THE SYNTHESIS, PROPERTIES AND CARCINOGENICITY OF 4,4'-BIS(p-DIMETHYLAMINOPHENYLAZO)PHENYLDISULFIDE

by

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INTRODUCTION

Interest in the cancer inducing ability of specific chemicals has increased greatly in recent years. The carcinogenicity of two classes of compounds has long been known. Certain azo dyes and certain polycyclic hydrocarbons are known to induce cancerous growth in certain tissues of experimental animals (8, 9). Cancer incidence can occur through external contact with the carcinogen and by ingestion of the carcinogen. Experimental azo dye carcinogenisis can generally be produced by feeding the tumor inducing factor to rats. Positive results appear as hepatomas.

The correlation between molecular structure and carcinogenicity has not been elucidated completely. To gain further insight into this problem, it was proposed to synthesize a compound, that by structural analogies to known carcinogens, might be able to induce cancer. Such a compound might also have a role in the protein-dye binding processes, which normally preceeds tumor formation, that is somewhat different from other carcinogens.

Such a molecule would incorporate a known carcinogenic radical and also a group that could in some way associate the entire molecule with the site where malignant growth should begin. Since growth is associated with mitosis, the cell proteins would seem to be the place to coordinate such factors. Organic disulfide compounds are known to associate with proteins. Hence it would be desirable for the carcinogen to contain a disulfide linkage (-S-S-).

The chemistry of disulfides has revealed that there is an interaction between disulfides and mercaptans. Mercaptans are easily oxidised to disulfides and many disulfides may be reduced to mercaptans. Mercaptans may reduce disulfides since the former may act as a hydrogen donor to the latter.

Such an interaction is here represented.

$$2\ R\text{-}S\text{-}H + R'\text{-}S\text{-}S\text{-}R' \rightleftharpoons R\text{-}S\text{-}S\text{-}R + 2\ R'\text{-}S\text{-}H$$

The position of the equilibrium in this exchange depends primarily on the relative stability of the products and any external redox potential.

This disproportionation reaction occurs widely in protein chemistry. The cystine - cysteine system is a good example. Indeed extraneous disulfides may enter the equilibrium. The "permanent" wave given to hair is due to the formation of new disulfide linkages in the protein fiber. Since the surface of the protein molecule may be regarded as providing a wealth of thiol groups and some disulfide groups, it seemed reasonable to assume that perhaps most any disulfide could be more or less chemically bound to a protein surface by such an interaction. Such "binding" of a foreign material might occur in vivo during detoxification in the liver. Hence the possibility of hepatoma.

$$R\text{-}S\text{-}S\text{-}R + H\text{-}S\text{-}Protein \rightleftharpoons R\text{-}S\text{-}S\text{-}Protein + R\text{-}S\text{-}H$$

$$R\text{-}S\text{-}H + Protein\text{-}S\text{-}S\text{-}Protein \rightleftharpoons R\text{-}S\text{-}S\text{-}Protein + Protein\text{-}S\text{-}H$$

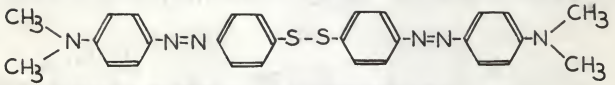
If the R group were cacrcinogenic, a compound containing both functions could provide a high incidence of tumor inducing potential at the site where malignant growth might start. However, carcinogenicity is probably not the mere juxtaposition of groups that bear the proper relation. It is undoubtedly more complex in nature.

There are other factors complicating this theoretical consideration. First such a compound would <u>not necessarily</u> have carcinogenic effects. Nor would it necessarily have the ability to associate with proteins to the extent postulated. It is not necessarily true that a chemical entity will have properties that are closely analogous to the summation of the properties of its component parts.

The nature of the binding between protein and dyes is not known completely. Association complexes and adsorption phenomena generally account for the binding

between protein and ordinary dyes. A dye with disulfide linkages should be bound chemically, thru covalent bonds, more than some other systems.

In view of these considerations, it was proposed to synthesize such a dye and observe the effects of feeding this dye to rats. Azo dyes such as p-dimethylaminoazobenzene, DAB, and m'-methyl-p-dimethylaminoazobenzene, m'-ME DAB, are known carcinogens. If the cancer inducing moiety of R-S-S-R were to be the DAB group, then such a compound would have this formula.



4,4'-bis-(p-dimethylaminophenylazo)phenyldisulfide (DS)

The synthesis of this compound and the study of its properties comprise the topic of this dissertation. This compound DS had not been prepared previously and the literature afforded few examples of azo disulfide compounds (11, 12, 18). Generally disulfides are formed by the oxidation of mercaptans or by the reduction of sulfonic and sulfinic acids. The azo linkage is susceptible to such processes and must be excluded when such reactions are performed. The synthesis therefore became somewhat complex. The DS was synthesized by coupling dimethylaniline to the appropriate tetrazonium compound in which the disulfide linkage had already been formed.

Disulfides in general may be cleaved to mercaptides or mercaptans. The cleavage of DS seemed interesting since the p'-sodium mercaptide of p-dimethylaminoazobenzene is the thiolate analog of methyl orange.

On the other hand, oxidative scission of DS should form a sulfinic acid. This compound, p-dimethylaminoazobenzene-p'-sulfinic acid, is the primary reduction product of the free acid of methyl orange.

EXPERIMENTAL

4,4'-Bis(p-dimethylaminophenylazo)phenyldisulfide

Preparation of Intermediates. The compounds that are intermediary in the synthesis of DS begin with a sulfur derivative that can be reduced to the disulfide. Such a molecule is p-acetaminobenzenesulfonyl chloride. This compound can be reduced to the desired disulfide, 4,4'-bis(acetamino)phenyl-disulfide. The amide may be easily hydrolysed to the amine. Tetrazotization of 4,4'-diaminophenyldisulfide will give the tetrazonium salt that can be coupled with dimethylaniline to form DS.

