LXXXVII. THE ROLE OF THE AROMATIC AMINO GROUP IN DERANGED PIGMENT METABOLISM

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THE recent widespread use in clinical practice of drugs of the benzenesulphonamide type has been followed by many reports of intoxication. Among the more dangerous conditions described are the cases involving the leucopoietic mechanism and resulting in fatal cases of granulocytopenia [Johnston, 1938]. This peculiar toxic action is shared by probably all the coal tar antipyretics and by drugs of the arsphenamine type. Kracke & Parker [1933] showed that the clinical onset of granulocytopenia was preceded by prolonged or intensive administration of drugs which contained an aromatic amino group. These workers describe cases involving the use of acetanilide, phenacetin or amidopyrine, and others with neorsphenamine or arsphenamine. They concluded that this toxic action depended on the oxidation reactions of the drugs. This view is supported by Archer & Discombe [1937] and Jennings & Southwell-Sander [1937], both of whom advance evidence that drugs containing an aromatic amino group are also capable of causing methaemoglobinaemia, and of catalysing sulphaemoglobinaemia.

Methaemoglobinaemia and sulphaemoglobinaemia

Equally disquieting are the toxic effects of this class of drugs involving the erythropoietic mechanism. Erysipelas patients treated with aminobenzene-sulphonamide have been reported by Snodgrass & Anderson [1937] to develop cyanosis in 30 % of cases, while of 106 puerperal cases cyanosis was observed in 56 % by Colebrook & Purdie [1937]. This cyanosis has been commonly attributed to methaemoglobin or sulphaemoglobin but it is claimed by Marshall & Walzl [1937] that it is unaccompanied by a decrease in the oxygen carrying capacity of the blood, and this claim is supported by King & Leslie [1938], who suggest that a black oxidation product of sulphanilamide may be the cause of the cyanosis and dark colour of the blood. Nevertheless, methaemoglobin and sulphaemoglobin formation following benzenesulphonamide administration have been reported by Colebrook & Kenny [1936] and confirmed by a number of workers [Discombe, 1937; Kane, 1937; Paton & Eton, 1937, 1, 2]. These apparently contradictory reports are illuminated by earlier work on acetanilide. Evidence has accumulated to show that methaemoglobin can be demonstrated in the blood for several hours after acetanilide has been ingested. It has been demonstrated spectroscopically by Dennig [1900], Cabot [1902], Payne [1935] and Todd [1926] and by reduction in the oxygen carrying capacity of the blood by McEllroy [1919]. At this stage phenols which can be detected in the blood stream are at their highest levels, and cyanosis is deepest, while at a later stage it may be impossible to demonstrate the spectrum of methaemoglobin although cyanosis is still observed [Payne, 1935]. These successive stages were thoroughly elucidated by the experiments of Payne [1935]. The persistent cyanosis observed when these drugs are ingested for long periods has been described variously as livid blue, grey or slatey in colour. Immediately following ingestion the livid cyanosis develops

and at this stage methaemoglobin is undoubtedly a factor, while as the drug is eliminated, slowly with sulphanilamide, phenacetin and amidopyrine, but quickly with phenazone, which is soluble, the livid blue gives way to a muddy-grey colour, which persists long after methaemoglobin and sulphaemoglobin have disappeared. Small quantities of *p*-aminophenol in the tissue have been repeatedly noticed and may account for this condition. If acetanilide is not ingested but introduced parenterally it may not be possible to demonstrate the presence of methaemoglobin even although cyanosis is seen [Young & Wilson, 1926]. Methaemoglobinaemia was observed by Snapper [1922] in dogs which had been given phenacetin, while if uncombined sulphur was given simultaneously, sulphaemoglobin developed instead. The cyanosis ceased on withdrawal of the drug and reappeared when the drug was resumed. Sulphaemoglobinaemia was also reported by Ivens & Vollenhoven [1925] in the case of a woman who took phenacetin for three months. Amidopyrine was reported by Kobert [1926] to produce methaemoglobin. In the many published reports of acute aspirin poisoning methaemoglobin and sulphaemoglobin formation have not been suspected and cyanosis has never been reported [compare Dyke, 1935; Wyllie, 1935; Neale, 1936].

