THE SYNTHESIS AND BIOLOGICAL PROPERTIES OF 3-(4'-DIMETHYLAMINOPHENYLAZO)PHENYL METHYL SULFIDE

by

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INTRODUCTION

Since 1906, when Fischer (9) observed that the injection of scarlet red into the ears of rabbits caused an atypical growth of epithelial tissue, the carcinogenic properties of many aminoazo dyes have been studied. In 1933 Yoshida (24) conclusively demonstrated the hepatic carcinogenicity of the azo dye, 2',3-dimethyl-4-aminoazobenzene, which is a portion of the scarlet red molecule. Three years later Kinosita (13) reported the high hepatic carcinogenicity of 4-dimethylaminoazobenzene (DAB). The carcinogenicity of this compound and its derivatives have been studied extensively by the Millers (11,15,16,17, 19).

When azo dyes are administered to rats tumor formation usually occurs in the liver, although histological changes sometimes occur in other organs. Inbred rats have been used extensively because of their high susceptibility.

Azo dyes are very useful in the study of the metabolic and physiological changes which occur during tumor formation and may thus be used to aid in the elucidation of the problem of cancer. The mode of action of azo dyes is, however, still largely unknown. Even with the great number and wide variety of compounds which have been found to be carcinogenic, the mode of action is still thought to be the same.

Miller and Miller (15) observed that liver protein from rats fed DAB was pink when suspended in acid and yellow in neutral or basic solvents. They concluded that the dye was bound to the protein by chemical linkages for the following reasons. The dye could not be removed by dialysis or extraction and was released upon hydrolysis of

the protein. Whether the dye is bound during protein synthesis or to already existing protein is not known. The Millers (15) also concluded that the dye was combined with the protein through a derivative of the $-N(CH_3)$ group or through some substituent on the ring bearing this group. Azo dyes without a dimethyl or monomethyl amino group are inactive as carcinogens.

In this laboratory the carcinogenicity of derivatives of DAB containing sulfur have been studied (2,3,4). These are of interest since they contain potential mercapto groups which are capable of forming disulfide linkages with protein. Bauer (2) has determined the carcinogenicity of 4-(4'-dimethylaminophenylazo)phenyl methyl sulfide (p-S-Me-DAB) and 2-(4'dimethylaminophenylazo)phenyl methyl sulfide (o-S-Me-DAB). His results showed the para compound to be active as a carcinogen whereas no tumors were observed in the rats fed the ortho dye. Millers (18) have prepared and tested the carcinogenicity of the corresponding methoxy compounds, including the meta compound which was found to be very active, causing severe gross cirrhosis.

In this investigation 3-(4'-dimethylaminophenylazo)phenyl methyl sulfide (m-S-Me-DAB) was synthesized and characterized. The carcinogenicity and the presence of the bound dye in the liver protein were determined.

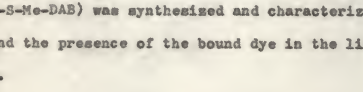

Fig. 1. m-S-Me-DAB

The preparation and characteristics of this dye as well as its hepatic carcinogenicity are reported in this thesis.

EXPERIMENTAL

Preparation of Intermediates

The preparation of the dye involved the synthesis of m-aminophenyl methyl sulfide which was then coupled to dimethylaniline to form the azo compound. The preparation of the intermediates was accomplished by established methods of organic chemistry. The synthesis of m-aminophenyl methyl sulfide involved a series of reactions which are described below. The starting material was m-nitrobenzenesulfonyl chloride obtained from Eastman Kodak.

Preparation of 3,3'-dinitrophenyldisulfide. The procedure used for this preparation was according to the method of Bauer and Cymerman (1). Hydroiodic acid was used to effect the reduction of sulfonyl chlorides to disulfides. Thirty grams of m-nitrobenzenesulfonyl chloride were dissolved in 300 ml. of glacial acetic acid and 150 ml. of 47 per cent hydroiodic acid added to the solution. The solution immediately turned dark brown due to the formation of molecular iodine. The reaction mixture was allowed to stand over night after which crystals of iodine were observed. Three hundred fifty ml. of 10 per cent sodium thiosulfate were added to convert the iodine to iodide. The solution was then carefully neutralized by adding sixty grams of anhydrous sodium carbonate in small portions. Ten per cent NaOH was added until the solution was distinctly basic. The crude brownish yellow precipitate was collected by filtration and recrystallized by dissolving the precipitate in hot ethanol and adding water until the cloud point was reached. The melting point of the yellow crystals of the disulfide after two recrystallizations was 82.5°C. (yield 12 grams - 60 per cent).

