and 12.5 ocular, one observes a fibrillar structure as if the gel were composed of needle crystals of exceedingly small cross section. In fact, the cross section of the fiber is so small as to not be apparent when an oil immersion objective (1.9 mm. fluorite) was used. These fibrillar areas, however, could not be demonstrated to be continuous throughout the gel structure. In certain of our preparations they appeared to be discontinuous and surrounded by an area of approximately equal extent which was optically void. It is possible that the fibers were present in such an area, but we could not demonstrate them with our relatively crude ultramicroscope. Then again the areas in which the fibers could be demonstrated may possibly have been formed by crystal growth following the true gel formation, and may not be essential to the gel structure. Certain of the fibrillar areas apparently grow in opacity and size as the gel ages, serving as nuclei for the crystalline benzoyl-cystine noted earlier. This growth is however to be expected from the Noyes-Nernst rule governing the growth of crystals.

All of the available evidence, however, points to the benzoyl-cystine gel as having a fibrilar structure, the fibrils being exceedingly minute crystals of benzoyl-cystine. The evidence is incomplete, but if 0.2 g. of the non-hydrophilic benzoyl-cystine can form a crystal gel retaining 100 g. of water, such a phenomenon certainly has a profound bearing on the subject of gel formation and gel structure in other systems.

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Received June 10, 1921.

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

THE RELATION BETWEEN THE MODE OF SYNTHESIS AND TOXICITY OF ARSPHENAMINE AND RELATED COMPOUNDS.[1]

BY WALTER G. CHRISTIANSEN.

Received June 21, 1921.

In the oldest and probably the most widely used method of preparing arsphenamine, 3-nitro-4-hydroxy-phenylarsonic acid is reduced by sodium hydrosulfite directly to arsphenamine base which is then converted into the dihydrochloride by solution in methyl-alcoholic hydrochloric acid and precipitated with ether. This procedure has led to wide variations in the toxicity of the product; some preparations kill at doses as low as 60 mg./kg., whereas others do not kill at 130 or 140 mg./kg. Believing that some

[1] This is the fourth 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

condition or set of conditions controlled the toxicity, a detailed investigation of the arsphenamine synthesis was undertaken. The toxicity discussed in this paper is that described under Type II in a paper by Dr. Reid Hunt[2] and is quite different from that due to the presence of 3-amino-4-hydroxyphenyl-arsenious oxide or to the physical nature of the solution.

The nitrohydroxy acid is obtainable by three commercially feasible processes,[3] and it was found that relatively toxic and nontoxic arsphenamine could be obtained from crude or purified nitro acid regardless of the method by which the latter was made. Moreover, the replacement of as much as 5% of the nitro acid by substances which might be present as impurities, *e. g.*, 3,5-dinitro-4-hydroxy-phenylarsonic acid, *o*-nitro-*p*-cresolarsonic acid, or a mixture of 3-nitro- and 5-nitro-2-hydroxy-phenylarsonic acids, caused only slight variations in the toxicity of the resulting arsphenamine. In this connection attention should be called to the fact that the presence of the dinitro acid, as an impurity, would lead to the formation of a tri-amino and not a tetra-amino arseno compound as has been suggested. This is a consequence of the formation of an unsymmetrical arseno compound in preference to two symmetrical ones when a mixture of two different arsonic acids is reduced. Previous investigators[4] have mentioned this, and work done in this laboratory is entirely in agreement with that of the others.[5]

Investigation of methods available for the conversion of the base into the dihydrochloride[6] revealed nothing which caused distinct variations in toxicity. In fact, when a sample of the base was converted half into the dihydrochloride by the methyl-alcohol—ether method and half into the dihydrosulfate by precipitation of the latter from a dilute solution of the base in aqueous hydrochloric acid with dil. sulfuric acid the two substances had identical toxicities. In another experiment, the dihydrochloride was dissolved in methyl alcohol and reprecipitated with ether

direction of Dr. Reid Hunt, who is also responsible for the biological tests reported in this paper. Additional aid has been received from the Committee of the Permanent Charity Fund, Incorp.

[2] Hunt, *J. Am. Med. Assoc.*, **76**, 854 (1921).

[3] For an outline of these syntheses see Lythgoe, *Chem. Age* (N. Y.), **28**, 390 (1920).

