blocking of the preferred site of ring hydroxylation would be expected with 2-substituted amines. This did not occur. Furthermore, rats, which hydroxylated aniline mainly in the 4-position, excreted as ring-hydroxylated metabolites at least as much after a dose of 4-chloroaniline (62%) as after 2-chloroaniline (54%) (Newell, Argus & Ray, 1960). It is therefore unlikely that the blocking of 4-hydroxylation accounts for the increased N-hydroxylation seen in 4-substituted anilines. Other factors, possibly related to enzyme fit, must be involved.

The high methaemoglobin-forming activity of 4-substituted anilines, and the low activity of the 2- and 3-isomers, may reflect the activities of their N-hydroxy derivatives. Despite a high rate of N-hydroxylation by dog liver microsomes, 3-aminopropiophenone formed little methaemoglobin *in vivo* because 3-hydroxylaminopropiophenone reacted very slowly with haemoglobin and was consumed in side reactions (Kiese & Rauscher, 1965). On the other hand, 4-aminopropiophenone was more rapidly N-hydroxylated than aniline and 4-hydroxylaminopropiophenone oxidized haemoglobin in the red cell at three times the rate of phenylhydroxylamine (Graffe, Kiese & Rauscher, 1964).

Steric factors did not seem to be very important in the activities of most of the substituted anilines tested. Although 2-Et, -OEt and -OMe anilines formed less methaemoglobin than the 3-isomers this is unlikely to be due to steric retardation of activity, since this effect was absent with the equally bulky -Cl, -Br and Me groups and there was no difference in the activity of 2,4- and 2,6-dimethylaniline. However, 2,6-dibromo- and 2,4,6-tribromoaniline were less active than the corresponding di- and trichloroanilines. This was presumably due to steric effects since the monobromoanilines were as active as the monochloroanilines.

The situation may be complicated by the metabolic reactions of the substituent groups. Replacement of chlorine (Betts, Bray & others, 1957) or fluorine (Renson, 1964; Daly, Guroff & others, 1968) in the 4-position of aromatic amines by hydroxyl has been reported as a minor metabolic pathway. This reaction is not extensive enough to account for e.g. the difference in the activities of 4-chloro- and 4-fluoroaniline. There was no evidence for hydroxylation-induced migration of halogen from the 4- to the 3-position ("The NIH Shift"; Guroff, Daly & others, 1967) in 4-fluoro- or chloroaniline or the corresponding acetanilides incubated with rabbit liver microsomes (Daly & others, 1968). Although metabolically very stable, the halogen substituents may affect the activity of the amine by blocking ring hydroxylation. For example, it is possibly because of the blocking of all the major sites of ring hydroxylation that 2,4,6-trichloro- and 2,4,6-tribromoaniline formed more methaemoglobin than the

corresponding 2,5-dihaloanilines. Alternatively, this may be simply due to the high activity associated with a -Cl or -Br in the 4-position.

Aromatic alkyl groups are oxidized *in vivo via* the alcohol to the carboxylic acid (Williams, 1959). 4-Acetotoluidide was oxidized to the carboxylic acid, whereas ring hydroxylation was the preferred metabolic reaction for the 2- and 3-acetotoluidides (Bray & Thorpe, 1948). Rapid conversion to the inactive aminobenzoic acid may be thought to explain the low activity of 4-methylaniline. However, it has been reported that after intravenous administration of equimolar doses of aniline and 2-, 3- and 4-methylaniline to dogs, the blood concentration of the amines fell at the same rate over 5 h and that 4-methylaniline produced a higher blood level of the *N*-hydroxy derivative than was found after aniline (Kiese 1963). Moreover, the methaemoglobin response to 2-, 3- and 4-methylaniline was unaffected by pretreatment with SKF 525 A [2-(diethylamino)ethyl-2,2-diphenylvalerate hydrochloride], a compound known to inhibit the oxidation of alkyl side-chains (Cook, Tonor & Fellows, 1954). Thus the different activities of the methylanilines seem to be related to differences in the intrinsic activities of the *N*-hydroxy compounds.

Daly & others (1968) found that 4-ethylacetanilide was metabolized mainly to 4-(1'-hydroxyethyl)acetanilide by rabbit liver microsomes. Since further oxidation of alcohols to ketones or aldehydes is thought to be due to the alcohol dehydrogenase in the soluble fraction of liver (Gillette, 1959), the possible formation of the *N*-hydroxy metabolite of 4-aminoacetophenone from 4-ethylaniline may explain the high activity of 4-ethylaniline over its 2- and 3-isomers. A similar mechanism, involving the *N*-hydroxy metabolites of the aminobenzaldehydes, may account for the high activity of 4-aminobenzylalcohol compared with its 2- and 3-isomers.

4-Phenetidine was much more active than 4-anisidine. This difference is not related to a difference in the rates of ether cleavage to 4-aminophenol since 4-OEt and 4-OMe acetanilide were cleaved at the same rate by rabbit liver microsomes (Axelrod, 1956). However, aminophenol metabolites may account for some of the activity of the alkoxy anilines since in the cat, 4-aminophenol was approximately as active as aniline, and 2-aminophenol was more active in forming methaemoglobin (Kiese & Rachor, 1964).

