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Studies Involving Sulfur-Containing Azo Dyes Related to Dimethylaminoazobenzene*

R. K. BURKHARD, R. D. BAUER,† AND D. H. KLAASSEN

From the Departments of Biochemistry and Chemistry, Kansas State University, Manhattan, Kansas
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Four sulfur-containing azo dyes related to dimethylaminoazobenzene—the 2-, 3-, and 4(4′-dimethylaminophenylazo)phenyl methyl sulfides and 4-phenylazophenyl methyl sulfide—were synthesized and tested in regard to their ability to induce hepatic tumor formation in the rat. Of these, only two were found to be active although all four were capable of being bound to liver protein. The nature of the bound dye resulting from the administration of one of these was studied in some detail, and it was revealed that the amount, the rate of formation, the intracellular distribution, and the mode of attachment to liver protein were comparable to those of similarly studied bound dyes. Certain of the data, however, suggested the possibility of binding through both halves of the dye molecule. An alternative method for the preparation of rat liver for subsequent bound-dye studies was developed and compared to a previously reported method. It was found that these two methods yielded bound dyes that were qualitatively similar but present in different amounts.

The induction of hepatomas in rats by the administration of azo dyes has been studied extensively for a number of years. These studies have included the relationship between molecular structure and carcinogenicity, cellular changes that accompany dye administration, metabolic fates of dyes, and possible mechanisms of carcinogenesis (Miller and Miller, 1953).

One of the attempts to relate carcinogenic activity and molecular structure has been made by Pullman (1946), who has proposed that this type of activity is associated with dyes having a critical density of electrons at the azo link. Using the Hammett substituent constant (Hammett, 1940) as a measure of this electron density, Badger and Lewis (1952) have examined the validity of this hypothesis by comparing the carcinogenic activities of certain 3′- and 4′-substituted 4-dimethylaminoazobenzenes to the appropriate Hammett substituent constants. Since the Hammett substituent constants for the *m*- and *p*-*S*-CH_3 groups are known (Hammett, 1940; Jaffe, 1953) and they fall in the critical region suggested for carcinogenic activity, it was thought that a further test of this hypothesis could be obtained from a study involving the 3- and 4(4′ - dimethylaminophenylazo) phenyl methyl sulfides (abbreviated as *m*′-*S*-Me-DAB and *p*′-*S*-Me-DAB, respectively, to indicate their relationship to dimethylaminoazobenzene, DAB). Furthermore, since slight variations in molecular structure can greatly affect the potency of a dye (Miller and Miller, 1953), and since studies involv-

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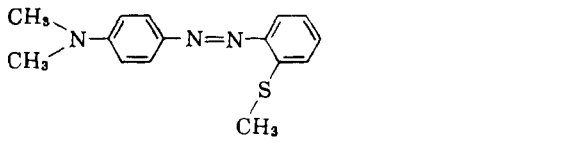
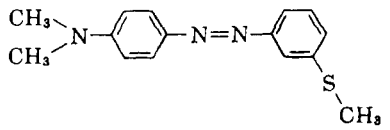
† Current address: Department of Chemistry, Long Beach State College, Long Beach, Calif.

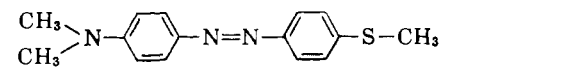
ing the *o'*-, *m'*-, and *p'*-oxygen analogues of these compounds have been reported (Miller *et al.*, 1957), it was also thought desirable to extend this study to include the 2-, 3-, and 4(4'-dimethylaminophenylazo)phenyl methyl sulfides (the *o'*-, *m'*-, and *p'*-*S*-Me-DAB's respectively) and 4-phenylazophenyl methyl sulfide (*p*-*S*-Me-AB).



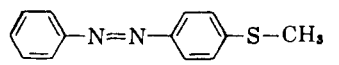
2(4'-dimethylaminophenylazo)-phenyl methyl sulfide
o'-S-Me-DAB



3(4'-dimethylaminophenylazo)phenyl methyl sulfide
m'-S-Me-DAB



4(4'-dimethylaminophenylazo)-phenyl methyl sulfide
p'-S-Me-DAB



4-phenylazophenyl methyl sulfide
p-S-Me-AB

Another problem pertaining to azo dye carcinogenesis concerns the nature of the protein-bound dye formed after dye administration. Considerable progress has been made in other laboratories regarding this, and good evidence has been presented for the involvement of the number two position of the dimethylamino portion of the dye molecule in its linkage to the protein (Nye and Luck, 1953; Rastogi *et al.*, 1956). Since all four of the dyes synthesized for this study contain the *S*-methyl group, one might wonder whether administration of this type of dye might result in the formation of a novel type of bound dye. Accordingly, a study of the nature of the bound dye resulting from administration of one of these sulfur-containing dyes was undertaken. This paper thus reports on the synthesis and biological testing of four sulfur-containing azo dyes, and on the mode of binding of one of these to rat liver proteins.