Preparation of 3-nitrothiophenol. Glucose was used for the reduction of the disulfide to the corresponding thiophenol using the method of Claasz (5). Five grams of the 3,3'-dinitrophenyldisulfide were mixed with 3.5 grams of glucose in 15 ml. of 95 per cent ethanol. After heating the mixture on a steam cone, a solution of 2.5 grams of NaOH in 5 ml. of water was added. The solution immediately turned a dark red due to the formation of the sodium salt of the mercaptan. The mixture was stirred while heating for 15 minutes after which it was cooled rapidly with the addition of ice. Upon acidification, the mercaptan was obtained as a dark oil which was extracted from the solution by repeated extractions with diethylether (five 50 ml. portions). Due to the instability of the mercaptan the ether was removed rapidly by vacuum distillation keeping the flask cold. The mercaptan was immediately methylated.

Preparation of m-nitrophenyl methyl sulfide. Immediately after the removal of the ether from the mercaptan, 4.65 grams of sodium hydroxide in 20 ml. of water and 3 ml. of dimethylsulfate were added to methylate the mercaptan. The procedure of Fricke and Spilker (10) was modified in that the solution was refluxed for three hours. The crude dark solution was then distilled with steam until a liter of distillate had been collected. The m-nitrophenyl methyl sulfide was extracted with diethylether and the ether evaporated on a steam cone. Three grams of the yellow-orange oil was obtained (yield - 55 per cent).

Preparation of m-aminophenyl methyl sulfide. The nitro group of m-nitrophenyl methyl sulfide was reduced to the corresponding amine by the use of iron powder (22). To 5.4 grams of m-nitrophenyl methyl sulfide was added an excess of iron powder (15 grams) and 100 ml. of

water. One-tenth of a ml. of glacial acetic acid was also added to make the solution slightly acidic. The mixture was stirred mechanically for ten hours at a temperature of 85-90°C. and then neutralized with sodium carbonate and filtered. The aqueous phase was extracted with five 100 ml. portions of benzene and the solid material extracted with five 50 ml. portions of hot benzene. The extracts were combined and dried over magnesium sulfate. The dried benzene solution of the amine was saturated with dry hydrochloric acid and the amine hydrochloride collected by filtration (yield - 4 grams 72 per cent). The amine hydrochloride may be recrystallized from an ethanol solution. After neutralization of the amine hydrochloride with base and extraction in benzene the amine may be distilled for purification. The pure amine is a yellow oil of which various derivatives were prepared.

Diazotization and Coupling

The final step in the preparation of the dye is the diazotization of m-aminophenyl methyl sulfide and the coupling to dimethylaniline. Zincke and Müller (25) reported that the amine could be easily diazotized and coupled to dimethylaniline as well as other amines. This was done according to the method of Giese, Miller and Baumann (11). Ten grams of the amine in 18 ml. of concentrated hydrochloric acid in 100 ml. of water was cooled to 0°C. and diazotized with a solution of 4.96 grams of sodium nitrite in 22 ml. of water. This mixture was poured, all at one time, into a cool solution of 8.7 grams of dimethylaniline and 11.8 grams of sodium acetate in 220 ml. of 70 per cent ethanol. Red crystals of the azo dye are formed in good yield. After two recrystallizations from methanol 14.6 grams of the dye are obtained (yield - 75 per cent).

The absorption spectrum of the dye was determined in absolute ethanol from 350-550 mμ and in a 1:1 ethanol concentrated hydrochloric acid solution over the range 400-600 mμ and the molar extinction coefficients calculated.