Preparation of p-acetaminobenzenesulfonyl chloride (17). One hundred and fifty ml. (265 gm.) of chlorosulfonic acid was maintained at 12-15°C. in a 500 ml. boiling flask fitted with a mechanical stirrer. Powdered acetanilide (60 gm) was added slowly with stirring over a period of thirty minutes. The temperature was not allowed to rise. The acetanilide dissolved slowly with evolution of hydrogen chloride as the reaction proceeded (HOOD). After all the acetanilide was added, the reaction mixture was allowed to warm slowly to room temperature. The flask was then warmed to 60 degrees and maintained until the evolution of hydrogen chloride ceased. The syrupy liquid was poured onto a liter of cracked ice and water. A white solid separated which was collected on a Büchner funnel and washed three times with water. The pasty material was allowed to air dry for 24 hours. The crude product (86 gm.) melted at 146-50° C. The yield was 82 percent of theory. The crude material may be recrystallized from benzene in small amounts. The crude product was used in the subsequent reactions.

Preparation of 4,4'-diaminophenyldisulfide (2). Ten grams of crude p-acetaminobenzenesulfonyl chloride was dissolved in 100 ml. of glacial acetic

acid. Fifty ml. of hydroiodic acid (48%) was then added. The solution darkened rapidily as iodine was liberated. The reaction mixture, upon standing over night with occasional shaking, gave a dark brown precipitate. The mixture was then placed in a large beaker and 350 ml. of 10 percent sodium thiosulfate were added. This removed the iodine and left a brown precipitate. Sixty gm. of sodium carbonate was then stirred in cautiously. Then 10 percent sodium hydroxide solution was added until the solution was alkaline to litmus paper. The brown-yellow material was collected on a funnel and washed with water. The crude product (6 gm., 90% yield) is bis(p-acetamino)phenyldisulfide. (M.P. 183-6°C.).

Hydrolysis of this amide to the amine was accomplished by refluxing the crude material with 40 ml. of ethyl alcohol and 60 ml. of concentrated hydrochloric acid for two hours. The hot solution was placed in a beaker and 40 ml. of concentrated hydrochloric acid were added. The resulting white precipitate was collected after cooling and washed with 1:1 hydrochloric acid. This crude dihydrochloride was recrystallized from 60 ml. of 1:1-hydrochloric acid containing sufficient ethyl alcohol to effect solution at the boiling point. This solution precipitated a fine network of white crystals upon cooling. The crystals were collected and dried in air. The dried needles (5 gm., overall yield 75%) had a melting point of 228-30°C. with slight decomposition. The free amine is precipitated from aqueous solution by alkali. Recrystallization of this yellow material from alcohol-water mixtures gave cream colored needles (M.P. 74-6°C.). However for the subsequent tetrazotization reaction, the amine hydrochloride is desirable and therefore the material was kept as the dihydrochloride salt.

Coupling of Dimethyaniline with 4,4'-Diamino-phenyldisulfide. The tetrazotization of the diamine to 4,4'-phenyldisulfidetetrazonium sulfate was accomplished with nitrosylsulfuric acid.(7)Sodium nitrite (0.69 gm.) was powdered

and dissolved by stirring slowly into 5 ml. of concentrated sulfuric acid. The temperature was maintained at zero degrees to avoid decomposition. The mixture was then slowly warmed to 60 degrees to effect complete solution without excessive decomposition. This solution of nitrosylsulfuric acid was cooled to zero degrees with crystals separating.

4,4'-Diaminophenyldisulfide dihydrochloride (1.61 gm.) was stirred into 7 ml. of concentrated sulfuric acid. The solution was cooled to zero degrees. This tended to solidify the material. The suspension of the amine salt was then added to the solution of nitrosylsulfuric. The temperature was maintained at less than zero degrees. Vigorous stirring and rapid addition minimized decomposition. The heat of the tetrazotization reaction is considerable. The addition was as rapid as the maintenance of the low temperature would allow and was completed in fifteen minutes. The resultant solution of the tetrazonium salt decomposed upon standing and exposure to air and was used in the coupling reaction as soon as possible.

Coupling of the tetrazonium compound was done by adding the cold sulfuric acid solution of the tetrazonium salt to a cold solution (5 degrees or less) of dimethylaniniline (1.21 gm.) in 200 ml. of 50 percent alcohol containing 60 gm. of sodium acetate as a buffer. Other buffers can complicate the reaction (7). The addition was dropwise with very vigorous stirring. Finely chipped ice was added to maintain the reaction mixture at five degrees or less. The pH_1 was followed with indicator paper. More sodium acetate buffer was added to maintain a pH of not less than five. The reaction mixture became very viscous due to the precipitation of inorganic salts. Alcohol was added to maintain an easily stirred mixture. The coupling occurred and precipitated an orange-red material. The final volume was approximately 600 ml. The coupling was accomplished in thirty minutes. Stirring was continued for thirty more minutes. One half

a gram of urea was added to decompose any excess tetrazonium salt. The mixture was filtered and washed four times with water to remove inorganic salts. The reaction liquor and washings were only slightly colored and were discarded. The reaction gave 2.1 gm. (82% yield) of crude product.

DS was purified by recrystallization from boiling pyridine with enough water added to effect a cloud point. A second recrystallization was effected from pyridine without the water by using more concentrated solutions. This product was then washed with ether till the odor of pyridine disappeared. DS was then dried in an oven at 115 degrees for 12 hours. The material was then washed with more ether by shaking and filtering. The DS was then dried in a vacuumn over sulfuric acid for twelve hours. DS is only slightly soluble in ordinary organic solvents. It dissolves fairly readily in hot ethylene chloride. This disulfide (DS) was recrystallized from ethylene chloride giving fine red needles with a melting point of 197-9 degrees Centigrade. This purified material was incorporated into the basal ration of the rats. An analytical sample was prepared by recrystallizing DS from ethylene chloride four different times. The initial crop of crystals were the only ones carried into the next recrystallization process. The melting point of pure DS was 198-199.5 degrees. The recovery of material though the processes including the first ethylene chloride recrystallization was about seventy percent.