Experimental

Synthesis of Dyes.—The substituted dimethylaminoazobenzenes were all prepared by the diazotization and coupling of the appropriate amines to dimethylaniline (Giese *et al.*, 1945). *o*-Aminophenyl methyl sulfide was prepared from *o*-chloronitrobenzene by its reaction with methyl mercaptan in alkali (Hodgson and Handley, 1927) followed by reduction of the resulting *o*-nitrophenyl methyl sulfide by the use of iron in dilute acetic acid (Waldron and Reid, 1923). Synthesis of the *meta* isomer involved the conversion of *m*-nitrobenzene sulfonyl chloride to the corresponding *m*-nitro mercaptide by means of consecutive hydriodic acid and alkaline glucose reductions (Bauer and Cymerman, 1949; Claasz, 1912) followed by methylation with methyl sulfate (Gilman and Beaber, 1925) and iron reduction. *p*-Aminophenyl methyl sulfide was prepared from *p*-chloronitrobenzene by conversion to *p*-nitrothiophenol (Waldron and Reid, 1923; Price and Stacey, 1945) followed as before by methylation with methyl sulfate and iron reduction. We found, as reported earlier (Hodgson and Handley, 1927), that *p*-nitrophenyl methyl sulfide could not be prepared by the one-step method employed for the *ortho* isomer. 4-Phenylazophenyl methyl sulfide was prepared by the reaction of nitrosobenzene with *p*-aminophenyl methyl sulfide in glacial acetic acid (Mills, 1895).

The introduction of radioactivity (S^{35}) into *p*-aminophenyl methyl sulfide was achieved by the use of radioactive sodium disulfide in the conversion of *p*-chloronitrobenzene into *p*-nitrothiophenol. The preparation of radioactive sodium disulfide involved first the incorporation of labeled elemental sulfur into iron sulfide by heating in the presence of iron filings, then formation of hydrogen sulfide by the addition of acid, and finally passage of this gas into an alkaline solution of sodium disulfide.

As far as we know only two of these compounds have been mentioned previously in the literature. Fox and Pope (1912) have reported the synthesis and melting point for *p*-*S*-Me-AB (83–84°). Zincke and Müller (1913), while not reporting any details, have mentioned that *m*-aminophenyl methyl sulfide can be diazotized and coupled to dimethylaniline to yield a red crystalline substance. We found the melting points for *p*-*S*-Me-AB and for *o'*-, *m'*-, and *p'*-*S*-Me-DAB to be 80–81°, 117–119°, 73.5–74°, and 177–178.5° respectively. The empirical formula for each dye was calculated from elemental analysis data[1] and each agreed with the appropriate formula.

Our reaction sequence for the synthesis of *m*-aminophenyl methyl sulfide (*m*-APMS) differed from that used by earlier investigators, and accordingly this one intermediate was characterized by determination of its refractive index (n^{20}_D = 1.6433) and the melting points of two derivatives, *N*-acetyl-*m*-APMS (76.5–77.5°) and *m*-aminophenyl methyl sulfone (134–136°), all of which were in good agreement with previously reported values (Gilman and Martin, 1952; Zincke and Müller, 1913). In view of the small number of reported derivatives for *m*-APMS two new derivatives were prepared, *N*-benzoyl-*m*-APMS and *N*-benzenesulfonyl-*m*-APMS, which

[1] Analyses performed by Weiler and Strauss, Oxford, England.

TABLE I
WAVE LENGTHS OF MAXIMUM ABSORPTION (λ_{max}) AND CORRESPONDING MOLAR ABSORBANCY INDICES (A_m) OF DYES

Dye	Solvent			
	Alcohol		Alcohol–Conc. HCl (1:1)	
	λ_{max} (mμ)	$A_m \times 10^{-4}$	λ_{max} (mμ)	$A_m \times 10^{-4}$
p-S-Me-AB	360	1.66 ± 0.02[a]	525	4.06 ± 0.06[a]
o'-S-Me-DAB	427	2.53 ± 0.01	535	0.99 ± 0.01
m'-S-Me-DAB	414	2.82 ± 0.01	526	4.39 ± 0.03
p'-S-Me-DAB	417	3.32 ± 0.02	555	4.02 ± 0.02

[a] Mean and standard error.

were found to melt at 94–95° and 79–81° respectively.

Biological Evaluation.—Male albino rats weighing approximately 175–200 g were fed *ad libitum* either a dye-free ration (Rusch *et al.*, 1945) or a dye-containing ration for specified periods of time. Each rat also received weekly one drop of halibut liver oil. In all cases except one the amount of any dye incorporated into a dye-containing ration was 0.06%. This level of *p'*-*S*-Me-DAB was found to cause an excessive number of deaths, and accordingly the amount of this one dye was reduced to 0.03% during the first 2 weeks of feeding. Gross examinations of the rat livers were made at the end of the feeding periods to determine the extents of tumor formation.

