STUDIES ON THE STRUCTURES OF POLAR DYES FROM LIVER PROTEINS OF RATS FED N-METHYL-4-AMINOAZOBENZENE: I. RETENTION OF THE METHYL GROUP

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Received August 21, 1967

Hepatic protein-bound aminoazo dyes are formed in the rat from dyes such as N-methyl-4-aminoazobenzene (MAB) or N,N-dimethyl-4-aminoazobenzene and may play a role in hepatocarcinogenesis by these amines (Miller and Miller, 1953, 1966). Enzymatic and alkaline hydrolysis of these protein-bound dyes yields four alkali-stable water-soluble or polar dyes (P1, P2a, P2b, and P3) (Terayama and Takeuchi, 1962; Higashinakagawa et al., 1966) which contain a secondary aromatic amino group and are ninhydrin-positive. Recently alkali was found to release the non-polar dye 3-methylmercapto-N-methyl-4-aminoazobenzene (3-CH_3S-MAB) from the liver protein of rats fed MAB (Scribner et al., 1965). Subsequently Higashinakagawa et al. (1966) and Terayama (1966) reported that the four polar dyes contain sulfur. P1 contained sulfur derived from methionine-^{35}S but not from cystine-^{35}S. The major component, P2b, also contained ^{35}S when the rat was administered methionine-^{35}S. Upon treatment with Raney nickel all the polar dyes yielded α-aminobutyric acid. P1 upon oxidation with CF_3CO_3H and subsequent reduction with $SnCl_2$ yielded p-phenylene diamine.* These data led Higashinakagawa et al. (1966) to conclude that the polar dyes are derived from protein-bound dye in which the S of methionine is attached to the methyl group of MAB, probably in the

* This finding is not conclusive evidence of the absence of ring substitution since CF_3CO_3H might oxidize a sulfur atom on the diamine ring to a sulfonic acid which could be hydrolyzed to form p-phenylene diamine.

form of a methylene bridge.

The data reported below show that the polar dyes contain unsubstituted methyl groups derived from the administered MAB and therefore do not support the structure proposed by Higashinakagawa et al. (1966).

Materials and Methods

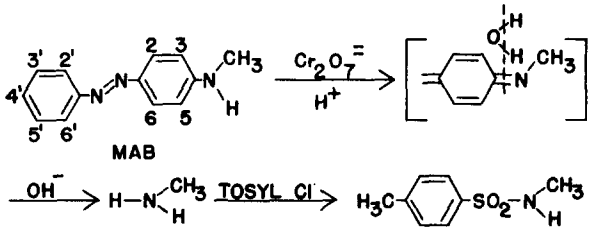
MAB($CH_2{}^3H$) + MAB ($^{14}CH_3$) was prepared from 1.3 mmoles (100 mc) of CH_3I-^3H plus 1.3 mmoles (1.95 mc) of CH_3I-^{14}C and 20 mmoles of 4-aminoazobenzene by the general procedure of Terayama et al. (1960). The chromatographed MAB ($CH_2{}^3H$) + MAB($^{14}CH_3$) (80% yield, 440 mg, m.p. 88-89°) had specific activities of 6.1×10^8 dpm/mg for ^3H and 8.1×10^6 dpm/mg for ^{14}C.

Male albino rats (Holtzman strain), 160-180 gm, were fed a purified diet (Andersen et al., 1964; 1 mg of riboflavin/kg) for 7 days before receiving by stomach tube 25 mg of a 1:1 mixture of labeled and unlabeled MAB dissolved in 1 ml of corn oil. The rats were exsanguinated 28 hrs later and the polar dyes were isolated from the pooled livers after enzymatic and alkaline hydrolysis (Terayama and Takeuchi, 1962; Hanaki and Terayama, 1962). As described by these authors the polar dyes were concentrated on a silica gel column and chromatographed on Whatman No. 1 paper in the aqueous phase of n-propanol: n-butanol: water (1:4:5 by volume) to give four components with R_f's as follows: P1 (0.55), P2a (0.63), P2b (0.34), P3 (0.75). The dyes were eluted with 50% methanol and 0.5 ml aliquots were mixed with 10 ml of ANPO scintillation mixture (α-naphthylphenyloxazole, 0.46 gm; diphenyloxazole, 46 gm; naphthalene, 738 gm; xylene, 3500 ml; dioxane, 3500 ml; and absolute ethanol, 2100 ml) for simultaneous determination of ^3H and ^{14}C in a Packard Tricarb liquid scintillation spectrometer. These measurements were corrected for background and quenching.

The enzymatic oxidative demethylation of the labeled MAB by normal rat liver homogenate was performed in the presence of 0.01 M semicarbazide

(Mueller et al., 1953). The contents of 20 flasks (60 ml containing homogenate equivalent to 1 gm of fresh liver) were pooled and acidified with 40 ml of 5% $HClO_4$. After centrifugation 2 mg of formaldehyde were added to the supernatant solution as carrier and 100 ml of the mixture were distilled into 50 ml of 0.2% dimedon solution. After standing overnight at 4° the dimedon derivative crystallized. The washed and dried crystals were dissolved in benzene and the radioactivities were determined in Liquifluor scintillation fluid (Pilot Chemicals, Watertown, Mass.)