Properties. Carcinogenicity. It is necessary to use rats that have been inbred to be susceptible to carcinogens for this experimental work. The cancer inducing ability of DS was determined by incorporating the dye at 0.06 percent in the basal ration fed to the male, Sprague-Dawley, albino rats and later inspecting the liver of each rat. The basal ration was essentially the

same as that used by the Wisconsin group (15), and has the following composition.

Casein	12%
Glucose	79%
Corn Oil (Mazola)	5%
Salts Mixture (15)	4%

The following supplements were added per kilogram of ration.

Thiamine chloride	3.0 mg.
Riboflavin	2.0 mg.
Pyridoxine hydrochloride	2.5 mg.
Calcium Pantothenate	7.0 mg.
Choline chloride	30.0 mg.

The dye was dissolved in the corn oil of the ration. Each rat was administered one drop of Halibut Liver Oil each month.

The rats were housed in individual cages made entirely of galvanized screen. The ration and water were fed "ad libitum" except for a short period near the sixth week. A temporary shortage of the ration forced a cutback in the amount of ration fed. A group of control rats were maintained on the basal ration without the DS.

The average weight of the rats at the beginning of the experiment was 203 grams. The rats gained weight rapidly from the start to reach an average weight of 305 grams after 40 days, and 325 grams after 12 weeks. The average weight of the control rats at the beginning was 202 grams and increased to 236 grams after 40 days. The average weight after 12 weeks was 261 grams. Both of these weight gains are well above the values observed for a known carcinogen m'-ME DAB. The growth rates can be compared by reference to Figure 1.

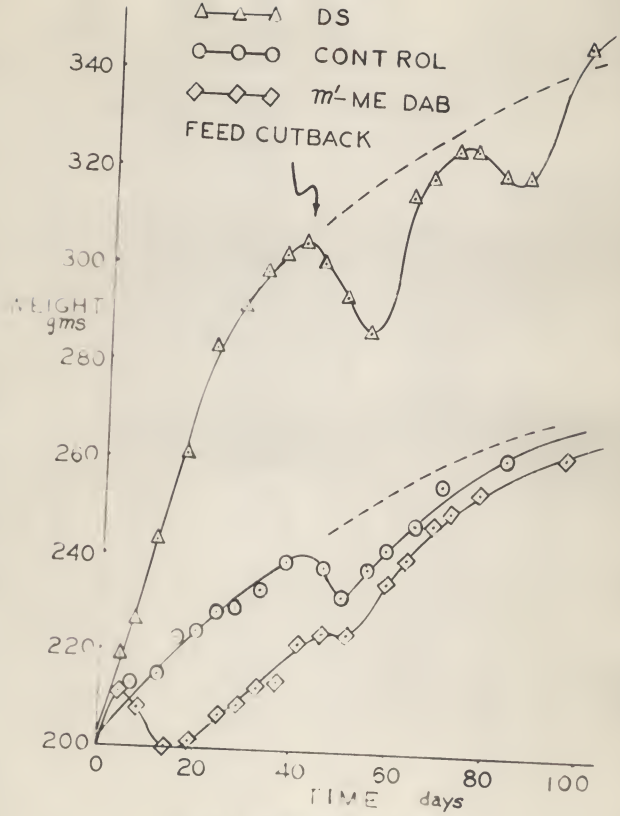


Figure 1. Comparative Growth Rates

The rats fed the diet containing DS appeared normal throughout the experimental period except for their large gain in weight. The rats were active and aggressive. No disorders or abnormalities, except the large structure of the animal, were detected by visual inspection at any time.

Visual inspection of the liver of each animal revealed no carcinoma. The livers appeared normal and healthy. The livers were larger than the controls but were in proportion to the body weight. The color was the ordinary dull red. The surface of the livers had a grained texture that was not seen, to the same extent, in the control rats. The grained texture was very slight and in no way indicated any liver damage. There was no indication of cirrhosis of the liver. Inspection of the other organs and tissues revealed no abnormalities. In general, the organs and tissue of the treated rat were much larger than those of the control, and there was no indication of carcinogenisis.

These observations made it evident that 4,4'-bis(p-dimethylaminophenylazo)-phenyldisulfide is not a carcinogen. However DS does effect the rat but in manner not originally perceived.

Spectrum. The absorption spectra of DS was determined as a characteristic of the compound that could be followed in protein-dye binding work by the ordinary methods of colorimetry. The spectra was determined in the upper regions of the ultra violet, through the lower regions of the visible spectra by using a Beckmann Model DU spectrophotometer. One centimeter quartz cells were used. The instrument was balanced against the blank solvent at each wavelength measurement by adjusting the slit width. This maintained sensitivity. The solvent used was ethylene chloride. Optical density values were determined for the range of 350 millimicrons to 520 millimicrons.

The optical densities may be converted to molecular extinction coef-

ficients, , through the use of the Beers-Lambert Law.

$$\frac{I}{I_o} = e^{-\epsilon cl}$$

or $$\log \frac{I_o}{I} = \epsilon cl = \text{optical density}$$

I_o is the intensity of the incident light and I is the intensity of the light transmitted through a depth l of the absorbing solution. Where c is the concentration of the dye in mols/liter, then ϵ is the extinction coefficient and is more characteristic of the solute alone than other functions. Since the cell length is one centimeter, the value of ϵ may be calculated from the equation:

$$\epsilon = \frac{\text{optical density}}{c(\text{mols/liter})}$$

The results of the absorption spectra are indicated by Figure 2. The absorption maximum is at 430 millimicrons, where the extinction coefficient is 30.4 units.

Analysis. An analytical sample of DS was prepared by repeated recrystallizations from ethylene chloride as indicated before. The pure compound crystallized as red needles. This compound was analyzed for nitrogen and for sulfur. For the compound, $C_{28}H_{28}N_6S_2$ (M.W.= 512.59), the following results are indicated.

