Studies on Structures of Polar Dyes Derived from the Liver Proteins of Rats Fed *N*-Methyl-4-aminoazobenzene. II. Identity of Synthetic 3-(Homocystein-*S*-yl)-*N*-methyl-4-aminoazobenzene with the Major Polar Dye P2b*

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ABSTRACT: The protein-bound dyes formed in the livers of rats fed the hepatocarcinogen *N*-methyl-4-aminoazobenzene were hydrolyzed to form a series of polar dyes. Thin-layer chromatography on cellulose revealed the presence of seven dye-containing components. The major polar dye, P2b, was isolated in pure form by repeated thin-layer chromatography on cellulose, elution with water, and extraction into 1-butanol. In all respects tested (infrared and electronic spectra; thin-layer chromatography on cellulose and silica in four solvent systems before and after chemical modification by methylation, oxidation, or both; and pK_a value), P2b was identical with synthetic 3-(homocystein-*S*-yl)-*N*-methyl-4-aminoazobenzene. The latter dye was synthesized by treating 3-iodo-*N*-methyl-4-aminoazobenzene in hot alkaline solution with DL-homocysteine thiolactone. 3-Iodo-*N*-methyl-4-aminoazobenzene was prepared by the iodination of *N*-methyl-4-aminoazobenzene with iodine monochloride or by six conventional steps from *o*-iodoaniline.

Hepatic protein-bound dyes and nucleic acid bound derivatives are formed in the rat from the hepatocarcinogen, *N*,*N*-dimethyl-4-aminoazobenzene (DAB),[1] and its equally carcinogenic metabolite, *N*-methyl-4-aminoazobenzene (MAB). These partially characterized bound metabolites may play roles in hepatocarcinogenesis by these dyes (Miller and Miller, 1953, 1966; Roberts and Warwick, 1966; Dingman and Sporn, 1967). Hydrolysis of hot ethanol-denatured liver proteins of rats fed dye has yielded four alkali-stable polar dye fractions separable by chromatography (P1, P2a, P2b, and P3) (Terayama and Takeuchi, 1962; Higashinakagawa *et al.*, 1966). These polar dyes contain a secondary aromatic amino group and are ninhydrin positive. Terayama and his associates (Kusama and Terayama, 1957; Terayama *et al.*, 1958, 1959; Terayama and Takeuchi, 1962) have suggested a variety of structures for these polar dyes, generally with substitution in the aromatic 3 position (*ortho* to the amino group) of the aminoazo dye component. Recent efforts to characterize the protein-bound dyes have implicated methionyl residues as sites of binding. This was first suggested by the finding that the nonpolar dye 3-methylmercapto-*N*-methyl-4-aminoazobenzene is released by alkali at room temperature from the extracted but nonheated liver proteins of rats fed MAB or DAB (Scribner *et al.*, 1965). This dye presumably arises from a sulfonium dye derivative of protein-bound methionyl residues. Subsequently, Higashinakagawa *et al.* (1966) detected sulfur in each of the polar dyes and Terayama (1967) noted that each polar dye yielded α-aminobutyric acid after treatment with Raney nickel. In the latter reports Terayama and his associates abandoned the concept that the polar dyes contain MAB substituted in the 3 position and presented preliminary evidence in favor of structures in which MAB is attached through a methylene bridge, derived from the *N*-methyl group, to the sulfur of methionine (or derivative polar groups). However, data obtained later with isotopic MAB containing *N*-methyl groups labeled with ^{14}C and ^{3}H showed that each polar dye contains an intact *N*-methyl group derived from the MAB (Lin *et al.*, 1967).

In the present study we report the synthesis of 3-(homocystein-*S*-yl)-MAB and its identity with the major polar dye P2b. Thin-layer chromatography revealed new components of the polar dye fraction.

Experimental Section

Spectrophotometric Methods. The electronic spectra of the dyes were determined in the appropriate solvents in a Beckman DB spectrophotometer equipped with a Sargent SR recorder. The infrared spectra of the synthetic compounds were determined in a Beckman IR-10 instrument with 0.5-in. diameter KBr disks pressed from an intimate mixture of 1 mg of compound with 150 mg

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: DAB, *N*,*N*-dimethyl-4-aminoazobenzene; MAB, *N*-methyl-4-aminoazobenzene; DMSO-d_6, deuterated dimethyl sulfoxide.

