CXVIII. PORPHYRINURIC ACTION OF DRUGS RELATED TO SULPHANILAMIDE. COMPARISON WITH REPORTED TOXICITY, THERAPEUTIC EFFICIENCY AND CAUSATION OF METHAEMOGLOBINAEMIA. DEFINITION OF THE STRUCTURE RESPONSIBLE FOR PORPHYRINURIC ACTION

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It has been shown [Rimington & Hemmings, 1938] that a marked increase in porphyrin excretion follows the daily oral administration to white rats of sulphanilamide at dosage levels of 0.4–1.5 g./kg. body weight. The porphyrins were isolated from urine and faeces and identified as coproporphyrin III together with small quantities of coproporphyrin I. Porphyrinuria due to these same two pigments was demonstrated in a series of human clinical cases undergoing a course of sulphanilamide treatment, and similar observations have more recently been reported by Long & Bliss [1938], and also by Silver & Elliott [1939].

Discussion of the mechanism of this porphyrinuria led to the conclusion that the drug, in the doses employed, exerts a toxic action most probably affecting both the liver and the bone marrow. The possibility was indicated that some patients might prove to be more susceptible than others and it was suggested that determination of the amount of porphyrinuria might serve as a means of assessing the toxic action of sulphanilamide upon this phase of haematopoietic activity and of revealing individual idiosyncrasy. That sulphanilamide is capable of causing profound disturbances in the pigment metabolism of the body is evident from the frequency with which cyanosis accompanied by demonstrable methaemoglobinaemia has been reported. Thus, in a review of the types of toxic manifestation occurring in 408 patients exhibiting sulphanilamide intoxication, Long *et al.* [1939] noted cyanosis in 90–100%, whilst most observers agree that at least half of all their patients treated with the drug show some degree of cyanosis. There has been much discussion as to whether methaemoglobin is alone responsible, or whether products derived from sulphanilamide may also contribute to the discoloration. Some observers have gone so far as to assert that methaemoglobinaemia is of comparatively rare occurrence; on the other hand, careful investigations by Lockwood *et al.* [1938], Hartmann *et al.* [1938] and others have shown that a close correspondence can be traced between the degree of cyanosis and the quantity of spectrophotometrically determined methaemoglobin. It cannot be too greatly emphasized that in particular those toxic effects revealed by changes in the pigment economy and metabolism of the organism are deserving of much more thorough investigation than has hitherto been accorded them [compare Rimington, 1939].

In the present communication, experiments are described by means of which answers to the following questions have been sought:

(1) In treatment with the sulphonamide series of drugs, is porphyrinuric activity an invariable accompaniment of therapeutic effectiveness or does this symptom parallel toxicity to the animal as a whole (minimal acute fatal dose)?

(2) Which chemical grouping or portion of the molecule is responsible for porphyrinuric activity?

(3) Is there any relation between methaemoglobin formation (compare sulphanilamide cyanosis) and increased excretion of porphyrin?

A series of substances related to sulphanilamide or possessing certain of the structural elements of this drug have been fed over periods of 1, 2 or 3 weeks to rats maintained, as in the previous experiments, upon a sufficient, synthetic diet. The doses were varied to allow of comparison as to porphyrinuric effect, and in all cases when a toxic action was manifest, post-mortem histological examinations have been made. The results demonstrate that porphyrinuric action is closely associated with ability to produce methaemoglobinaemia and runs roughly parallel with toxicity as a whole.

EXPERIMENTAL

Technique of animal experiments

Certain improvements in technique have been made since the publication of our first paper, and our present method will therefore be recorded in detail.

White rats weighing 150–200 g. were kept in groups of three in metabolism cages arranged for the separate collection of urine and faeces. These cages were made of metal, the funnel-shaped lower portion being carefully treated with molten paraffin wax at intervals of about a week, to prevent any possibility of the urine coming into direct contact with the metal surface. Toluene was added to the glass urine-collecting flasks.

The artificial diet was made up as follows, and was fed *ad lib.* Water was supplied *ad lib.* from Drummond bulbs:

Synthetic diet. Casein, 20%; rice starch, 73%; salt mixture, 2%; yeast powder, 5%; cod-liver oil, 20 μ l. per rat per week.

Salt mixture. NaCl, 41.6 g.; MgSO₄, 7H₂O, 130.2 g.; NaH₂PO₄, 83.2 g.; K₂HPO₄, 228.8 g.; CaHPO₄, 129.6 g.; ferric citrate, 47.2 g.; calcium lactate, 146.2 g.

The food was not placed in the metabolism cages, but the animals were removed and allowed to feed and drink in separate cages for 1 hr. in the morning and 1 hr. in the evening (a single period of 2 hr. on Sundays). A small quantity of urine was unavoidably lost during this time, but contamination of the collecting receptacle with debris was obviated. All animals were weighed at weekly intervals. The drugs were administered orally, unless otherwise stated, usually in the form of a suspension in 10% gum acacia solution. A requisite volume of this mixture was introduced daily into the oesophagus by means of a hypodermic syringe fitted with a blunted metal needle. Liquid drugs were introduced into the oesophagus in the same manner. Groups of normal rats, kept under similar conditions, served as controls.

