# Studies on Structures of Polar Dyes Derived from the Liver Proteins of Rats Fed *N*-Methyl-4-aminoazobenzene. III. Tyrosine and Homocysteine Sulfoxide Polar Dyes*

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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 give polar dyes separable by chromatography on silica and thin layers of cellulose. The polar dyes P1a, P1b, and P1c were characterized in this study. The synthetic ester *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene reacted at pH 7 with tyrosine to form two polar dyes which were identical with P1a and P1b in all respects tested (electronic spectra in acetate buffer and 6 N HCl; thin-layer chromatography on cellulose in a basic, an acidic, and two neutral solvent systems before and after methylation of the dyes; and tests with ninhydrin, nitrous acid, and phenol reagent). Through a variety of reactions the tyrosine polar dyes were provisionally characterized as *N*-(3-tyrosyl)-*N*-methyl-4-aminoazobenzene (P1a) and 3-(3-tyrosyl)-*N*-methyl-4-aminoazobenzene (P1b). Synthetic 3-(homocystein-*S*-yl)-*N*-methyl-4-aminoazobenzene, previously proved to be identical with the major polar dye P2b, was easily oxidized by $H_2O_2$ to a sulfoxide that was identical with the minor polar dye P1c. The four dyes P2b, P1a, P1b, and P1c accounted for 90% of the polar dyes obtained by the successive enzymatic and hot alkaline hydrolysis of the hepatic protein-bound dyes.

Neither the polar dye derived from the reaction of tryptophan with *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene nor radioactivity was detected among the polar dyes derived from the liver proteins of rats fed *N*-methyl-4-aminoazobenzene and tryptophan-2′,3′-$^3$H. *N*-Benzoyloxy-*N*-methyl-4-aminoazobenzene reacted with cysteine to form a polar dye that was destroyed completely by the hot alkaline hydrolysis used in the isolation of the polar dyes from the liver proteins.

*N*-Methyl-4-aminoazobenzene,[1] a hepatocarcinogen in the rat, is converted in this species into metabolites which react with hepatic proteins and nucleic acids to form covalently bound derivatives. These reactions and macromolecular bound forms may participate in the carcinogenic action of MAB and related dyes in the rat liver (Miller and Miller, 1953, 1966; Roberts and Warwick, 1966; Dingman and Sporn, 1967). The successive enzymatic and alkaline hydrolysis of the hepatic protein-bound derivatives yields several polar dyes which contain secondary aromatic amino groups and single α-amino acid residues (Terayama and Takeuchi, 1962; Higashinakagawa *et al.*, 1966). Previous papers from this laboratory (Lin *et al.*, 1967, 1968) have demonstrated that (a) the polar dyes contain the intact *N*-methyl group of the administered MAB, (b) the four polar dye fractions (P1, P2a, P2b, and P3) obtained by paper chromatography (Terayama and Takeuchi, 1962; Higashinakagawa *et al.*, 1966) are separable by thin-layer chromatography on cellulose into seven components (P1a, P1b, P1c, P2b, P3a, P3b, and P3c), and (c) the major polar dye P2b is identical with synthetic 3-(homocystein-*S*-yl)-MAB. In other work from this laboratory (Poirier *et al.*, 1967), the carcinogenic synthetic ester *N*-benzoyloxy-MAB was found to form polar dyes nonenzymatically at pH 7 and 37° in reactions with methionine, cysteine, tryptophan, and tyrosine. Likewise, studies *in vivo* and *in vitro* (Scribner *et al.*, 1965; Lotlikar *et al.*, 1966; Poirier *et al.*, 1967) suggested that esters of *N*-hydroxy-MAB are among the reactive metabolites of MAB that give rise to the protein-bound dyes in the rat liver.

In the following study the polar dyes P1a and P1b are shown to be identical with the reaction products of *N*-benzoyloxy-MAB with tyrosine, and provisional structures of these dyes are presented. Polar dye P1c is demonstrated to be 3-(homocystein-*S*-yl)-MAB sulfoxide. Studies are also reported on the polar dyes obtained from the reaction of *N*-benzoyloxy-MAB with tryptophan and cysteine.

## Experimental Section

*Preparation of Polar Dyes P1a, P1b, and P1c from Livers of Rats Fed MAB.* The procedures described in paper II of this series (Lin *et al.*, 1968) were followed for the isolation of the polar dyes by chromatography after successive Pronase and alkaline hydrolysis of the hepatic protein-bound dyes of 100 adult male albino rats that had received MAB (25 mg/ml of corn oil/200 g

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

body weight) by gastric intubation 30 hr previously. Analyses based on final chromatography of the freshly prepared polar dyes on thin layers of cellulose showed that 3-(homocystein-*S*-yl)-MAB (P2b) accounts for about half (54%) of the polar dyes obtained. P1a and P1b occur in a ratio of approximately 1:2 and together account for about one-third of the total polar dyes; P1c amounts to about 5% of the total. These four dyes thus account for at least 90% of the total polar dyes obtained by this procedure, and each chromatographed as a single spot in a variety of solvent systems. Storage of these dyes, even at 4°, for a few days produced small amounts of the P3 fractions, especially P3c. These quantitatively minor P3 fractions, which together amount to about 7% of the total, were not investigated further.

In the procedures that follow repeated reference is made to chromatography on thin layers of cellulose. Unless otherwise indicated this procedure refers to the use of Brinkman MN 300 cellulose with the aqueous phase of 1-propanol–1-butanol–water (1:4:5, v/v). This procedure gave good resolution of P1a, but some overlap of P1b and P1c occurred. To obtain purified samples of P1a the P1a zone was scraped from the plates and extracted with 20% methanol, and the dye was rechromatographed twice on cellulose. The final eluate of P1a was dried, and the residue was dissolved in water. The dye was then extracted into 1-butanol, the butanol was washed twice with water and removed under reduced pressure at 50°, and the P1a was stored in methanol solution.

To prepare P1b free of P1c the lower two-thirds of the P1b zone was extracted with 20% methanol and rechromatographed on cellulose. In this case, after the first development with solvent, the plates were dried with a stream of warm air and then redeveloped. After an additional chromatography on cellulose the P1b zone was further purified as described above for P1a.

For the isolation of P1c the polar dyes from 120 rats were chromatographed on paper, and the dye in the P2b zone was rechromatographed on cellulose thin layers to separate the P1c from the P2b (Lin *et al.*, 1968). The dye in the P1c zone was rechromatographed twice under the same conditions and was then purified as described above for P1a.