TABLE I: Chromatography of Polar Dyes Derived from Liver Protein of Rats Fed *N*-Methyl-4-aminoazobenzene.

On Paper [a,b]		On Cellulose Thin Layer[b]			
Fraction	R_F	Fraction	R_F	Color with HCl Vapor[c]	% of Total[d]
P2b	0.34	P2b	0.20	Pink-red	55
P2a	0.63[a]	P1a	0.27	Violet-red	15
P1	0.55[a]	P1b	0.34[e]	Red	17
		P1c	0.35[e]	Orange-red	
P3	0.75	P3a	0.52	Red	13
		P3b	0.61	Red	
		P3c	0.80	Red	

[a] Nomenclature of Terayama (1967) and Higashinakagawa *et al.* (1966). Components P2a and P1 appear as two indistinctly separated bands on paper but on thin layers of cellulose both bands contain P1a, P1b, and P1c. [b] By ascension on Whatman No. 1 paper or on thin layers of Brinkman MN 300 cellulose with the aqueous phase of 1-propanol–1-butanol–water (1:4:5, v/v). [c] Tests made on the thin-layer plate. [d] Calculated from the absorbance at 520 mμ in 5 N HCl after elution with methanol–water (1:1, v/v). These polar dyes had been stored at 4° for 4 weeks before analysis. The freshly prepared polar dyes contain negligible amounts of the P3 fractions while aged preparations contain increased amounts of these fractions and diminished amounts of P2b. [e] Components P1b and P1c are easily separated (R_F 0.17 and 0.22, respectively) on cellulose thin layers with the aqueous phase of 95% ethanol–1-butanol–water (1:2:7, v/v).

of KBr (IR grade) in an evacuated Beckman No. 5020 die. The infrared spectra of the polar dyes were determined in the same instrument equipped with a Beckman No. 46075 beam condenser. The purified dyes (50–100 μg) were deposited in the tip of a pear-shaped flask and the dye residue was rubbed with 40 mg of KBr. The dye-KBr mixture was pressed for 10 min into a 5-mm diameter disk in a Carle No. 4019 die and press (Carle Instruments, Anaheim, Calif.). The stainless-steel ring holding the disk was pressed into a hole of the same diameter in a piece of magnetic rubber and the disk was centered flush with the slit in a Beckman micropellet holder No. 24497. This holder was mounted in the beam condenser.

The dissociation constant, pK_a, was determined from absorbances measured in a Zeiss PMQ II spectrophotometer. The concentration of the dyes for this determination was between 1 and 5×10^{-5} M and the absorbance at 400 mμ was chosen as a measure of the concentration of the nonprotonated dyes (Hanaki and Terayama, 1962). Similarly, the polar dyes were considered to exist in the completely protonated and nonprotonated forms in 5 N HCl in water and in pH 4.7, 0.05 M acetate buffer in water, respectively. The absorbance, A, was measured at various pH values, pH_M, close to the pK_a value and the pK_a was calculated from the relationship of Flexser *et al.* (1935): $pK_a = pH_M + \log [(A_B - A_M)/(A_M - A_{BH^+})]$, where A_B and A_{BH^+} are the absorbances of the base (at pH 4.7) and salt (in 5 N HCl), respectively, and A_M is the absorbance of a mixture of base and salt at the intermediate pH, pH_M.

Isolation of Polar Dyes. Modifications of previous procedures (Miller and Miller, 1947; Higashinakagawa *et al.*, 1966) were employed in the preparation of the polar dyes. Male rats (Holtzman Rat Co., Madison, Wis.) weighing 160–200 g were fed a purified diet (Andersen *et al.*, 1964) low in riboflavin (1 mg/kg of diet) for 1 week. At this time each rat received by stomach tube 25 mg of MAB (Miller and Baumann, 1945) dissolved in 1 ml of corn oil. The rats were killed by decapitation 28–30 hr later. The livers were removed, pooled, and homogenized with an equal weight of water in a Waring Blendor. The homogenate was immediately mixed with ten volumes of 95% ethanol and the mixture was heated with stirring to 60° on a steam bath for 1 hr. After cooling, the precipitated crude liver protein was centrifuged and washed twice with ten volumes of absolute ethanol and then with the same volume of acetone. The defatted precipitate was dried in a desiccator at reduced pressure with continuous suction. The dry material was then homogenized in the blender with ten volumes each of 0.1 M sodium acetate and 0.1 M sodium bicarbonate in water. This suspension was heated to 80° for 1 hr on a steam bath. After cooling, 0.25% Pronase (Calbiochem, Los Angeles, Calif.) was added and the mixture was incubated at 37° for 60 hr in the presence of toluene and chloroform. Trace amounts of nonpolar dye were removed by extraction of the enzyme hydrolysate with five volumes of benzene. The polar dyes were obtained by two extractions with two volumes of 1-butanol. After removal of the butanol at 45–50° in a rotary flash evaporator, the polar dyes were dissolved in 3 N HCl in water. This pink acidic solution was then extracted twice with two volumes of ethyl acetate to remove impurities. After neutralization of the bright pink aqueous solution with solid sodium bicarbonate, the polar dyes were again extracted into 1-butanol, the

