Summary

1. When ketonic esters, which have a malonic ester residue in the β-position to the carbonyl group, are brominated in methyl alcohol, the only product is a monobromo compound which has bromine α to carbonyl.

2. When the same esters are brominated in chloroform or carbon tetrachloride, the result is a mixture of monobromo derivatives. In one of these the bromine is α to carbonyl, in the other it is in the malonic ester residue.

3. A third monobromo derivative was obtained by first introducing two bromine atoms α to carbonyl and then replacing one of these with hydrogen.

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THE SULFUR CONTENT OF ARSPHENAMINE AND ITS RELATION TO THE MODE OF SYNTHESIS AND THE TOXICITY. I[1]

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In the course of an investigation designed to bring out any existing relation between the sulfur content and the toxicity of arsphenamine, a large number of samples were prepared by hydrosulfite reduction of 3-nitro-4-hydroxyphenylarsonic acid using the same quantities of reagents, but reducing the nitro group under varying conditions.[2] Thus a series of specimens was obtained in which the toxicity, as determined by intravenous injection into white rats, varied from 50 to 150 mg./kg. The results are given in the accompanying graph (Fig. 1).

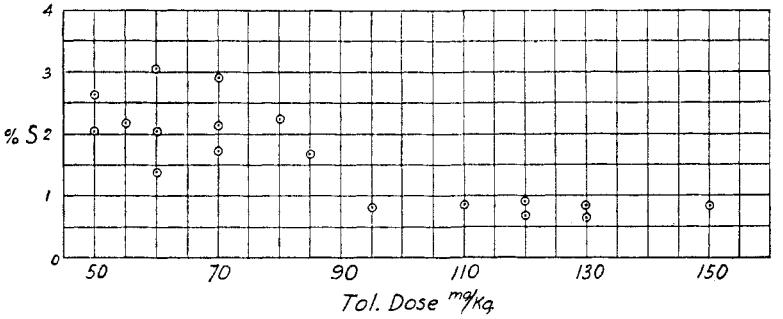


Fig. 1.—Sulfur content and tolerated dose of arsphenamine.

[1] This is the sixth of a series of studies on the properties contributing to the toxicity of arsphenamine being made under a grant from the United States Interdepartmental Social Hygiene Board to the Harvard Medical School; the work is under the general direction of Dr. Reid Hunt, who is also responsible for the biological tests reported in this paper.

[2] The conditions affecting the toxicity were discussed in THIS JOURNAL, **43**, 2202 (1921).

Although there is no direct relation between the toxicity and the total sulfur content, it is evident that the least toxic preparations have less than 1% of sulfur and the most toxic contain 1.5–3.0%. Inasmuch as 7 samples containing 0.66 to 0.94% of sulfur were tolerated in doses varying from 95 to 150 mg./kg., and 5 containing 2.04 to 2.25% of sulfur had tolerated doses varying from 50 to 80, and 3 which were tolerated at 60 mg. contained 1.40, 2.09, and 3.03% of sulfur, it follows that either the toxicity is only partly due to the presence of toxic sulfur compounds or the sulfur can be present in more than one state of combination and affects the toxicity differently when in different combinations. These results also prove that the extent to which sulfur compounds are formed depends upon the manner in which the nitro group is reduced.

This bears a striking resemblance to the relation found between the mode of reduction of the nitro group and the toxicity. Methods were developed for the production of relatively toxic and non-toxic arsphenamine from the nitro acid, and it was proved that the consistent production of the least toxic material required the use of the corresponding pure amino acid as starting material.[2] Upon analysis it was found that the sulfur content is lowest in material obtained from the amino acid,[3] slightly higher when the nitro acid is reduced under the conditions favoring the lowest toxicity, and much higher when the reverse conditions are used (Table I).