*Formation of Tyrosine Polar Dyes A and B from the Reaction of N-Benzoyloxy-MAB with Tyrosine.* A solution of *N*-benzoyloxy-MAB (20 mg, 60 μmoles) (Poirier *et al.*, 1967) in 50 ml of methanol was added to a solution of L-tyrosine (20 mg, 110 μmoles) in 50 ml of 0.06 M pH 7 phosphate buffer and the mixture was left at room temperature for 16 hr. After removal of the methanol at 40° in a rotary flash evaporator, the turbid orange solution was extracted with 60 ml of 1-butanol. The butanol extract was washed twice with 60-ml portions of water, and the butanol was removed under reduced pressure at 50°. The dye residue was dissolved in 10 ml of acetone and passed through a column of silica (1.6 × 20 cm, 50–200 mesh; G. Frederick Smith Chemical Co., Columbus, Ohio) previously equilibrated with acetone. The column was washed with 200 ml of acetone to remove large amounts of nonpolar dyes before the adsorbed polar dyes were eluted with 500 ml of methanol.

The methanol solution of the polar dyes was concentrated and streaked on thin-layers of cellulose. Chromatography yielded two yellow zones which are hereafter referred to as tyrosine polar dye A ($R_F$ 0.30) and tyrosine polar dye B ($R_F$ 0.38). These dyes were eluted with 50% methanol, and the amounts were determined spectrophotometrically at 400 mμ in 95% ethanol (assumed molar absorptivity $2.2 \times 10^4$) and at 520 mμ in 6 N HCl (assumed molar absorptivity $5.3 \times 10^4$). These molar absorptivities are those for the polar dye P2b or 3-(homocystein-*S*-yl)-MAB (Lin *et al.*, 1968) and the corresponding absorptivities for MAB are of the same order of magnitude (Cilento *et al.*, 1956). The yields of the purified dyes calculated at each wavelength agreed closely; approximately 0.6% of polar dye A and 0.7% of polar dye B were obtained from *N*-benzoyloxy-MAB in this reaction with excess tyrosine.

When the synthetic tyrosine polar dyes were hydrolyzed in hot alkali, extracted, and chromatographed in the manner used for the isolation of the polar dyes from rat liver, recoveries of 25% and 43% were obtained for tyrosine dyes A and B, respectively.

*Identities of Polar Dyes P1a and P1b with Tyrosine Polar Dyes A and B, Respectively.* Exact correspondences were obtained in all respects tested for these two pairs of dyes and their methylated derivatives. P1a and tyrosine polar dye A and their methylated derivatives and P1b and tyrosine polar dye B and their methylated derivatives exhibited identical $R_F$'s in four solvent systems (neutral, basic, and acidic) in thin-layer chromatography on cellulose (Table I). Similar correspondences were noted for the colors in HCl vapor and the responses of the dyes in the nitrous acid test for the type of aromatic amino group present, in the ninhydrin test, and in the Folin–Ciocalteau phenol test (Table I).

The electronic spectra of tyrosine polar dyes A and B at pH 4.6 and in 6 N HCl corresponded closely with the spectra of P1a and P1b, respectively, in the visible region (Figure 1). Higher absorption of ultraviolet light was noted with the preparation of P1a, but a good general correspondence was still noted in this region. The electronic spectra of tyrosine polar dye B and P1b under the same conditions showed even better correspondences. The spectra of all these dyes also exhibited maxima at approximately 275 mμ which are consistent with the presence of tyrosine residues in these compounds (Wetlaufer *et al.*, 1958).

The presence of tyrosine residues in these polar dyes was verified by experiments with DL-tyrosine-3,5-$^3$H (New England Nuclear Corp., Boston, Mass.) both *in vitro* and *in vivo*. Tyrosine polar dyes A and B were prepared by the reaction of *N*-benzoyloxy-MAB and tyrosine as described above but with the addition of labeled tyrosine (0.07 μg, 10 μCi). The radioactive polar dyes from the silica column (containing 16 μg of tyrosine polar dye A and 12 μg of tyrosine polar dye B in 2 ml of methanol) were added to normal defatted rat liver protein (2.5 g) and the polar dyes were recovered after the usual hydrolyses, purification, and chromatographic

TABLE I: Chromatographic Comparisons of Polar Dyes P1a, P1b, and P1c and Their Methylated Derivatives with Tyrosine Polar Dyes A and B and Oxidized 3-(Homocystein-*S*-yl)-*N*-methyl-4-aminoazobenzene and Their Methylated Derivatives, Respectively.

| Compound | $R_F$ on Thin Layers of Cellulose[a] in | | | | Color in HCl Vapor[c] | Aromatic Amine Type[d] | Ninhydrin Test[e] | Phenol Test[f] |
|---|---|---|---|---|---|---|---|---|
| | A[b] | B[b] | C[b] | D[b] | | | | |
| P1a | 0.32 | 0.40 | 0.90 | 0.40 | Violet | Tertiary | Gray-blue | Green-blue |
| Tyrosine polar dye A | 0.31 | 0.40 | 0.90 | 0.40 | Violet | Tertiary | Gray-blue | Green-blue |
| P1b | 0.39 | 0.50 | 0.89 | 0.52 | Pink | Secondary | Gray-blue | Green-blue |
| Tyrosine polar dye B | 0.39 | 0.50 | 0.89 | 0.52 | Pink | Secondary | Gray-blue | Green-blue |
| Methylated P1a[g] | 0.33 | 0.41 | 0.48 | 0.43 | Violet | Tertiary | Yellow | Gray-yellow |
| Methylated tyrosine polar dye A[g] | 0.34 | 0.40 | 0.47 | 0.43 | Violet | Tertiary | Yellow | Gray-yellow |
| Methylated P1b[g] | 0.29 | 0.70 | 0.47 | 0.33 | Pink | Secondary | Yellow | Gray-yellow |
| Methylated tyrosine polar dye B[g] | 0.29 | 0.70 | 0.46 | 0.34 | Pink | Secondary | Yellow | Gray-yellow |
| Tyrosine | | | | | Colorless | | Pink-blue | Dark blue |
| Methylated tyrosine[g] | | | | | Colorless | | Colorless | Gray |
| *N*-Methyl-4-aminoazobenzene | | | | | Red | Secondary | Yellow | Green-yellow |
| P1c | 0.37 | 0.54 | 0.68 | 0.48 | Orange-red | Secondary | Gray-blue | Gray-yellow |
| Oxidized 3-(homocystein-*S*-yl)-*N*-methyl-4-aminoazobenzene[h] | 0.38 | 0.55 | 0.69 | 0.49 | Orange-red | Secondary | Gray-blue | Gray-yellow |
| Oxidized P2b[h] | 0.38 | 0.55 | 0.68 | 0.48 | Orange-red | Secondary | Gray-blue | Gray-yellow |
| Methylated P1c[g] | 0.51 | 0.72 | 0.50 | 0.64 | Orange-red | Secondary | Yellow | Gray-yellow |
| Methylated, oxidized 3-(homocystein-*S*-yl)-*N*-methyl-4-aminoazobenzene[g,h] | 0.51 | 0.73 | 0.50 | 0.63 | Orange-red | Secondary | Yellow | Gray-yellow |
| Methylated, oxidized P2b[g,h] | 0.51 | 0.72 | 0.50 | 0.63 | Orange-red | Secondary | Yellow | Gray-yellow |