[4] Bertheim, *Chem. Ztg.*, **38**, 756 (1914); Karrer, *Ber.*, **49**, 1648 (1916); Fargher, *J. Chem. Soc.*, **118**, 865 (1920).

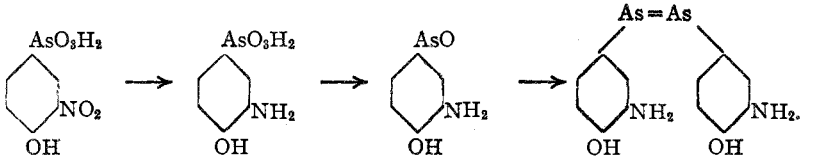
[5] The reduction of equivalent quantities of *p*-hydroxy-phenylarsonic acid and its mononitro or mono-amino derivative gave no acid insoluble arseno compound; the only substance obtained gave a hydrochloride which analyzed for mono-amino-dihydroxy-arsenobenzene hydrochloride. Also, the reduction of equivalent quantities of 3-nitro- or 3-amino-4-methoxy-phenylarsonic acid and 3-amino- or 3,5-dinitro-4-hydroxy-phenylarsonic acid gave no alkali-insoluble arseno compound. These results could be obtained only by the formation of an unsymmetrical molecule.

[6] Ehrlich and Bertheim, *Ber.*, **45**, 756 (1912); Kober, THIS JOURNAL, **41**, 442 (1919).

without any alteration in toxicity. Another portion was converted into the base by treating an aqueous solution with sodium carbonate and the toxicity of the base was equivalent to that of the original hydrochloride.

These results indicate that the toxicity of the arsphenamine is determined during the reduction of the nitrohydroxy acid to arsphenamine base and is not dependent upon the character or source of the nitro compound or upon the conversion of the base into the dihydrochloride.

Examination of the reaction by which arsphenamine base is obtained shows the presence of several known intermediates and the opportunities for undesired side reactions to occur.



At this point in the work the average tolerated dose of a large number of preparations made from the nitro compound by the standard method of reduction was 110 mg./kg.[7] and varied between 70 and 140 mg./kg. When the pure amino acid, from various sources, was reduced under the same set of conditions the arsphenamine consistently had a tolerated dose of 140–150 mg./kg. This figure agrees well with those obtained for arsphenamine prepared from the pure amino acid by other methods[8] and is probably as high as can be attained with any degree of regularity. Hydrosulfite reduction of the pure arsenious oxide gave a product tolerated in doses of approximately 130 mg./kg. It is evident from the wide variations encountered when the nitro acid is the starting material, from the consistency with which an excellent product may be secured by reducing the amino acid by the same method used for the nitro acid, and from the fact that the mechanism of the reaction is the same in both cases after the nitro group has been reduced, that the toxicity of the arsphenamine obtained by hydrosulfite reduction depends upon some variable process in the formation of the amino group. Later, when methods had been developed for the consistent production, from the nitro acid, of arsphenamine with a tolerated dose as low as 50–70 mg./kg., the application of these methods to the reduction of the pure amino acid gave a product with a tolerated dose of approximately 140 mg./kg.

That the toxicity of arsphenamine should be closely bound up with the reduction of the nitro group is not surprising when one considers the many intermediates and by-products encountered in the reduction of nitro compounds. When the quantities of reagents used in this reduction were

[7] The average tolerated dose of the arsphenamine of 6 manufacturers was found by Schamberg, Kolmer, and Raiziss, *Am. J. Med. Sci.*, **160**, 188 (1920), to be 105 mg./kg.

[8] Christiansen, THIS JOURNAL, **42**, 2402 (1920); **43**, 370 (1921).

varied no alterations were found in the toxicity of the resulting arsphenamine, but as soon as variations were made in the conditions under which the reaction took place marked variations in toxicity were obtained. Ultimately it was determined that the important factors are the degree to which high local concentrations of the nitro compound momentarily exist at the start of the reduction, the temperature at which the nitro group is reduced, and the speed at which the solution, after the reduction of the nitro group, is raised to 55°. When this point in the reaction is reached, the toxicity of the arsphenamine which will be produced has been settled. The procedure best suited for the development of low toxicity is very vigorous mechanical stirring while a cold solution of the nitro compound is slowly added to a cold solution of magnesium chloride and sodium hydrosulfite followed by rapid heating to 55°. Obviously the reverse conditions tend to develop high toxicity. That the degree of agitation affects the toxicity only at the time of reduction of the nitro group is shown by the fact that as soon as the color of the nitro group has been destroyed the rate of stirring can be increased to that used for the preparation of arsphenamine of low toxicity without decreasing the toxicity of the material.