Preparation of Liver Samples for Dye Analysis.—Blood was removed from each liver by perfusion *in situ* with cold physiologic saline while the rat was under anesthesia. The liver was then extirpated and any large tumors removed as rapidly as possible prior to freezing of the remaining tissue in acetone and dry ice. The livers were then stored in the frozen state until used. When desired these frozen livers were thawed, homogenized in a cold Waring Blendor, subjected to a 48-hour dialysis against continuously flowing distilled water at 0° with Visking Nojax regenerated cellulose casing (Visking Corporation, Chicago, Ill.), lyophilized, extracted for 48 hours with 95% alcohol in a Soxhlet extractor, and finally dried *in vacuo*.

It should be pointed out that this type of liver preparation represents the nondialyzable, alcohol-insoluble portion of rat liver and is not the same as has been used by the Wisconsin group (Miller *et al.*, 1949), which represents the trichloroacetic acid–precipitatable, alcohol-insoluble portion of rat liver. This alternative method of liver preparation was used because it was thought that it would selectively remove only the low-molecular-weight colored materials without concomitant alteration of protein-bound dyes. The period of time used in the dialysis portion of this preparative procedure was selected as a result of experimentation involving the rate at which the resistance of the dialysate from a rat liver homogenate increased. It was found that in this length of time the resistances of the dialysate and the water used for dialysis were essentially equal. The Industrial Instruments model RC-16 conductance bridge was used for all measurements of resistance. In view of the fact that this preparation differs from that used by the Millers and their co-workers it was deemed essential to compare these two preparative methods in regard to amount and type of bound dye, and to show that alteration of the bound dye did not occur to any appreciable extent during dialysis. The experiments by which these were done are outlined below.

Determination of Bound Dye.—Total bound dye was determined spectrophotometrically in the manner outlined by the Millers (1947) except that the final solvent for spectral determinations was 1:1 ethanol–concentrated HCl. All spectrophotometric determinations were made by use of either the Beckman model DU spectrophotometer or the Cary model 11 recording spectrophotometer. The absorption maxima and corresponding molar absorbancy indices for the dyes in ethanol and in 1:1 ethanol–concentrated HCl are given in Table I.

Determination of Radioactivity.—S^{35} determinations were made by use of a liquid scintillation counting technique subsequent to perchloric acid oxidation of the liver preparations as outlined by Jeffay *et al.* (1960). The Tri-Carb liquid scintillation spectrometer, model 314-DC, was used for all such determinations. Because of naturally occurring radioactivity in potassium salts, sodium salts were substituted for the corresponding potassium salts wherever analytical procedures analogous to the Millers' method for bound dye were employed.

Comparison of Liver Preparations.—Four general approaches were used to compare our liver preparation with that of the Millers and their co-workers. First, each type of preparation was analyzed for nitrogen (micro-Kjeldahl) and for amount and spectral properties of bound dye resulting from DAB administration. Secondly, after administration of radioactive *p'*-*S*-Me-DAB each type of preparation was analyzed for both total radioactivity and radioactivity that was extractable with alcohol and ether after hydrolysis. It should be noted that, although this latter determination is analogous to the colorimetric

determination of total bound dye, the measurement of radioactivity does not necessarily represent bound dye. Next, to determine whether it was likely that the amount of bound dye could be altered during a 48-hour dialysis one portion of a dye-containing homogenate (from DAB administration) was immediately prepared and analyzed for bound dye while the other portion was first kept for 48 hours at 0° before preparation and analysis. This experimental procedure does not duplicate dialysis, but it should yield meaningful data and even constitute a more stringent test for alteration of bound dye than could be accomplished by dialysis itself, since dialysis could remove certain co-factors that might be necessary for alterations of the bound dye. Finally, two experiments were performed to determine whether our procedure could cause either a loss of bound dye through dialysis or an increase due to the retention of bound dye that might not have been precipitated by the Miller procedure. Accordingly, a rat liver homogenate was dialyzed against an equal volume of water and the dialysate examined for protein. Also a filtrate from the trichloroacetic acid precipitation of rat liver protein (from DAB-treated rats) was examined for the presence of nondialyzable forms of dye. Protein detection was accomplished by the biuret test, the addition of equal volumes of 20% trichloroacetic acid or saturated picric acid, or nigrosin staining of Oxoid cellulose acetate strips (Consolidated Laboratories, Inc., Chicago Heights, Ill.) to which a very small drop of each solution had previously been applied and dried (Ortega, 1957; Kohn, 1958). For this last test similar amounts of an albumin solution (100 μg/ml) were treated in an identical manner for comparative purposes. Dye detection was accomplished by means of acidification of protein samples to be tested. For comparative purposes protein samples containing bound dye were similarly treated.