solvent was removed under reduced pressure, and the dye residue was dissolved in 3.5 N KOH in 1:1 methanol–water by volume. This mixture was refluxed at 90 ± 3° for 20 hr. This alkaline hydrolysate was neutralized with concentrated HCl and the polar dyes were extracted into 1-butanol as before. After removal of the butanol under reduced pressure the polar dyes were dissolved in a minimal volume of acetone–methanol (95:5, v/v). This solution was passed through a column of silica (G. Frederick Smith Chemical Co., Columbus, Ohio, 50–200 mesh) previously equilibrated with acetone. A column 20 cm long and 1.6 cm in diameter was used for the extract from 600 g of fresh rat liver. The column was washed thoroughly with acetone to remove traces of nonpolar dyes before the adsorbed polar dyes were eluted with pure methanol.

After removal of the methanol under reduced pressure concentrated solutions of the dyes in methanol were streaked on thin-layer plates of cellulose (Brinkman MN 300). The plates were chromatographed with the aqueous phase of the solvent system described in Table I (footnote *b*).

Chromatography of the polar dyes on thin layers of cellulose revealed at least seven components (Table I) compared with the four components (P1, P2a, P2b, and P3) noted on paper in this and previous work (Higashinakagawa *et al.*, 1966; Lin *et al.*, 1967) with the same solvent system. P2a appears to consist of three components also found among the three components noted in P1. Component P3 also appears to contain three components and these subfractions of P1 and P3 are designated by the letters a, b, and c. Fresh P2b forms a single zone on paper and on cellulose thin layers.

Several of the chromatographic fractions, particularly the major polar dye P2b, were further purified for spectrophotometric measurements. Component P2b first was rechromatographed on thin layers of cellulose three times as described above. Direct elution of this dye from the cellulose with organic solvents gave solutions with excessive absorption in the ultraviolet and infrared regions. This interference was avoided by first eluting the polar dye from the cellulose with distilled water and then extracting the dye from this eluate with 1-butanol. After removal of the butanol *in vacuo*, the dye was transferred into a small pear-shaped flask with small volumes of ethyl acetate. The solvent was removed carefully with a stream of nitrogen gas.

Synthesis of 3-(Homocystein-S-yl)-N-methyl-4-aminoazobenzene and Precursor Compounds (Figure 1). The melting points were determined in a Fisher-Johns apparatus and are uncorrected. Elementary analyses were made by Huffman Laboratories, Wheatridge, Colo.

N-Acetyl-o-iodoaniline (II). *o*-Iodoaniline (2 g) (Eastman Organic Chemicals, Rochester, N. Y.) was dissolved in 10 ml of acetic anhydride. Considerable heat was evolved and the mixture was gently refluxed for 20 min on a steam bath. Crystallization occurred on cooling. The crystalline needles were filtered and washed with water; upon drying a quantitative yield (2.4 g) of product resulted with a melting point of 106–107°. (Doht (1904) obtained mp 109–110°.)

