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THE HYDROXYLATION AND CARCINOGENICITY *IN VIVO* OF AMINOAZO DYES

J. W. WESTROP and J. C. TOPHAM

University of Sheffield, Cancer Research Unit, Western Bank, Sheffield 10

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Abstract—Various aminoazo dyes were administered to rats and the amount of 4′-hydroxylated metabolites in the liver was estimated.

For these dyes having an unsubstituted 4′-position the degree of 4′-hydroxylation did not correlate with their carcinogenic potency.

On the other hand, 4′-substituted aminoazo dyes suffered replacement of the substituent by a hydroxyl group to an extent which was proportional to their carcinogenic activity.

The results are interpreted as offering support for the proposal that the intermediate formation of arylhydroxylamines from aminoazo dyes has carcinogenic significance.

THE metabolic reactions of chemical carcinogens have been studied intensively[1, 2] with the object of discovering which of them, or indeed if any, are related to their carcinogenic activity. Probably the major success arising out of these endeavours is the demonstration that several carcinogens become covalently bound to tissue proteins together with the impressive body of data which correlates this phenomenon with carcinogenicity, especially for aminoazo dyes,[3–5] amines and amides,[3, 6, 7] and polybenzenoid hydrocarbons.[3, 8, 9] However, at least as far as aminoazo dyes are concerned, the exact nature of the precursor metabolic reactions which may be supposed to convert the carcinogen into a protein-reactive intermediate remain obscure. Thus, they undergo oxidative N-demethylation[10] and reductive fission of the azo linkage[11, 12] but neither reaction correlates with their carcinogenicity.[13–16] It is understandable why the remaining known metabolic reaction of aminoazo dyes, C-hydroxylation occurring mainly at the 4′-position,[11] has not been studied from this viewpoint since the products are non-carcinogenic[17] and their formation would seem to be merely a detoxification reaction. Nevertheless, such a study was undertaken partly because of the availability of a method of thin layer chromatography[18] which permitted the easy separation of aminoazo dye metabolites. An early outcome, reported elsewhere,[19] was the finding of the same 4′-hydroxylated metabolites in the liver of rats treated with either 4-dimethylaminoazobenzene (DMAB) or 4′-fluoro-DMAB despite the ostensibly blocked 4′-position in the latter. It was pointed out that the immediate importance of this result lay in the element of doubt which it attached to the conclusions derived from the use of fluorinated carcinogens in certain studies of carcinogenic mechanisms since they were based upon the assumption, now disputed, of the metabolic inertness of this type of carbon-fluorine bond.

Additionally, the unexpected defluorohydroxylation of 4′-fluoro-DMAB hinted

at the possibility that the 4′-hydroxylation of aminoazo dyes, although resulting in non-carcinogenic products, might be related in some obscure way to their carcinogenicity. That is to say, it might be consequent upon, and serve as a pointer to, the prior occurrence of an unknown metabolic reaction having carcinogenic significance. Such a reaction which suggested itself was that of N-hydroxylation. Since some arylhydroxylamines are known to rearrange to the isomeric *p*-hydroxy-amines,[20] it was supposed that if a major part of the total 4′-hydroxylation of aminoazo dyes were consequent upon their initial conversion to N-hydroxy-compounds (arylhydroxylamines), and if this held crucial importance for their activity as seems to be the case for a variety of aromatic amines and amides,[1, 2, 7, 21] then it could be predicted that the extent of the 4′-hydroxylation of an aminoazo dye should be related to its carcinogenicity. The present report is concerned with the experimental testing of this prediction.

EXPERIMENTAL

Groups of male Wistar rats (250–350 g body wt.) which had access to Diet No. 86 and water at all times received by stomach tube a dose of 20 mg of each azo dye in 1 ml olive oil per 100 g body wt. Five hours later the animals were killed by exsanguination under ether anaesthesia. The livers were removed, rinsed in cold saline, blotted dry and pooled for immediate analysis.