Care and Handling of Animals

Male albino rats were obtained from Sprague-Dawley, Madison, Wisconsin, and housed singly in screen bottomed metal cages. The ration and water was available ad libitum. The basal ration was essentially the same as that suggested by the Wisconsin group (21) and has the following composition:

Casein (vitamin free).....12 per cent
Glucose.....79 per cent
Corn oil (Mazola)..... 5 per cent
Salts mixture W (18)..... 4 per cent

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 incorporated into the ration at the level of .06 per cent by dissolving it in warmed corn oil and then mixing with the other ingredients. A group of five rats were fed the basal ration without the dye to serve as controls. The weight of each rat was recorded weekly. One drop of halibut liver oil was given on top of the ration each week.

Isolation and Spectrum of Bound Dye

After the rats had been fed the dye for six weeks a group of six rats was sacrificed for the bound dye analysis.

The rats were anesthetized with diethylether and then slit open on the ventral side exposing the thoracic and abdominal cavities. The liver was immediately perfused with physiological (.89 per cent) saline by inserting a needle through the inferior vena cava after severing the portal vein. The liver was removed intact and frozen immediately in acetone chilled with dry ice and kept frozen until the analysis was made.

The analysis was done with a modification of the procedure of Miller and Miller (15). After the livers were thawed they were homogenized in a Waring blendor and dialized for 48 hours in Visking casing bags against continuous flowing distilled water to remove the free aminoazo dyes and lipids. The homogenate was then lyophilized and extracted with absolute ethanol for 48 hours in a Soxhlet apparatus. It was ground up with a mortar and pestle to make it homogeneous. A 200 mg. portion was hydrolyzed in a mixture of 8 ml. of ethanol and 20 ml. of 4.5 normal potassium hydroxide at 80°C. for 20 hours. After hydrolysis 8 ml. of ethanol, 16 ml. of water and 16 ml. of 11 normal potassium hydroxide were added and the mixture extracted with 40 ml. of diethylether. Two more extractions were carried out using 24 ml. of a 1:5 ethanol ether mixture. The extract was evaporated until dry on a steam cone and the residue taken up in 5 ml. of ethanol and transferred to a 10 ml. volumetric flask. The solution was made up to volume (10 ml.) with concentrated hydrochloric acid and the absorption spectrum determined.

RESULTS AND DISCUSSION

Physical Constants of Intermediates

The physical constants of the intermediates as well as their derivatives were compared with those found in the literature. This data is summarized in Table 1. All melting points listed are corrected.

Physical Characteristics of the Dye

The dye is in the form of orange plates when recrystallized from methanol and its melting point is 73.5°C. The dye acts as an acid-base indicator being red in acid and yellow in neutral or basic solution. The color change occurs at pH 2-4.

The absorption spectra of the pure dye in absolute ethanol and in 1:1 ethanol concentrated hydrochloric acid are shown in Figure 2. In ethanol the spectrum shows a maximum peak at 414 mμ and the extinction coefficient of 2.80×10^4. In the 1:1 ethanol HCl solution the maximum absorption peak is at 526 mμ and the extinction coefficient is 4.39×10^4. This is within the normal range for acidified azo dyes.

The results from the elemental analysis as well as the theoretical and observed empirical formulas are shown in Table 2. Analytical results were supplied by Drs. C. Weiler and F. B. Strauss, 164 Banbury Road, Oxford, England. The empirical formula as calculated from the analytical results indicate that the compound is most likely the one desired.