Determination of urinary porphyrin

The urine sample, representing a 3-day collection from three rats (about 70 ml.), was filtered through glass wool into a separating funnel and the collecting flask rinsed with 20–30 ml. of water. To the combined filtrates were added 20 ml. of glacial acetic acid followed by 200 ml. of ether. The mixture was well shaken and the lower aqueous layer was run off as far as the small

gelatinous emulsion which is almost invariably found at the interface. Half an hour was allowed for this emulsion to contract into a more solid mass, from which the ether was decanted. The emulsion residue was now shaken again with 3–4 ml. of acetic acid and 100 ml. of ether, this solvent, after decantation, being used for a final extraction of the main bulk of the urine. The combined ether extracts were washed four times with 200 ml. of water, the first two washings containing 5 ml. of saturated potassium acetate solution adjusted, if necessary, to pH 7.0. After removal of the bulk of the dissolved acetic acid from the ether phase in this way, the porphyrins were extracted by shaking with successive lots of 10 ml. of 5% (by weight) HCl, allowing the acid phase to settle down completely each time before tapping off. Completion of extraction can be controlled by spectroscopic or fluoroscopic examination. Usually three or four extractions suffice to remove all the porphyrin. Such an acid extract will only contain the porphyrin in very great dilution and may also contain other colouring matters which interfere with quantitative determination, especially if a fluorescence method be used. It has been our practice, therefore, to purify and concentrate the porphyrin as follows.

To the 5% HCl extract in a smaller separating funnel are added 5 ml. of ether and sufficient saturated potassium acetate to render the reaction neutral to Congo red. (8 ml. are sufficient for 30 ml. of acid extract.) After vigorous shaking, the ether is transferred to a 20 ml. capacity funnel having a very short outlet tube, and the aqueous solution is extracted with two more successive 5 ml. lots of ether, which are combined with the first. This ether solution is washed once with 3 ml. of water, taking care to shake down drops adhering to the glass. The coproporphyrin is then extracted by shaking with 0.5 ml. lots of 0.5% by weight HCl until the acid absorption spectrum is no longer visible. Usually five shakings suffice to remove even as much as 100 μ g. The volume is then carefully adjusted to 3 ml. (or more, if much porphyrin is present) and the quantity of coproporphyrin present determined by measuring the light absorption in the region of the β band of the acid porphyrin spectrum (centre about 549 m μ .) on a Hilger-Nutting spectrophotometer previously calibrated against standard coproporphyrin solutions. Since the relation between porphyrin concentration and the photometer scale reading is linear, the exact concentration of porphyrin in the test solution is readily obtained. Concentrations as low as 3 μ g./ml. in the final solution are easily determined by this method.

It will be noticed that we have employed 0.5% HCl for the final extraction of the coproporphyrin. The advantage of this procedure is that any protoporphyrin present through accidental contamination of the urine specimen with faeces is thereby left behind in the ether, since protoporphyrin has a much higher acid number than has coproporphyrin.

The rats selected for any particular experiment were usually allowed to remain for 6–12 days upon the basal synthetic diet, whilst a "base line" of porphyrin excretion was being obtained. Daily dosing was thereafter commenced and continued for 9–24 days, according to the promptness of response. In many instances, the effect did not become very marked until about the ninth day, after which porphyrin excretion rose rapidly. It is possible that saturation of the organism with the drug, or the precursor of the porphyrin, occurs during the initial period, since the phenomenon of delayed response was particularly striking in the case of substances like sulphanilamide and prosectasine which are poorly absorbed and less evident or absent when materials like aniline or hydroxylamine were dosed. It is also possible that a hepatotoxic effect might develop during the course of some days [compare Espersen, 1937].

At the end of an experiment, the animals were killed by coal gas and specimens of liver, spleen and kidney (sometimes also bone-marrow) taken in corrosive-acetic fixative for histological examination.

All preparations were examined for stainable iron (prussian blue reaction) in addition to haematoxylin-eosin differentiation.

Isolation of the porphyrins

The urine extracts from experiments with any one drug were pooled and the ether-soluble porphyrins isolated in the following manner.

The 0.5% HCl solution was filtered into a small separating funnel and covered with a layer of ether. A few drops of glacial acetic acid were then added and sufficient saturated aqueous potassium acetate to render the mixture neutral to Congo red. Upon shaking, the porphyrins passed up into the ether layer which was separated, washed well by shaking with water and allowed to evaporate to dryness. The residue was washed once with light petroleum and then esterified by dissolving in methyl alcoholic HCl (saturated at 0°). After 24 hr., chloroform was added and much water in a separating funnel. The chloroform layer, containing the porphyrin esters, was washed well with dilute Na_2CO_3 and then water and evaporated to dryness. After rinsing with light petroleum, the dry residue was treated with cold, anhydrous ether until no more pigment passed into solution. This ether solution, containing the coproporphyrin III ester, was evaporated to a very small volume and set aside in a closed tube in the ice-box for crystallization. Any ether-insoluble pigment was dissolved in a little chloroform and to the concentrated solution about 5 vol. of hot methyl alcohol were added and the mixture set in the ice-chest. Any coproporphyrin I ester separating could be centrifuged down, but in many cases the quantity present was barely more than a trace, too small for identification by melting-point. The crystals of coproporphyrin III ester, which formed in the concentrated ether solution, were separated from mother liquor (removing the latter with a micro-pipette), washed sparingly with cold, anhydrous ether, then with light petroleum and dried over P_2O_5 *in vacuo*. Melting-points were recorded on the Kofler electrically-heated micro-melting-point apparatus.