TABLE I

THE MODE OF SYNTHESIS, TOXICITY AND SULFUR CONTENT OF ARSPHENAMINE

Arsphenamine prepared by hydrosulfite reduction of	Expt.	Av. tol. dose Mg./kg.	Limit of tol. dose Mg./kg	Av. S content %	Limit of S content %
Amino acid	2	145	140–150	0.43	0.41–0.46
Nitro acid I	3	137	130–150	0.80	0.66–0.87
Nitro acid II	7	62	50–85	2.08	1.40–3.03

Since high toxicity produced in this way is concomitant with high sulfur content the nature of the sulfur compounds present and their relation to the toxicity were taken under consideration.

It is only the sulfur compounds resulting from phenomena occurring during the reduction of the nitro group which may cause the excessive toxicity of these products. Since the mechanism of the reaction, after the nitro group is reduced is the same as in the reduction of amino acid, an amount of sulfur equal to that found in arsphenamine prepared from amino acid will be introduced during this part of the synthesis. Therefore, the sulfur content of arsphenamine prepared from the amino acid may be subtracted from that of material made from the nitro acid and we find that arsphenamine made from the nitro acid contains 0.37 or

[3] This agrees with the observation made by Fargher and Pyman, *J. Chem. Soc.*, **117**, 370 (1920).

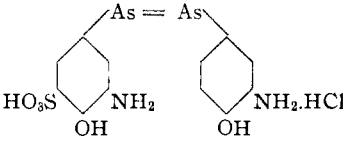
1.65% sulfur which is introduced during the reduction of the nitro group, depending upon the set of conditions used. Evidently when the nitro group is reduced under the most favorable conditions, very little sulfur enters the molecule.

Arsphenamine sulfate is present but can be formed as readily during the reduction of the amino acid as during the reduction of the nitro acid and is probably present in all samples to about the same extent.[4] Moreover, as the sulfate and hydrochloride prepared from the same base have equal toxicities, the formation of this substance could have no effect on the toxicity. Since it is formed after the nitro group has been reduced, it contributes its sulfur to that subtracted from the total sulfur in order to obtain the figure representing the sulfur introduced when the nitro group is reduced.

It has been suggested,[5] but not demonstrated, that sulfur compounds in which the sulfur is attached to the arsenic atom may be present. These substances, if present, would be formed during the reduction of the arsonic acid group, *i. e.*, after the nitro group has been reduced and they should constitute a fairly constant part of the total sulfur in arsphenamine prepared either from the amino acid or from the nitro acid. This, then, is also part of the sulfur subtracted from the total sulfur of arsphenamine made from the nitro acid.

Since arsphenamine prepared by hydrosulfite or hypophosphorous acid reduction of the pure amino acid has the same low toxicity, the sulfur introduced during hydrosulfite reduction can have no bearing upon the toxicity.

In the work of Fargher and Pyman[5] and later of King[6] sulfur compounds present as impurities in arsphenamine are discussed. The former investigators showed that a conc. methyl alcohol solution of arsphenamine, prepared by reduction of the nitro acid, deposited a precipitate which had a high sulfur content. A methyl alcohol solution of arsphenamine prepared from the amino acid deposited very little material on standing. Thus, the compound containing the sulfur introduced during the nitro group reduction could be separated. At first the precipitate was thought to be a sulfamic acid, but King subsequently showed that it was mainly a compound of the formula



I

contami-

[4] Except when the hydrochloride is precipitated from aqueous solution by conc. hydrochloric acid.

[5] Fargher and Pyman, Ref. 3.

[6] King, *J. Chem. Soc.*, **120,** 1107, 1414 (1921).

nated by a little arsphenamine sulfate. It is probable that the small quantities of solid which Fargher and Pyman obtained from solutions of the arsphenamine prepared from the amino acid consisted of arsphenamine sulfate. King states that (I) is the main sulfur impurity in arsphenamine and being more toxic than the latter is an undesirable impurity.