Ultimate fate of the aromatic amino group in the organism

From a purely chemical standpoint it is a simple matter to postulate a common degradation type for drugs of this series. Hydrolysis and oxidation of compounds containing the aromatic amino group will usually produce *p*-aminophenol. The ease with which it is produced will depend on the stability of the substituent side-chains or rings. Where the *o*-position is substituted as in arspenamine the conversion will proceed first through the less stable *o*-aminophenol. Where there is no amino group, as in aspirin, oxidation will be slow and the final product is likely to be quinol. Evidence in support of this claim is fragmentary and the only cases clearly proved are those of aniline, dealt with later, and acetanilide. Ellinger [1920] has isolated acetylphenylhydroxylamine from the blood of cats poisoned by acetanilide, while Michel *et al.* [1937] have demonstrated that an enzymic hydrolysis due to the acylase of Abderhalden splits acetanilide to acetic acid and aniline. Liver converts aniline into a brown pigment which they consider to be oxidized *p*-aminophenol. Dakin [1922] has shown that the end-point of acetanilide oxidation *in vivo* and *in vitro* is *p*-aminophenol and this product has been isolated from the urine by Herrick & Irons [1906] and from the blood plasma of dogs by Young & Wilson [1926]. The unchanged drug is excreted as a glycuronate according to Herrick & Irons [1906].

No mention has been made in the literature of the isolation or identification of *p*-aminophenol following the use of amidopyrine or phenazone but degradation products are known.

Jaffé [1901] has isolated the red-coloured compound from the urine of dogs treated with amidopyrine. This compound, rubazonic acid, is a condensation product of two oxidized amidopyrine molecules. Enklewitz [1935] has shown that the reducing action of urine following the administration of the drug is due to the conjugated glycuronate. Meyer [1937] has recently advanced evidence to relate the therapeutic efficiency of the aminobenzenesulphonamide molecule with the oxidation potential of the compound ultimately produced in the tissues. Marshall & Walzl [1937], and Rimington [1938] have both observed the presence of a brown oxidation product following sulphanilamide intoxication. Of aspirin, Sollmann [1936] says it is hydrolysed in the gastrointestinal tract to salicylic acid, the bulk of which is excreted as glycuronates and as sulphates,

while a small amount is oxidized to hydroxysalicylic acid and quinol. This oxidation must be small compared with that of drugs containing the readily oxidized amino group. Of the ultimate fate of *p*-aminophenol itself, Herrick & Irons [1906] say it is excreted by the kidneys as ethereal sulphate.

Methaemoglobin formation

The mechanism of methaemoglobin formation has been established by Heubner and his collaborators, working with the simple aniline molecule. Although phenylhydroxylamine occurs as an intermediary in the oxidation of aniline, it is not considered to be the actual methaemoglobin-former on account of the ease with which it is irreversibly converted *in vivo* into azoxybenzene [Heubner *et al.* 1923]. When aniline was given to cats by subcutaneous injection of a watery solution, Heubner & Schwedtke [1936] were able to demonstrate that eight times the theoretical molar equivalent of methaemoglobin was formed, at which stage an equilibrium was established when 37 % of the total blood pigment was oxidized. These results justify Heubner's conviction that phenylhydroxylamine was not responsible but that the reduction-oxidation system set up by *p*-aminophenol and *p*-iminoquinone was the effective agent. Iminoquinone would be capable of oxidizing one molecule of haemoglobin into methaemoglobin being itself reduced thereby; it could again be oxidized in the blood when it would be available to start again. This catalytic conversion would proceed until an equilibrium was established. To fill the role in this scheme a drug containing an aromatic amino group with a hydroxyl in the *o*- or *p*-position, or with a potential source of these, is required. It seems possible that a quinol:quinone system would fulfil the same function.