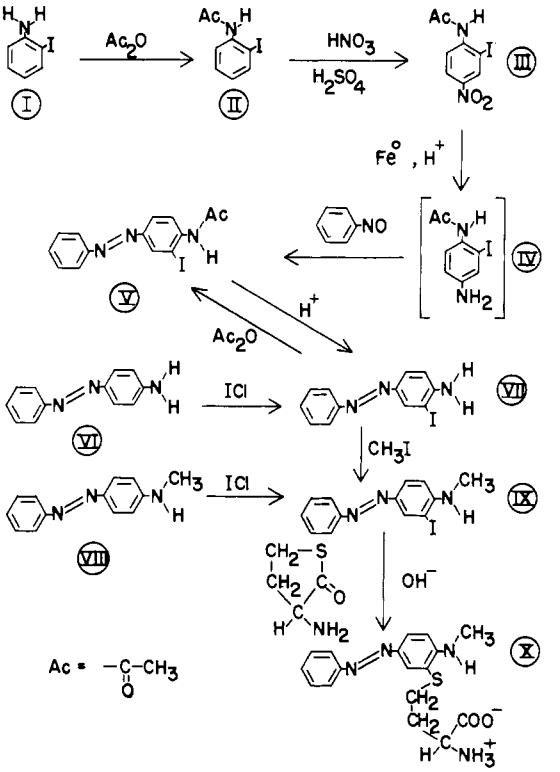


FIGURE 1: Syntheses of 3-iodo-*N*-methyl-4-aminoazobenzene (IX) and 3-(homocystein-*S*-yl)-*N*-methyl-4-aminoazobenzene (X).

N-Acetyl-p-nitro-o-iodoaniline (III). A solution of nitric acid (12 ml, density 1.5) in sulfuric acid (12 ml of concentrated) was added dropwise with vigorous stirring to a solution of 7.5 g of II in sulfuric acid (20 ml of concentrated) maintained at 5 ± 2°. The reaction mixture was allowed to warm nearly to room temperature for 30 min and it was then poured into 500 ml of a 1:1 ice–water mixture with vigorous stirring. After 5 hr the crystalline solid was filtered and recrystallized from ethanol (6.7 g, 72% yield, mp 120°). For identification a solution of the product (2 g) in 20 ml of ethanol was mixed with 4 ml of concentrated HCl and the mixture was refluxed on a steam bath for 1 hr. The reaction mixture was poured into a ice–water mixture and the solution was neutralized with NaOH solution. The crystalline precipitate was filtered and recrystallized from ethanol to give yellow needles (1.0 g) with a melting point of 101–103°. (Michael and Norton (1878) report a melting point of 105° for *p*-nitro-*o*-iodoaniline.)

N-Acetyl-3-iodo-4-aminoazobenzene (V). A. FROM III. Iron powder (10 g) was added slowly to a refluxing solution of III (6.3 g) in 0.35 N HCl (40 ml) and ethanol (120 ml). The mixture was refluxed for 1 hr, cooled, and made alkaline with solid sodium bicarbonate (11 g). The mixture was filtered and the clear filtrate was concentrated *in vacuo* to 60 ml. This solution was extracted with chloroform and after removal of the chloroform a brown-yellow residue of *N*-acetyl-*p*-amino-*o*-iodoaniline (IV) remained which was used in the following step without further purification. The residue of IV was dissolved in 20 ml of glacial acetic acid and this solution

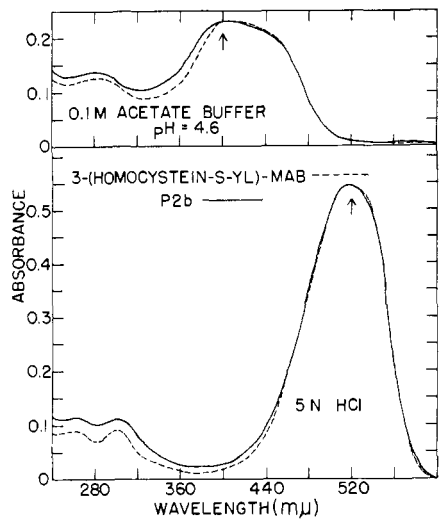


FIGURE 2: Electronic absorption spectra of the major polar dye P2b and 3-(homocystein-*S*-yl)-MAB in aqueous media. The spectra were arbitrarily matched at the wavelengths indicated by the arrows.

was added to a solution of nitrosobenzene (6 g) in 60 ml of glacial acetic acid. The coupling mixture was allowed to stand at room temperature (25°) for 48 hr. After this period the mixture was distilled *in vacuo* in a rotary flash evaporator to remove the glacial acetic acid and the excess nitrosobenzene. The moist yellow residue was mixed with 100 ml of absolute ethanol in the distillation flask and the mixture was distilled as before; this procedure was repeated with two more portions of ethanol. The final residue was extracted three times with *n*-hexane (Skelly B). The combined extracts were evaporated in the hood under a stream of nitrogen to give a yellow crystalline precipitate of V (5.2 g, over-all yield of 69%). Recrystallization from ethanol gave orange needles with a melting point of 165–166°.