Theoretical	Found (3)
12.16% S	12.0% S
15.96% N	15.8% N

Physical Constants. Bis-4,4'-(p-dimethylaminophenylazo)phenyldisulfide is a red solid. Its crystals are needle shaped. The melting point is 198-99.5 degrees (uncorrected). The disulfide is quite stable up to temperatures of about 300 degrees, where slight decomposition occurs.

DS is only slightly soluble in benzene, ether and other low polar solvents. It is somewhat soluble in alcohol and also in chloroform. Highly polar solvents

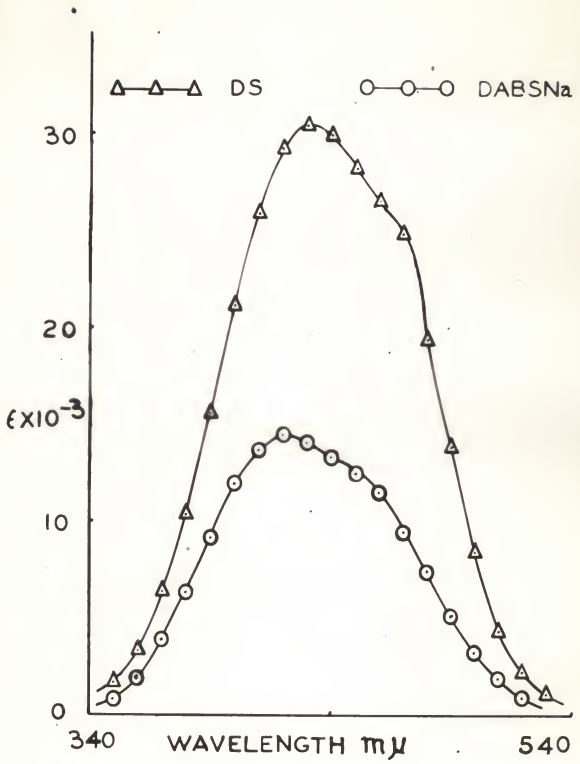
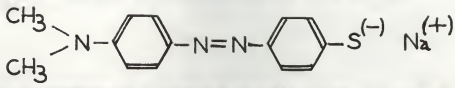


Figure 2. Absorption Spectra

such as ethylene chloride and pyridine dissolve the compound readily, especially when hot. DS exhibits a maximum absorption peak at 430 millimicrons, where the extinction coefficient is 14.5 units. The DS shows a color change in alcohol at a pH of about 2. The color changes from orange to purple.

Sodium 4-Dimethylaminoazobenzene-4'-Mercaptide.



This compound is not reported in the literature. As a part of the characterization of DS, it seemed of interest to find out if the disulfide could be cleaved to the mercaptide or to the mercaptan. It has been reported (13) that organic disulfides may be cleaved with inorganic salts. Burawoy and Turner (7) performed this cleavage on an azo disulfide by merely shaking an alcoholic suspension of the disulfide compound with salts as sodium sulfide. This procedure was followed in the cleavage of DS.

One gram of the disulfide was suspended in 55 ml. of absolute alcohol. Very little of it dissolved. A solution of two gm. of sodium sulfide nonahydrate in ten ml. of water was then added. The mixture was mechanically shaken for four hours at room temperature. The original mixture was orange in color. Upon shaking, the color of the mixture turned to an intense bright red. The reaction mixture was then diluted to 200 ml. with water. No precipitate of mercaptan or mercaptide formed. An attempt was then made to precipitate the free mercaptan by acidifying the solution. The reaction mixture was alkaline, (pH of 9). A precipitate formed at a pH of about 7, coagulated at a pH of 5 and changed to deep purple color at a pH less than two. The precipitate was collected and dried in air. This crude material (0.5 gm.) had a melting point of 195-7°. Recrystallization from a water-

pyridine system gave a melting point of 197-8° for this orange-red material. A mixed melting point with a pure sample of DS showed no depression with a melting point of 197-9°. This indicated that one half of the original DS was recovered from the reaction mixture unchanged. No other product could be isolated from the mixture although the filtrate was highly colored.

Another cleavage reaction was performed. All amounts were increased five fold and the time was lengthened to eight hours. The reaction mixture was then carefully acidified to a pH of 5. The precipitate obtained was recrystallized from pyridine. It was noted that the crude product decomposed slowly in the presence of boiling water. The product (25% of the theoretical yield) was then repeatedly recrystallized from alcohol-water mixtures. The purified product was yellow platelets with a melting point of 116-8°. A mixed melting point with a known sample of DAB gave a melting point of 116-7°. This indicated that the compound was p-dimethylaminoazobenzene (DAB). These yellow platelets were analyzed for nitrogen and for sulfur.

Theoretical	Found (3)
18.7% N	18.0% N
0.0% S	0.0% S

It was therefore concluded that this compound was DAB.

The reaction was repeated using the ratio of one mol of DS to four mols of sodium sulfide. The product was isolated by acidification and recrystallized from ethylene chloride. This yellow colored product had a melting point of 115-7°. It gave no depression of the melting point when mixed with DAB. The overall yield of DAB was 25 percent.

The reaction was repeated with one gram of DS and two grams of sodium sulfide. The reaction mixture was then cooled to -22°C. A precipitate formed and was filtered off. The material had a deep orange-red color. It was very

hydroscopic. It gave a positive test for sulfur by sodium fusion. The filtrate produced an orange material upon acidification which resembled the mixtures of DAB and DS as before. However, when the original filtrate was cooled to a -22°, a second crop of material precipitated. One tenth of a gram of material was obtained that resembled the first residue. A third crop of psuedo-crystals was formed during 16 hours at -22°. The intensity of the red color of the filtrate was much lessened this time. The product resembled the previous precipitations. It gave a strong sodium flame test and a positive sulfur test. Its dry weight was 0.12 gm. A solution of this last material in boiling alcohol formed a precipitate of DS that was about 10 percent of the material that was used. This product of the cleavage was quite soluble in alcohol and in water. It was generally insoluble in most other solvents. No crystals of any nature could be isolated.