Porphyrin excretion

The rash, which has been reported by Schwentker & Gelman [1937] in a proportion of cases of sulphanilamide intoxication has been shown to be connected with exposure to sunlight by Newman & Sharlit [1937] and Frank [1937]. The possibility that photosensitization was present was investigated by Brunsting [1937] who reported an increase of urinary porphyrin in two uncontrolled experiments. Porphyrin excretion in fever or liver injury was early observed by Garrod [1900], and is discussed by Gunther [1922] but isolation of the type excreted was not attempted. The normal daily human output of urinary porphyrin has been given as 0–50 μ g., and values outside this range are regarded as abnormal by Schreus & Carrié [1933] and Brugsch [1935]. Fink [1934] characterized the normal urinary porphyrin as coproporphyrin I, which was shown by Bingel [1937] to be a much more active photosensitizing agent than the corresponding series III isomerides. The view expressed by Rimington [1936] that urinary porphyrin excretion furnishes a reliable index of the extent of normal haematopoietic activity has received the support of Dobriner *et al.* [1937]. In a series of experiments with rats, Rimington [1938] showed that with doses of 0.4, 1.39 and 1.5 g. of sulphanilamide per kg. the urinary porphyrin excretion increased to 2.5, 7 and 8 times respectively. These porphyrins were isolated and identified as members of the series III, together with small quantities of series I. Earlier experiments with a drug giving a similar degradation product are those of Schreus [1935] who identified coproporphyrin III in the urine of patients treated with salvarsan.

In view of the close connexion between the drugs of the coal tar antipyretic series and drugs of the benzenesulphonamide series, it was decided to investigate

the effect of the former drugs when administered orally to normal healthy rats maintained on a constant artificial diet. The drugs which were examined were, acetanilide, phenacetin, phenazone, amidopyrine, *p*-aminophenol and aspirin.

EXPERIMENTAL WORK ON RATS

Groups of three adult male rats, of the albino strain maintained in this laboratory, and weighing 120–150 g. were used. They were kept in metabolism cages, the grids and sloping bases of which were coated with paraffin wax to avoid contamination with metals. Urine was collected every morning from conical flasks fitted with pear-shaped glass bulbs to avoid any contamination with faeces, and was analysed at 3-day periods. A few drops of toluene were always added to the cleaned flasks to inhibit bacterial growth. The rats were removed to feeding cages for 1 hr. during the forenoon and 1 hr. during the afternoon (a single 2 hr. period during the forenoon on Saturday and Sunday) and were offered unrestricted amounts of synthetic diet. Water was available in the metabolism cages, and in the food cages. The drugs were administered by stomach tube, first thing in the morning, and on an empty stomach and some 2 hr. before offering food. These drugs, finely powdered, were suspended in 2 % acacia mucilage in tap water which was administered as 5 ml. per 100 g. body weight. In the first experiments the dose level for all drugs was fixed at 25 % of the LD 50 which had been previously determined [Brownlee, 1939]. Since the excretion of coproporphyrin in the rat varies from animal to animal a pre-period was included for each group and a control group which was dosed throughout with 2 % acacia mucilage was included.

The details of the extraction of the pigment are reproduced later in this work. Quantitative determinations were made by comparing the intensity of the 550 $m\mu$ absorption band in acid solution with that of a standard. This was a solution of coproporphyrin in 0.5 % HCl containing 10 μ g. per ml. Comparison was made by diluting the unknown to match the standard, and viewing the solutions with a wave-length spectrometer, where the 550 $m\mu$ band only was observed.

The results presented in Table I show porphyrin excretion for two periods of 3 days without drug, and for three periods of 3 days with drug. For acetanilide the marked rise was immediate and reached 10 times the excretion pre-period. With phenacetin, a rise was not seen until the second 3-day period but the final figure was six times. Phenazone showed a decrease for the first 3-day period and finally rose to four times. This decrease which is noted here and is observed again later is probably due to a temporary depression of haematopoiesis by the drug. In the case of amidopyrine the rise is immediate and the final figure was eight times. With aspirin the first 3-day rise is of the order of 2.5 times, reaching 5 times on the second period. This dose of aspirin proved toxic; two of the animals dying on the first day of the third period, and the other, 2 days later. A post-mortem examination of these animals showed gastric haemorrhage [compare van Bodegom, 1925; Barbour & Dickerson, 1938], while the gross appearance of other organs seemed normal.

A second series of experiments was designed to determine whether the dose levels of the various drugs could be adjusted to give the same degree of porphyrin excretion. The details of the experiments are given in Table II while a summary of the porphyrin excretion only is given in Table III.