If this sulfonic acid derivative is the main sulfur impurity in arsphenamine, it would be possible, by allowing a conc. methyl alcohol solution to stand, to obtain from the mother liquor, after removal of the precipitate, arsphenamine with a sulfur content nearly as low as that found when the pure amino acid is used as a source of arsphenamine, *i. e.*, 0.43%. The difference between 0.43 and the sulfur found would be due to the solubility of the sulfonic acid. By reducing mixtures of 3-amino-4-hydroxyphenylarsonic acid and its 5-sulfonic acid,[6] arsphenamines of varying sulfur content were prepared in which the main sulfur impurity was necessarily the sulfonic acid (I). A solution of 1 g. in 4 cc. of absolute methyl alcohol was allowed to stand for 20 to 24 hours in the absence of air and moisture and then was centrifuged. The solid was washed once with methyl alcohol; the mother liquor and washing were united and poured into ether to precipitate the material which had remained in the alcoholic solution.

TABLE II

REMOVAL OF THE SULFONIC ACID DERIVATIVE BY FRACTIONATION IN METHYL ALCOHOL

Ratio of amino acid to amino-sulfonic acid used for reduction	Reduction Product		CH_3OH soluble part
	Tol. dose Mg./kg.	% S	% S
4.1 : 1	90	1.75	0.69
4.1 : 1	*ca.* 80	2.10	0.80
8.6 : 1	90	1.50	1.08
8.6 : 1	*ca.* 80	1.23	0.98
12 : 1	110 or above	0.99	0.77
12 : 1	100	1.03	..
17 : 1	*ca.* 90	0.95	0.79
			Av. 0.85

These results (Table II) show that arsphenamine so prepared that the main sulfur impurity is the sulfonic acid derivative will deposit this impurity from a methyl alcohol solution in varying degrees depending upon the degree of contamination and that the material which remains in solution has a fairly constant sulfur content, 0.85%. In the last experiment the precipitation was very slight, and since the original sulfur content, 0.95%, is only slightly higher than the average sulfur of the material recovered from the mother liquor, arsphenamine containing less sulfonic acid than this would not deposit a solid under the conditions used. When methyl

alcohol solutions of arsphenamine prepared from the nitro acid by the two methods previously developed were treated as above, it was found that the low sulfur-containing (0.83, 0.58%), slightly toxic, specimens gave no insoluble matter. This would be expected from the fact that the sulfur content is below that found in the above alcohol-soluble material. However, upon similar examination of the high sulfur-containing, highly toxic samples, a precipitate insoluble in methyl alcohol was obtained, but the sulfur contents were lowered from 2.09, 1.64 and 1.75% to only 1.49, 1.13 and 1.31% respectively. Again, the sulfur content of the alcohol-soluble material is fairly constant, averaging 1.31%, but is considerably higher than that found for specimens to which a known amount of the sulfonic acid had been added. The difference between 0.85 and 1.31, *i. e.*, 0.46, is the average percentage of sulfur in high sulfur-containing arsphenamine which is introduced during the reduction of the nitro group but which cannot be removed from concentrated methyl alcohol solution as sulfonic acid.

In King's work these experiments were carried out using 1 g. of arsphenamine to 3 cc. of methyl alcohol, but in order to facilitate the preparation of the solutions, 4 cc. of alcohol was used in the work here reported. This might introduce a slight variation in the absolute sulfur content but would not affect the difference noted between the two sets of experiments. To determine the extent to which differences in concentration affected the results, 1 g. of arsphenamine produced by reducing a mixture of 1.4 parts of amino acid and 1 part of the sulfonic acid was treated with 4 cc. of methyl alcohol in one case and 10 cc. in another. The original sulfur content was 2.10% and that of the material recovered from the alcoholic mother liquor 0.80 and 1.21% in the respective experiments. Evidently, the difference between using 3 cc. and 4 cc. of methyl alcohol would be very slight.