The influence of each of these conditions is shown in the following table. When nothing is specified it indicates that the importance of the condition had not been discovered and was only approximately constant in successive experiments. All experiments recorded here were conducted identically after 55° had been reached.

TABLE I.

INFLUENCE OF CONDITIONS OF PREPARATION UPON TOXICITY.

Rate of stirring.	Rate of addition of nitro solution.	Temp. of solutions. °C.	Rate of heating.	Expt.	Average tol. dose. Mg./kg.	Limit of tol. dose. Mg./kg.	Average as content. %
R[a]	..	..	..	10	110	100–120	30.27
S[b]	..	..	..	3	73	50–100	30.25
R	S	..	..	6	115	100–130	30.99
S	R	..	..	6	73	60–100	30.60
R	S	10	..	9	118	110–130[c]	30.96
S	R	30	..	9	79	60–90[c]	29.82
R	S	10	R	5	134	130–150[d]	31.15
S	R	30	S	2	55	50–60[d]	29.83

[a] R = Rapid.

[b] S = Slow.

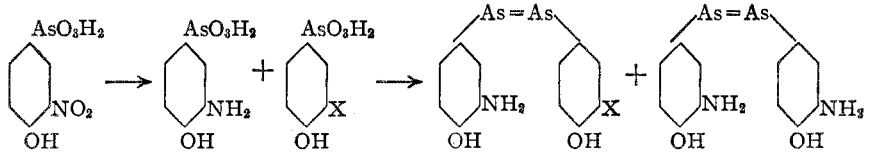
[c] This series includes preparations made: (a) from crude and recrystallized nitro acid made from phenol; (b) from recrystallized nitro acid made from aniline by the oxalyl method; (c) with different commercial brands of hydrosulfite; (d) by different methods of conversion of the base into the dihydrochloride.

[d] This series includes preparations made from crude nitro acid made from phenol and from aniline by the oxalyl method.

Since it has been established that the crucial part of the synthesis lies in the reduction of the nitro group, preliminary work has been done toward ascertaining what undesired secondary process takes place during this reaction. The nitro acid was reduced with hydrosulfite between $-10°$ and 0, in order to slow down this very exothermic reaction and produce conditions similar to prolonged high local concentrations of the nitro compound in the presence of hydrosulfite. The crude amino acid, after isolation, was further reduced with hydrosulfite and yielded arsphenamine tolerated in doses of 120–130 mg./kg. However, when a small quantity of pure amino acid was added to the mother liquor from the crude amino acid separated above and the solution reduced with hydrosulfite, the arsphenamine obtained had a tolerated dose of about 53 mg./kg.; this is the average of 11 experiments in which the tolerated dose varied from 30 to 70. It is evident that the secondary reduction product remains for the most part in the solution when the crude amino acid is isolated. The small quantity of pure amino acid was added to the mother liquor in order that the secondary reduction product may unite, upon reduction, with a molecule of pure amino acid to form the unsymmetrical arseno compound since this is what must happen in the formation of arsphenamine where the ratio of the amino acid to the secondary product is large.

A number of experiments have been made with this solution apparently containing the secondary reduction product and work is now in progress toward the determination of the nature of this product.

The following explanation for the variations found in the toxicity of arsphenamine is suggested.



(I). (II). (III).

The toxicity of arsphenamine depends upon the quantity of (III) present, which in turn depends upon the amount of (II) formed at the beginning of the reaction and this is very variable. By regulation of the conditions as discussed above the formation of (II) can be accelerated or retarded but apparently not always entirely inhibited because the average of the best preparations from the nitro acid is below that of preparations from the amino acid. The success of the arsphenamine synthesis is due to the fact that (I) is the major and (II) the minor reduction product.

In order to verify this explanation and to see whether it could be applied to substances related to arsphenamine, mono-, tri-, and tetra- amino dihydroxy-arsenobenzenes were prepared from the nitro and amino acids.