Cellular Distribution of Bound Dye.—The administration of dye for cellular distribution studies was carried out according to the procedure of Gelboin *et al.* (1958), who found that intraperitoneally administered dye gave rise during the first 24 hours to bound dye that was very similar to that formed after feeding of the dye for one or more weeks. The nuclear, mitochondrial, microsomal, and supernatant fractions of the rat liver were obtained according to the method of Schneider (1948). Prior to bound-dye analyses these fractions were subjected to the preparative procedure outlined for the liver homogenates (*i.e.*, dialysis, lyophilization, extraction, and drying).

Administration of Radioactive Preparations.—In the interest of personal safety all radioactive substances (*p'*-*S*-Me-DAB and *p*-aminophenyl methyl sulfide) were administered by intraperitoneal injection. Each rat received a dose of either 50 mg of dye or the equimolar amount of amine suspended in corn oil and was then sacrificed 24 hours later.

Reduction of Bound Dye and Characterization of Benzene-Extractable Products.—Bound dye was reduced, and the reduction mixture was neutralized, mixed with a solution of 1,2-naphthoquinone-4-sulfonate, and extracted with benzene as outlined by the Wisconsin group (Miller *et al.*, 1949). The absorption spectra of the resulting solutions and those obtained by reacting purified amines with 1,2-naphthoquinone-4-sulfonate were obtained by use of the Cary model 11 recording spectrophotometer.

The benzene-extractable reduction products and purified amines were also subjected to descending filter paper chromatography on Whatman No. 1 paper with an amine solvent suggested by Ekman (1948) consisting of methanol 40%, isoamyl alcohol 20%, benzene 20%, and water 20%. R_F' values (Evans *et al.*, 1949) were used throughout this study because measurement of the leading edge of a spot was found to be more consistent than measurement of the center of gravity of the spot. Spray application of a freshly prepared solution of 1,2-naphthoquinone-4-sulfonate (0.1% in phosphate buffer, *p*H 6.8) was used to develop the spots.

It was found by use of radioactive bound dye that it made little difference in the amount of extractable radioactivity if the reduction products were first reacted with 1,2-naphthoquinone-4-sulfonate and then extracted or simply extracted with benzene.

Results and Discussion

Biological Evaluation.—Of the four dyes studied only *m'*- and *p'*-*S*-Me-DAB induced tumor formation when tested in the manner employed. In the case of *m'*-*S*-Me-DAB at the end of 16 weeks of feeding 16 of 19 rats possessed liver tumors, and with the *para'* isomer at the end of a 20-week feeding period 13 of 16 rats possessed liver tumors. No tumors were observed in either of the two groups of 21 rats which were fed *o'*-*S*-Me-DAB or *p*-*S*-Me-AB for 23 and 25 weeks respectively.

These results are in accord with those from other laboratories which have shown that *o'*-substituted DAB's and azo dyes lacking the dimethylamino group are relatively inactive (Miller and Miller, 1953). These data are also in agreement with the suggestion of Badger and Lewis (1952) regarding activity of substituted DAB's and Hammett substituent constants, since the constants for the *m*- and *p*-*S*-CH_3 groups (0.144 and −0.047 respectively) (Hammett, 1940; Jaffe, 1953) fall into the suggested critical region (−0.2 to 0.8). Finally these data also support the suggestion of Pullman (1946) regarding activity and electron density at the azo link if one uses, as Badger and Lewis did, this substituent constant as a measure of the electron density.

Comparison of Liver Preparations.—It was found that our preparation contained less nitrogen and bound dye than did the Miller-type preparation (Table II). Furthermore, it was found that

TABLE II
ANALYSIS OF RAT LIVER PREPARATIONS

Determination	Type of Preparation	
	Burkhard	Miller
Nitrogen[a]	15.4 ± 0.3	17.0 ± 0.2
Bound dye from DAB administration[b]	0.038 ± 0.001	0.066 ± 0.001
Sulfur from *p'*-S-Me-DAB administration[c]	0.302 ± 0.008	0.367 ± 0.001
Sulfur in "bound-dye" fraction from *p'*-S-Me-DAB administration[c]	0.149 ± 0.002	0.175 ± 0.004
Benzene-extractable sulfur after reduction of "bound-dye" fraction from *p'*-S-CH_3-DAB administration[c]	0.053 ± 0.001	—
Bound dye from *p'*-S-Me-DAB administration[d]	0.046 ± 0.004	—
Sulfur from *p*-APMS administration[e]	0.052 ± 0.001	—
Sulfur in "bound-dye" fraction from *p*-APMS administration[e]	0.018 ± 0.001	—

