Effect of Azo Dyes on Rat Serum Para-Phenylene Diamine Oxidase Activity

Recently, MILLER, MILLER, and FINGER[1] noted that whereas 4'-methyl-4-dimethylaminoazobenzene (4'-MeDMAAB) has feeble carcinogenicity, 4'-ethyl-4-dimethylaminoazobenzene (4'-EtDMAAB) is an extremely potent liver carcinogen for rats. The authors state that 'no large difference in simple chemical reactivity of these dyes would be expected and the reason (i.e. for the great difference in carcinogenicity) seemingly must be sought in decisive metabolic and steric differences'.

A striking difference has now been found in the effect of these azo dyes on the para-phenylene diamine (PPD) oxidase activity of rat serum. Adult male albino rats were tail-bled ($\sim$ 1·5 ml) under ether anaesthesia and the PPD oxidase activity of the fresh serum was determined by the method of RAVIN[2]. 24 h later, pairs of rats received intraperitoneal injections of arachis oil (0·6 ml/100 g body weight [b.w.]) and of solutions in arachis oil (0·6 ml/100 g b.w.) of the powerful liver carcinogen 3'-methyl-4-dimethylaminoazobenzene (3'-MeDMAAB; 16·5 mg/100 g b.w.), of 4'-MeDMAAB (16·5 mg/100 g b.w.) and of 4'-EtDMAAB (17·5 mg/100 g b.w.). At intervals therafter, serum PPD oxidase levels were determined. The results are given in Table I.

After arachis oil injection, rat serum PPD oxidase activity increased during 2–4 days but, by 21 days, the levels had returned to normal. The same effects were obtained with the feeble carcinogen, 4'-MeDMAAB. However, the powerful hepatocarcinogens, 3'-Me- and 4'-Et-DMAAB strongly suppressed serum PPD oxidase activity during the 4-day period following injection.

Injections of arachis oil solutions of the powerful 'local' carcinogens, 3,4-benzpyrene and 20-methylcholanthrene (MC) and of the non-carcinogenic pyrene or the application of whole body X-irradiation failed to suppress rat serum PPD oxidase activity, and it is of interest that N,N-dimethyl-*p*-(1-naphthylazo) aniline which is known to be a rapid sarcoma-inducing agent for female rats[3] but which has a long induction time for hepatoma formation[4] was also inactive in this respect. Indications have been obtained that injection of MC into male rats which received simultaneously 3'-MeDMAAB, speeds the return of serum PPD oxidase levels to normal, and we have noted that the PPD oxidase levels of female rats injected with 3'-MeDMAAB return more quickly to normal than is the case with male rats. MC is known to protect rats against hepatocarcinogenesis by 3'-MeDMAAB[5] and it has been found that male rats are more suceptible than females to the azo dye[6].

In view of the above-mentioned results and unpublished observations for other azo dyes, it seems that there is a close correlation between the hepatocarcinogenicity of an azo dye for rats and its ability, after intraperitoneal injection, to lower and maintain at a low level serum PPD oxidase activity. Our results point to a possible method for rapid screening of azo hepatocarcinogens and indicate that these substances may attack the liver précursors of caeruloplasmin, the copper-containing serum globulin which is probably identical with PPD oxidase. Confirmation that copper metabolism is profoundly influenced by hepatocarcinogenic azo dyes has been found in the observations (see Table II) that 3 days after injection of 3'-MeDMAAB, 4'-MeDMAAB and arachis oil only, serum copper levels as determined by the method of GUBLER *et al.*[7] are strongly depressed in adult female rats which received 3'-MeDMAAB, but not at all in those injected with the feeble carcinogen 4'-MeDMAAB or with arachis oil only.

Addition of copper salts to the diet is known to protect rats against hepatoma formation by 3'-MeDMAAB[8] and it was this fact as well as the following considerations which prompted the present investigation of the action of azo dyes *in vivo* on some rat serum enzymes which depend for activity on trace elements such as copper. MILLER *et al.*[1] noted that an unsubstituted 2-position is one of the requirements for carcinogenic activity of azo dyes and earlier NYE and LUCK[9] had obtained evidence that 3'-

[1] J. A. MILLER, E. C. MILLER, and G. C. FINGER, Cancer Res. *17*, 387 (1957).

[2] H. A. RAVIN, Lancet *1956*, 726.

[3] A. S. MULAY and R. W. O'GARA, J. nat. Cancer Inst. *18*, 843 (1957).

[4] A. S. MULAY and H. I. FIRMINGER, J. nat. Cancer Inst. *13*, 35 (1952).