This orange red material gave a negative test for inorganic sulfide ion which boiled with dilute acid under lead acetate paper. Lead ion formed a brown precipitate from an alcoholic solution of the impure product. It was assumed that this brown precipitate was a lead mercaptide and the process was used to prepare a lead derivative to determine the purity of the product. A weighed sample of the impure product was dissolved in 50 ml. of water. A calculated amount of lead ion was added. (30 and 50 ml. of 0.0053 N $Pb(OAc)_2$). The precipitate was allowed to stand for two hours, then collected on a fritted glass filter of medium porosity. The precipitate was dried in an oven at 100 degrees for 12 hours, cooled in a dessicator and weighed. The following data is in evidence of the composition of this precipitate.

Sample weight of impure mercaptide	30.45 mg.	55.36 mg.
Weight of the lead derivative	33.40	56.70
Theoretical yield of lead derivative	39.23	71.21
Indicated purity	77.60%	79.60%

The average purity of the product was thus shown to be 78.60 percent. The theoretical yield of lead derivative was calculated as follows:

$$30.45 \text{ mg.} \times \frac{(\text{DAB-S-Pb-S-DAB})}{(2\ \text{DABSNA})} = \frac{719.8}{558.6} (30.45) = 39.23 \text{ mg.}$$

The equivalent weight of the compound was then determined. A potentiometric titration was employed for this purpose. A sample of the impure mercaptide (16.28 mg.) was dissolved in 75 ml. of water. This solution was titrated against 0.00933 N HCl. A Beckmann pH meter, model H2, was used to follow changes in the pH of the solution. This instrument was standardized against a phosphate buffer of pH 6.8. The pH of boiled, distilled water was 6.0. An atmosphere of nitrogen was maintained to avoid oxidation and carbonation by air. The titration curve is shown in Figure three.

A differential plot of pH versus volume of acid indicated the end point to be 4.90 ml. The equivalent weight was then calculated.

$$\text{Eq. Wt.} = \frac{(16.28 \times 78.6/100) \text{ mg.}}{(4.90 \times 0.00933) \text{ equivalents.}} = 280$$

Theory calls for an equivalent weight of 279. This indicated that the mercaptide analog of methyl orange was obtained by the cleavage of DS with sodium sulfide. However, it could not be purified by the methods which were used.

The absorption spectrum of this impure mercaptide was determined by the procedure noted before for the DS spectrum. The sample taken was corrected for its percentage composition. This made its concentration nearly the same as that of the disulfide. The solvent used for the mercaptide was absolute ethyl alcohol. The results are indicated in Figure 1. The absorption maximum appeared at 420 m.microns with an intensity of 14.5 units of molecular extinction coefficient.

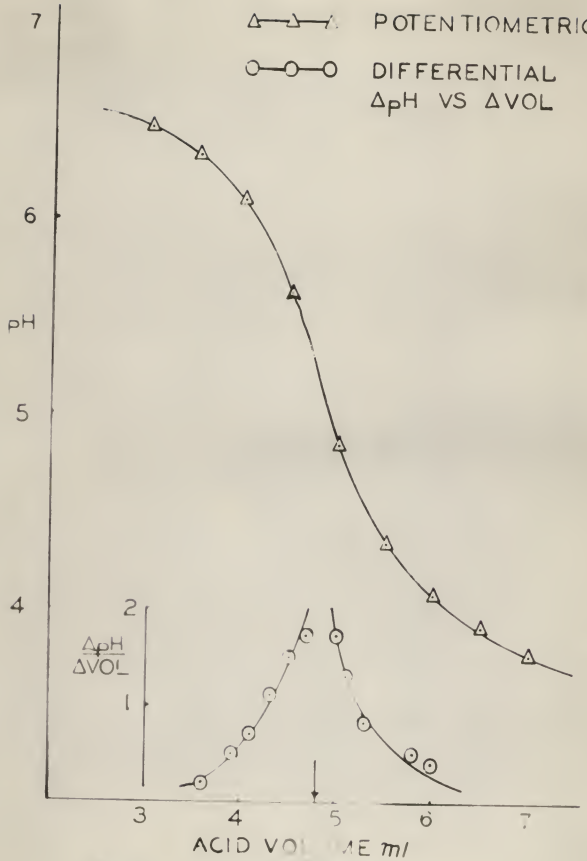
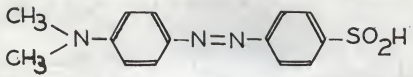


Figure 3. Potentiometric Titration

4-Dimethylaminoazobenzene-4'-Sulfinic Acid



The oxidative cleavage disulfides to sulfinic acids has been noted in the literature (7). Burawoy obtained sulfinic acids from azo disulfides compounds by refluxing them in alcoholic alkali. If the sulfinic acid analog of methyl orange could be prepared from DS by this procedure, it would further characterize DS.

One gram of DS was refluxed with 80 ml. of a 1-1 ethyl alcohol-water solution containing four grams of sodium hydroxide. The reaction mixture was filtered. The filtrate was then slowly acidified. An orange colored precipitate formed. This product was recrystallized from ethylene chloride. The melting point was 195-8° and showed no depression when mixed with a pure sample of DS. This precipitate gave no color test for the sulfinic acid group.(19). p-Acetaminobenzenesulfinic acid (16) was prepared for comparison in this test. The filtrate and the residue from the reaction mixture gave negative tests for the presence of the sulfinic acid group.

The reaction was repeated using corresponding amounts of potassium hydroxide in a 70% alcohol solution. Again no evidence of sulfinic acid formation could be detected. Further experiments in this line were discontinued.

RESULTS AND DISCUSSION

The compound, 4, 4'-(p-dimethylaminophenylazo)phenyldisulfide, DS, was prepared by the methods of synthetic organic chemistry. Since this compound has not been reported previously in the literature, methods used for its synthesis, its biological behavior, physical nature and degradtion products were studied critically.

The synthesis of DS was fairly straight forward. The five successive reactions needed to produce DS resulted in an overall yield of 45 percent.