It is seen that phenacetin and phenazone are porphyrin-producers of the same order, amidopyrine is twice as potent and acetanilide four times. The case of aspirin has proved difficult to interpret. On the previous experiments it

Table I. *Total urinary porphyrin in $\mu\text{g.}$ for three rats for 3 days, and erythrocyte counts in millions per $\mu\text{l.}$ on the first day of each period. Drugs were given once daily from 29 March to 6 April*

Date	Acetanilide 200 mg. per kg.		Phenacetin 300 mg. per kg.		Phenazone 400 mg. per kg.		Amidopyrine 300 mg. per kg.		Aspirin 300 mg. per kg.		Controls 2 % acacia only	
	R.B.C. count $\times 10^6$	Porphyrin $\mu\text{g.}$	R.B.C. count $\times 10^6$	Porphyrin $\mu\text{g.}$	R.B.C. count $\times 10^6$	Porphyrin $\mu\text{g.}$	R.B.C. count $\times 10^6$	Porphyrin $\mu\text{g.}$	R.B.C. count $\times 10^6$	Porphyrin $\mu\text{g.}$	R.B.C. count $\times 10^6$	Porphyrin $\mu\text{g.}$
23 March	8.2	28	6.7	36	10.0	24	8.0	18	8.8	18	8.8	12
	8.0		8.5		8.5		7.2		7.9		10.0	
	8.8		9.2		8.8		9.0		8.3		8.5	
26 March	8.0	24	7.4	32	9.6	20	8.2	18	8.5	28	8.6	18
	9.0		8.6		8.8		7.5		7.5		9.0	
	8.4		9.0		8.8		8.8		8.0		7.8	
29 March	8.5	172	7.2	32	8.2	12	8.2	80	8.6	60	8.6	24
	8.2		8.5		8.2		7.2		7.8		9.4	
	8.2		9.2		8.4		8.8		8.5		8.0	
1 April	7.2	280	6.5	140	8.2	60	6.0	90	3.6	120	8.0	24
	8.4		8.0		8.0		5.4		4.5		9.5	
	7.0		8.2		7.2		6.0		3.5		8.8	
4 April	4.5	330	5.0	200	5.6	72	5.2	140	—	—	8.4	22
	4.5		4.2		5.6		5.0		Dead		9.4	
	5.5		5.5		3.2		3.2		Dead		8.2	
7 April	4.0	—	Dead	—	5.2	—	3.2	—	—	—	8.2	—
	5.2		4.0		5.0		Dead		—		9.2	
	6.0		5.0		Dead		Dead		—		8.6	

Table II. Total urinary porphyrin in $\mu\text{g.}$ for three rats for 3 days and erythrocyte counts in millions per $\mu\text{l.}$ on the first day of each period. Two pre-periods of 3 days without drugs are followed by four 3-day periods with drugs. Then follow two 3-day periods when the drugs were withdrawn and finally a period when the drugs were resumed

