# Model Studies for Azo Dye Carcinogenesis

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The isomeric 2'-, 3'-, and 4'-hydroxymethyl-4-dimethylaminoazobenzenes have been prepared. The corresponding chloromethyl compounds have been isolated from reactions of the 3'- and 4'-isomers with thionyl chloride, but rapid formation of the indazole ring system precluded the isolation of a chloromethyl derivative from the 2'-isomer. The 3'- and 4'-chloromethyl and iodomethyl derivatives reacted with DNA *in vitro*.

COVALENT binding of 4-dimethylaminoazobenzenes to cellular macromolecules *in vivo* [1] is the most likely first step of their carcinogenic action. A covalent interaction does not occur *in vitro*, and this binding is probably mediated by metabolism of the azo dyes. A two-step enzymic activation mechanism of *N*-hydroxylation at the amino-group followed by esterification of the *N*-hydroxy-function to give a reactive sulphate ester has been suggested.[2] For the methyl-4-dimethylaminoazobenzenes, either the same mechanism or an analogous activation at the ring methyl group could be envisaged.[3] The present studies are relevant to the latter possibility and describe model compounds for the postulated reactive sulphate esters derived from the 2'-, 4'-, and 3'-methyl-4-dimethylaminoazobenzenes. The last compound is a much more potent hepatocarcinogen than either of the first two,[4] so that if metabolism at the ring methyl group is involved in the carcinogenic action of these compounds, differences might be expected either in the extent or in the mode of reaction of these model compounds with cellular nucleophiles, such as DNA.

The 2'-, 3'-, and 4'-hydroxymethyl-4-dimethylaminoazobenzenes were readily prepared either by diazotization of the appropriate aminobenzyl alcohol and coupling with *NN*-dimethylaniline, or by reduction of the methyl ester of the appropriate dye-carboxylic acid with sodium bis-(2-methoxyethoxy)aluminium hydride.[5] Reactions with thionyl chloride converted the 3'- and 4'-hydroxymethyl derivatives into chloromethyl derivatives, and from these the iodomethyl compounds were readily obtained. 4-Dimethylamino-2'-hydroxymethylazobenzene was converted by treatment with thionyl chloride into 2-(*p*-dimethylaminophenyl)indazole. This finding is consistent with the use of azobenzenes with a hydroxymethyl group *ortho* to the azo linkage as starting materials for indazole syntheses.[6] Treatment of the hydroxymethyl compounds with toluenesulphonyl chloride in dimethylformamide, with *N*-ethylmorpholine as base, gave the same products as the reported reactions with thionyl chloride. However, the yields of the 3'- and 4'-chloromethyl derivatives were lower, since aralkylation of the morpholine also occurred.

TABLE 1

Spectral properties of azo dye derivatives

| | In ethanol | | In 5N-HCl–ethanol (1 : 15) | |
|---|---|---|---|---|
| | $\lambda_{max.}$/nm | $10^{-3}\varepsilon$ | $\lambda_{max.}$/nm | $10^{-3}\varepsilon$ |
| Methyl 4'-dimethylaminoazobenzene-4-carboxylate | 433 | 33·7 | 512 | 52·4 |
| | 275 | 12·4 | 292 | 11·7 |
| Methyl 4'-dimethylaminoazobenzene-3-carboxylate | 415 | 29·7 | 510 | 30·8 |
| | 271 | 7·9 | 315 | 11·0 |
| | | | 293 | 11·0 |
| 4-Dimethylamino-2'-hydroxymethylazobenzene | 409 | 27·2 | 522—524 | 9·5 |
| | 313 | 4·3 | 323 | 16·3 |
| | 247 | 6·5 | 229 | 9·3 |
| 4-Dimethylamino-3'-hydroxymethylazobenzene | 406 | 30·6 | 519 | 38·0 |
| | 255 | 9·9 | 318 | 9·6 |
| | | | 232 | 7·0 |
| 4-Dimethylamino-4'-hydroxymethylazobenzene | 407 | 31·0 | 524 | 37·1 |
| | 255 | 10·0 | 325 | 11·0 |
| | | | 232 | 6·7 |
| 2-(*p*-Dimethylaminophenyl)-indazole | 325 | 22·0 | 300 | 19·1 |
| | 272 | 2·4 | 238 | 23·4 |
| 3-Chloromethyl-4'-dimethylaminoazobenzene | 410 | 28·9 | 516 | 36·4 |
| | 265 | 10·6 | 318 | 10·0 |
| 4-Chloromethyl-4'-dimethylaminoazobenzene | 413 | 29·4 | 520 | 37·8 |
| | 265 | 9·5 | 321 | 10·7 |
| 4-Dimethylamino-3'-iodomethylazobenzene | 410 | 28·9 | 517 | 37·2 |
| | 270 | 13·2 | 315 | 10·8 |
| | | | 295 | 11·3 |
| 4-Dimethylamino-4'-iodomethylazobenzene | 411 | 29·0 | 524 | 37·8 |
| | 255 | 10·2 | 324 | 12·1 |