[a] Brinkman MN 300 cellulose. [b] Aqueous phase of solvents (A) 1-propanol–1-butanol–water (1:4:5, v/v), (B) glacial acetic acid–1-propanol–1-butanol–water (1:2:8:10, v/v), (C) triethylamine–1-propanol–1-butanol–water (1:1:4:5, v/v), and (D) 95% ethanol–1-butanol–water (1:2:10, v/v). [c] Test made on plate. [d] Nitrous acid test of Terayama and Takeuchi (1962). [e] Plate sprayed with ninhydrin 3 (Sigma Chemical Co.) and heated at 110° for 10 min. [f] Plate sprayed with Folin–Ciocalteau phenol reagent (E. H. Sargent and Co.) and then sprayed with 20% sodium carbonate in water. [g] 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 dye was extracted with 1-butanol. Tyrosine was methylated under the same conditions. [h] 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.

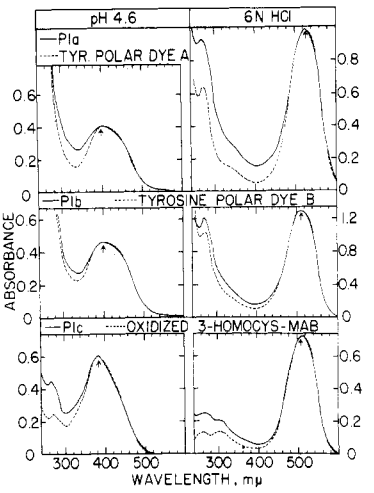


FIGURE 1: Electronic absorption spectra of polar dyes P1a and P1b, tyrosine polar dyes A and B, polar dye P1c, and oxidized 3-(homocystein-*S*-yl)-*N*-methyl-4-aminoazobenzene in aqueous media. The spectra were arbitrarily matched at the wavelengths indicated by the arrows.

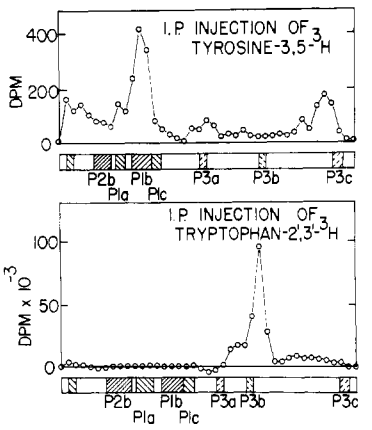


FIGURE 2: Plots of the radioactivities on thin-layer chromatograms on cellulose of polar dye fractions from liver proteins of rats fed *N*-methyl-4-aminoazobenzene and tyrosine-3,5-$^3$H or tryptophan-2',3'-$^3$H.

procedures employed with the liver proteins of rats fed MAB (Lin *et al.*, 1968). After development the final thin-layer plate was dried and cut into 40 equal sections. Each section was transferred to a 20-ml counting vial and mixed with 1 ml of 95% ethanol. The mixture was shaken for 5 min and then mixed with 10 ml of ANPO scintillation mixture (Lin *et al.*, 1967). The radioactivities associated with these sections were compared with the positions of the dyes on the plate. Radioactivity was associated only with the positions of the two synthetic polar dyes A and B in the region occupied by the polar dyes. A ninhydrin-positive zone devoid of dye occurred at a high $R_F$ that corresponded to that of tyrosine.

In the experiment *in vivo*, ten rats were each administered intraperitoneally at zero time 1.0 ml of DL-tyrosine-3,5-$^3$H (0.8 μg, 0.15 mCi) in 0.05 M pH 7 phosphate buffer; 5 hr later, each rat received intraperitoneally another 1 ml of the same buffer containing the same labeled amino acid (1.1 μg, 0.2 mCi). At the same time 1 ml of corn oil alone or containing 25 mg of MAB was administered by stomach tube to each rat in the control (five rats) and dye-fed (five rats) groups, respectively. The rats were killed 29 hr later, and the polar dyes from the dye-fed rats and the corresponding fractions from the control rats were isolated. Equal aliquots of these fractions were chromatographed side by side on a cellulose thin layer. The radioactivities of 40 equal sections of the chromatograms of the polar dyes and the corresponding control fraction were determined as described above. The radioactivity of each chromatographic section from the control rat livers was subtracted from that of the dye-fed rat livers and compared with the positions of the dyes (Figure 2). Radioactivity was concentrated in the zones occupied by the polar dyes P1a and P1b. Radioactivity was also noted in a low $R_F$ region where some nonpolar dye was present and in a high $R_F$ ninhydrin-positive zone (fractions 33–38) containing little dye and corresponding to the position occupied by tyrosine in this system.

The numerous correspondences under the various conditions noted above provide convincing evidence that the polar dyes P1a and P1b obtained from the livers of rats fed MAB are identical with the tyrosine polar dyes A and B, respectively, obtained from the reaction at pH 7 of *N*-benzoyloxy-MAB with tyrosine.

*Structural Features of the Tyrosine Polar Dyes*

*The Ratios of Tyrosine Residues to MAB Residues.* Polar dyes A and B were prepared from *N*-benzoyloxy-MAB and tyrosine as described above but with the addition of DL-tyrosine-3'-$^{14}$C (side chain labeled) (0.12 mg, 9 μCi, New England Nuclear Corp.) to the reaction mixture. After chromatographic isolation of the two polar dyes the moles of tyrosine residue and the moles of MAB residue in aliquots of each dye were determined by scintillation counting of radioactivity and by spectrophotometric measurements, respectively (Table II). The molar ratio of tyrosine residue to MAB residue in each of the tyrosine polar dyes appears to be one.

*The Degree of Substitution on the Aromatic Amino Groups.* The infrared spectra of the tyrosine polar dyes were determined in 5-mm diameter KBr disks as described previously (Lin *et al.*, 1968) (Figure 3). The lack of absorption of tyrosine polar dye A in the 3300–3500 $cm^{-1}$ region confirmed the results of the nitrous acid tests on this dye (Table I) which indicated the presence of a tertiary aromatic amino group. Similarly, the presence of a strong absorption band in the same region of the infrared spectrum of tyrosine polar dye B confirmed the conclusion reached from the nitrous acid tests on this dye (Table I) that it is a secondary aromatic amine.