Date	Acetanilide 75 mg. per kg.			Phenacetin 300 mg. per kg.			Phenazone 400 mg. per kg.			Amidopyrine 150 mg. per kg.			Aspirin 150 mg. per kg.			Controls 2 % acacia only		
	R.B.C.			R.B.C.			R.B.C.			R.B.C.			R.B.C.			R.B.C.		
	count $\times 10^6$	Porphyrin $\mu\text{g.}$	Porphyrin $\times 10^6$	count $\times 10^6$	Porphyrin $\mu\text{g.}$	Porphyrin $\times 10^6$	count $\times 10^6$	Porphyrin $\mu\text{g.}$	Porphyrin $\times 10^6$	count $\times 10^6$	Porphyrin $\mu\text{g.}$	Porphyrin $\times 10^6$	count $\times 10^6$	Porphyrin $\mu\text{g.}$	Porphyrin $\times 10^6$	count $\times 10^6$	Porphyrin $\mu\text{g.}$	Porphyrin $\times 10^6$
8 May	8.2 8.0 8.8	15 10.0 8.5	7.9 10.0 8.5	8.6 8.2 8.5	32 8.2 8.5	8.0 8.8 8.8	8.8 8.5 9.0	8.8 8.5 9.0	8.8 8.5 9.0	8.8 8.5 9.0	8.8 8.5 9.0	8.8 8.5 9.0	8.8 8.5 9.0	8.8 8.5 9.0	8.8 8.5 9.0	8.8 8.5 9.0	20 10.0 8.5	20 10.0 8.5
11 May	8.0 9.0 7.9	12 8.0 8.8	8.0 9.8 8.2	8.2 7.8 7.8	28 8.2 8.5	8.2 8.5 9.0	8.2 8.5 9.0	7.6 7.8 9.0	22 8.5 9.0	7.6 7.8 9.0	6 6 6	6 6 6	7.6 7.8 9.0	6 6 6	7.6 7.8 9.0	9.0 10.0 8.6	24 24 24	24 24 24
14 May	8.4 9.2 8.2	18 9.2 8.2	8.2 9.8 7.8	7.8 8.4 8.4	Nil Nil Nil	7.6 7.8 9.4	7.6 7.8 9.4	8.2 8.2 9.4	Nil Nil Nil	8.2 8.2 9.4	3 3 3	3 3 3	8.2 8.2 9.4	3 3 3	8.2 8.2 9.4	8.8 10.2 8.2	24 24 24	24 24 24
17 May	7.2 6.2 6.0	24 6.2 6.0	7.2 6.8 5.8	5.0 5.0 5.2	36 6.8 5.8	5.8 5.6 7.0	5.8 5.6 7.0	5.8 5.6 7.0	40 40 40	5.8 5.6 7.0	12 12 12	12 12 12	7.6 8.4 9.5	12 12 12	9.0 9.8 8.0	32 32 32	32 32 32	32 32 32
20 May	6.2 5.4 5.6	45 5.4 5.6	8.0 5.2 4.4	4.5 5.6 5.2	50 5.2 4.4	5.2 5.2 6.2	5.2 5.2 6.2	4.6 5.5 6.2	60 60 60	5.2 5.2 6.2	24 24 24	24 24 24	4.6 5.5 6.2	24 24 24	8.5 9.5 7.2	22 22 22	22 22 22	22 22 22
23 May	6.6 5.6 5.8	65 5.6 5.8	7.5 5.0 4.2	5.0 5.4 4.2	75 5.0 4.2	5.0 5.2 6.2	5.0 5.2 6.2	4.8 5.2 6.4	110 110 110	5.2 5.5 4.0	80 80 80	80 80 80	4.8 5.2 6.4	80 80 80	9.0 9.2 7.2	20 20 20	20 20 20	20 20 20
26 May	6.6 5.6 5.4	38 5.6 5.4	6.2 5.4 4.0	5.5 4.2 3.8	40 4.2 3.8	5.0 5.2 6.2	5.0 5.2 6.2	5.5 5.5 6.2	62 62 62	5.0 5.2 4.5	138 138 138	138 138 138	5.5 5.0 5.5	138 138 138	9.2 9.4 7.8	22 22 22	22 22 22	22 22 22
29 May	6.8 5.4 5.2	28 5.4 5.2	6.2 5.2 3.0	5.0 5.2 4.8	30 5.0 4.8	5.8 5.8 5.2	5.8 5.8 5.2	4.5 5.8 6.0	54 54 54	5.8 5.8 5.2	90 90 90	90 90 90	4.5 5.8 6.0	90 90 90	9.4 9.2 7.5	28 28 28	28 28 28	28 28 28
1 June	—	32	—	—	35	—	—	60	50	—	140	140	—	140	—	—	22	22

Table III. *Increase of coproporphyrin excretion in urine from groups of three rats for 3-day periods, following administration of antipyretic drugs*

Porphyrin excretion in multiples of the pre-period								
Drug	Daily dose in mg. per kg.	Drugs given				Drugs withdrawn		Drugs resumed
		1	2	4	5	3	2	
Acetanilide	75	1	2	4	5	3	2	2.5
Phenacetin	300	1.5	2.5	3.5	5	3	2	2
Phenazone	400	nil	1	3	5	2.5	1	2
Amidopyrine	150	nil	2	3	5	3	2.5	2.5
Aspirin	150	0.5	2	4	10	15	11	15
Controls	—	1	1.5	1	1	1	1.5	1

appeared to be as potent as phenacetin or amidopyrine while on this occasion it shows a potency of many times that of amidopyrine. Attempts to repeat these experiments with a dose level of 75 mg. per kg. gave an entirely negative response while doses of 150–300 mg. per kg. gave figures which varied from a faint positive to a figure of 15 times normal. These larger figures were always accompanied by haemoglobinuria and the animals very quickly lost tone and died. Post-mortem examination always showed gastric haemorrhage.

Further experiments with these drugs and with *p*-aminophenol were made on groups of twelve adult rats, with the object of isolating the pigments. The protocols are not reproduced again since the results are similar and confirm those given above. In the case of *p*-aminophenol the dose level of one quarter of the average lethal dose (LD 50 = 600 mg. per kg.) was well tolerated. The excretion of porphyrin during the preperiod of 3 days for four rats was found to be 8 μ g. There was a decrease for the first 3-day period of dosing and the second and third periods showed a small rise of 1.5 times. For the fourth period a jump to 11 times was recorded, while the fifth and sixth periods both gave 22 times the pre-period.