In the preceding table (II) it appears that reduction of mixtures of the amino acid and its 5-sulfonic acid gives products which are more toxic than arsphenamine obtained from the pure amino acid, but instead of having a direct relation between the amount of sulfonic acid added and the toxicity all the samples have approximately the same tolerated dose. This may indicate that the increased toxicity is not due directly to the presence of the sulfonic acid compound but the presence of the latter substance may cause variations in the properties (colloidal) of arsphenamine, thus acting indirectly; a small quantity present during the synthesis might be sufficient to effect such a change. The amino acid used in the above experiments gave, when reduced by itself, a product of low toxicity.

When the nitro acid is reduced under the most favorable conditions, the arsphenamine has on an average 0.80% of sulfur and a tolerated dose

of 137 mg./kg. indicating that 0.37% of sulfur is introduced during the reduction of the nitro group. Even the presence of only enough sulfonic acid to cause arsphenamine made from the amino acid to have a sulfur content of 0.95% instead of the customary 0.43% results in a decrease in tolerated dose to about 90 mg./kg.; consequently the 0.37% of sulfur introduced with the nitro group cannot have entered the molecule in a sulfonic acid group.

It was proved above that the high sulfur arsphenamines contain on an average 0.46% of sulfur which is introduced during the reduction of the nitro group but which cannot be removed from conc. methyl alcohol solution as sulfonic acid, and it now appears that 0.37% of sulfur is introduced under the most favorable conditions for reduction of the nitro group, does not enter the molecule as sulfonic acid and does not materially alter the toxicity. These figures agree well within the experimental error, and it is entirely probable that this amount of sulfur will enter the molecule in this form no matter how the nitro group is reduced.

It is now possible to state in what form the sulfur is present in arsphenamine, the extent of each form, and its relation to the toxicity if the method of preparation is known (Table III).

TABLE III

DISTRIBUTION OF SULFUR IN ARSPHENAMINE

Source of Arsphenamine	S Content %	Remarks
(a) Pure Amino Acid	0.43	Arsphenamine sulfate and possibly arsenic-sulfur compounds; no effect on toxicity.
(b) Nitro Acid I	0.43	Present as in (a).
	0.37	Introduced as a result of reduction of the nitro group; not present as sulfonic acid group; little effect on toxicity.
	0.80	
(c) Nitro Acid II	0.43	
	0.37–0.46	
	0.80–0.89	Present as in (b).
Total S less above (Av. 0.85) = 1.23 (Av.)		Introduced as a result of reduction of nitro group, removable as sulfonic acid; very closely related to the toxicity.

Therefore, in high sulfur arsphenamine obtained by reduction of the nitro acid approximately 20% of the total sulfur is not a result of the reduction of the nitro group; 20% is a result of this reduction but does not affect the toxicity; while 60% results from this reduction, is closely related to the toxicity and could, if the 5-sulfonic acid derivative of ars-

phenamine were completely insoluble in methyl alcohol, be removed as such.

Fargher and Pyman[5] calculated from chlorine values and neutralization values the amount of sulfur present in arsphenamine as sulfamic acid; this, according to King,[6] is present as sulfonic acid. In 10 commercial samples the average total sulfur was 1.50% (1.26–1.77%) and calculation showed 0.75% (0.27–1.37%) of sulfur as 'sulfamic acid'; therefore their figures show an average of 50% of the total sulfur present as 'sulfamic' acid. This figure agrees well with the average value (60%) calculated above for the sulfur introduced by improper reduction of the nitro group and separable as sulfonic acid.

A comparison of the toxicity which it is possible to obtain by reduction of the nitro acid (62 mg./kg., varying between 50 and 85) with the toxicity of products obtained by reducing mixtures of the amino acid and its sulfonic acid (about 90 mg./kg.), shows that the presence of the sulfonic acid derivative of arsphenamine can account for only part of the toxicity which is found.