*The Presence of Free α-Amino Acid Groups.* The infrared spectra of the tyrosine polar dyes (Figure 3) contain bands in the 1500–1600- and 2000–3000-$cm^{-1}$ regions characteristic of ionic amino and carboxyl groups (Bellamy, 1964, pp 234–236). Both tyrosine

TABLE II: The Ratios of *N*-Methyl-4-aminoazobenzene Residues to Tyrosine Residues in Tyrosine Polar Dyes A and B.

| | Tyrosine Polar Dye A | Tyrosine Polar Dye B |
|---|---|---|
| *N*-Methyl-4-aminoazobenzene residues[a] (μmoles) | 280 (400 mμ)<br>230 (520 mμ) | 270 (400 mμ)<br>230 (520 mμ) |
| Tyrosine residues[b] (μmoles) | 230 | 250 |
| *N*-Methyl-4-aminoazobenzene residues/tyrosine residues | 1.0–1.2 | 0.9–1.1 |

[a] The moles of *N*-methyl-4-aminoazobenzene residues were determined spectrophotometrically on an aliquot of each dye in ethanol or in ethanolic HCl, as described in the preparation of the tyrosine polar dyes. [b] Determined from measurements of radioactivity on the same aliquots of dye used for spectral determinations.

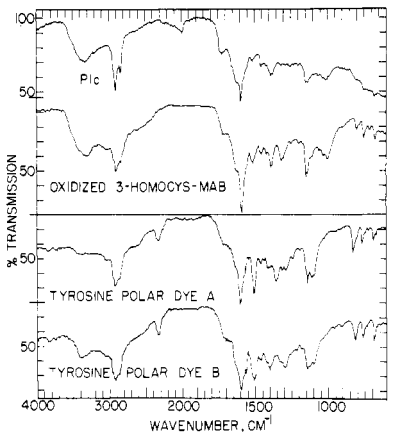


FIGURE 3: Infrared absorption spectra in KBr of polar dye Plc, oxidized 3-(homocystein-*S*-yl)-*N*-methyl-4-aminoazobenzene, and tyrosine polar dyes A and B.

polar dyes gave gray-blue colors with ninhydrin (Table I). Methylation caused the loss of reactivity toward ninhydrin. The same observations were made for tyrosine and methylated tyrosine, respectively (Table I). It appears that both tyrosine polar dyes contain free α-amino acid groups.

*The Presence of Free Phenolic Groups.* Both tyrosine polar dyes gave green-blue colors with the Folin–Ciocalteau phenol reagent (Table I). The methylated dyes gave only gray-yellow colors in this test. Similar results were obtained with tyrosine before and after methylation. The presence of free phenolic groups in the tyrosine polar dyes was confirmed by spectrophotometric measurements of the spectral shifts which occurred when dilute NaOH was added to aqueous ethanol solutions of these dyes. Alkali caused a shift of maximum light absorption from 270 mμ to longer wavelengths (290–330 mμ) with both of these dyes and with tyrosine (Beaven and Holiday, 1952). These shifts were absent from the spectra of the methylated dyes and methylated tyrosine determined under the same conditions.

*The Positions of Substitution in the Tyrosine Residues.* Doubly labeled tyrosine polar dyes A and B were prepared from *N*-benzoyloxy-MAB and tyrosine as described above with the addition of DL-tyrosine-3,5-$^{3}$H (0.03 μg, 25 μCi) and DL-tyrosine-3′-$^{14}$C (0.17 mg, 13 μCi) to the reaction mixture. In a second preparation the ratios were 0.6 μg and 108 μCi of tritiated tyrosine to 0.12 μg and 9 μCi of tyrosine-$^{14}$C. The $^{3}$H/$^{14}$C ratio of each tyrosine polar dye isolated from these reaction mixtures would be the same as that of the tyrosine if substitution in the tyrosine residue is not in the 3,5 positions. On the other hand, this ratio would be one-half of the initial ratio if substitution occurred in one of these positions. In fact, the $^{3}$H/$^{14}$C ratios in the two polar dyes extracted from the cellulose thin-layer chromatograms were one-half of that of the tyrosine used in the reaction (Table III). Thus, the 3-position of the tyrosine residues in both dyes must be substituted.

TABLE III: Ratios of $^{3}$H to $^{14}$C in Tyrosine Polar Dyes Derived from Reaction of Tyrosine-3,5-$^{3}$H-3′-$^{14}$C with *N*-Benzoyloxy-*N*-methyl-4-aminoazobenzene.

| Expt | Compounds[a] | $^{3}$H/$^{14}$C[a] |
|---|---|---|
| 1 | Tyrosine, before reaction | 2.1 |
| | Tyrosine polar dye A | 1.3 |
| | Tyrosine polar dye B | 1.1 |
| 2 | Tyrosine, before reaction | 12 |
| | Tyrosine polar dye A | 6.4 |
| | Tyrosine polar dye B | 5.2 |

[a] See text for details of these reactions.

*The Positions of Substitution in the MAB Residues.* Evidence obtained by resynthesis of the tyrosine polar dyes after reduction of the azo linkages indicated that the prime ring and the β-azo nitrogen in these dyes are unsubstituted. A solution of tyrosine polar dye A or B (16–20 μg) in 2 ml of absolute ethanol was mixed with 0.3 ml of glacial acetic acid and 30 mg of nickel boride (Brown and Brown, 1963). Hydrogen was bubbled gently through the mixture while it was heated on a steam bath until the yellow solution was completely decolorized (about 25 min). After cooling and centrifugation the clear supernatant fluid was transferred to a dry glass-stoppered test tube. A solution of nitrosobenzene (4 mg) in 0.2 ml of absolute ethanol was added and the mixture was warmed on the steam bath for 10 min and then left at room temperature for 16 hr. The polar dyes were recovered and chromatographed as

described above for the reaction mixtures of *N*-benzoyloxy-MAB and tyrosine. Starting with either tyrosine polar dye A or tyrosine polar dye B the original polar dye was recovered in amounts averaging 25–30% in several runs. No other polar dyes were detected in the resynthesized dyes.

These data therefore indicated that the position of substitution in the MAB residues of the tyrosine polar dyes was limited to the 2 position (or the equivalent 6 position), the 3 position (or the equivalent 5 position), or the amino nitrogen. Substitution of the *N*-methyl group was ruled out previously by isotopic data showing that the polar dyes from the livers of rats fed MAB contain in intact form the *N*-methyl groups of the administered MAB (Lin *et al.*, 1967).

*Provisional Structures of the Tyrosine Polar Dyes.* The experiments described above suggest strongly that the sites of monosubstitution in the tyrosine residues in these dyes are limited to the 3 position and that the sites of substitution in the MAB residues are limited to the amino nitrogen and the ring positions 2 and 3. The most probable forms of the cations formally derivable from *N*-benzoyloxy-MAB (Poirier *et al.*, 1967) indicate that the 3 position is a far more likely substitution site by a nucleophile than the 2 position in the MAB residue. The lack of an absorption band near 900 $cm^{-1}$ in the infrared spectrum of polar dye A suggests that 3 substitution (Bellamy, 1964, p 79) is absent from this compound. This is consistent with the tertiary nature of the aromatic amino group in this dye. Thus the 3-tyrosyl group is considered to be attached to the amino nitrogen of the MAB and tyrosine polar dye A is provisionally assigned the diphenylamine structure shown in Figure 4. The infrared spectrum of tyrosine polar dye B shows an absorption band at 900 $cm^{-1}$. This suggestion of a substitution at position 3 in the MAB residue and the secondary nature of the aromatic amino group in tyrosine polar dye B thus indicate strongly that the 3-tyrosyl group in this dye is attached to the 3 position of the MAB residue. On this basis the biphenyl structure shown in Figure 4 is provisionally assigned to tyrosine polar dye B. Synthesis of these structures by unequivocal routes will be required to establish these assignments.