General observations

Throughout the experiments a daily cycle of events was observed. Before dosing, the animals were quite playful and appeared normal, and at this stage blood was taken from the tail for the blood counts and for methaemoglobin determinations. After dosing, all the animals receiving drugs were depressed, and in the case of animals receiving aspirin, amidopyrine, phenazone and *p*-aminophenol typical muscular spasms were seen. In all cases except that of aspirin, cyanosis developed but passed off in the course of 6–8 hr. During the course of the experiments a tolerance to these drugs was acquired except in the case of aspirin, where the rats became markedly more irritable. All the animals showed losses in weight but the controls always finished with a good appetite, while rats receiving aspirin, phenacetin and amidopyrine showed little interest in food. At the close of the experiments the animals were killed by a blow on the head and the spleens taken for histological examination.

Urine. Urine from rats receiving acetanilide, phenacetin and *p*-aminophenol was dark with a greenish fluorescence. On exposure to air it darkened from the top down and rapidly became black. Rats receiving amidopyrine voided a dark red urine, and those receiving phenazone a light pink; both darkened on exposure to air. By qualitative tests all these urines showed increased amounts of protein, bile salts and urobilin. The urine from rats receiving aspirin was dark and darkened a little on standing, and while excess protein was frequently found, urobilin and bile salts were normal. Increases in the volumes of urine, such as Rimington [1938] observed with sulphanilamide were not seen. Blood from all

animals was examined spectroscopically for methaemoglobin and sulphaemoglobin, by approved methods, but only in the case of one animal treated with acetanilide and one receiving *p*-aminophenol was the characteristic spectrum of methaemoglobin identified. This examination was made 22 hr. after administration of the drug when only persistent methaemoglobinaemia would have been identified.

Blood picture. A gradual fall, amounting to a progressive anaemia, was observed in the circulating erythrocytes during the course of these experiments. The counts are recorded in Tables I and II.

Photosensitization. Animals treated with amidopyrine and phenazone lost all fur from the back and sides of the body. All the drugs examined were fed to groups of two rats for 9 days. The backs of these animals and of two normal rats were shaved and exposed to the ultraviolet irradiation from a mercury vapour arc at 24 in. for 15 min. on 3 successive days. All the rats receiving drugs developed typical red weals readily distinguishable from the erythema of the controls. The photosensitization was, however, of a mild order.

Histological examination of spleens. The spleens of all animals receiving the drugs were enlarged and slaty black in colour. Sections from animals receiving acetanilide, phenacetin, phenazone, amidopyrine and *p*-aminophenol showed a common microscopic picture. The sinuses were engorged with red blood cells, while the cells which lined them showed a marked reticular hyperplasia. The prussian blue reaction showed a quantity of stainable iron but much more conspicuous were large deposits of brownish non-staining pigment in the pulp.

Sections of spleens from animals treated with aspirin showed smaller amounts of stainable iron and of brown non-staining pigment. The sinuses and cells of the pulp presented a grossly abnormal picture being packed solidly with red cells, most of which were abnormal in outline and size.

The sections when examined by ultraviolet fluorescence microscopy showed some orange fluorescence but this was not associated with the characteristic brown pigment deposits.

Extraction of the porphyrins from urine

Methods of extraction of porphyrins were outlined by Garrod [1894] and were developed by Fischer [1924, 1, 2; 1926] and Fischer & Duesberg [1932] and by Watson [1932]. An excellent scheme for separation and identification of porphyrins was given by Dobriner [1936], and a method more suited to small amounts by Rimington [1936]. The following is the scheme used in this investigation and is drawn from the last two sources.

The urine is strained through glass wool and washings added; 0.1 vol. glacial acetic acid is added and the solution extracted with 1.5 vol. ether by shaking vigorously for 10 min. continuously. The ether phase is separated, and extraction repeated, but this time with 0.05 vol. glacial acetic acid. The ether is separated, and a third extraction made with 1 vol. ether alone, separated and the three ether-soluble porphyrin extracts are mixed. Trouble may be experienced at this stage by formation of emulsions. These are best broken by carefully floating a few ml. of glacial acetic acid on the surface and leaving undisturbed for 30 min. Acetic acid is now washed out by adding 0.5 vol. distilled water and a quantity of saturated potassium acetate equal to one-fifth of the total volume of acetic acid used. This is repeated once, then followed twice by distilled water alone.