Anal. Calcd for $C_{14}H_{12}IN_3O$: C, 46.05; H, 3.32; I, 34.75; N, 11.52; O, 4.38. Found: C, 46.23; H, 3.48; I, 34.08; N, 11.34; O, 4.90.

The positions and intensities of the absorption maxima in the infrared spectrum of V in KBr were: 3260 m, 1660 s, 1585 m, 1568 w, 1520 s, 1510 s, 1435 w, 1380 s, 1310 s, 1292 s, 1275 m, 1245 w, 1200 m, 1160 m, 1069 w, 1029 m, 965 w, 915 m, 882 m, 828 s, 762 s, 715 w, 682 s, 605 m, 575 m, 540 w, and 440 w cm^{-1}.

B. From iodination of 4-aminoazobenzene (VI). All solutions used in this preparation were precooled to 0–3°. A solution of 1.97 g (0.01 mole) of VI (Eastman Organic Chemicals) in 20 ml of ethyl ether and 20 ml of glacial acetic acid was added to a solution of anhydrous sodium acetate (1.20 g) in 10 ml of glacial acetic acid. This mixture was then mixed with a solution of iodine monochloride (1.59 g, 0.01 mole) in 10 ml of glacial acetic acid and 10 ml of ethyl ether. This reaction mixture was stored overnight in the dark at 4°. After this time it was taken to dryness *in vacuo* in a rotary flash evaporator at 40°. The residue was repeatedly extracted with *n*-hexane and the combined extracts were evaporated to give a dark orange oily residue of crude 3-iodo-4-aminoazobenzene (VII). Pure VII can be obtained from this residue by chromatographing it on alumina columns in *n*-hexane–benzene mixtures; under these conditions compound VII is eluted ahead of VI. Compound V can also be prepared readily from the crude VII. Acetic anhydride (20 ml) was added to the crude VII and the mixture was allowed to stand at room temperature for 20 hr. The crystalline solid that formed was filtered and washed with water to give orange needles of V (2.3 g, 72%). After crystallization from ethanol the compound melted at 165–166° and gave no depression of melting point when admixed with the preparation of V from A above.

Anal. Calcd for $C_{14}H_{12}IN_3O$: C, 46.05; H, 3.32; I, 34.75; N, 11.52; O, 4.38. Found: C, 46.36; H, 3.51; I, 34.77; N, 11.64; O, 5.01.

The infrared spectrum of this preparation was an exact match of the spectrum of V prepared in A above.

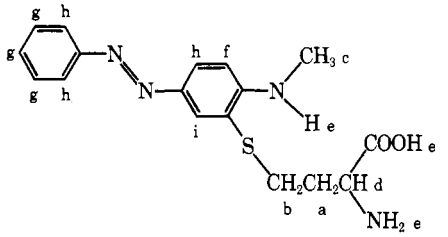
3-Iodo-N-methyl-4-aminoazobenzene (IX). A. From iodination of VIII, *N*-methyl-4-aminoazobenzene. All solutions used in the following preparation were precooled to 0–3°. A solution of 4.2 g (0.02 mole) of VIII in 80 ml of ethyl ether and 80 ml of glacial acetic acid was mixed with a solution of anhydrous sodium acetate (2.8 g) in 40 ml of glacial acetic acid. This mixture was then mixed with a solution of iodine monochloride (3.6 g, 0.022 mole) in 40 ml of glacial acetic acid and 40 ml of ethyl ether. The reaction mixture was allowed to stand with occasional shaking at 4° for 18 hr. After this time 100 ml of benzene and 560 ml of water were added and the mixture was extracted twice with 500-ml portions of *n*-hexane. The hexane extracts were combined and washed with 300 ml of 2 N NaOH and then twice with 200-ml portions of water. The hexane extract was dried by passing it through a bed of large granules of alumina. After removal of the hexane *in vacuo*, the dark orange residue was dissolved in a minimal volume of benzene and the solution was chromatographed on a column of alumina. The column was eluted with *n*-hexane and the first colored band was collected. After removal of the solvent orange needles of IX formed (5.9 g, yield 90%, mp 82–83°).