*Identity of Polar Dye P1c with an Oxidation Product of 3-(Homocystein-S-yl)-MAB with $H_2O_2$.* The major polar dye P2b or 3-(homocystein-*S*-yl)-MAB was noted to yield on exposure to air small amounts of another polar dye with chromatographic and other properties similar to those of P1c. Greatly increased conversion of P2b into this P1c-like dye was obtained upon mild oxidation with $H_2O_2$. A solution of crystalline 3-(homocystein-*S*-yl)-MAB (20 mg) in 16 ml of methanol was mixed with 0.9 ml of 30% $H_2O_2$, and after 7 days at room temperature about 95% of the synthetic P2b ($R_F$ 0.20) was found by chromatographic analysis to have been converted to a polar dye with an $R_F$ of 0.38. A trace amount of a more polar dye with an $R_F$ of 0.80–0.85 was also detected. The major component with an $R_F$ of 0.38 was purified by two successive chromatographic separations on cellulose before final elution with 50% methanol.

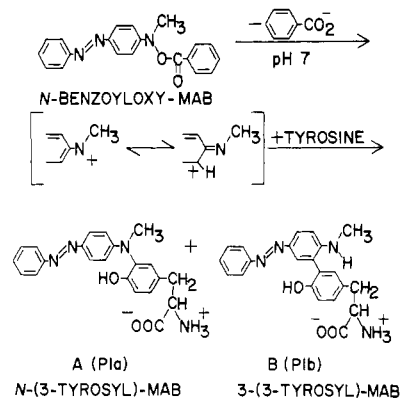


FIGURE 4: Provisional structures of tyrosine polar dyes A (P1a) and B (P1b) that result from the reaction of *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene with tyrosine.

The identity of this oxidation product of P2b with P1c was determined in several ways. Thin-layer chromatography of P1c and the oxidation product and their methylated derivatives in four solvent systems (neutral, basic, and acidic) showed that the parent dyes or their methylated derivatives could not be separated from each other in these systems (Table I). Correspondences were also noted in the ninhydrin and nitrous acid tests on these dyes (Table I).

Similarly, the electronic spectra of P1c and the oxidation product at pH 4.6 and in 6 N HCl corresponded closely (Figure 1). Greater absorption of light below 400 mμ was noted in the preparation of P1c, but this impurity did not alter the character of the absorption spectrum (Figure 1). Entirely similar findings were noted for the corresponding spectra of the methylated products of the two polar dyes.

Further definitive evidence of the identity of P1c and the oxidation product of synthetic 3-(homocystein-*S*-yl)-MAB was obtained by comparison of their infrared spectra (Figure 3) in which close correspondences were noted over the entire observed region of 600–4000 $cm^{-1}$.

*The Sulfoxide Nature of the Oxidation Product of 3-(Homocystein-S-yl)-MAB with $H_2O_2$.* Under the same mild conditions employed to convert P2b into P1c (1% $H_2O_2$ at room temperature) Toennies and Kolb (1939) obtained a 95% yield of methionine sulfoxide from methionine with no detectable formation of the sulfone. These data suggest that P1c is the sulfoxide of P2b, and this structural formulation was supported by the data which follow.

The infrared spectrum of P1c in KBr (Figure 3) has a strong absorption band at 1000 $cm^{-1}$ and a weak band at 1030 $cm^{-1}$ which are not present in the spectrum of the parent dye P2b (Lin *et al.*, 1968). For comparison the corresponding oxidation product of 3-methylmercapto-MAB (Scribner and Miller, 1967) with $H_2O_2$ was prepared by incubating a solution of this dye (5 mg) in 2 ml of ethanol with 0.12 ml of 30% $H_2O_2$ for 6 days at room temperature. The product ($R_F$ 0.12) was separated from the 3-methylmercapto-MAB ($R_F$ 0.00) by thin-layer chromatography on cel-

lulose. The infrared spectrum of this oxidation product also exhibited a broad absorption band at 1010–1030 $cm^{-1}$ that is absent from the spectrum of the parent dye. Likewise, a preparation of methionine sulfoxide (Calbiochem, Los Angeles, Calif.) gave a strong band at 1025 $cm^{-1}$ which is lacking in the spectrum of either methionine or its sulfone obtained from the same commercial source. Similarly, many simple sulfoxides give strong absorption bands in the region of 1020–1040 $cm^{-1}$ in KBr (Bellamy, 1964, p 357). However, Amstutz *et al.* (1951) found that *o*-hydroxydiphenyl sulfoxide has only a weak band at 1034 $cm^{-1}$ and a strong absorption band at 994 $cm^{-1}$ in the solid state. They attributed the shift to the latter band in this compound to hydrogen bonding between the adjacent hydroxyl group and the sulfoxide oxygen atom. In the present study the lower frequency of these absorption bands of both oxidized dyes appears to be likewise attributable to hydrogen bonding of the secondary amine hydrogen in these dyes with the sulfoxide oxygen atom.

Sulfoxides can be reduced to sulfides under relatively mild conditions and attempts were made to reduce P1c to P2b. While reduction of P1c to P2b with ascorbic acid or sodium borohydride failed, a low but definite yield of P2b was obtained by reduction with cysteine. The latter reaction was performed in a thick-walled tube in which a solution of P1c (30 $\mu$g) in 0.5 ml of water was mixed with 0.5 ml of a freshly prepared solution of DL-cysteine (0.2 M pH 7 phosphate buffer). This mixture was flushed with nitrogen and frozen in liquid nitrogen and the tube was sealed *in vacuo* and heated in a steam bath for 5 hr. The mixture was then extracted with 1-butanol and analyzed by thin-layer chromatography on cellulose. A yield of 3% of P2b was obtained. In addition 10% of the original amount of P1c was recovered, but no other dyes were noted on the chromatogram. Better yields of P2b were obtained with the reduction of both the sulfoxide and azo groupings to yield a diamine which could be coupled with nitrosobenzene. A solution of P1c (10 $\mu$g) in 3 ml of glacial acetic acid was stirred with zinc granules (40 mg), and a gentle stream of hydrogen was passed through at atmospheric pressure while the mixture was heated on a steam bath. The yellow color disappeared in 10 min, but the reduction was continued for 1 hr. After cooling, the clear solution was transferred to a dry glass-stoppered test tube and 2 mg of nitrosobenzene in 0.1 ml of absolute ethanol was added. The mixture was gently warmed on a steam bath for 10 min and allowed to stand at room temperature for 16 hr. After removal of the acetic acid and excess nitrosobenzene at 50° in a rotary flash evaporator, the dye residue was dissolved in 3 ml of water and extracted with 3 ml of 1-butanol. After washing twice with water, the butanol was removed under reduced pressure. The dye residue was chromatographed on a thin-layer of cellulose, and the yield of P2b (approximately 15%) was determined spectrophotometrically. Only unchanged P1c and the reduction product P2b were recovered from this reaction.