RESULTS

Economy of space prevents the full details being given for each substance tested. The data for experiments with sulphanilamide and aniline are given in full in the protocol in the Appendix; all the findings are presented in summary form in Tables I and II.

The following drugs were investigated:

- (1) *p*-Aminobenzenesulphonamide ("sulphanilamide"):

- (2) Disodium 4'-sulphonamidophenyl-2-azo-7-acetamino-1-hydroxynaphthalene-3:6-disulphonate ("prontosil soluble"):

(3) Sodium *p*-aminobenzenesulphonyl-*p*'-aminobenzenesulphonate ("sodium sulphanilyl-sulphanilate"):

(4) *p*-Aminobenzenesulphonyl-*p*'-aminobenzenesulphonamide hydrochloride ("disulphanilamide hydrochloride"):

(5) *p*-Benzylaminobenzenesulphonamide ("proseptasine"):

(6) 4-Sulphanilylamidoacetophenone:

This substance was kindly placed at our disposal by the manufacturers, Messrs E. Merck, in consultation with the Therapeutic Trials Committee of the Medical Research Council. We are grateful to Messrs Merck and to the above-named Committee for permission to publish our findings.

(7) *p*-Aminobenzenesulphonyl-*p*'-aminobenzenedimethylsulphonamide ("uliron"):

(8) *p*-Aminobenzenesulphonyl-*p*'-aminobenzenemethylsulphonamide ("disseptal B"):

(9) 4:4'-Diaminodiphenylsulphone:

(10) 4:4'-Diacetaminodiphenylsulphone:

(11) Sulphanilic acid.

(12) *p*-Aminobenzoic acid.

(13) *p*-Phenylenediamine.

- (14) Aniline, purified by redistillation.
- (15) Monomethylaniline (prepared by hydrolysis of the crystalline acetyl compound, "exalgin", and purified by redistillation).
- (16) Dimethylaniline (purified by repeated recrystallization and finally redistilled).
- (17) 2:4:6-Trichloroaniline.
- (18) Phenetidine (purified by redistillation).
- (19) Benzidine.
- (20) *o*-Toluidine.
- (21) 5-Chloro-*o*-toluidine.
- (22) Nitrobenzene (purified by redistillation).
- (23) Methylacetanilide ("exalgin").
- (24) Hydroxylamine hydrochloride.
- (25) Phenylhydroxylamine (freshly prepared by Imperial Chemical Industries and stored in N₂ in the dark).
- (26) Sodium nitrite.
- (27) *iso*Amylamine.

DISCUSSION

A discussion of our results will be much facilitated by reference to the structural formulae on pp. 963-4 and to Tables I and II in which the main findings are summarized.

It will be seen that porphyrinuric activity is by no means limited to sulphanilamide, but is shared by most of the drugs belonging to the sulphonamide series. In addition, a number of the simple aromatic amines tested, particularly aniline and its derivatives, were found to possess this property to a marked degree.

No doubt, were it possible to give sufficiently high doses, it would be found that a great number of substances would cause porphyrinuria, but on the basis of the dosage rates employed in this investigation it would seem permissible to draw certain general conclusions, relating porphyrinuric action and therapeutic efficiency to chemical constitution, as follows.

(1) *There is no quantitative relation between therapeutic efficiency and porphyrinuric action.* Within the series of sulphonamide drugs, it is not always easy to compare therapeutic action with toxicity on account of the fact that many of the substances are sparingly soluble and poorly absorbed so that only a small proportion of the ingested dose actually finds its way into the blood stream. Nevertheless, substances like sulphanilamide and disulphanilamide, for example, are considered to exert comparable therapeutic effects and yet the latter drug does not cause any increase in porphyrin excretion in the dosage attainable. Similarly, many of the simpler substances related to the sulphonamides have been shown to increase very markedly the urinary porphyrin excretion and yet they are devoid of therapeutic action in experimental infections.