In each case the products prepared by reduction of the amino aryl arsonic acids were less toxic than those prepared from the analogous nitro acids. Interesting biological observations on the effect of increasing the number of amino groups in the hydroxy-arsenobenzene have been made by Drs. Reid Hunt and G. P. Grabfield.[9]

In general the amino arseno compounds vary in toxicity according to the methods of preparation and are obtained in the purest form by reduction of amino arsonic acids. When the nitro acids serve as the starting material more toxic products result owing to the formation of secondary reduction products during the reduction of the nitro group.

The toxicity determinations were made by intravenous injection into white rats and the arsenic determinations by a modification of Lehmann's method.

Fargher and Pyman,[10] in a paper on the "Composition of Salvarsan," showed that arsphenamine contains two molecules of water of crystallization and small variable amounts of occluded methyl alcohol. In this laboratory 4 carefully prepared samples of arsphenamine base were found to contain the following percentages of arsenic: 37.33, 38.68, 37.78, 38.96; and one sample prepared by precipitation of an aqueous solution of the dihydrochloride with sodium carbonate contained 37.39%. The calculated percentage for $C_{12}H_{12}O_2N_2As_2$ is 40.98; for 1 H_2O, 39.06; for 2 H_2O, 37.32. Apparently water in variable amounts is firmly attached to the base when the latter forms and is not lost during the drying at room temperature or conversion into the dihydrochloride. If some phenomenon like this did not occur it would be hard to understand how a seemingly dry product when dissolved in anhydrous alcoholic solutions and precipitated with ether could obtain water of crystallization.

In the cases reported in this paper of compounds very closely related to arsphenamine the calculated and actual arsenic contents agree well if it is assumed that these substances also have 2 molecules of water of crystallization.

Experimental.

Reduction of 3-Nitro-4-hydroxy-phenylarsonic Acid to Arsphenamine.

A. To a solution of 5.3 g. of magnesium chloride hexahydrate in 130 cc. of water at 8°, 30 g. of sodium hydrosulfite is added and a cold (8°) solution of 2 g. of the nitro acid in 46 cc. of water containing 0.66 g. of sodium hydroxide is added gradually during 1–1.5 min. with very vigorous mechanical stirring. One g. of Superfiltchar[11] is added and the mixture is heated to 40° as rapidly as possible and then filtered.[12] The

[9] These observations will be published in a later paper.

[10] Fargher and Pyman, *J. Chem. Soc.*, **117**, 370 (1920).

[11] A high grade of vegetable decolorizing carbon, a supply of which was placed at our disposal by the Industrial Chemical Co., N. Y.

[12] Filtering at this point as suggested by Kober, Ref. 6, is of great assistance since it renders the subsequent conversion of the base into the hydrochloride much easier. The carbon helps to remove the more finely divided insoluble matter which often tends to pass through the filter but does not seem to have any bearing upon the toxicity of the arsphenamine.

clear colorless filtrate is rapidly heated with stirring to 55° and maintained at 55–60° for 90 minutes. The base is removed by filtration and, after washing and drying *in vacuo* over caustic soda, is converted into the dihydrochloride by dissolving in 7.5 cc., of absolute methyl alcohol containing the calculated amount of dry hydrogen chloride filtering and pouring into 10 volumes of ether[13] cooled to 0–5°. The precipitate is washed with ether and dried *in vacuo* over caustic soda. Yield, 1.2 g. of yellow powder easily soluble in water. Five experiments gave a product which had an average tolerated dose of 130 mg./kg. and arsenic content of 31.15%; that calculated for $C_{12}H_{14}O_2N_2Cl_2As_2.2H_2O$ is 31.58%.

B. The quantities are the same as in A., but the solutions are brought to 30° before mixing, the stirring is very slow when the hydrosulfite and nitro solution are added, and the latter is added in 5 seconds. As soon as the color due to the nitro group disappears the rate of stirring is increased to that used in A and as soon as the hydrosulfite has completely dissolved the material is filtered.[14] The filtrate is heated to 55° during 40 minutes with vigorous stirring and maintained at 55–60° for 1.5 hours. The remaining part of the experiment is like A. The product of 2 experiments gave an average tolerated dose of 55 mg./kg., and arsenic content of 29.82%.

Reduction of 3-Amino-4-hydroxy-phenylarsonic Acid.