Anal. Calcd for $C_{13}H_{12}IN_3$: C, 46.30; H, 3.59; I, 37.63; N, 12.47. Found: C, 46.43; H, 4.07; I, 37.04; N, 12.84.

The positions and intensities of the absorption maxima in the infrared spectrum of IX in KBr were: 3410 m, 2900 w, 1588 s, 1510 m, 1460 m, 1420 m, 1325 m, 1290 w, 1254 m, 1204 m, 1175 w, 1158 s, 1138 s, 1065 w, 1010 m, 930 w, 910 w, 888 m, 842 w, 798 s, 765 s, 705 m, 684 s, 656 w, 550 m, 492 w, and 450 w cm^{-1}.

B. From methylation of 3-iodo-4-aminoazobenzene (VII). A solution of VII (9.6 mmoles) in methanol (30 ml) was mixed with 0.6 ml (9 mmoles) of methyl iodide in an ice bath. This mixture was apportioned and sealed in six glass tubes. The tubes were heated at 60 ± 3° for 16 hr. The contents of the tubes were pooled and neutralized with concentrated ammonium hydroxide. After removal of the solvent *in vacuo* the residue was chromatographed as described above in A. The orange needles of IX (0.66 g, yield 20%, mp 81–82°) thus obtained gave no melting point depression when mixed

TABLE II: Nuclear Magnetic Resonance Spectrum of 3-(Homocystein-*S*-yl)-*N*-methyl-4-aminoazobenzene in DMSO-d_6.[a]



Chemical Shift (ppm[b])	Integrated Area (mm^2)	No. of Protons	Proton Assignment
2.10	16	2	a
2.99, 3.07	38	5	b, c
3.50	8	1	d
4.50–6.68	32	4	e
7.00	13[c]	(1)	f
7.80	25	3	g
8.02	22	3	h
8.22	9	1	i

[a] Taken by Simon Research Laboratory, Elgin, Ill. 60122. [b] With tetramethylsilane as an internal reference. [c] The high area of this proton peak could not be accounted for by known possible impurities such as MAB.

with the preparation from A above. This preparation also gave an infrared spectrum in KBr that was identical with that obtained above in A.

DL-3-(*Homocystein-S-yl*)-*N-methyl-4-aminoazobenzene* (*X*). A solution of IX (1.56 g) in methanol (91 ml) was mixed with 3.12 g of DL-homocysteine thiolactone hydrochloride (Aldrich Chemical Co., Milwaukee, Wis.) and 9.1 ml of 11 N KOH in water. This mixture was flushed with nitrogen gas and apportioned equally among 13 thick-walled glass tubes. The tubes were frozen in liquid nitrogen and sealed *in vacuo*. The sealed tubes were heated at 120 ± 3° for 48 hr in iron protector tubes. The tubes were opened after being frozen in liquid nitrogen. The contents of the tubes were pooled and the solvents were removed *in vacuo*. The residue was mixed with 180 ml of water and the mixture was heated on the steam bath for 10 min. A small amount of orange precipitate formed and was removed by filtration. The orange aqueous filtrate was extracted twice with 400-ml portions of a 1:1 mixture of benzene–*n*-hexane and then twice with similar portions of *n*-hexane alone. Concentrated hydrochloric acid was added to the clear orange solution until the pH was 6.0. At this point the color of the solution turned to a bright yellow and the solution became cloudy. This mixture was allowed to stand at 0° for 2 hr. At this time the yellow crystalline precipitate was filtered, washed with 3 ml of cold water and 30 ml of *n*-hexane, and dried

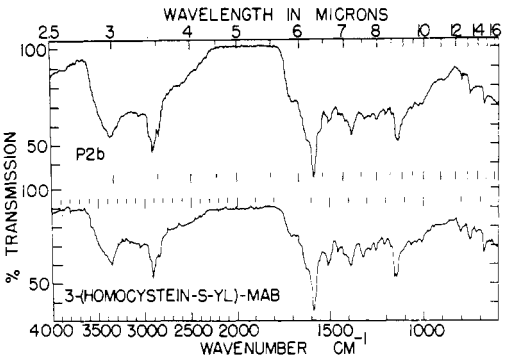


FIGURE 3: Infrared absorption spectra of the major polar dye P2b and 3-(homocystein-*S*-yl)-MAB in KBr. Approximately 100 μg of each dye was chromatographed on a thin layer of cellulose and eluted and prepared for analysis as described in the text. Beckman IR-10 spectrophotometer with beam condenser, slow scan, and gain set at 7.