The porphyrins are extracted from the ether by repeated vigorous shakings with 5 % HCl in distilled water. Completeness of extraction is controlled by

spectroscopic examination, or more easily, by fluorescence in ultraviolet light. A final extraction with 20 % HCl in distilled water, afterwards diluted, may be essential. The 5 % HCl is neutralized to Congo red by dropwise addition of saturated potassium acetate together with a few drops of acetic acid. The process of driving the porphyrins back and forth between ether and 2 % HCl in distilled water is repeated twice or until a clear ruby solution is obtained. This solution in HCl is shaken once with chloroform and separated to remove blue bile pigments and protoporphyrins. The porphyrins are again transferred to 5 ml. of ether and washed with half this volume of distilled water which is carefully and completely separated with the aid of a small funnel as advised by Rimington. The ether is vigorously extracted with 0.5 ml. quantities of 0.5 % HCl until no band in the green is observed in the acid shakings and in the ether. It is possible to extract completely in 4-5 shakings.

Isolation and identification of porphyrins as methyl esters

The accumulated acid shakings are transferred to a small volume of ether and evaporated over a sand bath. To this small residue are added 5 ml. of saturated HCl in dry methyl alcohol, and the mixture rotated, stoppered and left 24 hr. at room temperature. To the 5 ml. of esterification mixture are added 5 ml. of chloroform and 50 ml. of ice-cold water; this is repeated with fresh chloroform to carry down the droplets completely and separated. The chloroform is shaken with 2 % Na_2CO_3 until the chloroform solution shows the "alkaline porphyrin" spectrum. This solution is repeatedly washed with small amounts of distilled water, filtered and evaporated to dryness. In all the present cases the material at this stage was amorphous; these yields were washed with hot light petroleum and then taken up in 2 ml. of dried ether. To this were added 2 ml. of dried methyl alcohol and the volume reduced to 0.25 ml. by warming on a sand bath. The porphyrin esters precipitated in typical form after standing; they were washed free from mother liquor with methyl alcohol and allowed to dry at room temperature. Melting point and mixed melting point determinations with the methyl ester of authentic coproporphyrin III kindly supplied by Dr C. Rimington were then made on the six samples obtained. The figures obtained are shown in Table IV.

Table IV. *Melting points of the methyl esters of the isolated coproporphyrins*

Drug	M.P.: Re-M.P. ° C.	Mixed M.P. ° C.
Acetanilide	142 : 172	142-146
Phenacetin	145 : 168	144-145
Phenazone	143 : 170	142-144
Amidopyrine	152 : 170	142-145
Aspirin	146 : 172	143-146
p-Aminophenol	142 : 170	140-144

Thus the excreted porphyrins have been characterized as coproporphyrin III by spectra, characteristic crystal form and by mixed melting point determination. Fischer & Treibs [1926] gave for coproporphyrin I methyl ester M.P. 253°, and for coproporphyrin III methyl ester 142° they noted that the latter first melted at 142° and remelted after cooling at 172°.

DISCUSSION

The administration of drugs of the coal tar antipyretic group to healthy adult rats results in a marked porphyrinuria. These pigments belong to the series III type, together with smaller amounts of series I, and therefore these experiments

fall into line with those of Rimington [1938] with aminobenzenesulphonamide, and of Schreus [1935] with salvarsan.

The reported intoxications following the use of these drugs are all strikingly similar. Drugs containing the aromatic amino group are able to produce methaemoglobinaemia, while the incidence of granulocytopenia following benzenesulphonamide types must now be classified with the so-called idiosyncrasies of amidopyrine, acetanilide, phenacetin and the arspenamine compounds.

It is possible that *p*-aminophenol is the common degradation product for drugs containing a potential source of this compound and in some cases its presence has been demonstrated. This stage once reached, the oxidation-reduction system suggested by Heubner & Schwedtke [1936] satisfactorily explains the production of methaemoglobin.

Aspirin contains no amino group and is unlikely to give rise to a vigorous oxidation-reduction system even when repeatedly ingested. Moreover, it is not suspected of causing methaemoglobinaemia in clinical use. The most likely solution is that this drug does cause an abnormal degradation of haemoglobin, possibly through methaemoglobin.