The possible presence of an azoxy group in P1c was ruled out by (a) the absence of an absorption band at 1480 $cm^{-1}$ (Yukawa, 1965) in the infrared spectrum of this dye (Figure 3), and (b) the failure of $\alpha$- and $\beta$-*N*,*N*-dimethyl-4-aminoazoxybenzene (Anderson, 1952) to form colored tautomers in acid solution (Cilento *et al.*, 1956) as noted with P1c (Figure 1). These data and those discussed above indicate that the mild oxidation of P2b with $H_2O_2$ to form P1c and the reduction of P1c to P2b occur only in the sulfur atoms of these dyes. P1c therefore appears to be the sulfoxide of 3-(homocystein-*S*-yl)-MAB.

*The Polar Dye Derived from the Reaction of N-Benzoyloxy-MAB with Tryptophan.* The ability of the carcinogenic ester *N*-benzoyloxy-MAB to react with tryptophan (Poirier *et al.*, 1967) suggested that some of the polar dyes derived from the liver proteins of rats fed MAB might contain tryptophan residues. As an aid in investigating this possibility a polar dye containing MAB and tryptophan was prepared. A solution of *N*-benzoyloxy-MAB (60 mg, 180 $\mu$moles) in 100 ml of methanol was mixed with DL-tryptophan (450 mg, 2.2 mmoles) in 70 ml of 0.1 M pH 7 phosphate buffer, and the mixture was incubated at room temperature for 20 hr. Some white precipitate formed during this incubation and was removed by filtration. The methanol was removed under reduced pressure and the dyes were extracted into 100 ml of 1-butanol. After the butanol was washed twice with 60-ml portions of water, it was removed in a rotary flash evaporator at 50°. The dye residues were dissolved in 20 ml of acetone and passed through a column of silica previously equilibrated with acetone. The column (1.6 × 25 cm, 50–200 mesh) was washed thoroughly with acetone to remove a considerable amount of nonpolar dye before the adsorbed tryptophan polar dye was eluted with methanol. Chromatography on a thin layer of cellulose yielded a tryptophan polar dye with an $R_F$ of 0.18; this dye was rechromatographed twice in the same system before it was studied further.

While this tryptophan polar dye has an $R_F$ close to that of P2b it has different properties. The latter dye gives a pink color in HCl vapor while the tryptophan polar dye gives a violet color in acid. The nitrous acid test (Table I, footnote *d*) shows that the tryptophan polar dye is a tertiary amine. It is also negative in the Ehrlich test with *p*-dimethylaminobenzaldehyde in acid and thus appears to be substituted in the imidazole ring of the amino acid.

The stability of the synthetic tryptophan polar dye under the conditions used for the isolation of the polar dyes from the liver proteins of rats was determined with tritiated dye. A solution of *N*-benzoyloxy-MAB (13.5 mg, 40.5 $\mu$moles) in 3 ml of methanol was mixed with DL-tryptophan-2′,3′-$^3$H (side-chain labeled) (0.19 mg, 0.18 mCi; New England Nuclear Corp.) in 0.5 ml of 0.1 M phosphate buffer (pH 7). After 2 min, 80 mg of nonradioactive DL-tryptophan was added in a mixture of methanol (15 ml) and 0.1 M phosphate buffer (pH 7, 10 ml). The final reaction mixture was shaken for 20 min to aid in dissolving the tryptophan and then left for 24 hr at room temperature. The tryptophan polar dye was isolated as described above.

The tritiated tryptophan polar dye (36 μg) was added to defatted rat liver protein (2.2 g), the mixture was hydrolyzed with Pronase, and the polar dyes were then hydrolyzed with alkali, purified, and chromatographed as previously described (Lin *et al.*, 1968) in proportion to the amount of protein taken. Approximately 20% of the added dye was recovered in the chromatographic zone ($R_F$ 0.18) noted above, and the properties of the dye in this zone agreed with those noted above for the tryptophan polar dye. Furthermore, the radioactivity on the plate was associated essentially only with this zone.

*The Failure to Find a Tryptophan Derivative Among the Polar Dyes Derived from the Proteins of Rats Fed MAB.* Ten male albino rats, 180–200 g and prefed a purified diet for 2 weeks (Lin *et al.*, 1968), were each given at zero time and at 5 hr by intraperitoneal injection 0.5 ml of 0.06 M phosphate buffer (pH 7) which contained 0.19 mg of DL-tryptophan-2′,3′-$^3$H (0.18 mCi). At 5 hr, each of five rats (group I) was fed by stomach tube 25 mg of MAB dissolved in 1 ml of corn oil whereas each of the other five rats (group II) was fed 1 ml of corn oil alone. The animals were killed 30 hr later and the livers from each group were pooled. The polar dyes from the livers of group I and the corresponding fractions from the livers of group II were isolated after enzymatic and alkaline hydrolysis and chromatography on thin-layer plates as previously described (Lin *et al.*, 1968). The polar dyes from group I and the corresponding fractions from group II were chromatographed side by side on the same thin-layer plate and serial sections of this plate were assayed for dye and radioactivity as described above for the tyrosine polar dyes. The radioactivity of each section from group II was subtracted from that of each corresponding section from group I. As shown in Figure 2 all of the polar dye fractions were located in a region in which only a negligible amount of radioactivity was found. A significant amount of radioactivity was found in a region which was devoid of dye and had an $R_F$ corresponding to that of tryptophan.

Thus, while no tryptophan residues were detected in the polar dyes derived from the liver proteins of rats fed MAB and labeled tryptophan in these experiments, these data cannot rule out the possibilities that low levels of the polar dye sought or that other tryptophan polar dyes escaped detection because of lability to the hydrolysis procedures or for other reasons.