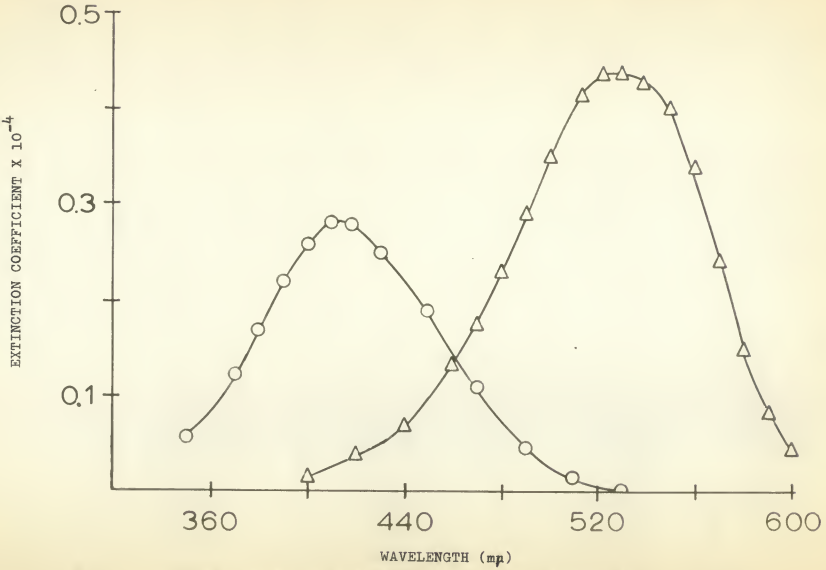

Fig. 2. Spectra of pure dye in ethanol (circles) and ethanol:acid (triangles)

Table 1. Physical Constants of Intermediates and Derivatives

Compound	Physical Constant	Observed Value	Literature Value	Reference
3,3'dinitrophenyldisulfide	m.p.	82.5°C	82°C	(14)
			83°C	(6)
			84°C	(8)
m-aminophenyl methyl sulfide	b.p.	150°C (10 mm Hg)	163-165°C (16 mm Hg)	(25)
			101.5-102.5°C (.3 mm Hg)	(12)
	n_D^{20}	1.6433	1.6427	(12)
a cetyl derivative of amine	m.p.	76.5-77.5°C	75°C	(25)
			78-78.5°C	(12)
sulfone of acetyl derivative of amine	m.p.	134-136°C	137.5-139°C	(12)
			137°C	(25)
benzoyl derivative of amine *	m.p.	94-95°C	--------	new derivative
benzenesulfonyl derivative of amine *	m.p.	79-81°C	--------	new derivative

* recrystallized from cyclohexane

Table 2. Elemental analysis and empirical formulas of the pure dye

Element	Calculated Results Per Cent	Formula	Analytical Results Per Cent	Formula
C	66.38	15	66.23	15.00
H	6.32	17	6.35	17.26
N	15.49	3	15.30	2.96
S	11.81	1	11.80	1.00

Effect of Dye on Rats

Of the thirty-seven rats which were obtained seven died of undetermined causes during the first two weeks. At the end of the first week the dye was incorporated into the diet of all but five of the rats which were fed the basal ration and kept as controls. After six weeks of dye administration six rats were sacrificed. Nineteen animals were kept on the dye containing diet for 16 weeks.

The growth curves for both groups of rats are shown in Figure 3. The weight of the control rats increased steadily from an average of 177 grams to 356 grams after 16 weeks. The weight of the rats fed the dye decreased slightly for the first two weeks after which it increased slowly. The initial average was 177 grams and the final was 243 grams. These results are typical of this type of experiment. The controls consumed considerably more ration than did the rats which were fed the dye, which very likely accounts for the difference in growth. The rats fed the dye looked rough and shabby and many showed a loss of hair especially on the hind quarters.

At the end of 16 weeks all rats were sacrificed and the livers

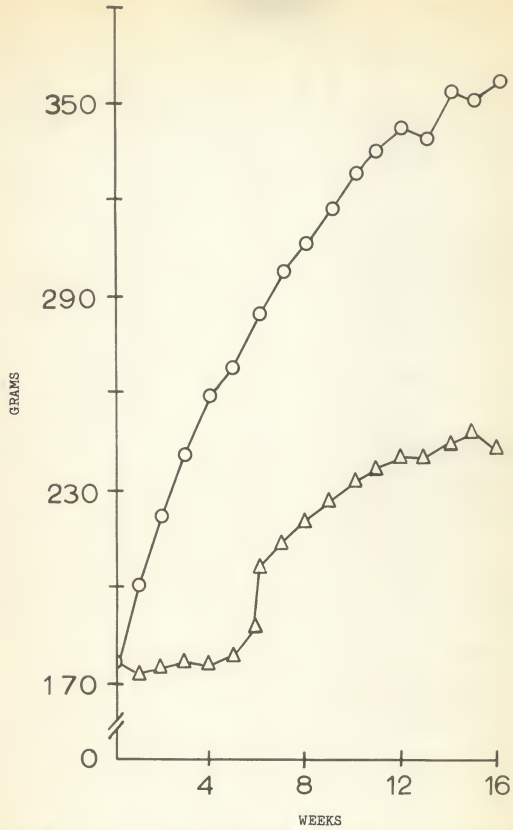

Fig. 3. Growth curve of rats, controls (circles), those on diet including dye (triangles)