Aminoazo dyes

DMAB was a commercial product and the other azo dyes, some of which were generous gifts of Dr. W. J. P. Neish of these laboratories, were synthesized by published procedures and recrystallized before use. Each dye was checked for the absence of contaminating C-hydroxylated or N-demethylated derivatives by thin layer chromatography on Silica Gel G using 10% methanol in benzene as solvent. In this system, these derivatives have appreciably different mobilities from those of the

TABLE 1. SEPARATION OF DMAB FROM SOME OF ITS DERIVATIVES BY THIN LAYER CHROMATOGRAPHY ON SILICA GEL G

Azo dye	Solvent	Solvent migration (cm)	R_f azo dye / R_f DMAB
4′-Fluoro-DMAB	Xylene	15	1·22
2′-Methyl-DMAB	Benzene	10	1·18
2-Methyl-DMAB	Xylene	10	1·13
4′-Methyl-DMAB	*O*-dichlorobenzene	15	1·10
4′-Ethyl-DMAB	Petrol.ether*/acetone, 7:3 v/v	15	1·07

* Boiling range 40–60.

parent dyes and their presence therein would have been detected easily. For those substituted DMAB compounds for which we were able to find solvent systems which permitted their separation from the adventitious presence of any unsubstituted DMAB (Table 1), the latter was below the level of detection (0·025 μg DMAB in 2 μg of total dye) in each case.

Estimation of 4'-hydroxylated metabolites

The preparation, from the liver, of the ether extract of the total free dye metabolites, the removal therefrom of interfering lipids, the separation of the metabolites by thin layer chromatography and their visualisation by exposure to HCl, were as described previously.[18, 19] Likewise, the determination of the total 4'-hydroxylated metabolites from the amount of *p*-aminophenol produced on reductive cleavage of the total dye metabolites. In some instances, this determination was supplemented by estimations of the individual 4'-hydroxylated metabolites, 4'-hydroxy-DMAB, 4'-hydroxy-4-methylaminoazobenzene, 4'-hydroxy-4-aminoazobenzene and 4'-hydroxy-4-acetylaminoazobenzene, following their elution from replicated chromatograms and direct spectrophotometric comparison, in acid solution, with authentic specimens. The procedure was modified slightly for certain of the azo dyes studied. Thus, the amphoteric (4'-hydroxylated) metabolites in the ethereal extracts obtained from the liver of rats treated with 4'methoxy-, 4'-ethoxy- and 4'-phenoxy-DMAB were removed with dilute NaOH before being subjected to reductive fission. Otherwise, the alkyloxy- or aryloxy- anilines, obtained as fission products of the administered dyes and their non-4'-hydroxylated metabolites, interfered in the determination of *p*-aminophenol. Finally, *p*-aminophenol was replaced as comparison standard by the appropriate 3-methyl- and 2-methyl-4-aminophenol in the estimation of the aminophenols produced by fission of the metabolites of 3'-methyl- and 2-methyl-DMAB, respectively.

RESULTS AND DISCUSSION

A qualitatively similar pattern of metabolites was obtained for all of the azo dyes examined excepting those which were not 4'-hydroxylated. The thin layer chromatograms, typical examples of which have been reproduced elsewhere,[19] showed the presence of the administered dimethylaminoazo dye, its N-monomethylated- and N-demethylated metabolites, the 4'-hydroxylated derivatives of all three and a novel metabolite, 4'-hydroxy-4-acetylaminoazobenzene. Hence, each of these latter contributed some proportion towards the total 4'hydroxylation. In the case of DMAB and those of its derivatives (4'-fluoro-, 4'-methyl- and 4'-ethyl-DMAB) for which each of the 4'-hydroxylated metabolites was determined separately, it was evident that the contribution to the total made by 4'-hydroxy-4-acetylaminoazobenzene was large and that by 4'-hydroxy-4-methylamino-azobenzene was small. No other generalisation could be made regarding the relative proportions of the 4'-hydroxylated metabolites yielded by the different azo dyes.

The total quantities of the 4'-hydroxylated metabolites found in the liver of rats 5 hr after the administration of various aminoazo dyes having a free or substituted 4'-position are given in Tables 2 and 3, respectively. For the former azo dyes it was apparent that the extent of 4'-hydroxylation did not correlate with carcinogenic activity. It will be recalled, however, that this predicted correlation was expected to obtain only if a major part of the total 4'-hydroxylation was effected via the proposed intermediate arylhydroxylamine. Presumably, any contribution by this latter mechanism to the overall hydroxylation of azo dyes having a free 4'-position has been obscured by the preponderance of that due to the competitive mechanism of direct aromatic hydroxylation.[22] In this connection, it may be noted that despite the greater carcinogenic potency of 3'-methyl-DMAB it was 4'-hydroxylated to a lesser extent

than DMAB. Perhaps for steric or other reasons the direct hydroxylation of 3′-methyl-DMAB is inhibited with the result that a disproportionately large share of the total hydroxylation of this compound derived from the proposed arylhydroxylamine rearrangement process.