TABLE 2

Reaction of chloromethyl- and iodomethyl-4-dimethylaminoazobenzenes with DNA

| | $E_{320}/E_{260}$ for DNA after reaction with azo dye derivatives | | | |
|---|---|---|---|---|
| Time (h) | 4'-chloro- | 4'-iodo- | 3'-chloro- | 3'-iodo- |
| 0·2 | 0·05 | 0·29 | | |
| 1·0 | 0·37 | 0·62 | | 0·12 |
| 1·5 | 0·39 | 0·71 | | |
| 2·7 | 0·42 | 0·70 | | |
| 5·0 | | | | 0·25 |
| 6·5 | 0·32 | 0·68 | | |
| 7·75 | | | 0·02 | 0·30 |
| 23 | 0·19 | 0·54 | 0·03 | 0·33 |
| 49 | 0·14 | 0·51 | 0·13 | 0·34 |
| 94 | 0·13 | 0·39 | 0·24 | 0·36 |

The spectral characteristics of the new compounds prepared are recorded in Table 1; Table 2 presents

[1] J. J. Roberts and G. P. Warwick, *Internat. J. Cancer*, **1966**, **1**, 573; C. W. Dingman and M. B. Sporn, *Cancer Res.*, **1967**, **27**, 938; G. P. Warwick, *European J. Cancer*, **1967**, **3**, 227; J. J. Roberts, *Jerusalem Symposia on Quantum Chemistry and Biochemistry*, **1969**, **1**, 229.

[2] L. A. Poirier, J. A. Miller, E. C. Miller, and K. Sato, *Cancer Res.*, **1967**, **27**, 1600.

[3] A. Dipple, P. D. Lawley, and P. Brookes, *European J. Cancer*, **1968**, **4**, 493.

[4] J. A. Miller and E. C. Miller, *Adv. Cancer Res.*, **1953**, **1**, 339.

[5] J. Vit, B. Casensky, and J. Machacek, Fr.P., 1,515,582; M. Cerny, J. Malek, M. Capka, and V. Chvalovsky, *Coll. Czech. Chem. Comm.*, **1969**, **34**, 1025.

[6] L. C. Behr, in 'Pyrazoles, Pyrazolines, Pyrazolidines, Indazoles and Condensed Rings,' ed. R. H. Wiley, Interscience, New York, **1967**, p. 302.

data obtained on the reactions of the 3'- and 4'-chloromethyl and iodomethyl derivatives with DNA. After treatment with the azo dye derivatives, DNA exhibits a new absorption maximum in the 320 nm region, so that the ratio $E_{320}/E_{260}$ is a measure of the relative extent of reaction of the dye derivative with DNA at various times. This ratio for both 4'-derivatives eventually decreases with time, which suggests the formation of labile products, possibly 7-substituted guanine derivatives.[7] Consistent with this is the observation that the DNA precipitates were particulate rather than fibrous (indicative of a decrease in molecular weight) in the experiments with the 4'-derivatives. This was not the case for the 3'-derivatives, nor was a decrease in the $E_{320}/E_{260}$ ratio with increasing time observed.