(2) *In general, porphyrinuric action runs roughly parallel with general toxicity.* An apparent exception to this generalization is the case of 4:4'-diacetamidodiphenylsulphone, which is tolerated in single doses > 40 g./kg. but which produces quite a pronounced porphyrinuria when administered daily at the level of 0.18 g./kg. These amines and their acetyl derivatives are known to come into a state of dynamic equilibrium *in vivo* and hence one might expect the toxicity of the diacetyl derivative of the sulphone to be much greater (approaching that of the parent amine) when given regularly than when administered on a single occasion [compare Nitti *et al.* 1938]. The post-mortem histological changes suggest a similar action. Moreover, it must again be pointed out that the

Table I

No.	Drug	Porphyrinuric action					M.P. and re-M.P. of Me ester of porphyrin isolated	MetHb formation	P.M. histological findings	Remarks
		M.Pro.D.* g./kg.	M.L.D.† g./kg.	Dose g./kg.	Porphyrin excretion	(× normal)				
1	Sulphanilamide	0.25-1	4	0.4	+	2.5	142-4°,	+	Liver, small amount of stainable iron and some brownish pigment in convoluted tubules. Spleen, intensely engorged, much stainable iron, also brownish pigment	There was a marked increase in water intake and in urine vol. accompanied by loss in body wt.
				0.93	+	4	166°			
				1.23	+	5				
				1.39	++	7				
				1.42	++	6.5				
				1.5	+++	8				
2	Prontosil soluble	1	4 (i.m.†)	1.25 (i.m.)	++	6	146°,	+	Spleens of groups on larger dose were engorged, contained brown amorphous pigment and stainable iron. Remaining organs and second group normal	Slight loss in body wt. Urine vol. unchanged. Slight local reaction at site of s.c. injection
				2.10	+++	13	168°			
3	Na sulphanilyl-sulphanilate	s.c.§ in-effective; per os 2	20	0.05 0.25 1.0 (s.c.)	- - -			-		Animals gained in wt. High M.L.D. and absence of porphyrinuric action, probably due to poor absorption with resulting low concentration in blood [Feinstone <i>et al.</i> 1938]
4	Disulphanilamide hydrochloride	0.25-1	20	0.53 0.80	- -			-		

5	Proseptasine	0.5	20	1.0	+	2	Slight engorgement of spleen with some deposits of stainable iron
6	4-Sulphanilyl-amidoacetophenone	0.5	>10	0.55 1.15	++ ++	7.6 7.1	Much brown pigment and stainable iron in spleen
7	Uliron			1.0	±		Loss of body wt. Urine vol. unaltered
8	Disseptal B			1.0	+	3	Marked loss in wt. and moderate increase in urine vol. Coats became rough and staring Wt. and urine vol. unchanged. Uliron has caused fatal funicular degeneration with an inflammatory reaction of the spinal cord [Schubert, 1938]. Both nos. 7 and 8 cause polyn neuritis [Hüllstrung & Krause, 1938, 1, 2] but cyanosis only infrequently
9	4,4'-Diaminodiphenylsulphone	0.0025 -0.01	0.25-0.5	0.028 0.045 0.080 0.15	++ ++ + Hburia + (deaths)	3.5 3.5	Small deposits of stainable iron in the spleen, but in general much less marked than in the case of sulphanilamide
10	4,4'-Diacetaminodiphenylsulphone	0.25-0.1	>40	0.18 0.59	++	4 5	Slight deposit of stainable iron in spleen Much less toxic than no. 9

† M.L.D. = Minimal lethal dose, mouse or rat.

|| Repeated doses.

* M.Pro.D. = Minimal protective dose, mouse or rat.
† i.m. = Intramuscularly. § s.c. = Subcutaneously.

Table II

No.	Substance	Porphyrinuric action				MetHb formation	P.M. histological findings	Remarks
		Dose g./kg.	Porphyrin excretion	($\times$ normal)	M.P. and re-m.p. of porphyrin isolated			
11	Sulphanilic acid	1.4 1.8	- -			-		Smaller dose administered as Na salt, dissolved in drinking water. Lack of effect and of therapeutic activity may be due to high solubility or to dipole nature of the molecule Substance also a dipole
12	<i>p</i> -Aminobenzoic acid	1.25	-			-		Causes dermatitis. The urine became very dark in colour
13	<i>p</i> -Phenylene-diamine	0.18 0.27	$\pm$ $\pm$			-		Well-known cause of methaemoglobinemia and of bladder tumours. Porphyrinuric action not previously discovered. Urine vol. increased by about 60%, very dark in colour but Hb absent. Nicotinic acid (0.1 g. per rat per day) had no effect on the porphyrinuria
14	Aniline	0.35 0.59	+ +	3 12	139°	+	Spleens engorged; slight increase in stainable iron. One liver contained a little stainable iron	
15	Monomethyl-aniline	0.025 0.125	+ +	3 5.5	133-7°	+	Slight engorgement of spleen, but no abnormal quantity of stainable iron	
16	Dimethylaniline	0.054 0.26	$\pm$ +	1.6 1.8	158°	+	Fairly abundant deposits of brown pigment in spleen and moderate increase in stainable iron	Dimethylaniline can be demethylated <i>in vivo</i> by the dog, cat and rabbit [Horn, 1936, 1, 2], the latter, however, only removing one Me group
17	2:4:6-Trichloro-aniline	0.01 0.054 0.625	- $\pm$ +			-	No increase in stainable iron in either liver, spleen or kidney	Substances dosed dissolved in olive oil. Both <i>o</i> - and <i>p</i> -positions are occupied thereby, preventing oxidation <i>in vivo</i> to an <i>o</i> - or <i>p</i> -iminoquinone
18	Phenetidine	0.044 0.22	+ +	2 3.5	137°	+	Engorgement of spleen but no increase in stainable iron	