The oxidative demethylamination of the labeled dyes was based on the following reactions:



Two to 20 μg of dye, 200 mg of $CH_3NH_2 \cdot HCl$, 100 mg of $K_2Cr_2O_7$ and 140 ml of 0.5 N HCl were refluxed for 30 min. After cooling in ice 10 gm of Na_2CO_3 were added. The mixture was then distilled into 50 ml of 1 N HCl until 100 ml of distillate was collected. The distillate was condensed to 40 ml and mixed with 0.8 gm of $CH_3NH_2 \cdot HCl$, 2.9 gm of tosyl chloride and 2.4 gm of NaOH. This mixture was heated until the tosyl chloride dissolved, filtered, and acidified slowly with conc. HCl. The white precipitate which formed was filtered off, washed with cold water, dried over conc. H_2SO_4, and repeatedly crystallized from ethanol-water and benzene-hexane mixtures. Each crystalline tosylmethylamide (m.p. 78°) sample was dissolved in 0.5 ml of ethanol and added to 10 ml of ANPO scintillation mixture for radioactivity determinations.

Results and Discussion

The MAB($CH_2{}^3H$) + MAB($^{14}CH_3$) administered to the rats and the free MAB recovered from the livers had similar $^3H/^{14}C$ ratios (Table 1). All the polar dyes isolated from the liver proteins had similar and somewhat higher ratios of ^3H to ^{14}C. According to the structure proposed by Higashinakagawa et al. (1966) the $^3H/^{14}C$ ratio could not exceed 51, unless selection had occurred in vivo among the two types of methyl-labeled MAB molecules in the formation of the protein-bound dyes. Theoretically this might occur in the oxidation of MAB to the reactive N-hydroxymethyl intermediate proposed by Higashinakagawa et al. (1966) as the precursor of the protein-bound dye. However, as shown in Table 2, rat liver demethylase is not able to distinguish between MAB ($CH_2{}^3H$) and MAB($^{14}CH_3$) in the oxidation of these dyes to the N-hydroxymethyl derivative (Mueller et al., 1953) that decomposes to yield formaldehyde.

Table 1

Radioactivity Ratios of Polar Dyes from Liver Protein of Rats Fed MAB($CH_2{}^3H$) + MAB($^{14}CH_3$)

	$^3H/^{14}C$	
	Exp. 1	Exp. 2
MAB($CH_2{}^3H$) + MAB($^{14}CH_3$), fed	76	76
" " , from liver	79	78
Polar dyes, total	83	82
P1	81	81
P2a	82	79
P2b	83	81
P3	82	-
Theoretical: Intact, $-NH-CH_3$	76	
Methylene bridge, $-NH-CH_2-S-$ (Higashinakagawa et al., 1966)	51 (for random removal of ^1H and ^3H)	

Table 2

Enzymatic Oxidative Demethylation of MAB($CH_2{}^3H$) + MAB($^{14}CH_3$) by Rat Liver Homogenate

	$^3H/^{14}C$
MAB($CH_2{}^3H$) + MAB($^{14}CH_3$) substrate	76
HCHO formed: Exp. 1	51
Exp. 2	52
Theoretical ratio for random removal of ^1H and ^3H	51

The formaldehyde had a $^3H/^{14}C$ ratio that was two-thirds that of the labeled substrate. This is the expected ratio if oxidation of the ^3H and ^1H atoms in the ^{12}C-methyl groups and of the ^1H atoms in the ^{14}C-methyl groups occurred at random.

Further evidence that intact methyl groups from MAB are present in the polar dyes was obtained from the oxidative demethylamination of the dyes (Table 3). The methylamine from each polar dye had $^3H/^{14}C$ ratios similar to

Table 3

Oxidative Demethylamination of Labeled MAB and Polar Dyes

	$^3H/^{14}C$	
	Dye	Tosylmethylamide[a] (1st to nth crystallization)
MAB($CH_2{}^3H$) + MAB($^{14}CH_3$)	76	88, 88
Polar dyes, total	85	85, 91, 91, 93, 90, 90
P1	81	81, 87, 86, 89
P2a	80	81, 93, 91, 90
P2b	84	83, 87, 86, 86
P3	80	81, 85, 82, 86

a) Over-all yields of methylamine were 30-35%.

that of the methylamine from the doubly-labeled MAB. A small isotope effect in this oxidation raised the $^{3}H/^{14}C$ ratios of the tosylmethylamide samples 10-15% above that of the dyes.

The data in this communication demonstrate that all the polar dyes contain in intact form the methyl group of the administered MAB. Previous data (Miller et al., 1949; Terayama and Kanda, 1960) show that high yields of volatile primary aromatic amine are formed upon reduction of the polar dyes. Thus the polar group(s) must be attached to the "diamine" or non-prime ring of MAB. The 3-position (ortho to the N-methyl group) of the MAB appears to be the most likely site of attachment since an appreciable fraction of the protein-bound dye in the liver can be released as 3-CH_3S-MAB (Scribner et al., 1965). The latter dye appears to be derived from a methionine sulfonium protein-bound dye. If this sulfonium form became S-demethylated in vivo or during the preparation of the polar dyes (Lin et al., 1967), or both, MAB bound to the S atom of protein-bound homocysteine would result. An alkali-stable polar dye would result upon hydrolysis of the protein. It is suggested that the major polar dye P2b consists of MAB attached at its 3-carbon to the sulfur atom of homocysteine. Oxidation in vivo or in vitro, or both, would yield related polar dyes. Studies on the synthesis and properties of these dyes are in progress.

Acknowledgment

This research was supported by grants from the National Cancer Institute (CA-07175), the Jane Coffin Childs Memorial Fund for Medical Research, and the Alexander and Margaret Stewart Trust Fund.

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