[a] Percent nitrogen (micro-Kjeldahl) and standard error. The value reported here for the Miller-type preparation is for one prepared by our hands. The Millers report an average of 15.3 for their own preparation (Miller and Miller, 1947). [b] Bound dye expressed as net absorbancy and standard error of the solution obtained by dissolving in 5 ml of 1:1 alcohol–conc. HCl the alcohol-ether extractable material contained in the hydrolysate of 50 mg of rat liver preparation. The absorbancies of the analogous solutions from normal rat liver preparations have been subtracted from the original data. Wave length: 520 mμ; absorption cell: 1 cm. The livers used for this study were obtained 24 hours after intraperitoneal injection of DAB. [c] Sulfur expressed as microgram-atoms per gram of liver preparation and standard error. These values were obtained from appropriate calculations following measurements of radioactivity. The livers used for this study were obtained 24 hours after intraperitoneal injection of radioactive *p'*-S-Me-DAB. The "bound-dye" fraction is the alcohol-ether extractable material in the hydrolysate of a rat liver preparation and corresponds to the fraction used for colorimetric determination of bound dye. [d] Bound dye expressed as in *b* but with the radioactive liver preparation in *c*. [e] *p*-APMS represents *p*-aminophenyl methyl sulfide. Sulfur determined and expressed as in *c* but with livers obtained 24 hours after intraperitoneal injection of radioactive *p*-APMS.

although the absorption spectra of the two bound-dye preparations were almost identical (Fig. 1*A*) the spectra of the corresponding blanks differed (Fig. 1*B*) and accordingly also the corrected spectra (Fig. 1*C*). In spite of this difference it should be noted that the two bound-dye preparations absorbed maximally at the same wave length; this fact suggests that they were similar.

Secondly, the studies involving S^{35} determinations gave results similar to those above (Table II). The Miller-type preparation contained both more total radioactivity and more radioactivity extractable with alcohol and ether after hydrolysis.

Next, it was found that storage of rat liver homogenate for 48 hours at 0° did not result in an alteration in the amount of bound dye in the original homogenate. The ratio of bound dye after storage to bound dye before storage was found to be 1.02:1. This result is in accord with the observations of Mueller and Miller (1948, 1949), who have shown that only fortified rat liver homogenates metabolize dyes to any great extent and that certain of this metabolic activity is easily destroyed, even by placing the homogenate in distilled water.

Finally it was found that no or at most only a trace of protein is lost from rat liver homogenate during dialysis. Evidence for this includes the lack of a positive biuret test, lack of precipitate formation upon addition of trichloroacetic acid or picric acid solutions, and the observance of an extremely light nigrosin-stained spot when the dialysate from this homogenate was examined. Furthermore, examination of the dialyzed filtrate from the trichloroacetic acid precipitation of rat liver protein revealed that although it did contain protein there was no indication of dye associated with it. Evidence for this includes the observance of a dark spot with the nigrosin-staining technique and the lack of pink coloration when the protein contained in this dialysate was placed in an acid solution. The observation that trichloroacetic acid did not precipitate all of the protein in rat liver homogenate was expected and is in agreement with the observation of Hiller and Van Slyke (1922) that this acid is not a very effective precipitant for low molecular weight proteinaceous material. Thus, since no protein is lost during dialysis and since the addition of trichloroacetic acid does not precipitate all the protein in a rat liver homogenate, it would appear that our preparation differs principally from that of the Millers in that it contains a larger amount of relatively low molecular weight proteinaceous material. This fact, combined with the observation that the protein which escapes trichloroacetic acid precipitation is essentially dye-free, allows a plausible explanation for the observation that our preparation contains less bound dye than the Miller-type preparation in as much as this dye-free proteinaceous material could dilute the bound dye characteristic of the Miller-type preparation. That this suggestion is reasonable is indicated by