The sulfur determinations were made by carbonate-peroxide fusion;[1] after addition of the barium chloride the solution was evaporated to 150–200 cc. and allowed to stand 40–48 hours before filtration. Owing to the low sulfur content and the fact that it was seldom possible to use over 0.3 g. of substance for each analysis, the barium sulfate obtained was always less than 60 mg. and averaged 30 mg. The solution from which this is obtained contains a large quantity of inorganic salts, about 17 g. of sodium chloride being the main one. To determine the effect of this upon the weight of the barium sulfate, weighed amounts of carefully purified potassium sulfate were fused and analyzed by the above procedure. The following 8 determinations (Table IV) were made; to eliminate any personal factor they were made by two investigators.

TABLE IV
ANALYSES OF POTASSIUM SULFATE

K_2SO_4 taken	$BaSO_4$ found	$BaSO_4$ calc.	Diff.
Mg.	Mg.	Mg.	Mg.
6.3	13.5	8.4	5.1
9.2	16.8	12.2	4.6
11.7	20.3	15.7	4.6
16.6	26.6	22.2	4.4
18.8	30.5	25.2	5.3
24.6	37.2	32.9	4.3
27.1	40.4	36.3	4.1
29.9	44.4	40.1	4.3
			4.6

In this method of analysis there is a constant error which causes the barium sulfate found to be 4.6 mg. too high and this correction must be applied.

In preparing arsphenamine intentionally contaminated by sulfonic acid the mixture of the amino acid and its sulfonic acid was reduced by the method used for the pure amino acid. The base after drying *in vacuo* was treated with methyl-alcoholic hydrochloric acid and this solution,

[7] Meyer, "Analyse Organischer Verbindungen," J. Springer, **1909**, p. 223.

except in cases where the amount of sulfonic acid was very high, could be filtered without the sulfonic acid separating. The material was precipitated by ether in the usual way.

Summary

1. A careful study of the mode of synthesis, toxicity and sulfur content shows that arsphenamine prepared (*a*) from the amino acid is the least toxic and has the lowest sulfur content; (*b*) from the nitro acid under the most favorable conditions is slightly more toxic and has a slightly higher sulfur content; (*c*) from the nitro acid under least favorable conditions is much more toxic and has a much higher sulfur content.

2. There is no direct relation between the total sulfur and the toxicity.

3. Only the sulfur in excess of that introduced when the nitro acid is reduced under the most favorable conditions has any great effect upon the toxicity.

4. Only from those preparations made from the nitro acid under the least favorable conditions can the sulfonic acid derivative of arsphenamine described by King be isolated, and the amount which the sulfur content can be lowered by removal of this substance is nearly the same as the excess sulfur introduced by improper reduction of the nitro group.

5. The presence of this sulfonic acid derivative cannot account for the whole of the high toxicity which is obtainable.

I wish to express my appreciation of Dr. Reid Hunt's work in determining the toxicity of the substances used in this paper, and of Mr. Arthur J. Norton's assistance in determining the correction to be applied in the sulfur analyses.

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[CONTRIBUTION FROM THE DEPARTMENT OF PHARMACOLOGY, HARVARD MEDICAL SCHOOL]

THE SULFUR CONTENT OF ARSPHENAMINE AND ITS RELATION TO THE MODE OF SYNTHESIS AND THE TOXICITY. II[1]

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While comparing high sulfur-containing, highly toxic arsphenamine obtained by the hydrosulfite reduction of 3-nitro-4-hydroxyphenylarsonic acid[2] with high sulfur-containing samples[3] prepared from known mixtures

[1] This is the seventh of a series of studies on the properties contributing to the toxicity of arsphenamine being made under a grant from the United States Interdepartmental Social Hygiene Board to the Harvard Medical School; the work is under the general direction of Dr. Reid Hunt who is also responsible for the biological tests reported in this paper.

[2] This type of arsphenamine will be referred to as arsphenamine I; for preparation, see THIS JOURNAL, **43**, 2202 (1921).

[3] Arsphenamine II; for preparation, see preceding paper.