Fischer and his school have established that blood and bile pigments are members of the series III isomerides. The theoretical precursor of haemoglobin is aetioporphyrin III. That this synthesis may involve the formation of small quantities of coproporphyrin series I as a by-product which is found in the urine, is suggested by Rimington [1936]. If this theory is correct it is clear that increased coproporphyrin I excretion must be associated with an increase, or derangement of normal bone-marrow haematopoiesis. Abnormal excretion of coproporphyrin I is found in congenital porphyria, sulphonamide poisoning and in many cases where the normal degradation products of haemoglobin are not removed from the blood stream, as in catarrhal jaundice, obstructive jaundice, haemolytic jaundice and in atrophic cirrhosis of the liver [Dobriner, 1936].

The excretion of the series III isomeride has been shown in pigment cirrhosis and liver tumour [Dobriner, 1936]. Its presence following lead intoxication has been widely quoted, for example by Grotepass [1932], Fischer & Duesberg [1932], and Mertens [1937], and it occurs after salvarsan [Schreus, 1935]. Recently its presence has been noted by Rimington [1938], while the results of the present observations must be added to the list.

It is improbable that normal erythrocyte breakdown would ever give rise to coproporphyrin III, for Lemberg [1935] considers that haemoglobin during its normal degradation to bile pigment is oxidized while iron and protein are still in combination, and thus never passes through a porphyrin stage.

While the precise relationship between methaemoglobin and coproporphyrin III has yet to be experimentally demonstrated, a general hypothesis can be stated. Lemberg [1935] has formulated a series of reactions leading from haemins to "green haemins" and thence to biliverdins. This conversion provides an authentic model for the system responsible for the formation of bile pigment in the body. In this conversion the rupture of the ring system is catalysed by iron which is present in an unoxidized form. It seems probable that where the iron is already oxidized, as in methaemoglobin, it cannot play its part in this conversion. Degradation of haemoglobin through methaemoglobin therefore results in coproporphyrin III and not bilirubin.

During the course of the experiments described, an increased breakdown of erythrocytes was taking place as shown by the progressive red cell anaemia and

the abnormal urobilin output. It is possible that the increased coproporphyrin I output implied by the photosensitization of the animals was directly due to haematopoiesis, stimulated by the increased degradation of haemoglobin.

SUMMARY

1. Acetanilide, phenacetin, phenazone, amidopyrine, aspirin and *p*-aminophenol have been administered by stomach tube to normal healthy rats in daily doses which corresponded to 25 % of the average lethal dose. The animals developed a porphyrinuria which in some cases reached values equivalent to twelve times the normal. During the period of dosing the animals lost weight and developed a progressive red cell anaemia. A mild degree of photosensitization was observed in all cases.

2. Equivalent doses of phenacetin and phenazone produce the same degree of porphyrinuria; amidopyrine and aspirin are twice as potent, and acetanilide four times, while *p*-aminophenol is more potent than acetanilide. These effects run approximately parallel with the acute toxicities of the drugs.

3. Animals taken for histological examination at the close of the experiments showed grossly abnormal spleens. These were enlarged and slaty-black in colour. The sinuses were much engorged and quantities of stainable iron were seen. Conspicuous were large deposits of brownish, non-staining pigment in the pulp.

4. A marked degree of blood-cell destruction is caused by these drugs as shown by the progressive anaemia and the abnormal urobilin output, but a more fundamental disturbance in blood-pigment metabolism is postulated to account for the progressive train of events which is described.

5. The urinary porphyrins have been isolated and identified as coproporphyrin III in each case. Smaller amounts of coproporphyrin I were also present. The significance of these findings is discussed and attention is drawn to the common chemical relationship of drugs known to cause coproporphyrin III excretion.

6. Evidence to support the claim that *p*-aminophenol is the common active degradation product for drugs containing a phenylamine group is reviewed, and where the amino group is absent the view is advanced that quinol is the probable degradation product.

7. Support is given to the claim that the oxidation-reduction systems set up by aminophenol or quinol are responsible for the oxidation of haemoglobin to methaemoglobin.

8. The hypothesis is advanced that where haemoglobin is oxidized to methaemoglobin the normal conversion into bilirubin cannot occur but is replaced by degradation to coproporphyrin III.

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