In this process there is no particular need of careful attention to the details at the beginning as no reaction occurs until the solution has become hot.

To a solution of 5.3 g. of magnesium chloride hexahydrate in 130 cc. of water, 25 g. of sodium hydrosulfite is added with stirring and then a solution of 1.8 g. of the pure amino acid in 40 cc. of water and 5 cc. of saturated sodium carbonate[15] solution. The solution is heated to 30°, filtered, and the experiment is completed as in the other cases. Yield, 1.46 g. of yellow powder easily soluble in water. Four experiments gave a product having an average tolerated dose of 140–150 mg./kg. and arsenic content of 31.55%.

Reduction of the Nitro Acid to the Amino Acid.

A solution of 2 g. of the nitro-hydroxy-phenylarsonic acid in 15 cc. of water and 1.5 cc. of sodium hydroxide (sp. gr. 1.295) is cooled in a freezing mixture (−16°) with rapid stirring. At about −2° the sodium salt of the nitro acid separates as fine crystals, with the evolution of heat. When the mush is at about −3°, 5 g. of hydrosulfite is added, the heat generated is partly absorbed as the nitro compound redissolves and if the freezing mixture is efficient the temperature can be kept below 0°. After the color of the nitro group has disappeared the temperature falls to −10°, and the amino acid starts to separate. At the end of ½ hour 0.6 cc. of glacial acetic acid is added and the material is left in the ice box for 1 hour. After filtration, washing, and air-drying, 1.2 g. of the crude amino acid is obtained.

The mother liquor is treated with 1.5 cc. of sodium hydroxide, and a solution of 0.5 g. of pure amino acid in 10 cc. of water and 1.5 cc. of saturated sodium carbonate solution is added. This solution is added to one of 2.6 g. of magnesium chloride hexahydrate in 20 cc. of water to which 11 g. of hydrosulfite has been added. The reduction and conversion of the base into the dihydrochloride is carried out as in the case of the pure amino acid. The average of the products from 9 experiments is 0.2 g. of brownish-yellow powder which dissolves very readily in water and is tolerated in doses of 53 mg./kg.

[13] Absolute ether is unnecessary.

[14] Carbon is not necessary as the fine particles coagulate rapidly at this temperature and are easily filtered out.

[15] Sodium carbonate is used in place of hydroxide as the aminohydroxy acid oxidizes much less rapidly in the carbonate solution.

Reduction of the crude amino acid by the method used for the pure acid gives 1.3 g. of arsphenamine with a tolerated dose of 120 mg./kg. or above and an arsenic content of 31.05%.

3-Amino-4,4'-dihydroxy-arsenobenzene Hydrochloride.

I. A mixture of 0.9 g. of anhydrous monosodium *p*-hydroxy-phenylarsonate and 0.9 g. of 3-amino-4-hydroxy-phenylarsonic acid is reduced by the method used in preparing arsphenamine from the amino acid. One and one-tenth g. of base is obtained which is completely soluble in acids. When a filtered methyl-alcoholic solution of the hydrochloride is poured into ether an oily substance separates which after decantation of the ether is dissolved in water and precipitated by pouring into 1:1 hydrochloric acid. After filtering, washing with acid and drying *in vacuo* over caustic soda, 0.7 g. of reddish-orange powder is secured which is soluble in water and is tolerated in doses above 130 mg./kg.

Analysis. Calc. for $C_{12}H_{12}O_2NClAs_2.2H_2O$: As, 35.43. Found: 35.39.

II. A mixture of 0.9 g. of monosodium *p*-hydroxy-phenylarsonate and 1 g. of 3-nitro-4-hydroxy-phenylarsonic acid is reduced by Method A used to prepare arsphenamine from the nitro acid but without using carbon, without particularly rapid heating to 55°, and filtering at 30° instead of 40°.[16] One g. of tan colored base is obtained. By dissolving it in 30 cc. of water and 0.3 cc. hydrochloric acid (sp. gr. 1.19) and pouring the filtered and cooled solution into 60 cc. of cold 1:1 hydrochloric acid the hydrochloride is precipitated. Continuing as in Procedure I one obtains 0.9 g. of reddish-orange solid, the tolerated dose of which is 120 mg./kg., and whose arsenic content is 36.12%.