[5] H. L. RICHARDSON and L. CUNNINGHAM, Cancer Res. *11*, 274 (1951).

[6] H. W. RUMSFIELD, W. L. MILLER, and C. A. BAUMANN, Cancer Res. *11*, 814 (1951).

[7] C. J. GUBLER, M. E. LAHEY, H. ASHENBRUCKER, G. E. CARTWRIGHT, and M. M. WINTROBE, J. biol. Chem. *196*, 209 (1952).

[8] E. PEDRERO and F. L. KOZELKA, Amer. med. Ass. Arch. Path. *52*, 455 (1951). – H. J. KING, J. D. SPAIN, and C. C. CLAYTON, J. Nutr. *63*, 301 (1957).

[9] W. NYE and J. M. LUCK, Abstr. Amer. chem. Soc., Meeting Sept. *1953*, 11C.

Table I

Para-phenylene diamine oxidase activity[2] (expressed as optical density at 530 mμ) of serums from pairs of rats

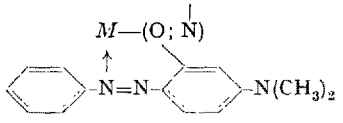
At time	Before and after injection of			
	Arachis oil only	Arachis oil solutions of		
		3'-MeDMAAB	4'-MeDMAAB	4'-EtDMAAB
24 h before injection	0·242; 0·369	0·369; 0·312	0·341; 0·328	0·327; 0·318
24 h after injection	0·332; 0·466	0·366; 0·297	0·418; 0·400	0·294; 0·285
48 h after injection	0·526; 0·598	0·263; 0·222	0·532; 0·548	0·254; 0·238
97 h after injection	0·394; 0·577	0·123; 0·171	0·532; 0·580	0·257; 0·187
21 days after injection	0·257; 0·341	died; 0·356	0·336; 0·348	0·336; 0·352

Serum (0·1 ml) is incubated with 2 ml of acetate buffer (pH 6) + 1 ml of PPD reagent (0·5 g PPD · 2 HCl/100 ml of water) for 1 h at 38° C. A serum-reagent blank containing in addition 1 ml of 0·1 % sodium azide was incubated at the same time. After reaction, 1 ml of 0·1% sodium azide was added to the first tube, the contents of both tubes were diluted to 10 ml with 3% sodium chloride and the colour read at 530 mμ (1-cm cell) in a Unicam SP 500 Spectrophotometer.

Table II

Substance injected in arachis oil	Rat No.	Serum Copper Level (μg/100 ml)	
		24 h before injection	72 h after injection
3'-MeDMAAB	1	224	57
	2	281	30
Nil	3	174	191
	4	144	178
4'-MeDMAAB	5	154	241
	6	157	198

MeDMAAB becomes attached to proteins by linkage through its 2-position with a protein amino group. Since azoxy compounds are known to be transformed readily *in vitro* into *o*-hydroxyazo derivatives[10], it was thought that similar reactions might occur *in vivo* leading to 2-hydroxy- and even 2-imino products of the metabolizing azo dyes. Molecules of this kind could then chelate with metals[11] (*M*):



a reaction which might be responsible for PPD oxidase inhibition.

As yet, no clear evidence has been obtained that such a mechanism is involved. Thus, 1-phenylazo-2-naphthol, a compound which could chelate with copper in the manner depicted, failed to inhibit rat serum PPD oxidase *in vivo*, and its solution in normal rat serum *in vitro* did not influence the oxidase activity of this serum. Crude metabolites of 3'-MeDMAAB from rat urine failed to inhibit rat serum PPD oxidase *in vitro* as did the parent azo dye, but in one experiment with 4'-EtDMAAB metabolites some inhibition was observed.

Injection of 8-hydroxyquinoline into rats stimulated strongly serum PPD oxidase activity. This compound like some *o*-aminophenolic substances, acts as a local carcinogen for the bladders of mice, and BOYLAND and WATSON[12] have suggested that a chelation mechanism may well be involved in this process. We have found that the hepatocarcinogen, 2-aminofluorene caused a feeble temporary decline in rat serum PPD oxidase activity, while its non-carcinogenic isomer, 4-aminofluorene was completely inactive. The potent liver carcinogen, 4-dimethylaminostilbene, proved to be as powerful an inhibitor of PPD oxidase *in vivo* as the azo carcinogen, 3'-MeDMAAB. Perhaps in the case of these amino carcinogens *o*-aminophenolic derivatives capable of chelation are produced in the liver.

Our results will be reported in detail elsewhere together with an account of the effects of azo carcinogens etc. on rat serum xanthine oxidase, an enzyme which requires molybdenum for its activity and which is known to be susceptible to inhibition by copper ions[13].