Carcinogenicity

The determination of the carcinogenicity of DS produced results that were of great interest but different from those expected. DS did in some way effect the growth of the rats. Recently a growth factor, "α-lipoic acid", has been reported (6, 14). This compound contains a disulfide linkage and has stimulated the growth of the intestinal organism E. Coli. In this experiment, the disulfide (DS) may have stimulated the growth of the intestinal organisms that produce some of the B complex vitamins that the body uses for general growth. This could have enabled the rat to receive an abundance of the vitamins that were needed for the excessive growth.

Furthermore, disulfides are known to operate as protective agents as evidenced in the case of feeding cystine to aid in detoxification processes. Hence DS could aid in its own detoxification. Whether a given disulfide, such as cystine, exhibits all its effects by aiding metabolic detoxification or by increasing the level of vitamins in the body, cannot be separated as two different results. These two processes are interrelated since one affects the other and they both tend to achieve the same result. In any event, this indicates that DS may have been detoxified before it could exert any carcinogenic effects.

The effects of various sulfur compounds on growth have been observed (5). It has been shown that thiols stimulate the growth of tumors that were already developed by transplantation. Thiols such as sodium thioglycolate and sodium thiomalate, administered by injection, had mild stimulating effect on such tumors. It is interesting to note that true mercaptans, like cysteine and thiolactic acid, do not have this stimulating effect. Indeed tumor growth was retarded by thiol inhibitors such as sodium iodoacetate. This retardation, however, did not prevent the establishment of the transplanted tumors. The compound, DS, through its disulfide linkage, could act as a thiol inhibitor, thus retarding its own carcinogenic potential.

There is a high degree of correlation between carcinogenicity and growth retardation in general (1). Intraperitoneal injection of 1,1'-dinaphthyldisulfide had a very slight retarding effect on the further development of transplanted tumors. DAB had a much stronger effect. At least the dinaphthyldisulfide did not increase the size of the tumor. 2-Mercaptobenzthiazole reversed the effect of both of these substances. Thus the administering of organic sulfur compounds caused the growth to develop further.

In general it has been noted that the inhibition of growth is correlated with the decrease in the amount of sulfur containing amino acids available in the body. This is due to detoxification processes using excessive amounts of compounds such as methionine and cystine. Indeed, when general growth was retarded by administering a carcinogen such as methyl cholanthrene or DAB, the inhibition was promptly reversed by feeding cystine or methionine. Subcutaneous injections of glutathione had the same results. This is an example of increasing growth through the use of a disulfide (cystine).

There are other possibilities that would result in this growth phenomenum. The anterior lobe of the pituitary gland is the master growth regulator. It is

not very likely that DS would affect this gland directly. It is possible that the DS could have an effect on the hormone cycle that emanates from this gland. The results of such an interaction would be rather difficult to predict.

Another consideration is esthetic. The rats may have merely liked the taste of the ration with the disulfide dye in it. However in this case one could be led to believe that the rat would gorge himself. This would result in deposition of excess fatty tissue. The fat deposition in the abdominal cavity was quite normal in amounts and in some cases was less than that seen in the smaller control rats.

That DS could increase the growth of rats was partially explained in the previous discussion. Two main ideas are presented. First a disulfide could increase the level of vitamins in the body that are concerned with growth. Secondly organic sulfur compounds aid the body in detoxification processes. The lack of carcinogenicity is inferred from the previous discussion to be the result of the possible ease of detoxification of DS. However growth is complex and cannot be fully explained on the basis of the few considerations developed here.

Spectrum

The absorption spectrum of DS is in accord with the spectra for comparable azo dyes. Its absorption peak appears at 430 m. microns. This accounts for its slightly orange-yellow color in concentrations of 10^{-5} molar. Incomparing DS to DAB one would expect the absorption peak to be shifted to longer wavelengths (deepening the color towards the red). This is due to the disulfide linkage. This group exerts both chromophoric and auxochromic effects on the entire molecule. The resonance stabilization gained by sulfur expanding its valence shell to ten electrons would be sufficient to cause some of the shift towards the red. Here the sulfur acts as a chromophore. In the auxochromic

function, the electron density of sulfur is decreased in favor of the chromophoric azo group. This slight resonance extension accounts for the rest of the shift towards the red. This resonance contribution is small and the shift is correspondingly small.

DS (ethylene chloride)	DAB-S-Na (in alcohol)	Methyl Orange (in water)	DAB (alcohol)
30.4	14.5	25.0	18.5
430	422	465	410

It should be noted that the color is not intensified by the great length of the molecule. None is expected since the disulfide linkage cannot conjugate both azobenzene groups, thus eliminating resonance conjugation of the entire system.

In comparing DS to methyl orange (the free acid), it is seen that the disulfide linkage is not nearly the electron "sink" (chromophore) that the sulfonic acid function is. This conclusion is evidenced by the fact that methyl orange is a much deeper red in color. The sodium mercaptide (DAB-S-Na) has a color comparable to the sodium salt of methyl orange. The sulfonate ion does not show the chromophoric properties of the nonionized group. The negative charge greatly reduces the ability of the chromophore to attract electrons, this reduces the resonance, and shift the absorption peak to shorter wavelengths. This causes a shift in the color from red to the yellow. The mercaptide has lost the chromophoric function of a disulfide group since the electron density of a negative ion cannot be increased for the purposes of resonance. This leaves the molecule with only the auxochromic function of the disulfide group donating electrons to the azo group and benzene ring. Hence the absorption of the mercaptide should differ from DS and should absorb at shorter wavelengths. The absorption is less than that of DS by 8 m.microns. The solvents were different but of comparable dipole moments, thus minimizing any great solvent effect.