TABLE 2. THE 4′-HYDROXYLATION OF AZO DYES HAVING AN UNSUBSTITUTED 4′-POSITION BY RAT LIVER *in vivo*

Azo dye	Relative carcinogenic activity*	Rats per expt.	4′-hydroxylated metabolites (m μmoles/g liver)
DMAB	6	6	47·7
2-Methyl-DMAB	0	3	24·3
3-Methyl-MAB†	1	2	18·7
2′-Methyl-DMAB	2–3	3	20·4
3′-Methyl-DMAB	10–12	3	18·4

* See ref. 17.
† MAB—4-methylaminoazobenzene.

TABLE 3. THE 4′-HYDROXYLATION OF AZO DYES HAVING A 4′-SUBSTITUENT BY RAT LIVER *in vivo*

Azo dye	Relative carcinogenic activity *	Rats per expt.	4′-hydroxylated metabolites (m μmoles/g liver)
4′-Nitro-DMAB	0	3	0
4′-TrifluoromethylDMAB	0	3	0
4′-Methyl-DMAB	1	3	6·6
4′-Chloro-DMAB	1–2	3	5·0
4′-Methoxy-DMAB	3	1	10·0
4′-Ethyl-DMAB	10	3	21·6
4′-Fluoro-DMAB	10–12	6	16·2
4′-Phenoxy-DMAB	—	4	0
4′-Ethoxy-DMAB	—	1	6·6

* See ref. 17.

The situation was quite different for the azo dyes which possessed a blocking group in the 4′-position. Thus, the two noncarcinogenic azo dyes were not 4′-hydroxylated at all and the three weakly carcinogenic azo dyes were hydroxylated appreciably less than the two strongly carcinogenic ones. Especially noteworthy was the greater extent of hydroxylation of the highly carcinogenic 4′-ethyl-DMAB relative to that of the much weaker carcinogen, 4′-methyl-DMAB. It had been anticipated that for this series of 4′-substituted dyes a direct 4′-hydroxylation should not be possible. Therefore, whatever 4′-hydroxylation did occur could result only from some other mechanism, for example the proposed arylhydroxylamine rearrangement. Since the correlation of carcinogenic activity with 4′-hydroxylation which was predicted for these dyes has been realised, it suggests that the suppositions upon which it was based are probably correct. One of these was that the metabolic conversion of aminoazo dyes into arylhydroxylamines is an important factor in carcinogenesis by these compounds. Substantiation of the proposition requires the availability for carcinogenic assay of an

N-hydroxylated methyl aminoazo dye but their synthesis has not been achieved as yet.[23] It would receive some support if it could be shown for a carcinogenic amine or amide, known to owe its activity to its conversion *in vivo* into an N-hydroxy-compound, that fluorine substitution at a preferred site of *in vivo* hydroxylation was unable to prevent hydroxylation. This has been achieved in preliminary work which has indicated that carcinogenic 7-fluoro-2-acetylaminofluorene which, like the unsubstituted amide, is metabolised to the N-hydroxy-derivative[1] affords a conjugated 7-hydroxy-2-acetylaminofluorene in the urine of appropriately dosed rats and guinea pigs. The proposition could be further tested by an assessment of the carcinogenic potency of 4′-ethoxy- and 4′-phenoxy-DMAB which, from the extent of their 4′-hydroxylation, might be predicted to be low and zero, respectively.

Although the 4′-hydroxylation of 4′-methoxy-DMAB correlates well with its carcinogenicity, this could be fortuitous in respect of the present concept since the O-demethylation of this compound occurs possibly by a mechanism[24] which differs from that proposed here for the other 4′-substituted azo dyes. Finally, the initial metabolism of aminoazo dyes to the N-oxide followed by its rearrangement to give the ortho-hydroxy derivative has been proposed by Terayama[25] as having significance for their carcinogenic activity. The possibility of rearrangement to the "extended para" (4′) position, discussed in the present report, was not considered.

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