3-METHYLMERCAPTO-N-METHYL-4-AMINOAZOBENZENE: AN ALKALINE-DEGRADATION PRODUCT OF A LABILE PROTEIN-BOUND DYE IN THE LIVERS OF RATS FED N,N-DIMETHYL-4-AMINOAZOBENZENE*

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The stepwise demethylation of the hepatocarcinogenic dye N,N-dimethyl-4-aminoazobenzene (DAB) to N-methyl-4-aminoazobenzene (MAB) and 4-aminoazobenzene (AB) and the methylation of MAB to DAB in the liver of the rat were reported 20 years ago (Miller, Miller and Baumann, 1945). The conclusion that the latter reaction occurs now appears to be incorrect. In these early studies the livers were digested in ethanolic alkali and the dyes were extracted with hexane. The dyes, after purification by acid extraction, were chromatographed on alumina. When either DAB or MAB was fed, similar microgram quantities of DAB, MAB, and AB appeared to be present in the liver. These dyes were characterized by cochromatography with the authentic dyes and by their spectra in acid solution. Recently, by gas chromatography, we have found that when DAB is fed, the "DAB" fraction on alumina columns is a mixture of DAB and a new dye. Furthermore, when MAB is fed, the new dye appears to be the sole constituent of the "DAB" fraction. Studies on the new dye reveal that it is:

a) 3-methylmercapto-N-methyl-4-aminoazobenzene, and b) an alkaline-

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degradation product of a labile protein-bound dye formed in the livers of rats fed either DAB or MAB.

Male albino (Holtzman) rats (180-200 g) were fed DAB (0.06%) or MAB (0.056%) in a semi-synthetic diet (Andersen et al., 1964) for 3 days to 4 weeks. After exsanguination the livers were homogenized and digested in ethanolic KOH at room temperature, and the dyes extracted and chromatographed on alumina as previously described (Miller et al., 1945). Careful removal of suspended lipid from the intermediate acid extract in this procedure by extraction with 30% benzene in hexane permitted satisfactory gas chromatography of the combined dyes. Gas chromatography of the dye extracts and dye bands from alumina columns was performed with a Barber-Colman Model 10 gas chromatograph on columns of Gas-Chrom S containing methyl silicone gum SE-30 as previously described (Andersen et al., 1964). The column temperature was 182°C and the dyes were injected in ethyl acetate solutions. When the dyes extracted from alkaline digests of livers of rats fed DAB were examined on these columns, AB, MAB, and DAB emerged as symmetrical peaks with retention times of 4.0, 5.4, and 6.0 min. respectively. In addition, a new dye peak was noted at 13.8 min. with an area equal to or greater than that of the DAB peak. When MAB was fed the livers contained AB, MAB, and the new dye, but no DAB. Mixtures of the new dye and DAB could not be resolved by chromatography on alumina, but could be resolved on carboxymethyl cellulose ether dispersed in a 1:4 mixture by volume of 95% ethanol and 0.1 M citric acid respectively. This solvent was used to apply the dyes and to develop the column; DAB formed a pink band that moved ahead of an orange-brown band of the new dye. The latter band was dispersed in 5% K_2CO_3 and the dye removed by repeated extraction with ethyl ether.

The new dye was acetylated by acetic anhydride at 60°C, and the relatively polar acetyl derivative was easily separated from DAB by chromatography on alumina in hexane. This derivative did not give a pink

color in acid as did the new dye; thus N-acetylation of a p-aminoazo dye had occurred. After treatment with nitrous acid, acid solutions of the dye were colorless and would not couple with α-naphthol (Silverstone, 1948); this indicated that the dye is a secondary aromatic amine. After reduction of the N-acetyl derivative with mossy tin in refluxing 1 N HCl the resulting amines were gas-chromatographed on a column of Apiezon L on Chromsorb W. Only aniline could be detected under conditions that gave satisfactory separation of mixtures of aniline and N-methyl-p-phenylene diamine. All these results suggested that the new dye might be MAB substituted in the diamine ring or on the N-methyl group.