Thus, although both the 3'- and 4'-halogenomethyl derivatives react with DNA, the data indicate that these agents attack different sites on the DNA. This could account for the different carcinogenic potencies of the parent 3'- and 4'-methyl-4-dimethylaminoazobenzenes, if metabolic conversion into a reactive centre at the ring methyl groups is involved in their carcinogenic action. Also, the low carcinogenic potency of 4-dimethylamino-2'-methylazobenzene is consistent with this mechanism, since the introduction of a leaving group on to the 2'-carbon atom would lead to indazole formation in preference to alkylation of cellular nucleophiles.

## EXPERIMENTAL

M.p.s are corrected and were determined with a microscope hot-stage apparatus. Absorption spectra were recorded with a Unicam SP 800 spectrophotometer. Analyses were performed by Dr. F. B. Strauss, Microanalytical Laboratory, Oxford.

Methyl *m*-aminobenzoate and *p*-aminobenzoate and *o*-aminobenzyl alcohol were obtained commercially. *m*-Aminobenzyl alcohol was prepared (82%) by reduction of *m*-aminobenzoic acid in benzene with 3 mol. equiv. of sodium bis-(2-methoxyethoxy)aluminium hydride (R. N. Emanuel, Wembley) at 80° for 90 min.

*4-Dimethylaminoazobenzenes.*—*NN*-Dimethylaniline (1 mol. equiv.) in ethanol was added to a solution of the appropriately substituted diazotized aniline. The solution was then neutralized by addition of sodium acetate in portions, and the precipitated azo dye was collected and crystallized.

*Methyl 4'-dimethylaminoazobenzene-4-carboxylate* was obtained as shiny red plates (65%), m.p. 195—196° (from ethyl acetate) (Found: C, 67·55; H, 5·95; N, 14·3. $C_{16}H_{17}N_3O_2$ requires C, 67·8; H, 6·05; N, 14·85%). *Methyl 4'-dimethylaminoazobenzene-3-carboxylate* was obtained (84%) as orange needles, m.p. 99·5—101·5° [from light petroleum (b.p. 60—80°)] (Found: C, 68·45; H, 6·2; N, 14·9%). *4-Dimethylamino-2'-hydroxymethylazobenzene* was obtained (56%) as orange needles, m.p. 103—104° (from ethanol–water) (Found: C, 70·7; H, 6·65; N, 16·2. $C_{15}H_{17}N_3O$ requires C, 70·6; H, 6·7; N, 16·45%). *4-Dimethylamino-3'-hydroxymethylazobenzene*, obtained similarly in 80% yield or by reduction of the appropriate ester with sodium bis-(2-methoxyethoxy)aluminium hydride in benzene at room temperature (yield 81%), gave orange crystals, m.p. 121·5—123° (from ethanol–water) (Found: C, 70·05; H, 6·7; N, 16·2%). *4-Dimethylamino-4'-hydroxymethylazobenzene* was obtained by reduction of the appropriate ester in 85% yield, m.p. 178·5—180·5° (from ethanol) (Found: C, 70·1; H, 6·6; N, 16·4%).

*Reaction of 2'-, 3'-, and 4'-Hydroxymethyl-4-dimethylaminoazobenzenes with Thionyl Chloride.*—Each isomer (1 mmol) was separately treated with thionyl chloride (1 ml). After 20 min at room temperature the red solutions were evaporated to dryness and the residues were slurried in acetone (20 ml) and treated dropwise with 10% (v/v) pyridine in acetone until all the solid had dissolved and the original red colour had changed to orange. The solutions were again evaporated to dryness, the residues were extracted six times with hot benzene, and the extracts were dried ($MgSO_4$) and evaporated to dryness. The final residues were crystallized.