*Properties of the Polar Dyes Derived from the Reactions of N-Benzoyloxy-MAB with Cysteine and Homocysteine.* Cysteine, like methionine, tryptophan, and tyrosine, reacts readily with *N*-benzoyloxy-MAB at pH 7 (Poirier *et al.*, 1967) and a cysteine polar dye was prepared by this reaction for further study. A solution of *N*-benzoyloxy-MAB (14 mg, 42 μmoles) in 30 ml of methanol was mixed with a solution of cysteine hydrochloride (100 mg, 630 μmoles) in 20 ml of 0.1 M phosphate buffer (pH 7) and kept at room temperature for 24 hr. Some white precipitate formed during this time and was removed by filtration. The methanol in the clear filtrate was removed under reduced pressure, and the residual turbid aqueous solution was diluted with 20 ml of water and extracted twice with 50-ml portions of benzene–*n*-hexane (1:1, v/v) to remove nonpolar substances. The cysteine polar dye was extracted from the aqueous phase into 1-butanol, the butanol was removed under reduced pressure at 50°, and the dye residue was chromatographed on thin layers of cellulose.

The cysteine polar dye had an $R_F$ of 0.22 and in acid gave a pink solution with an absorption maximum at 520 mμ. In these respects it closely resembled the polar dye P2b or 3-(homocystein-*S*-yl)-MAB. However, unlike the latter dye, the cysteine polar dye was completely destroyed in hot alkali. In this test a solution of the cysteine polar dye (3 μg) in 0.5 ml of methanol was mixed with 1 ml of 4.5 N KOH in water and refluxed at 95° for 20 hr. After cooling, the hydrolysis mixture was adjusted to pH 6 with concentrated HCl and solid sodium bicarbonate. The mixture was extracted with 1-butanol and, after the butanol was washed twice with water, the butanol was removed under reduced pressure at 50°. The residue was dissolved in methanol and chromatographed on a thin layer of cellulose. Although as little as 0.05 μg of the cysteine polar dye could be detected visually on a cellulose thin layer after exposure to HCl vapor, no cysteine polar dye was detected. Some unidentified dyes occurred in the $R_F$ region of 0.7–0.9. This cysteine polar dye would thus be destroyed in the alkaline hydrolysis generally used in preparations of the polar dyes from the liver proteins of rats fed MAB.

The homocysteine polar dye was prepared in a manner analogous to the above preparation of the cysteine derivative. A solution of *N*-benzoyloxy-MAB (5 mg, 15 μmoles) in 5 ml of methanol was mixed with DL-homocysteine (10 mg, 74 μmoles, Nutritional Biochemical Corp.) in 10 ml of water which had been adjusted to pH 8.0 with 1 N KOH and incubated for 20 hr at room temperature. The polar dye thus formed was extracted with 1-butanol, separated on a column of silica, and finally chromatographed on a thin layer of cellulose as described above for the tyrosine polar dyes. Approximately 1.5% of the *N*-benzoyloxy-MAB was recovered as the homocysteine polar dye.

As expected, the homocysteine polar dye proved to be chromatographically and spectroscopically identical with P2b or synthetic 3-(homocystein-*S*-yl)-MAB (Lin *et al.*, 1968). It also proved to be highly resistant to the alkaline hydrolysis procedure described above for the cysteine polar dye. A recovery of 85% of the homocysteine polar dye from this hydrolysis was obtained. These results on the alkali lability of the cysteine polar dye and the alkali stability of the homocysteine polar dye are consistent with the known alkali lability of *S*-aryl derivatives of cysteine (Clarke and Inouye, 1931) and with the lability of *S*-ethylcysteine to alkali in contrast to the alkali stability of its isomer methionine (Mueller, 1923).

*The Failure of the Homocysteine, Homocysteine Sulfoxide, Cysteine, Tyrosine, and Tryptophan Polar Dyes to Form Nonpolar Dyes upon Treatment with Alkali.* It has been known since the discovery of the protein-bound dyes in the rat (Miller and Miller, 1947) that hot alkaline hydrolysis of the hot ethanol-extracted liver proteins of rats fed MAB or *N,N*-dimethyl-4-

aminoazobenzene yields small amounts of the nonpolar dyes MAB and 4-aminoazobenzene (accounting together for approximately 10% of the total protein-bound dyes) in addition to the polar dyes. Recently Matsumoto *et al.* (1968) noted that unfractionated preparations of polar dyes obtained after enzymatic hydrolysis of the liver proteins of rats fed 3'-methyl-DAB also yielded the nonpolar dyes 3'-methyl-MAB and 3'-methyl-AB after treatment with alkali. The temperature of this treatment and the yield of nonpolar dyes were not stated. Following the report by Matsumoto *et al.* (1968) 20–100 μg of each of the various synthetic polar dyes was subjected to the hot alkaline hydrolysis procedure used in the preparation of the polar dyes from hepatic protein (Lin *et al.*, 1968). The hydrolysis mixtures were extracted with benzene to remove any nonpolar dye that might have been formed, and the extracts were analyzed by gas chromatography (Scribner *et al.*, 1965) with samples of authentic MAB and AB as standards. These dyes are stable to hot alkaline hydrolysis (Miller and Miller, 1947) and this procedure could have detected the formation of 0.5% of either MAB or AB from each of the polar dyes. However, none of the polar dyes derived from homocysteine or its sulfoxide, cysteine, tyrosine, or tryptophan yielded any detectable amounts of MAB or AB after hot alkaline hydrolysis. Thus it appears that the MAB and AB released by alkali from the liver proteins of rats fed MAB or from unfractionated polar dye preparations produced by enzymatic hydrolysis must arise from alkali-labile dye derivatives which are not identical with any of the polar dyes thus far isolated from the proteins or prepared by reactions of amino acids with *N*-benzoyloxy-MAB.

## Discussion

The total amount of the polar dyes obtained by present methods from the liver proteins of the rat at any time during the feeding of MAB for hepatocarcinogenesis does not appear to exceed 50 to 100 μg of dye/10 g of fresh liver (Miller and Miller, 1947; Terayama, 1967). Similar levels of these dyes are obtained after large doses of MAB or related dyes are administered intragastrically or intraperitoneally to rats (Hultin, 1956; Gelboin *et al.*, 1958; Lin *et al.*, 1968). Thus the small amounts of the individual polar dyes that are available, even from the hydrolyses of the pooled liver proteins of hundreds of rats, have imposed severe limitations on approaches to the purification and characterization of these dyes (*cf.* Terayama, 1967). However, the reactivity of the synthetic carcinogenic ester *N*-benzoyloxy-MAB with tyrosine and homocysteine has fortunately provided derivatives which are identical with four of the polar dyes from the liver. It now appears possible to assign final or provisional structures to these four dyes which account for 90% of the polar dyes obtained by present methods from the liver proteins of rats fed MAB. Furthermore, these data suggest that the reactive forms of MAB *in vivo* include esters of *N*-hydroxy-MAB.

The failure to find a tryptophan derivative among the polar dyes obtained from the liver proteins of rats fed MAB and labeled tryptophan may reflect the relatively low amounts of this amino acid in many proteins and the lability of the tryptophan polar dye to hot alkali. It is also evident that the hydrophobic tryptophan residues in globular proteins tend to occur inside the folded peptide chain (Kendrew, 1962; Blake *et al.*, 1965; Dopheide and Jones, 1968). Thus the active forms of MAB *in vivo* may not be able to interact with many tryptophan residues in proteins.