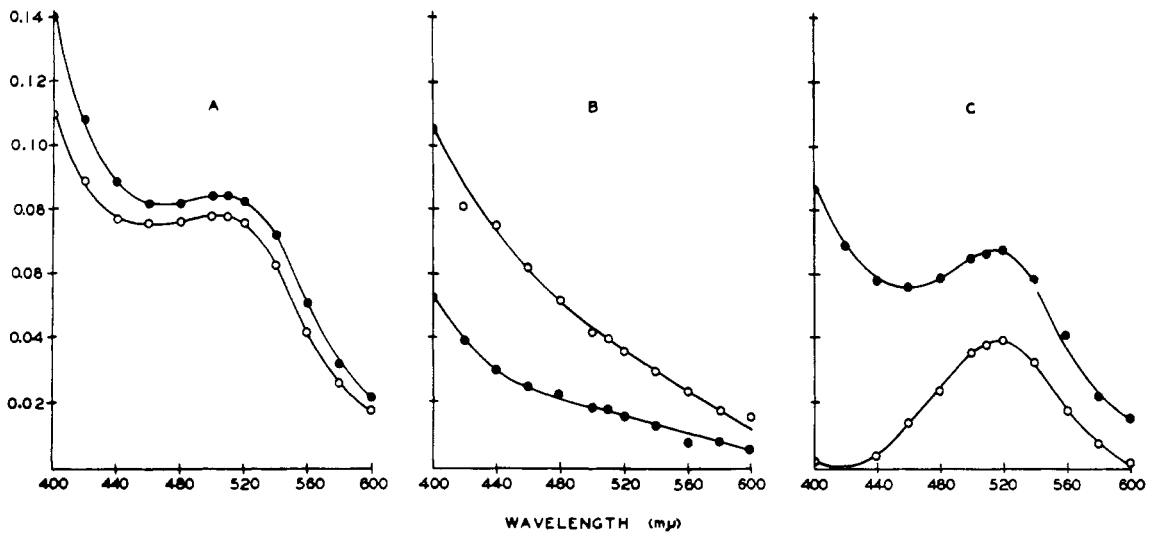


FIG. 1.—*A*, Spectra of "bound dye" fractions from Burkhard and Miller type preparations of rat liver 24 hours after intraperitoneal administration of DAB. *B*, Spectra of "bound dye" fractions from Burkhard and Miller type preparations of normal rat liver. *C*, Corrected spectra (*A* − *B*) of bound dye in Burkhard and Miller type preparations 24 hours after intraperitoneal injection of DAB. Ordinate: Absorbancy of the solution obtained by dissolving in 5 ml of 1:1 alcohol–conc. HCl the alcohol-ether extractable material contained in the hydrolysate of 50 mg of rat liver preparation. Burkhard-type preparation —O—; Miller-type preparation —●—.

the studies of Soroff *et al.* (1954), who have shown that the majority of the bound dye resulting from administration of azo dyes is associated with proteins having an average molecular weight of 50,000. Proteins of this molecular weight would undoubtedly be retained by both preparative methods but could be mixed with a larger amount of low molecular weight material in our preparation.

Nature and Distribution of Bound Dye.—Although only two of the dyes studied resulted in tumor formation it was found that all four were bound to liver proteins, as indicated by the fact that in each case upon acidification of the alcohol and ether extractable materials obtained after hydrolysis of a rat liver preparation a pink-colored solution resulted. This is in agreement with earlier work by the Millers and their coworkers, who have shown that although carcinogenic dyes are bound to rat liver proteins this alone is not a guarantee of carcinogenic activity; what is more important is the rate at which the dye is incorporated into these proteins (Miller *et al.*, 1949).

Studies involving the intracellular distribution of bound *p'*-*S*-Me-DAB showed that 24 hours after intraperitoneal injection the nuclear, mitochondrial, and supernatant fractions contained less dye than the microsomal fraction (Table III). These results are not in complete agreement with those of other workers (Price *et al.*, 1948, 1949, 1950; Hultin, 1956; Gelboin *et al.*, 1958).

The Wisconsin group was first to show that after the feeding of azo dyes the supernatant fraction contained the largest amount of bound dye. Later Hultin and still later Gelboin *et al.* showed that the intracellular distribution of bound dye changed during the first few hours following intraperitoneal administration of dye, with the largest amount in the microsomal fraction shortly after administration and in the supernatant at later times. With 3'-Me-DAB it was shown for example by the Wisconsin group that between 5 and 24 hours after administration the ratio of microsomal-bound dye to supernatant-bound dye changed from 1.5:1 to 0.9:1. In our experiment only one time interval was used (24 hours), and at this time the ratio was approximately 2:1.

Either or both of the following considerations could account for the above results. First, it might be that *p'*-*S*-Me-DAB gives rise to bound

TABLE III
BOUND DYE IN CELLULAR FRACTIONS TWENTY-FOUR HOURS AFTER ADMINISTRATION OF *p'*-S-Me-DAB

Fraction	Bound Dye[a]	Relative Amounts
Nuclear	0.097	0.6
Mitochondrial	0.105	0.7
Microsomal	0.159	1.0
Supernatant	0.081	0.5

[a] Bound dye expressed as net absorbancy of the solution obtained by dissolving in 5 ml of 1:1 alcohol-conc. HCl the alcohol-ether extractable material contained in the hydrolysate of 50 mg of rat liver fraction. The absorbancies of the analogous solutions from normal rat liver fractions have been subtracted from the original data. Wave length: 520 mμ; absorption cell: 1 cm. The livers used for this study were obtained 24 hours after intraperitoneal injection of *p'*-S-Me-DAB.

dye at a slower rate than 3'-Me-DAB, and hence 24 hours after injection the status of this bound dye might be analogous to its status in the early stages of 3'-Me-DAB binding, where the ratio of microsomal bound dye to supernatant bound dye was greater than 1:1. In this regard it was found that when fed at the same level both *p'-S*-Me-DAB and DAB gave rise to their maximal levels of bound dye in approximately 5 weeks, but at the end of 2 weeks the bound dye from *p'-S*-Me-DAB was only 51–55% of its maximal value while that from DAB was 71–84% of its maximal value. This information, along with the fact that 3'-Me-DAB is known to form bound dye more rapidly than DAB (Miller *et al.*, 1949), suggests that at a time interval greater than 24 hours different results would have been obtained (*e.g.*, the ratio of microsomal-bound dye to supernatant-bound dye might be less than 1:1). No study was made to determine whether this might be true. Secondly, one should keep in mind that our method for sample preparation differs from earlier methods and accordingly the observed differences in intracellular distribution of dye might be due to this factor. If the supernatant fraction in contrast to the microsomal fraction contains a larger amount of relatively low molecular weight, dye-free proteinaceous material, then one could also easily account for these results. No study was made to determine whether this suggestion might be true.