(170 mg, 11% yield). After recrystallization from methanol–water mixtures golden yellow crystals were obtained that softened and decomposed at about 225°.

Anal. Calcd for $C_{17}H_{20}N_4O_2S$: C, 59.29; H, 5.85; N, 16.27; O, 9.29; S, 9.29. Found: (1) C, 59.04; H, 5.80; N, 15.42; O, 9.39; S, 9.36. (2) C, 59.27; H, 5.76; N, 16.05.

It was necessary to transfer and weigh this hygroscopic substance (previously dried *in vacuo* at 60° over P_2O_5) in dry nitrogen for these analyses; otherwise the results (note first nitrogen analysis) tended to be low. Determination of the molecular weight of X in camphor solutions by the Rast method gave values in agreement with the empirical formula calculated from the elementary analyses. Compound X formed yellow solutions in water and it was ninhydrin positive. The electronic absorption spectra of X (Figure 2) were those of a typical *p*-aminoazo dye in acidic and neutral solutions (Miller *et al.*, 1948). In 95% ethanol its molar absorptivity was 2.2×10^4 at 400 mμ; in 4.2 N HCl in 50% ethanol in water this value was 5.3×10^4 at 520 mμ. Compound X behaved as a secondary aromatic amine in tests with HNO_2 (Terayama and Takeuchi, 1962) and as shown from the single absorption band at 3360 cm^{-1} (Figure 3). The correspondence of the infrared spectrum of X (Figure 3) with that of 3-methylmercapto-MAB (Scribner *et al.*, 1965) in the aromatic ring-substitution region of 600–900 cm^{-1} confirmed the position of the polar group in X. The above data and the nuclear magnetic resonance spectrum of X (Table II) confirmed that it was 3-(homocystein-*S*-yl)-*N*-methyl-4-aminoazobenzene.

Identity of the Major Polar Dye P2b with 3-(Homocystein-S-yl)-MAB. The identity of the major polar dye P2b derived from the liver proteins of rats fed MAB with the 3-(homocystein-*S*-yl) derivative of MAB was determined in several ways. The most definitive structural comparison of the two dyes was obtained with their infrared spectra (Figure 3) in which exact correspondence of the absorption bands was noted over the entire observed region of 600–4000 cm^{-1}. Similarly, the electronic spectra of the two dyes from 240 to 600 mμ (Figure 2) in both neutral and acidic solutions cor-

TABLE III: Chromatographic Comparisons of 3-(Homocystein-*S*-yl)-*N*-methyl-4-aminoazobenzene (X) and Polar Dye P2b and Their Derivatives.

Compound[a]	R_F on Thin Layers of Cellulose[b] A[c]	B[c]	C[c]	Silica[b] D[c]	Ninhydrin Test[d]	Color in HCl Vapor[e]
X	0.40	0.20	0.42	0.38	+	Pink-red
P2b	0.40	0.20	0.42	0.38	+	Pink-red
Methylated X[f]	0.55	0.37	0.62	0.13	−	Pink-red
Methylated P2b[f]	0.55	0.37	0.62	0.13	−	Pink-red
Oxidized X[g]	0.55	0.35	0.56	0.31	+	Orange-red
Oxidized P2b[g]	0.55	0.35	0.56	0.31	+	Orange-red
Methylated, oxidized X[f,g]		0.54	0.73		−	Orange-red
Methylated, oxidized P2b[f,g]		0.54	0.73		−	Orange-red