On the other hand, cysteine residues in some enzyme proteins are in relatively exposed positions for reactions with substrate molecules and might be expected to react with active metabolites of MAB in the rat liver. However, the great lability to alkali of the cysteine polar dye derived from *N*-benzoyloxy-MAB and cysteine makes it difficult, with present methods of isolation of the polar dyes, to approach the question of the presence of this type of cysteine-bound dye in the liver proteins of rats fed MAB. A recent abstract by Ketterer and Christodoulides (1968) states that cysteine–dye binding occurs in the basic azo dye binding protein from the livers of rats given 3'-methyl-DAB and cysteine-$^{35}$S. Pronase and alkaline hydrolysis were employed in this study. However, it is not clear from this preliminary communication how the radioactivity in the "azo dye bound protein fragments" was shown to be in cysteine residues attached to dye. Alkaline hydrolysis could lead to the breakdown of dye–cysteine residues to dye-$^{35}$S derivatives and thus provide evidence for the binding of dye to protein-bound cysteine *in vivo*.

As noted in the present paper considerable destruction of several of the other polar dyes also occurs in the relatively harsh hot alkaline hydrolysis employed in the present isolation procedures. Further progress may depend on finding a gentle and reasonably complete nonalkaline hydrolysis procedure, probably enzymatic in nature, for the liver proteins of dye-fed rats that will yield dye derivatives containing single amino acid residues. Perhaps only in this way will determinations be made of the true extent to which dye is bound to the various amino acids in these proteins.

## References

Amstutz, E. D., Hunsberger, I. M., and Chessick, J. J. (1951), *J. Amer. Chem. Soc. 73*, 1220.

Anderson, W. (1952), *J. Chem. Soc.*, 1722.

Beaven, G. H., and Holiday, E. R. (1952), *Advan. Protein Chem. 7*, 324.

Bellamy, L. J. (1964), The Infra-red Spectra of Complex Molecules, New York, N. Y., Wiley.

Blake, C. C. F., Koenig, D. F., Mair, G. A., North, A. C. T., Phillips, D. C., and Sarma, V. R. (1965), *Nature 206*, 757.

Brown, C. A., and Brown, H. C. (1963), *J. Am. Chem. Soc. 85*, 1003.

Cilento, G., Miller, E. C., and Miller, J. A. (1956), *J. Am. Chem. Soc. 78*, 1718.

Clarke, H. T., and Inouye, J. M. (1931), *J. Biol. Chem. 94*, 541.

Dingman, C. W., and Sporn, M. B. (1967), *Cancer Res. 27*, 938.
Dopheide, T. A. A., and Jones, W. M. (1968), *J. Biol. Chem. 243*, 3906.
Gelboin, H. V., Miller, J. A., and Miller, E. C. (1958), *Cancer Res. 18*, 608.
Higashinakagawa, T., Matsumoto, M., and Terayama, H. (1966), *Biochem. Biophys. Res. Commun. 24*, 811.
Hultin, T. (1956), *Exptl. Cell Res. 10*, 71.
Kendrew, J. C. (1962), *Cold Spring Harbor Symp. Quant. Biol. 15*, 216.
Ketterer, B., and Christodoulides, L. (1968), *Biochem. J. 109*, 37P.
Lin, J.-K., Miller, J. A., and Miller, E. C. (1967), *Biochem. Biophys. Res. Commun. 28*, 1040.
Lin, J.-K., Miller, J. A., and Miller, E. C. (1968), *Biochemistry 7*, 1889.
Lotlikar, P. D., Scribner, J. D., Miller, J. A., and Miller, E. C. (1966), *Life Sci. 5*, 1263.
Matsumoto, M., Takata, H., and Teramaya, H. (1968), *Gann 59*, 231.
Miller, E. C., and Miller, J. A. (1947), *Cancer Res. 7*, 468.
Miller, E. C., and Miller, J. A. (1966), *Pharmacol. Rev. 18*, 805.
Miller, J. A., and Miller, E. C. (1953), *Advan. Cancer Res. 1*, 339.
Mueller, J. H. (1923), *J. Biol. Chem. 56*, 156.
Poirier, L. A., Miller, J. A., Miller, E. C., and Sato, K. (1967), *Cancer Res. 27*, 1600.
Roberts, J. J., and Warwick, G. P. (1966), *Intern. J. Cancer 1*, 179.
Scribner, J. D., and Miller, J. A. (1967), *J. Org. Chem. 32*, 2348.
Scribner, J. D., Miller, J. A., and Miller, E. C. (1965), *Biochem. Biophys. Res. Commun. 20*, 560.
Terayama, H. (1967), *Methods Cancer Res. 1*, 399.
Terayama, H., and Takeuchi, M. (1962), *Gann 53*, 293.
Toennies, G., and Kolb, J. J. (1939), *J. Biol. Chem. 128*, 399.
Wetlaufer, D. B., Edsall, J. T., and Hollingworth, B. R. (1958), *J. Biol. Chem. 233*, 1421.
Yukawa, Y., Ed. (1965), *in* Handbook of Organic Structural Analysis, New York, N. Y., Benjamin, p 421.

# Antibody Binding and Complement Fixation by a Liposomal Model Membrane*

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ABSTRACT: Previous studies have shown that liposomal model membranes, prepared with fractions obtained from sheep erythrocytes, release trapped glucose in the presence of rabbit antisheep erythrocyte serum and unheated guinea pig serum. The present experiments demonstrate that: (1) these liposomes can bind (neutralize) antibodies in the antiserum, which are responsible for sheep erythrocyte hemolysis, but only when the appropriate antigen has been incorporated into the liposomal structure, and (2) liposomes, which contain antigen, are able to fix guinea pig complement but only in the presence of antiserum.

These observations further support the feasibility of using liposomes as an artificial membrane system for studying certain aspects of complement mechanism.

In recent years, the major emphasis of studies dealing with immune lytic mechanisms has been directed toward isolation of complement components and establishing the order in which they react to produce a structural defect in cell membranes. Although notable progress along these lines has been made in numerous laboratories (see review by Muller-Eberhard, 1968), it seems apparent that explanation of complement-induced lysis on a molecular basis is also contingent on the identification of the membrane constituents which participate in this process and the manner in which these are organized in cell membranes before and after lysis.

The possibility that artificial membranes might provide some useful information on this point prompted us to attempt construction of a liposomal model membrane from sheep erythrocyte lipids that would respond to antibody and complement (Haxby *et al.*, 1968). The choice of this artificial membrane system, and starting material, was dictated by several considerations. Liposomes rather than lipid monolayers or thin lipid (bilayer or "secondary black") films were employed because their preparation does not require any unique equipment, and permeability alterations can be conveniently determined by a simple spectrophotometric procedure. This method utilizes glucose

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