III. The mixture of nitrohydroxy and hydroxy acids is reduced by Method B for the preparation of arsphenamine from the nitro acid but the filtered solution is not heated particularly slowly to 55°. The hydrochloride is obtained as above. Yield: 0.8 g. of reddish-orange solid which is tolerated in doses of 80 mg./kg. and has an arsenic content of 35.98%.

In each of the following experiments the arseno base is converted into the hydrochloride by the methyl alcohol-ether method used for arsphenamine.

3,5,3'-Tri-amino-4,4'-dihydroxy-arsenobenzene Trihydrochloride.

I. A mixture of 0.9 g. of 3-amino-4-hydroxy-phenylarsonic acid and 0.95 g. of 3,5-diamino-4-hydroxy-phenylarsonic acid is reduced by the method used to reduce the mono-amino acid to arsphenamine. Yield, 1.5 g. of yellow powder which is very readily soluble in water and is tolerated in doses of 80 mg./kg.

Analysis. Calc. for $C_{12}H_{16}O_2N_3Cl_3As_2.2H_2O$: As, 28.49. Found: 28.68.

II. A mixture of 1 g. of 3-nitro-4-hydroxy-phenylarsonic acid and 1.17 g. of 3,5-dinitro-4-hydroxy-phenylarsonic acid is reduced as in Preparation II of the mono-amino dihydroxy-arsenobenzene. Yield, 1.2 g. having a tolerated dose of 80 mg./kg. and an arsenic content of 28.23%.

III. The above mixture of mono- and dinitro acids is reduced as in Preparation III of the mono-amino-dihydroxy-arsenobenzene. Yield, 1.15 g.; tolerated dose, 60 mg./kg.; arsenic content, 28.70%.

3,3',5,5'-Tetra-amino-4,4'-dihydroxy-arsenobenzene Tetrahydrochloride.

In the following experiments only that portion of the base soluble in methyl alcoholic hydrochloric acid[17] was used.

[16] This work was done before the influence of the rate of heating had been investigated.

[17] Raiziss and Gavron, THIS JOURNAL, 43, 582 (1921).

I. One and nine-tenths g. of 3,5-diamino-4-hydroxy-phenylarsonic acid is reduced by the method used in reducing the mono-amino acid to arsphenamine. Yield, 0.6 g. yellow powder which is very easily soluble in water and which is tolerated in doses of 70 mg./kg.

Analysis. Calc. for $C_{12}H_{18}O_2N_4Cl_4As_2.1H_2O$: As, 26.78; calc. for $2H_2O$: 25.95. Found: 27.29.

II. Two and three-tenths g. of 3,5-dinitro-4-hydroxy-phenylarsonic acid is reduced as in Preparation II of the mono-amino analog. Yield, 0.4 g.; tolerated dose, questionable at 50 mg./kg.; arsenic content, 25.55%.

III. The dinitro acid is reduced as in Preparation III of the mono-amino analog. Yield, 0.4 g.; tolerated dose, 50 mg./kg.; arsenic content, 26.10%.

Summary.

1. It has been proved that the toxicity of arsphenamine prepared by hydrosulfite reduction of 3-nitro-4-hydroxy-phenylarsonic acid is determined during the reduction of the nitro group.

2. By altering the conditions under which the nitro group is reduced the toxicity of the arsphenamine can be made to fluctuate between wide limits. Two sets of conditions have been determined which enable one to produce arsphenamine of widely different toxicity; one set results in a tolerated dose of 130 mg./kg. and the other of 55 mg./kg.

3. For the consistent production of arsphenamine of the lowest toxicity it is advisable to use pure 3-amino-4-hydroxy-phenylarsonic acid as the starting material.

4. It appears to be general that the amino aryl arseno compounds vary in toxicity with the method of preparation and are obtained in the least toxic condition by the reduction of amino arsonic acids. Due to the formation of by-products during the reduction of the nitro group, more toxic products result if the nitro acids serve as the starting material. These secondary reduction products unite with the amino acid in the subsequent reduction of the arsonic acid group to form unsymmetrical arseno compounds.

I take this opportunity to thank Dr. Reid Hunt for his kindness in determining the toxicity of the large number of samples involved in this work, and Miss Helen A. Pratt for her careful work in preparing some of the intermediates used.

BOSTON, MASSACHUSETTS.