[a] All compounds acted as secondary aromatic amines in the HNO_2 test of Terayama and Takeuchi (1962). [b] Brinkman MN 300 cellulose and silica gel, HF_{254}. [c] Aqueous phase of solvents (A) 1-propanol–1-butanol–water (1:2:7, v/v), (B) 1-propanol–1-butanol–water (1:4:5, v/v), and (C) 95% ethanol–1-butanol–water (1:2:7, v/v). (D) Organic phase of solvent B. [d] Plate sprayed with ninhydrin 3 (Sigma Chemical Co.) and heated at 110° for 10 min. [e] Test made on plate. [f] Methyl sulfate (0.5 ml) was added dropwise to an aqueous solution of dye while the mixture was heated on a steam bath. When a pink color developed 2 N NaOH was added until the yellow color returned. This process was repeated until the methyl sulfate was consumed. The methylated product was extracted with 1-butanol. [g] The dye was dissolved in 50% methanol containing 1% hydrogen peroxide and the mixture was allowed to stand at room temperature for 20 hr. The oxidized product was extracted with 1-butanol.

responded almost exactly with each other. Some extra absorption of light below 400 mμ was noted in the preparation of P2b but this impurity did not alter the general character of the absorption spectrum in this region. Further evidence of identity was noted in the thin-layer chromatography of P2b and 3-(homocystein-*S*-yl)-MAB, or their methylation, oxidation, or methylated oxidation products (Table III). The parent dyes or their derivatives could not be separated in four solvent systems, three on cellulose, and one on silica. Finally, corresponding pK_a values of 2.20 and 2.25 were obtained for the synthetic dye and P2b, respectively, in aqueous solution.

Discussion

The data in this report establish that the major polar dye P2b derived from the liver proteins of rats fed MAB is 3-(homocystein-*S*-yl)-MAB. The protein-bound sulfonium dye, 3-(methion-*S*-yl)-MAB, appears to be the most probable precursor of both this polar dye and the nonpolar dye, 3-methylmercapto-MAB (Scribner *et al.*, 1965), that are obtained by hot alkaline hydrolysis and cold alkaline treatment of the liver proteins, respectively. Both of these modes of breakdown of the protein-bound sulfonium dye probably occur *in vivo*. The level of the protein-bound sulfonium dye formed *in vivo*, as measured by the release of 3-methylmercapto-MAB by alkali, is maintained at a low level of approximately 0.5 μg/g of fresh liver (Scribner *et al.*, 1965; C. Whelan, E. C. Miller, and J. A. Miller, unpublished data) during the feeding of MAB to rats for 6 weeks. Under the same conditions, the amount of the polar dyes obtained by alkaline hydrolysis of the liver proteins exceeds the level of the sulfonium protein-bound dye after the first few days of dye feeding and increases to a maximum of up to 5 μg/g of fresh liver at about 1 month (Miller and Miller, 1953). Thus, the latter data point to S demethylation *in vivo* of the protein-bound sulfonium dye. Alternatively, loss of the amino acid side chain of the sulfonium dye by nucleophilic attack probably also occurs *in vivo* to yield free 3-methylmercapto-MAB and peptide-bound homoseryl residues, possibly with cleavage of the peptide chain. This mode of breakdown seems likely from the decomposition at neutral pH of the sulfonium reaction products of *N*-benzoyloxy-MAB and methionine or methionylglycine to form 3-methylmercapto-MAB and homoserine lactone or homoserine lactone plus glycine, respectively (Lotlikar *et al.*, 1966; Poirier *et al.*, 1967). The latter studies and closely related studies with the carcinogen, 2-acetylaminofluorene, and its derivatives (Lotlikar *et al.*, 1966; DeBaun *et al.*, 1967; Miller and Miller, 1968) point to esters of *N*-hydroxy-MAB as the reactive metabolites of MAB that give rise to the hepatic protein-bound dyes in the rat.

The findings that the polar dyes P1, P2a, and P3 obtained by paper chromatography contain sulfur and yield α-aminobutyric acid with Raney nickel (Higashinakagawa *et al.*, 1966; Terayama, 1967) indicate that they include derivatives of 3-(homocystein-*S*-yl)-MAB, possibly by oxidation of this dye. However, since these dyes actually contain at least six components it is possible in addition that other amino acid residues in proteins are represented in these fractions. This is strongly

suggested by the reactivity of the carcinogenic synthetic ester, *N*-benzoyloxy-MAB (Poirier *et al.*, 1967). This ester not only reacts with methionine *in vitro* at pH 7, but it also reacts under these conditions with tryptophan, tyrosine, and cysteine to form polar dyes. Thus, the hepatic protein-bound dyes formed *in vivo* from MAB may also involve these amino acids. Studies on these possibilities are in progress in this laboratory.

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