examined for tumors. Two rats died during the last week and both had large liver tumors. Thus 16 (including the two that died) of the 19 rats showed severe tumors.

The Millers (18) determined the carcinogenicity of the corresponding methoxy dye and found it to be more active than the para or ortho dye. It is generally found that the meta substituted isomers are more active as a carcinogen than either the para or ortho compounds.

Bound Dye

The presence of bound dye in the liver protein of rats fed the dye for six weeks was demonstrated by the addition of acid to the extract of the dye which was prepared as described earlier. The color of the extract changed from yellow to pink immediately upon the addition of acid whereas a similar extract from the normal rat liver exhibited no change in color.

The spectrum (Fig. 4) of the clear extract also shows a broad but definite absorption peak at 510 mμ which is the normal range for acidified azo dyes. The peak at 510 mμ is shifted slightly from the peak of 526 mμ of the pure dye. This is probably due to the effect of metabolism of the dye by the rat. Thus a quantitative determination of the bound dye is difficult since the exact extinction coefficient cannot be obtained. Another difficulty is the cloudiness of the extract of the normal liver, although prepared exactly like the dye containing liver extract which was clear.

SUMMARY

The azo dye, 3-(4'-dimethylaminophenylazo)phenyl methyl sulfide was

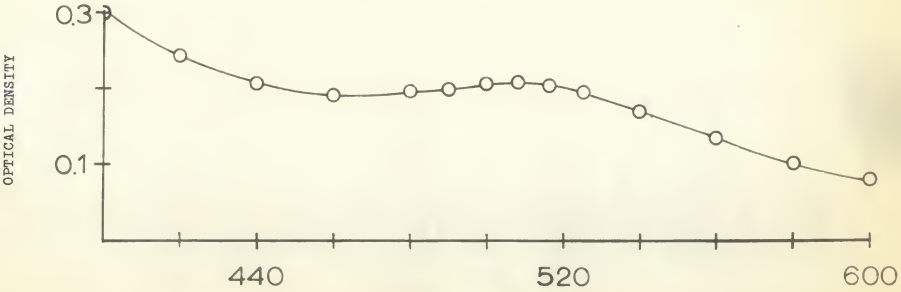

Fig. 4. Spectrum of bound dye

synthesized and characterized. The properties of this dye have not been reported earlier, although Zincke and Müller (25) reported the synthesis.

Previously unreported derivatives of one of the intermediates, *m*-aminophenyl methyl sulfide, were prepared and their melting points recorded. These derivatives were the benzoyl and benzenesulfonyl.

It was demonstrated that the dye was bound to the liver protein and that it was a very active carcinogen producing large tumors in 16 of 19 rats. The carcinogenicity of this dye was expected since the para isomer was shown to be active by Bauer (2), and the meta isomers are usually more carcinogenic than the para isomers.

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APPENDIX

Spectrum and Extinction Coefficients of Dye in Ethanol

Wavelength (mμ)	Optical Density	Extinction Coefficient x 10^{-4}
350	.105	.57
360	.155	.83
370	.226	1.21
375	.268	1.44
380	.314	1.69
385	.360	1.99
390	.408	2.20
395	.444	2.39
400	.479	2.58
405	.505	2.72
410	.522	2.80
414	.529	2.84
416	.527	2.83
418	.522	2.80
420	.516	2.77
422	.508	2.73
426	.489	2.63
430	.463	2.49
440	.410	2.20
450	.351	1.89
460	.279	1.50
470	.202	1.09
480	.136	.73
490	.084	.45

Spectrum and Extinction Coefficients of Dye in Ethanol (concl.)