The low activities of benzocaine and procaine are likely to be due to their rapid hydrolysis *in vivo* to the inactive 4-aminobenzoic acid (Williams, 1959). Aminobenzoic acids, being ionized as zwitter ions, presumably do not reach the sites of *N*-hydroxylation in the endoplasmic reticulum (Brodie, 1964). The very weak activity of the polyfluoroanilines is possibly due to their low water solubility, and therefore poor absorption (Brodie & Hogben, 1957). 2,4,6-Trimethylaniline confirmed the trend that increasing the number of methyl substitutents diminishes methaemoglobin formation by aniline, possibly because the *N*-hydroxy derivatives are less active.

REFERENCES

ALLEN, D. W. & JANDL, J. H. (1961). *J. clin. Invest.*, **40**, 454–475.
AXELROD, J. (1956). *Biochem. J.*, **63**, 634–639.
BERITIC, T. (1965). *Blood*, **15**, 999–1008.

BETTS, J. J., BRAY, H. G., JAMES, S. P. & THORPE, W. V. (1957). *Biochem. J.*, **66**, 610–621.

BEUTLER, E. (1962). *Nature, Lond.*, **196**, 1095–1096.

BODANSKY, O. (1951). *Pharmac. Rev.*, **3**, 144–196.

BRAY, H. G. & THORPE, W. V. (1948). *Biochem. J.*, **43**, 211–216.

BRODIE, B. B. (1964). *Physico-chemical factors in drug absorption, in Absorption and Distribution of Drugs*. Editor: T. B. Binns, p. 15–48, London: Livingstone.

BRODIE, B. B. & HOGBEN, A. M. (1957). *J. Pharm. Pharmac.*, **9**, 345–380.

COOK, L., TONOR, J. J. & FELLOWS, E. T. (1954). *J. Pharmac. exp. Ther.*, **111**, 131–141.

DALY, J. W., GUROFF, G., UDENFRIEND, S. & WITKOP, B. (1968). *Biochem. Pharmac.*, **17**, 31–36.

GILLETTE, J. R. (1959). *J. biol. Chem.*, **234**, 139–143.

GRAFFE, W., KIESE, M. & RAUSCHER, E. (1964). *Arch. exp. Path. Pharmak.*, **249**, 168–175.

GUROFF, G., DALY, J. W., JERINA, D. M. RENSON, J., WITKOP, B. & UDENFRIEND, S. (1967). *Science, N.Y.*, **157**, 1524–1530.

HARLEY, J. D. & MAUER, A. M. (1960). *Blood*, **16**, 1722–1735.

VON ISSEKUTZ, B. V. (1939). *Arch. exp. Path. Pharmak.*, **193**, 551–566.

VON JAGOW, R., KIESE, M. & RENNER, G. (1966). *Biochem. Pharmac.*, **15**, 1899–1910.

KIESE, M. (1963). *Arch. exp. Path. Pharmak.*, **244**, 387–404.

KIESE, M. (1966). *Pharmac. Rev.*, **18**, 1091–1161.

KIESE, M. & KAESKE, H. (1942). *Biochem. Z.*, **312**, 121–149.

KIESE, M. & RACHOR, M. (1964). *Arch. exp. Path. Pharmak.*, **249**, 225–236.

KIESE, M. & RAUSCHER, E. (1965). *Ibid.*, **251**, 201–202.

KIESE, M. & SOETBEER, M. (1950). *Ibid.*, **210**, 305–310.

LESTER, D. (1943). *J. Pharmac. exp. Ther.*, **77**, 154–159.

MCLEAN, S. (1968). Ph.D. Thesis, University of Sydney, pp. 87–111.

MCLEAN, S., MURPHY, B. P., STARMER, G. A. & THOMAS, J. (1967). *J. Pharm. Pharmac.*, **19**, 146–154.

MCLEAN, S., ROBINSON, J., STARMER, G. A. & THOMAS, J. (1967). *Ibid.*, **19**, 803–809.

MÜLLER-OERLINGHAUSEN, B. & BAETHKE, R. (1966). *Arch. exp. Path. Pharmak.*, **253**, 327–335.

NEWELL, M., ARGUS, N. & RAY, F. E. (1960). *Biochem. Pharmac.*, **5**, 30–38.

PARKE, D. V. (1960). *Biochem. J.*, **77**, 493–503.

RENSON, J. (1964). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **23**, 325.

RENTSCH, G. (1968). *Biochem. Pharmac.*, **17**, 423–427.

ROBIN, H. & HARLEY, J. D. (1964). *Australas. Ann. Med.*, **13**, 313–319.

ROBIN, H. & HARLEY, J. D. (1966). *Aust. J. exp. Biol. med. Sci.*, **44**, 519–526.

ROTHBERG, H., CORALLO, L. A. & CROSBERG, W. H. (1959). *Blood*, **14**, 1180–1186.

SCHMAUCH, G. (1899). *Arch. Anat. Physiol.*, **156**, 201–244.

SPICER, S. S. (1950). *J. Pharmac. exp. Ther.*, **99**, 185–194.

WILLIAMS, R. T. (1959). *Detoxication Mechanisms*, 2nd edn, London: Chapman Hall.