The absorption maxima of the Schiff bases obtained by reacting 1,2-naphthoquinone-4-sulfonate with known amines and the benzene-extractable reduction products obtained from *p'-S*-Me-DAB are listed in Table IV. Although not unequivocal, the absorption maximum associated with the bound dye reduction products suggested that *p*-aminophenyl methyl sulfide was released upon reduction. The fact that this spectrum was not too definitive suggested, moreover, that more than one reduction product was present in this system. That this was the case was shown by chromatographic separation of three benzene-extractable reduction products. Comparison of the R_F' values and colors of the three spots obtained by this separation indicated that one of these substances was *p*-aminophenyl methyl sulfide (Table IV). It thus appears that the binding of *p'-S*-Me-DAB, as has been shown earlier for DAB and 3'-Me-DAB, can involve the dimethylamino-bearing half of the dye (Nye and Luck, 1953; Rastogi *et al.*, 1956). The substances responsible for the two remaining chromatographic spots have not been identified, and their identification may necessitate our reevaluation of the nature of bound *p'-S*-Me-DAB.

TABLE IV

R_F' VALUES FOR VARIOUS SUBSTANCES; THE COLORS AND WAVE LENGTHS OF MAXIMUM ABSORPTION (λ_{max}) OF THE SCHIFF BASES FORMED BY REACTION WITH 1,2-NAPHTHOQUINONE-4-SULFONATE

Substance(s)	R_F' of Substance(s)	Color of Schiff Base(s) on Chromatogram	λ_{max} ($m\mu$) of Schiff Base(s) in Benzene
Aniline	0.86	red	450
p-Phenylene diamine	0.68	purple	540
N-Methyl-*p*-phenylene diamine	0.80	purple	570
N,N-Dimethyl-*p*-phenylene diamine	0.79	purple	585
p,p'-Diaminophenyl disulfide	0.72	red	470
p-Aminophenyl methyl sulfide	0.92	red	495
Benzene-extractable substances obtained by reduction of bound *p'*-S-Me-DAB	0.92	red	480–490
	0.72	purple	—
	0.53	purple	—
Benzene-extractable substance obtained by reduction of bound *p*-S-Me-AB	0.92	red	—

The utilization of radioactive substances permitted additional studies which it was hoped would be helpful in the elucidation of the nature of these protein-bound dyes. We dealt with only two of the various questions that such studies might answer: Does the sulfur atom of *p'-S*-Me-DAB become incorporated into liver proteins in any other forms than bound dye? Does the reduction of labeled bound dye permit the complete extraction of the radioactivity by use of benzene?

In regard to the first question, it was found that 24 hours after injection of either radioactive *p'-S*-Me-DAB or *p*-aminophenyl methyl sulfide radioactivity could be detected in both the liver homogenate preparations and the corresponding alcohol-ether extracts of their hydrolysates. However, the amount of sulfur due to dye administration was much greater than that due to amine administration, and each of these amounts was about two to three times larger than that in the corresponding extract of the hydrolysate (Table II). In view of the fact that the alcohol-ether extract mentioned above is the same as that used for the spectrophotometric determination of total bound dye, these findings lead to the conclusion that about half of the sulfur originally administered as *p'-S*-Me-DAB is incorporated into the rat liver proteins in forms other than bound dye. Furthermore, since *p*-aminophenyl methyl sulfide can give rise to bound sulfur, some of the radioactivity measured in the dye experiments could have been due to bound amine that might arise from *in vivo* reduction of the dye. It should be

It may be of some interest to note that in the case of the bound dye from *p-S*-Me-AB administration similar analysis resulted in the detection of only one spot, which again corresponded to *p*-aminophenyl methyl sulfide (Table IV). It thus appears that this dye can be bound to the rat liver protein through the unsubstituted ring of the dye.

noted that these findings are quite similar to those of Salzberg *et al.* (1951), who have shown that after alkaline hydrolysis of bound dye from labeled 3′-Me-DAB a considerable amount of radioactivity is not extractable (ca. 50% of that which was extractable).

Additional information bearing on the first question raised above was obtained by comparison of spectrophotometric and radiotracer analyses of the bound dye in a labeled liver preparation (Table II). If one assumes that the maximum molar absorbancy index for the bound dye in 1:1 ethanol-concentrated HCl is in the range of 3.0–5.0 $\times 10^4$ (see Table I), then there is between 0.09 and 0.15 μmoles of bound dye per gram of homogenate preparation. On the other hand, if one assumes that all of the radioactivity in this fraction is due to dye, then there is 0.15 μmoles of bound dye per gram of homogenate preparation. In view of the uncertainty of each assumption the agreement between these two calculations is surprisingly good, but it still seems probable that not all of the radioactivity in the bound dye fraction is truly due to bound dye. These results should be compared with those of Salzberg *et al.* (1951), who have reported that with labeled 3′-Me-DAB colorimetric and tracer techniques gave essentially the same amounts of bound dye, and finally with those of the Millers and their coworkers, who have indicated (as read from Fig. 3 in Miller *et al.*, 1949) that 1, 2, and 4 weeks after administration of DAB there are found approximately 0.10, 0.15, and 0.25 μmoles of bound dye per gram of protein.