19	Benzidine	0.25	++	7.5	153°	+	Engorgement of spleen with fairly abundant deposits of stainable iron. Some also present in liver	Well known to be a cause of cyanosis and intoxication among industrial workers, who inhale the light powdered substance
20	o-Toluidine	0.11	+	3.5		+	Slight deposition of stainable iron in spleen	
21	5-Chloro-o-toluidine	0.15	+	3.5		+	Some vacuolation of liver cells. Spleen engorged and contained much stainable iron	Substance dosed dissolved in olive oil. Causes both cyanosis and dermatitis in man. All animals lost wt.
22	Nitrobenzene	0.125	+	2	146°, 170°	+		Well known to cause cyanosis and "anilism" among industrial workers. The nitro compounds can be reduced <i>in vivo</i> to the corresponding hydroxylamines or amines
23	Methylacetanilide	0.11 0.13	- -			-		Animals exhibited nervous excitability and twitching. The drug differs from acetanilide in that having the amino group completely substituted, it does not cause methaemoglobinæmia
24	Hydroxylamine-hydrochloride	0.075 0.15	++ ++	4.5	145°, 170-1°	+	Liver and kidneys contained brownish black particles in which the iron stain was positive. Spleens engorged, but otherwise normal	The substance is an active hæmolytic agent [de Langen & Grotepass, 1938]
25	Phenylhydroxylamine	0.014	+	3.3		+		Animals markedly cyanosed. This substance or a derivative is postulated as an intermediate in the oxidation of all the amines here investigated (see Discussion)
26	Na nitrite	0.18	-			+	Liver showed engorgement with vascular dilatation. Otherwise organs normal	Administered 10 mg. <i>per os</i> daily and a 0.4% solution in the drinking water. Porphyrin excretion decreased to nearly nil. Urine colour normal. Possibly some degree of anaemia
27	isoAmylamine	0.11	-			-		

figures quoted by various workers for the minimal fatal dose of these sparingly soluble compounds are apt to be misleading. The quantity of drug reported as being tolerated in a single dose may sound impressive, but in all probability the greater part of the material is not absorbed.

(3) *In general, all those substances found to exert a porphyrinuric action are also capable of producing methaemoglobinaemia in vivo and*

(4) *one can therefore define the chemical grouping necessary for increasing porphyrin excretion in the same terms as allotted to the structure giving rise to methaemoglobin formation—viz. the presence of an aromatic amino group, unsubstituted or potentially free and preferably in such a system that it is capable of undergoing oxidation with the formation of a hydroxylamine derivative or a reversibly oxidizing system such as a p- or o-iminoquinone.*

The role of the primary amino group in sulphanilamide in determining the toxicity of this substance has already been suspected [Jennings & Southwell-Sander, 1937], largely as a result of the work of Kracke & Parker [1933–4].

More recently, a striking occurrence reported by Rogers [1938] would appear to support the idea that the amino group is the structural feature, possessed by the sulphonamide drugs in common with certain other substances, which is largely responsible for their specific toxic effects. A woman of 58 was receiving sulphanilamide treatment for erysipelas. Some years previously the local anaesthetic "procain" (*p*-aminobenzoyldiethylaminoethanol hydrochloride) had been administered with evident indications of sensitization. After ingestion of as little as 1.6 g. of sulphanilamide, reactions occurred at all the old sites of injection in a roughly quantitative manner. Patch tests were performed and proved consistently positive to procain but not to sulphanilamide. The author, however, concluded that the feature which these chemical substances exhibited in common was the possession of an aromatic primary amino group. It appears to the writers unfortunate that patch tests were not tried also with phenylhydroxylamine and *p*-aminophenol.

It will be seen, when conclusion no. 4 is examined more carefully with reference to the summarized results in Table I, that not only is there a qualitative correspondence between methaemoglobin production and porphyrinuric action, but that the relationship is also roughly quantitative. Thus aniline, *o*-toluidine and benzidine are well known by experience to cause severe methaemoglobinaemia and they also cause a prompt rise in porphyrin excretion at a relatively low level of dosage. Dimethylaniline, although fully substituted, yet causes a mild degree of methaemoglobinaemia [compare Horn, 1936, 2], and even most carefully purified material was also found to be effective in causing a relatively weak porphyrinuria. *p*-Phenylenediamine, in spite of its tendency to cause dermatitis etc., induces neither methaemoglobin formation nor porphyrinuria. The drug "exalgin" (methylacetanilide) is similarly ineffective, and 2:4:6-trichloroaniline, in which both *p*- and *o*-positions are substituted, is practically ineffective in both respects. The finding with nitrobenzene must also be mentioned. The nitro-compounds are known easily to suffer reduction in the animal body and give rise to methaemoglobinaemia just as do the corresponding amines; in fact the nitro-compounds are perhaps somewhat more dangerous. In the animal experiment it was found by us that so low a dose of nitrobenzene as 0.125 g./kg. brought about a distinct and prompt porphyrinuria. It will be noticed that prontosil is relatively more effective in causing increased porphyrin excretion than is sulphanilamide, when the doses are compared upon the basis of potential equivalence in sulphanilamide content. Prontosil is known to be