The absorption intensity of DS is much greater than DAB and even more than for methyl orange. Considering the structure of DS as a whole, it is seen that the molecule will have much greater resonance energy than any of the other systems. Change in color is dependent on the resonance interaction of groups and not on the sum of the total resonance of isolated groups. The height of the peak is a function of the molecular extinction coefficient which is calculated on the basis of the entire molecule. In view of this it is observed that the intensity of absorption of DS is approximately twice that of the mercaptide. The intensity of the mercaptide is even less than that for DAB. This is explained on the basis that the field effect of the negative charge on the sulfur atom will prevent polarization of the azo group, which in turn prevents the resonance shift of electrons from the amino nigrogen to the azo chromophore.

Analysis

The quantitative analysis of DS indicated that the product of the series of reactions was the compound expected. The results were not in complete agreement with the theory. However, the error was not considered sufficient to disqualify the formula indicated ($C_{28}H_{28}N_6S_2$). The discrepancies for both nitrogen and sulfur are of the same magnitude. This indicates that the cause of the error is common to the analysis of both elements. It was assumed that a trace of the solvent was present when the analysis was performed.

The analysis of the yellow platelets obtained from the abnormal cleavage of DS with sodium sulfide clearly indicated that the material was DAB. The percentage of nitrogen was low compared to the calculated amount, but it was not considered enough to invalidate the empirical formula, ($C_{14}H_{15}N_3$). The mixed melting point and the analysis showed that this compound was p-dimethylaminoazobenzene.

Physical Constants

The results of these observations are straight forward as indicated before. One conclusion was drawn concerning the disulfide dye. The red crystals were of good form and had a sharp melting point for azo dyes (range 1.5). This indicated that the product submitted for analysis was merely contaminated with a small amount of volatile material and not by any products of possible side reactions.

Sodium 4-Dimethylaminoazobenzene-4'-Mercaptide. This compound served as a characterization derivative for DS. This substance was not isolated in pure form, so its properties could not fully be determined. The only significant results are the spectral data and equivalent weight. The spectrum produced results that are in accord with theory. The equivalent weight, which was determined rather indirectly, was considered the best indication of the formation of the compound of formula $C_{14}H_{14}N_3SNa$. The chemistry of the reaction that produced the mercaptide at first seemed normal, but some anomalies were noted. The cleavage of the DS to the mercaptide was considered as the normal reaction. The most bizarre reaction was the formation of a product that contained no sulfur, DAB. The mechanism of the reaction by which this compound was formed is not completely understood. One bit of evidence was the recovery of DS from the reaction mixture filtrate where it was originally insoluble.

DS dissolved in the reaction mixture very slowly. As the mixture was shaken, the color of the solution intensified and less and less of the material remained undissolved. This reaction mixture could then be filtered giving a deep, red solution. The residue from this filtration was shown to be DS. Acidification of the filtrate with non oxidizing acid (HCl), was generally observed to produce two products, DAB and DS. Evidently the mercaptide is

present since it can be isolated from the reaction mixture without acidification.

It has been reported that the basic cleavage of disulfides in alcohol form initially the mercaptide and the sulfenic acid. (18). Sulfenic acids would cause the reverse of the original cleavage by uniting with mercaptide ion and eliminating hydroxyl ion in the presence of acid to form water. A mechanism that would explain how the carbon - sulfur bond is broken is not too evident. In the process of the decomposition of the sulfenic acid it is possible to form a positively charged sulfur system. This could react with any $NaS_n^{(-)}$ present in the solution. The resultant polysulfide system could be cleaved at the carbon - sulfur bond by stronger acid concentrations. The literature (11, 12, 18) showed no (2) diaryl disulfide systems that had been cleaved at the carbon - sulfur linkage by such mild conditions (very weak base, Na_2S; or dilute acid in dilute solution of the mercaptide or the sulfenic acid. A clear mechanism did not present itself and so the process was left as a unique cleavage of carbon - sulfur bonds.

4-Dimethylaminoazobenzene-4'-Sulfinic Acid. The attempted synthesis of this compound gave no indication of producing any of the actual sulfinic acid by the method that was tried. Such an oxidative cleavage would have characterized the DS more fully. The failure of the reaction as performed was probably the result of the insolubility of the DS in the reaction medium. The product of the reaction would have been soluble if it had been formed. The same reaction conditions had produced results with an analogous compound (6). Also, if any of the desired product had formed it was no doubt altered to forms undesirable of isolation under the vigorous conditions (refluxing for 8 hours in alcoholic potassium hydroxide. Since this reaction did not lend itself to the further characterization of DS, investigations along this line were discontinued.

SUMMARY

The synthesis of 4,4'-bis(p-dimethylaminophenylazo)diphenyldisulfide (DS) has been accomplished. The last of a series of reactions that produced this compound was the coupling of dimethylaniline with the tetrazonium sulfate of 4,4'-diaminophenyldisulfide. The absorption spectra of this compound was observed and its physical constants were determined.

The cleavage of the above compound to sodium 4-dimethylaminoazobenzene-4'-mercaptide was effected by hydrolytic scission with sodium sulfide. This reaction aided in the characterization of the disulfide compound (DS). The spectra of this mercaptide was studied.

The carcinogenicity of 4,4'-bis(p-dimethylaminophenylazo)phenyldisulfide was established. This compound is not a hepatic carcinogen.

However, this compound (DS) does affect the growth of a rat. Ingestion of DS by the rat was seen to cause the animal to rapidly gain weight. Over an experimental period of fourteen weeks, the rats fed DS gained weight at a rate of two and one half times the weight gain of a group of control animals.

ACKNOWLEDGMENT

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APPENDIX

SPECTRUM OF DS Beckmann Spectrophotometer model DU

Temp. 25°C. 4.77 mg. in a liter of solution. 0.932×10^{-5} molar.

100% transmission at optical density of -0.02. Corr. Factor is .02.