Wavelength (mμ)	Optical Density	Extinction Coefficient x 10^{-4}
500	.047	.25
510	.024	.13
520	.012	.06
530	.009	.03

Spectrum and Extinction Coefficients of Dye in Ethanol:Acid

Wavelength (mμ)	Optical Density	Extinction Coefficient x 10^{-4}
400	.015	.16
410	.025	.27
420	.034	.37
430	.047	.51
440	.063	.68
450	.088	.95
460	.121	1.30
470	.162	1.74
480	.213	2.29
490	.265	2.85
500	.321	3.45
505	.346	3.72
510	.369	3.97
512	.378	4.06
514	.385	4.14
516	.391	4.20
518	.397	4.27
520	.402	4.32
522	.405	4.36
524	.407	4.38
526	.407	4.38
528	.406	4.37
530	.406	4.37
532	.405	4.36

Spectrum and Extinction Coefficients of Dye in Ethanol:Acid (concl.)

Wavelength (mμ)	Optical Density	Extinction Coefficient x 10^{-4}
534	.403	4.34
536	.400	4.30
538	.397	4.27
540	.393	4.23
545	.384	4.13
550	.369	3.97
560	.312	3.36
570	.221	2.38
580	.137	1.47
590	.075	.81
600	.040	.43

Average Weight of Rats

No. of weeks of feeding	m-S-Me-DAB		Control	
	No. of rats	Ave. Weight	No. of rats	Ave. Weight
0	26	177	5	178
1	26	173	5	200
2	25	175	5	222
3	25	177	5	241
4	25	176	5	260
5	25	179	5	268
6	25	188	5	284
7	19	214	5	297
8	19	220	5	306
9	19	226	5	317
10	19	233	5	328
11	19	238	5	336
12	19	241	5	343
13	19	240	5	338
14	19	244	5	354
15	19	248	5	351
16	18	243	5	356

Spectrum of Bound Dye

Wavelength (mμ)	Optical Density
400	.300
410	.268
420	.244
430	.225
440	.209
450	.200
460	.193
470	.194
480	.195
490	.200
496	.205
498	.206
500	.207
502	.208
504	.209
506	.209
508	.209
510	.208
512	.208
514	.207
516	.206
520	.202
525	.196
530	.188

Spectrum of Bound Dye (concl.)

Wavelength (mμ)	Optical Density
540	.172
550	.155
560	.137
570	.116
580	.100
590	.087
600	.080

THE SYNTHESIS AND BIOLOGICAL PROPERTIES OF 3-(4'-DIMETHYLAMINOPHENYLAZO)PHENYL METHYL SULFIDE

by

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AN ABSTRACT OF THESIS

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Manhattan, Kansas

1961

Many compounds related to the known carcinogen, dimethylaminoazobenzene (DAB) have been used to study the effect of molecular structure on carcinogenicity. Previously, the sulfur containing dyes 4-(4'-dimethylaminophenylazo)phenyl methyl sulfide (p-S-Me-DAB) and 2-(4'-dimethylaminophenylazo)phenyl methyl sulfide (o-S-Me-DAB) have been prepared and their carcinogenicity determined. The para compound was found to be carcinogenic. In this investigation the meta isomer 3-(4'-dimethylaminophenylazo)phenyl methyl sulfide (m-S-Me-DAB) was synthesized and administered to rats to determine its hepatic carcinogenicity.

m-Aminophenyl methyl sulfide was prepared from m-nitrobenzenesulfonyl chloride by a series of reactions including reductions by hydroiodic acid, glucose, and iron powder and methylation by dimethylsulfate. The physical constants of the amine were determined and various derivatives prepared. The amine was diazotized and coupled to dimethylaniline to form the desired compound.

The azo dye was characterized and administered in the ration, ad libitum, to 19 rats at the level of .06 per cent for 16 weeks. Gross tumors were found in the livers of 16 of the rats. The dye was also shown to be bound to the liver protein.

The meta substituted dye was found to be more active as a carcinogen than either the para or ortho isomers as is generally the case.