To answer the second question raised above, radioactive bound dye was reduced with tin and HCl and after the addition of alkali the benzene extractable radioactivity was determined. It was found that approximately a third of the radioactivity in this fraction was removed by such treatment (Table II). These data appear to be unequivocal, but their interpretation unfortunately is not.

If the amount of bound dye in this radioactive sample is truly in the range estimated spectrophotometrically (0.09–0.15 μmoles per gram) and if one uses the lower of these two figures it is still possible to account for only slightly more than half of the corresponding radioactivity in the benzene extract of the bound-dye reduction mixture. Then if one assumes that the only reason for the benzene insolubility of a bound dye reduction product is its attachment to an amino acid residue, it appears likely that a portion of the bound dye is bound in some manner other than simple involvement of the dimethylamino-bearing half of the dye molecule. The binding of *p′-S*-Me-DAB solely through the other half of the dye molecule, however, is not too likely in view of the dearth of evidence for the release of *N,N*-dimethyl-*p*-phenylene diamine, *N*-methyl-*p*-phenylene diamine, or *p*-phenylene diamine upon reduction of bound dye and the fact that the bound dye from *p-S*-Me-AB administration did not release aniline upon reduction. Thus it appears reasonable, although certainly not unequivocal, to suggest that once *p′-S*-Me-DAB is bound to rat liver protein through a link involving the dimethylamino-bearing half of the molecule subsequent alteration of this bound dye might occur, resulting in the formation of a mixed type of bound dye involving both halves of the dye molecule. Further work, however, will have to be done to determine the exact nature of the bound dye resulting from *p′-S*-Me-DAB administration.

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Physical Characteristics of Proteins of the Electron Transfer System and Interpretation of the Structure of the Mitochondrion*

R. S. CRIDDLE,† R. M. BOCK, D. E. GREEN, AND H. TISDALE

From the Department of Biochemistry (R.S.C. and R.M.B.) and the Institute for Enzyme Research (D.E.G. and H.T.), University of Wisconsin, Madison 6, Wisconsin

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A protein has been isolated in large amounts from beef heart mitochondria which satisfies various criteria for a structural protein. The homogeneity of the isolated structural protein has been established by ultracentrifugal, electrophoretic, and end-group analysis. At neutral *p*H the structural protein forms a water-insoluble polymeric aggregate, but at *p*H 11, or in the presence of anionic detergents, a monomeric form of the protein can be demonstrated (m.w. 2×10^4–3×10^4). Cytochromes *a*, *b*, and c_1 show analogous behavior with respect to the polymer-monomer transition. Structural protein forms one-to-one water-soluble complexes with each of the cytochromes, which are soluble in aqueous media at *p*H 7; the identity of these complexes has been rigorously established. The hydrophobic bond is the predominant type responsible both for the polymerization phenomenon and for complex formation between the monomeric species of the structural protein and of the cytochromes. Structural protein is capable of binding phospholipid; this property is shared by the three cytochromes. The interactions between structural protein and cytochromes and between structural protein and lipid have considerable relevance to the problem of mitochondrial organization.

For many years it has been recognized that the oxidation of substrates of the citric acid cycle by molecular oxygen depends on the structural integrity of a catalytic unit constituting a multienzyme system (Green, 1956–1957). Several observations have led to the postulate that the transfer of electrons involved in this oxidation takes place within an array of oxidation-reduction enzymes that are organized not only in an enzymatic sense but also in a structural sense. The very fact of an organized system of enzymes suggests a complex in which specific chemical interactions at the molecular level stabilize the molecular array. The study of mitochondrial proteins can, therefore, yield information on both the structure of the organized enzyme system and the nature of the specific forces involved in stabilizing the system.

Inherent in the concept of the mitochondrion as an organized and structured system of enzymes has been the assumption that all the component parts (proteins, lipids, and coenzymes) fulfill some role relevant to the function of the mitochondrion (Green, 1956–1957). It was only when the program of systematic isolation of all the protein components of the mitochondrion had neared completion and when a rough balance sheet could be prepared that it became possible to put this assumption to the test. The amounts of oxidation reduction protein components in the mitochondrion were insufficient by a very large margin (they constituted no more than 25%) to account for the total protein of the mitochondrion (Green *et al.*, 1961b). This conclusion was reinforced by our observations that in the isolation of cytochromes *a*, *b*, and c_1 the principal contaminant was a colorless protein without any oxidation-reduction groups and that this protein formed such tight complexes with each of the cytochromes

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† Present address: Department of Biochemistry, University of California, Davis, Calif.