transformed, in part at least, into sulphanilamide in the animal body [Fuller, 1937; Jauerneck & Gueffroy, 1937] and could theoretically yield 29.27% of its weight of sulphanilamide.

	Dosage g./kg.	Sulphanilamide equiv. g./kg.	Increase in porphyrin times normal
Prontosil soluble	1.25	0.366	6
	2.1	0.62	13
Sulphanilamide	0.4	0.4	2.5
	0.93	0.93	4

The transformation in the living organism would involve a reduction of the azo linkage giving, in addition, another amino-compound thus:

Since one of the conclusions reached as a result of the present investigation is that aromatic amino compounds are potentially capable of producing increased porphyrin excretion, the greater magnitude of the effect produced by prontosil soluble may be due to the fact that both the sulphanilamide and the naphthylamine derivative produced during its biological fission contribute towards porphyrin excretion and the net result is therefore additive. Attention, however, must also be drawn to the fact that the prontosil was given intramuscularly and the sulphanilamide by mouth.

The discovery of this relationship between methaemoglobin-forming properties and porphyrinuric action would seem to be of considerable significance. Not only does it define the chemical structure in the drug responsible for the effect and thereby throw open the possibility of synthesizing a compound free from this adverse characteristic, but possibly still capable of exerting the required chemotherapeutic effects (attempts in this direction have already been made by Michaux *et al.* [1938] who prepared the Na methylenesulphonate derivative of sulphanilamide, and by McLeod [1938]), but it also serves to associate these two manifestations of the toxic action of the sulphonamide drugs upon the haemopoietic system.

Primary aromatic amines, e.g. aniline, can undergo oxidation in the animal body with the formation of phenolic derivatives such as *p*-aminophenol, a reaction in which the hydroxylamine derivative is probably an intermediate. Thus Young & Wilson [1926] were able to detect *p*-aminophenol in the blood plasma and urine after administration of acetanilide to rabbits, whilst Ellinger [1920] recovered acetylphenylhydroxylamine from the blood of acetanilide-poisoned cats, and Lipschitz [1920] has demonstrated the reduction of *m*-dinitrobenzene to *m*-nitrophenylhydroxylamine by minced animal tissues *in vitro*. Heubner, however, does not consider the hydroxylamine to be the actual methaemoglobin-former, since it is irreversibly oxidized *in vivo* with ease to

azoxybenzene [Heubner *et al.* 1923], and his own studies have shown, in agreement with the work of others, that aniline introduced into the blood stream may bring about the formation of many times its equivalent of methaemoglobin; in fact it appears as if an equilibrium is established when approximately one-third of the total blood pigment has been oxidized. Aminophenol, on the other hand, is known to pass very readily and reversibly into *p*-iminoquinone. This could oxidize one equivalent of haemoglobin into methaemoglobin, becoming reduced in the process, be oxidized again by the tissues and enter into reaction with a further molecule of haemoglobin. It would thus act as a catalyst, converting more and more of the blood pigment into methaemoglobin until an equilibrium was eventually established, or it itself became destroyed. The scheme is illustrated by the following diagram:

Should a similar mechanism be entertained as responsible also for methaemoglobin formation in the case of sulphanilamide and its homologues (as first suggested by Rimington [1939]), then evidence must be adduced that the primary aromatic amino group of these compounds is susceptible to such an *in vivo* oxidation as that postulated. Ottenberg & Fox [1938] have shown that on irradiating dilute aqueous solutions of sulphanilamide with ultra-violet light, a rapid change takes place, the solution becoming first purple-coloured and then yellow-brown. Simultaneously there is a decrease of 30–60% in diazotizable amine, determined by Marshall's method. Ottenberg and Fox did not determine the type of change affecting the amino group (see also Main *et al.* [1938]), but repetition of their experiments has convinced us that oxidation plays a prominent part since (1) no colour change takes place when all air is replaced by nitrogen; (2) aeration during the process of irradiation greatly accelerates and intensifies the reaction; (3) the purple-coloured product is reversibly decolorized by a reducing agent such as sodium hydrosulphite (the yellow-brown is not and would appear to represent a further stage of greater complexity, possibly analogous to the transformation of aniline into aniline black); and lastly, (4) methylene blue may replace oxygen in the system as a hydrogen acceptor. Irradiation of sulphanilamide solution under nitrogen and in presence of dilute methylene blue leads to decoloration of the latter.

From these observations we feel justified in regarding the irradiation phenomenon as one of oxidation in which the amino group is involved.