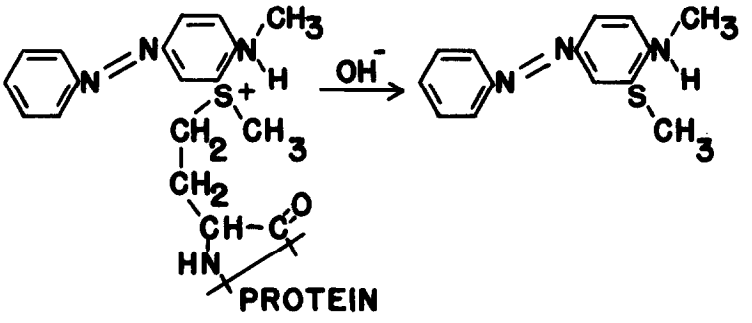
The presence of a substituent in the new dye in a position ortho to the amino group was suggested by the close similarities of the UV and IR absorption spectra of the new dye and its N-acetyl derivative with those of 3-methyl-MAB and its N-acetyl derivative. Distinctly different spectra were obtained with 2-methyl-MAB and its N-acetyl derivative. The IR spectrum of the new dye in KBr showed absorption maxima at 3386, 2920, 2850, 1730, 1589, 1560, 1510, 1460, 1426, 1391, 1317, 1286, 1252, 1208, 1174, 1155, 1143, 1120, 1068, 1018, 967, 909, 810, 762, 728, 687, 590, 510, 459, 420, and 394 cm^{-1}. The spectrum of 3-methyl-MAB did not show absorption at 1155 cm^{-1}. The IR spectrum of the new dye was also obtained in CCl_4 solution; the N-H stretching frequency for the new dye in both KBr and CCl_4 solution is 3386 cm^{-1}. In contrast, the absorption maxima for this group in 3-methyl-MAB are 3344 and 3442 cm^{-1} in KBr and 3468 cm^{-1} in CCl_4 solution. The independence of the N-H frequency in the new dye with respect to solvent and its low polarity as shown by cochromatography with DAB on alumina suggested that the N-H group in this secondary aminoazo dye was intramolecularly hydrogen-bonded to an atom in the ortho position. Thus it appeared that some hetero atom, probably nitrogen, oxygen or sulfur, was present in the 3-position of the new dye.

Several model dyes were studied and a dye containing sulfur in the

3-position of MAB, 3-methylmercapto-N-methyl-4-aminoazobenzene (3-CH_3S-MAB), corresponded exactly with the new dye in every respect: cochromatography in the gas chromatograph and on alumina, IR spectra of the free amine and of the N-acetyl derivative, and UV spectra of the free amine in 7 N HCl (λ_{max} = 527 mμ) and in 95% ethanol (λ_{max} = 402 mμ). 3-CH_3S-MAB was synthesized by the general diazoamino rearrangement previously described (Miller and Miller, 1948). The N-formyl derivative of 2-methylmercapto-aniline (Aldrich) was reduced to the N-methylamine with $LiAlH_4$. This amine was coupled with diazotized aniline to give the diazoamino derivative. A low (5%) yield of 3-CH_3S-MAB as an impure oil was obtained from the rearrangement. The N-benzoyl derivative was purified by chromatography on alumina to give a crystalline derivative, m.p. 191.5-192.5°C. Calculated for $C_{21}H_{19}N_3OS$: C, 69.79; H, 5.30; N, 11.63; S, 8.84. Found: C, 69.69; H, 5.44; N, 11.24; S, 9.41. Reduction of the N-benzoyl derivative with $LiAlH_4$ gave 3-CH_3S-MAB as an oil. Calculated for $C_{14}H_{15}N_3S$: C, 65.36; H, 5.88; N, 16.33; S, 12.44. Found: C, 64.95; H, 5.98; N, 15.31; S, 13.01.