The 2'-isomer was thus converted into 2-(p-*dimethylaminophenyl)indazole* (80%), as pale yellow needles, m.p. 187° (from benzene) (Found: C, 75·8; H, 6·3; N, 17·7. $C_{15}H_{15}N_3$ requires C, 75·95; H, 6·35; N, 17·7%). The reactions of the 3'-isomer and the 4'-isomer gave the corresponding chloromethyl compounds (78 and 76%, respectively). *3-Chloromethyl-4'-dimethylaminoazobenzene* gave orange needles, m.p. 101—102·5° [from light petroleum (b.p. 100—120°)] (Found: C, 65·85; H, 5·9; N, 15·2. $C_{15}H_{16}ClN_3$ requires C, 65·8; H, 5·9; N, 15·35%), and *4-chloromethyl-4'-dimethylaminoazobenzene* had m.p. 149—150° [from light petroleum (b.p. 100—120°)] (Found: C, 65·95; H, 5·9; N, 15·25%).

*Reactions of 2'-, 3'-, and 4'-Hydroxymethyl-4-dimethylaminoazobenzenes with Toluene-p-sulphonyl Chloride.*—Each isomer (1 mmol) in dimethylformamide (0·5 ml) and *N*-ethylmorpholine (0·5 ml) was separately treated with tosyl chloride (1·3 mmol) and then kept at 37°.

*2'-Isomer.* After 17 h the deposited crystals were collected, washed with water, dried, and recrystallized from benzene to give pale yellow needles of 2-(*p*-dimethylaminophenyl)indazole (78%), identical with that already described [m.p., u.v. spectrum, and t.l.c. (three solvent systems)].

*3'-Isomer.* After 2·5 h the mixture was diluted with water (5 ml) and extracted four times with ethyl acetate. The organic layers were dried and applied to a column of silicic acid. 3-Chloromethyl-4'-dimethylaminoazobenzene (60%), identical with that already described (u.v. spectrum, m.p., and t.l.c.), was eluted with benzene. The aqueous layer slowly deposited crystals of N-[3-(4-*dimethylaminophenylazo)benzyl*]-N-*ethylmorpholinium tosylate* (15%), m.p. 209—211° (from water) (Found: C, 63·55; H, 6·85; N, 10·7. $C_{28}H_{36}N_4O_4S$ requires C, 64·1; H, 6·9; N, 10·7%).

*4'-Isomer.* After 17 h the deposited crystals were collected and recrystallized from water to give N-[4-(4-*dimethylaminophenylazo)benzyl*]-N-*ethylmorpholinium tosylate* (49%), m.p. 195—197° (decomp.) (Found: C, 63·5; H, 6·95; N, 10·35%). The presence of 4-chloromethyl-4'-dimethylaminoazobenzene in the original filtrate was demonstrated by chromatography but it was not isolated in this experiment.

*Iodomethyl Derivatives.*—Both the 3- and the 4-chloromethyl dyes reacted smoothly with sodium iodide in acetone under reflux. *4-Dimethylamino-3'-iodomethylazobenzene* was obtained (72%) as pale orange plates (from n-hexane), m.p. (darkened at 93°) 113—114° (Found:

[7] P. D. Lawley and P. Brookes, *Biochem. J.*, 1963, **89**, 127.

C, 49·6; H, 4·5; N, 11·55. $C_{15}H_{16}IN_3$ requires C, 49·35; H, 4·4; N, 11·5%). *4-Dimethylamino-4′-iodomethylazobenzene* (62%) gave orange-red plates (from acetone) which darkened at 136° and decomposed above 138—140° (Found: C, 49·2; H, 4·4; N, 11·4%).

*Reaction of Chloromethyl and Iodomethyl Derivatives with DNA.*—To a solution (40 ml) of salmon sperm DNA (0·5 mg per ml in 0·025M-sodium acetate, pH 5·8) was added acetone (35 ml) containing the appropriate dye derivative (0·11 mmol). The solution was incubated at 37° and at various times DNA was precipitated from samples (2·5 ml) by two volumes of ethanol. The DNA was washed twice in ethanol, twice in acetone, twice in ether, dried, and redissolved in water. U.v. absorption spectra of these solutions were recorded; since reaction with the azo dye derivative introduced a new peak in the 320 nm region the extent of reaction of the dye with DNA was monitored by the ratio $E_{320}/E_{260}$ (Table 2).

This investigation was supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council and Cancer Research Campaign.

[1/1692 *Received*, 26*th October*, 1971]