Levaditi *et al.* [1938], following a similar line of thought, have tested experimentally a variety of substances of the quinonoid type of structure for chemotherapeutic activity. Certain of these, such as quinol, diacetylquinol, *p*-aminophenol etc., had a definite though slight effect in gonococcal and meningococcal infections. Mayer [1938], on the other hand, claims to have demonstrated an antistreptococcal activity in *p*-hydroxylaminobenzenesulphonamide equal to that of sulphanilamide itself. This substance (the simple hydroxylamine corresponding to sulphanilamide) was active *in vitro* as well as *in vivo* and Mayer considers that it is in reality the therapeutic agent formed when sulphanilamide is administered to a patient. His conclusion is supported by the fact that the *p*-nitro-derivative, which would be reducible *in vivo* to the hydroxylamine, was also active.

There would appear to be a considerable amount of evidence, therefore, to support the supposition that the amino group of sulphanilamide may undergo oxidation *in vivo*.

Indisputable evidence as to the removal of the sulphonamide grouping *in vivo* is not as yet forthcoming, although on theoretical grounds such a reaction is not improbable. Were these transformations to take place, all the necessary requirements would be fulfilled for the easy production from these drugs of a catalytic oxidizing system capable of forming considerable quantities of methaemoglobin.

Such far-reaching alterations in the molecular structure may not, however, be essential for the manifestation of this activity if the theories put forward by Schwartzbach & Michaelis [1938] be considered.

These authors have pointed out that certain molecular structures, as exemplified by the indamine and indophenol redox dyestuffs, are capable of electronic rearrangements during a stepwise oxidation, whereby one electron is lost and a partially oxidized or "semiquinone" type of molecule is produced. A resonance system may be set up within the molecule itself, whereby sufficient stability is conferred to allow of the existence of the semiquinone in aqueous solution. Even fully substituted groups, such as the dimethylamino group may participate in this reaction, as illustrated by the simple case of Wurster's blue (see below). Schwartzbach & Michaelis point out that, so far, all the semiquinones studied contain two equal atoms (other than carbon) symmetrically located in the molecule. They are able to formulate electronically the change of a dyestuff like phenolindophenol from the fully reduced form through the intermediate semiquinone stages to the fully oxidized form without recourse to the more usual benzenoid and quinonoid schemes of representation.

When sulphanilamide is similarly represented, as in the accompanying figure, and its electronic configuration examined, it will be seen that it also could be visualized as suffering stepwise oxidation by the loss of an electron. There appears to be no reason why resonance should not occur throughout the structure from nitrogen to nitrogen, which would materially increase the stability of such a semiquinone type of molecule. On the other hand, were the tissue systems with which it was in contact so adjusted that this intermediate could act as a link in an oxidation-reduction chain, then the requirements for its activity as a catalyst would be met. Depicting haemoglobin as the acceptor, catalytic oxidation to methaemoglobin could occur just as in the case of *p*-aminophenol, previously discussed:

The conclusion that oxidation of a primary amino group is responsible for the porphyrinuric action of the sulphonamide drugs, etc., is strongly supported by the finding of Brownlee (personal communication of unpublished results) that a similar increase in urinary porphyrin excretion is caused by another class of pharmaceuticals, namely the antipyretic drugs, phenacetin, phenazone etc., which contain a free or substituted aromatic amino group.

Important though our conclusions concerning the phenomena of methaemoglobin formation and porphyrinuric action appear to us to be for a general understanding of the toxicity of the sulphonamide drugs and their action upon the haemopoietic system, yet we feel that our work would not be complete without some discussion of the possible origin of the extra porphyrin excreted.

Obviously the question which first presents itself is whether excreted coproporphyrin is actually derived from the methaemoglobin of the blood stream. In all instances, the porphyrins isolated after the administration of the drugs we have employed, belonged to the isomeric series III, so that such an origin would be chemically possible. It has, however, become customary to accept the view of haemoglobin degradation put forward by Lemberg [1935] in which the haematin moiety of haemoglobin is pictured as suffering oxidative ring scission whilst the iron and globin are still attached to the molecule and so passing to biliverdin without at any time going through a porphyrin stage. Porphyrins are thus unlikely to arise from the normal breakdown of haemoglobin and there is much physiological evidence to support the correctness of this viewpoint. Recently, however, we have been studying the fate of methaemalbumin formed by injecting haematin into the blood stream of rabbits, man or monkeys and we have evidence that this pigment ultimately appears, in part at least, as extra proto- and copro-porphyrin in the faeces. That haematin injection does not lead to increased bile pigment formation, as does the injection of haemoglobin, has been known for some time [cf. Duesberg, 1933-4]. An increased excretion of coproporphyrin III has also been observed by several workers to occur in certain types of hepatic disease, and in many of these, as reported by Schumm [1927], haematinaemia has been demonstrated.