3-CH_3S-MAB proved to be an artifact of the alkaline digestion procedure. The livers from rats fed DAB were homogenized in 95% ethanol at room temperature and extracted exhaustively with this solvent at the same temperature. When the extracts were made alkaline as in the procedure used for the dyes in whole liver DAB, MAB and AB, but not 3-CH_3S-MAB were found. The liver residue, however, when made alkaline, readily yielded the usual amounts (~0.5 μg/g fresh liver) of 3-CH_3S-MAB. However, heating this residue to 60°C in 95% ethanol greatly diminished the amount of 3-CH_3S-MAB that was obtained after alkaline digestion. This extraction procedure has been used to remove lipid in the determination of protein-bound dye in liver (Miller and Miller, 1947). Extraction of the total nucleic acids from liver by phenol (DiGirolamo, Henshaw and Hiatt, 1964) yielded protein residues which still retained the precursor of 3-CH_3S-MAB. The CH_3S-group in the new dye and the facile release of this dye from the total

crude liver protein by alkali strongly suggest that the precursor is protein in which the sulfur atom of a methionine side chain is attached to the 3-carbon atom of MAB as in:



Recent work in this laboratory (Poirier, Sato, Miller and Miller, 1965) indicates that N-hydroxy-MAB is a precursor of this protein-bound dye and is a proximate carcinogenic metabolite of MAB and DAB. Matsumoto (1965) reported the presence of small amounts of a new primary aminoazo dye (F-1) containing an unknown substitutuent in the extracts of alkaline digests of livers of rats force-fed large amounts of AB. In similar experiments we obtained very small amounts of this primary aminoazo dye and found it to have a gas-chromatographic retention time of 11.0 min. Synthetic 3-methylmercapto-AB and the dye obtained by enzymatic N-demethylation (Mueller and Miller, 1953) of 3-CH_3S-MAB have the same chromatographic property. Matsumoto's F-1 dye thus appears to be 3-CH_3S-AB.

The consequences of the presence of the above protein-bound sulfonium dye in the livers of rats fed DAB or MAB are being explored. This new type of protein-bound dye might act as a foreign "active" methionine and lead to abnormal methylation of cellular constituents such as nucleic acids and proteins. The protein carrier could confer considerable specificity to such methyl transfers. After methyl transfer (i.e., in vivo or after heating in ethanol as in present bound-dye determinations) the dye would remain bound to protein through an alkali-stable sulfide bond and

would have properties similar to the protein-bound dyes long known to be present in the livers of dye-fed rats (Miller and Miller, 1947; Terayama and Takeuchi, 1962). The presence of sulfur in these protein-bound dyes was previously reported (Miller and Miller, 1961; Andersen, 1964).

REFERENCES

Andersen, R. A., Ph.D. Thesis, Univ. Wisconsin, 1964.
Andersen, R. A., Enomoto, M., Miller, E. C., and Miller, J. A., Cancer Res., 24, 128 (1964).
DiGirolamo, A., Henshaw, E. C., and Hiatt, H. H., J. Mol. Biol. 8, 479 (1964).
Matsumoto, M., J. Jap. Biochem. Soc. (Seikagaku) 37, 37 (1965).
Miller, E. C., and Miller, J. A., Cancer Res. 7, 468 (1947).
Miller, J. A., and Miller, E. C., J. Exp. Med. 87, 139 (1947).
Miller, J. A., and Miller, E. C., Canad. Cancer Conf. 4, 468 (1961).
Miller, J. A., Miller, E. C., and Baumann, C. A., Cancer Res. 5, 163 (1945).
Mueller, G. C., and Miller, J. A., J. Biol. Chem. 202, 579 (1953).
Poirier, L. A., Sato, K., Miller, E. C., and Miller, J. A., in preparation.
Silverstone, H., Cancer Res. 8, 301 (1948).
Terayama, H., and Takeuchi, M., Gann 53, 293 (1962).