Wavelength	Optical Density	Extinction coefficient $\times 10^{-3}$
350 m.micron	.018	1.72 (1/mol)
360	.033	3.33
370	.061	6.33
380	.099	10.4
390	.147	15.6
400	.200	21.2
410	.243	25.9
420	.274	29.2
425	.281	29.9
428	.283	30.2
430	.285	30.4
432	.284	30.3
435	.283	30.2
438	.281	29.9
440	.279	29.8
445	.270	28.8
450	.265	28.2
460	.249	26.5
470	.233	24.8
480	.182	19.3
490	.132	13.9
500	.081	8.47
510	..042	4.30
520	.023	2.26
530	.012	1.07

SPECTRUM OF DABSN$_a$ Beckmann Spectrophotometer model DU

Temp. 25°C. 3.93 mg.(70.6% pure) in a liter of solution 1.11×10^{-5} molar

Correction factor to be subtracted is 0.02.

Wavelength	Optical density	Extinction coefficient $\times 10^{-3}$
350 m.microns	.009	0.7 (1/mol)
360	.021	1.9
370	.041	3.9
380	.066	6.4
390	.094	9.2
400	.122	12.0
410	.139	13.7
415	.143	14.1
418	.146	14.4
420	.146	14.5
423	.147	14.5
425	.146	14.4
427	.145	14.3
430	.143	14.1
433	.139	13.9
435	.137	13.7
440	.135	13.3
450	.126	12.4
460	.117	11.5
470	.096	9.4
480	.076	7.4
490	.053	5.1
500	.034	3.2
510	.021	1.9
520	.011	0.9
530	.006	0.4

POTENTIOMETRIC TITRATION — Beckmann pH meter model H2

Temp. 25°C. — N HCl= 0.00933 — 16.28 mg. DABSNa in 75 ml of water

pH	ml acid	pH	ml acid
8.63	0.00	6.28	3.60
8.53	0.10	6.30	3.70
8.41	0.20	6.22	3.80
8.29	0.30	6.17	3.90
8.13	0.40	6.10	4.00
7.95	0.50	6.03	4.10
7.65	0.60	5.97	4.20
7.42	0.70	5.86	4.30
7.39	0.80	5.77	4.40
7.38	0.90	5.62	4.50
7.32	1.15	5.48	4.60
7.30	1.22	5.33	4.70
7.28	1.30	5.17	4.80
7.20	1.40	5.00	4.90
7.16	1.50	4.83	5.00
7.11	1.60	4.70	5.10
7.02	1.70	4.58	5.20
6.98	1.80	4.50	5.30
6.90	1.90	4.42	5.40
6.85	2.00	4.34	5.50
6.80	2.10	4.28	5.60
6.75	2.20	4.22	5.70
6.63	2.30	4.17	5.80
6.58	2.40	4.11	5.90
6.52	2.50	4.07	6.00
6.49	2.60	4.02	6.10
6.46	2.70	3.99	6.20
6.43	2.80	3.96	6.30
6.40	2.90	3.93	6.40
6.37	3.00	3.90	6.50
6.35	3.10	3.87	6.60
6.31	3.20	3.83	6.70
6.40	3.30	3.81	6.80
6.36	3.40	3.79	6.90
6.30	3.50	3.76	7.00
		3.72	7.10
		3.68	7.20

FEEDING DATA

SPRAGUE-DAWLEY RATS

DYES AT 0.06%

DS (7 ♀ rats)		CONTROL (4 ♀ rats)		M'-ME DAB (5 ♀ rats)	
Days	Avg.wt.	Days	Avg.wt.	Days	Avg.wt.
0	203	0	202	0	204
4	219	6	213	4	212
11	226	12	215	8	208
17	261	16	223	14	199
22	283	20	224	19	201
28	291	28	229	25	207
32	299	33	233	29	209
36	302	38	239	33	213
40	305	46	238	37	214
44	301	50	232	41	222
49	294	55	238	46	224
54	287	59	242	51	224
62	315	65	247	59	235
66	319	70	256	63	240
71	325	84	261	68	247
75	325			72	250
81	321			78	254
86	320			82	259
98	347			96	262
ALL LIVERS NORMAL		ALL LIVERS NORMAL		ALL LIVERS CANCEROUS	

THE SYNTHESIS, PROPERTIES AND CARCINOGENICITY OF 4,4'- BIS(p-DIMETHYLAMINOPHENYLAZO)PHENYLDISULFIDE

by

DONALD EUGENE SETTER

B.S., KANSAS STATE COLLEGE, 1951

AN ABSTRACT

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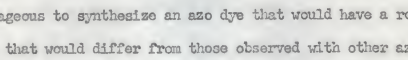
The problem of correlating the cancer inducing ability of certain chemicals to their molecular structure has avoided complete elucidation. The analagies are few that can be drawn between the carcinogenicity and the structure of an azo dye.

Induced tumor formation is generally preceded by processes which bind an azo dye to a protein. In order to gain further insight to this problem, it was deemed advantageous to synthesize an azo dye that would have a role in these binding processes that would differ from those observed with other azo dyes.

The compound chosen for this study was 4,4'-bis(p-dimethylaminophenylazo)-phenyldisulfide.



DS

The disulfide linkage of DS should enable the molecule to be more or less chemically bound to a protein surface. The disulfide group (-S-S-) should undergo an exchange reaction with the thiol groups on the surface of a protein molecule. The moeity of DS that could be carcinogenic is the p-dimethylaminoazobenzene group. It is known that p-dimethylaminoazobenzene itself is carcinogenic.

The compound DS was prepared for this purpose by the method of synthetic organic chemistry. The physical properties of this compound were determined. The chemical properties of DS center around its scission to the corresponding mercaptide, sodium p-dimethylaminoazobenzene-4'-mercaptide, by the use of sodium sulfide. The spectrum of this latter compound was observed.

The dye (DS) was fed to rats at 0.06% in a basal ration for 14 weeks. Inspection of the livers after this time revealed no carcinoma. The rats appeared quite normal and healthy throughout the experimental period. No tissue or organ damage whatsoever was observed. However, one startling abnormality was the increase in weight. The rats fed DS gained weight throughout

the 14 week period at a rate two and one half times the weight increase of a group of control rats.

It was concluded that DS is not a carcinogen. It was observed that DS in some way stimulates the growth of rats.