It would appear, therefore, that not only does there exist the haemoglobin-verdohaemochromogen-biliverdin mechanism of blood pigment degradation, but that, in addition, an alternative route exists by which haematin, the oxidized haem component detached from globin, is metabolized in pathological conditions, at least, but perhaps also normally [compare Thomas, 1938]. It is conceivable that methaemoglobin, if formed in quantity, might follow the same route. Heubner considers that the rate of reconversion of methaemoglobin into haemoglobin *in vivo* is not so great as the earlier experiments of Sakurai [1925] would lead one to suppose.

From histological examination of the organs, and particularly the spleens, of the rats made porphyrinuric by the dosing of sulphanilamide, aniline etc., it is highly probable that an increased red cell destruction has been caused by these drugs. The spleens exhibit a considerable degree of haemosiderosis with dilatation and engorgement [compare Hageman, 1937; Schwartz *et al.* 1938, etc.]. Machella & Higgins [1939] have recently demonstrated the production of severe anaemia by the dosing of sulphanilamide to rats.

Tentatively, therefore, we put forward the view that these drugs, capable of suffering oxidation at the amino group *in vivo*, by causing methaemoglobinaemia and increased disintegration of erythrocytes, bring about an increased excretion of type III porphyrins derived from the broken down blood pigment. What role, if any, impairment of normal liver function may play in this sequence of events

we are at present not able to state with certainty. A severe hepatotoxic action of sulphanilamide has been reported in cases by Garvin [1938] and by Cline [1938].

SUMMARY

The finding, already published, that sulphanilamide, when dosed to rats, causes an increase in the urinary and faecal porphyrin excretion, has been confirmed and amplified by extending the investigation to include other drugs of the sulphonamide series and also simpler related chemical substances. From the results which are now reported in detail, with chemical and pathological findings, the following general conclusions may be drawn.

(1) Within the series of sulphonamide drugs tested, there is no quantitative relation between therapeutic efficiency and porphyrinuric action.

(2) Porphyrinuric action runs roughly parallel with general toxicity.

(3) In general, all those substances found to exert a porphyrinuric action are also capable of producing methaemoglobinaemia *in vivo*.

(4) One can therefore define the chemical grouping necessary for increasing porphyrin excretion in the same terms as allotted to the structure giving rise to methaemoglobin formation—viz. *the presence of an aromatic amino group, unsubstituted or potentially free and preferably situated in such a system that it is capable of undergoing oxidation with the formation of a hydroxylamine derivative or a reversibly oxidizing system such as a p- or o-iminoquinone*.

The last generalization has been discussed in the light of Heubner's work upon the mechanism of methaemoglobin formation following administration of aniline. Both aniline and nitrobenzene (which can suffer reduction *in vivo*) have been shown to be very effective porphyrinuric agents and attention has been drawn to the possible importance of this finding in connexion with the control of the health of industrial workers.

The evidence has been considered which supports the hypothesis that sulphanilamide, etc., may to some extent undergo oxidation at the amino group *in vivo*.

The source of the extra coproporphyrin III excreted as a result of the administration of these substances, has also been considered, and the hypothesis is put forward that it is derived from an increased breakdown of blood pigment. It is suggested that methaemoglobin, when once formed, is degraded, in part at least, by a mechanism which leads ultimately not to bile pigment but to porphyrin. Haematin may possibly represent one of the intermediary stages in this transformation. Further work is in progress designed to elucidate the exact nature of the changes involved.

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APPENDIX

Protocol of experiment with sulphanilamide

Three male rats, av. wt. approximately 200 g., in each group. Groups 36 and 37 and control gained in wt. during the exp., whilst group 38 lost in wt.

	Group 38 (0.93 g./kg.)		Group 36 (1.23 g./kg.)		Group 37 (1.42 g./kg.)		Controls	
	Porph. μg.	Vol. ml.	Porph. μg.	Vol. ml.	Porph. μg.	Vol. ml.	Porph. μg.	Vol. ml.
Pre-period								
i-iii	9.8	40	Trace	50	15.3	52	20.7	23
iv-vi	11.2	62	6	62	5.0	69	30.2	34
vii-ix	10.8	61	13.5	63	10.8	88	29.9	37
x-xii	14.1	66	11.4	53	12.8	66	32.9	31
Days on drug								
1-3	19.1	166	27.2	186	29.9	175	32.7	25
4-6	15.9	97	30.5	182	29.9	131	23.3	27
7-9	46.8	81	35.6	120	40.8	105	30.2	24
10-12	38.6	97	44.0	169	29.1	198	38.7	29
13-15	53.6	82	63.4	169	68.0	124	31.1	29
16-18	45.0	74	64.9	169	62.6	121	—	—

Protocol of experiment with aniline

The average body wt. decreased slightly during the exp.

Group 13. Aniline 0.59 g./kg. per day.

	Urinary porphyrin in μg.		Urine vol. in ml. Group 13
	Group 13 3 young ♂	Controls 3 ♀	
Pre-period (mean)	9	10	73
Days on drug 1-3	110	6	106
4-6	110	7.5	120
7-9	110	7.5	103
10-12	110	7.5	137
13-15	130	7.5	105

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