[10] G. M. BADGER and R. G. BUTTERY, J. chem. Soc. *1954*, 2243.

[11] H. D. K. DREW and J. K. LANDQUIST, J. chem. Soc. *1938*, 292. – M. L. ERNSBERGER and W. R. BRODE, J. org. Chem. *6*, 331 (1941). – V. I. MUR, J. gen. Chem., Moscow (Consultants Bureau Translation) *26*, 405 (1956).

[12] E. BOYLAND and G. WATSON, Nature *177*, 837 (1956).

[13] F. PASQUIER and J. POLONOVSKI, Bull. Soc. Chim. biol. *39*, 1465 (1957).

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Cancer Research Unit, The University, Sheffield, April 28, 1958.

Résumé

On a trouvé que les azoïques 3'-méthyl- et 4'-éthyl-4-diméthylaminoazobenzène, substances cancérigènes puissantes pour le foie du rat, empêchent fortement l'activité de la *p*-phénylènediamine oxydase du sérum sanguin. Au contraire, 4'-méthyl-4-diméthylaminoazobenzène avec pouvoir cancérigène très faible n'a aucune activité. L'azoïque 3'-méthyl-4-diméthylaminoazobenzène, mais pas son isomer 4'-méthyl, diminue fortement le cuivre sérique.

Über einen Cortison-äquivalenten Katalysator der Autoxydation der Linolsäure *in vitro*

Die nichtenzymatische Oxydationskatalyse der Linolsäure mit Cortison und anderen Corticosteroiden, als körpereigene Hormone, ist von praktischem und theoretischem Interesse[1]. Das bei der Cortisonkatalyse der Linolsäure entstehende und mit der TBA-Reaktion proportional dem Sauerstoffverbrauch nachweisbare[2] Reaktionsprodukt ist, ebenso wie nach UV.-Bestrahlung dieser Fettsäure, ein Hydroperoxyd, dem biologische Bedeutung zukommt: Es ist bekannt, dass Linolsäurehydroperoxyd bestimmte Fermente, wie Succinoxydase, Cytochromoxydase und Cholinoxydase zu hemmen vermag[3], dass es die Atmung und Glykolyse von Ehrlichs Ascitestumorzellen hemmt[4], dass es die Fähigkeit besitzt, Desoxyribonukleinsäure zu depolarisieren[5], und dass es bestimmte Zellteilungen blockiert[6]. In diesem Zusammenhang ist erwähnenswert, dass die Oxydationskatalyse der Linolsäure mit Cortison, unter Bildung von Linolsäurehydroperoxyd, durch andere Steroide, solche mit Follikelhormonwirkung, gehemmt wird[7].

Die Linolsäureoxydationskatalyse durch bestimmte Corticosteroide ist eine für deren Hormoncharakter ungewöhnliche Wirkung, deren Bedeutung für die Hormoneffekte unklar bleibt. Sie steht in Zusammenhang mit Metall-katalytischen Phänomenen, denn für ihr Zustandekommen sind Metallionen, besonders Cu^{2+}, nötig, so dass ein Cortison-Cu-Komplex als massgeblich angenommen werden muss[1]. Die Veresterung der Seitenkette der Corticosteroide hebt deren katalytische Wirkung auf. Diese ist somit für die Katalyse massgebend. Ein Beweis hierfür sind auch unsere Befunde, dass niedermolekulare, aliphatische, konstitutionell der Corticosteroidseitenkette ähnliche Verbindungen, wie Dioxyaceton usw., die Linolsäureoxydation katalysieren können[8]. Allerdings besteht zwischen diesen und den Corticosteroiden ein grundsätzlicher Unterschied. Die niedermolekularen, bisher ge-

[1] W. SCHULER und R. MEIER, Hoppe-Seylers Z. *302*, 236 (1955).

[2] W. SCHULER, unveröffentlichte Befunde.

[3] F. BERNHEIM, M. K. WILBUR und C. B. KENASTON, Arch. Biochem. Biophys. *38*, 177 (1952). – A. OTTOLENGHI, F. BERNHEIM und K. M. WILBUR, Arch. Biochem. Biophys. *56*, 157 (1955).

[4] C. W. SHUSTER, Proc. Soc. exp. Biol. Med. N.Y. *90*, 423 (1955).

[5] W. D. FISHER and K. M. WILBUR, J. Elisha Mitchell Soc. *70*, 124 (1954).

[6] K. M. WILBUR, G. B. KENASTON, N. WOLFSON, A. OTTOLENGHI und M. E. GAULDEN, Anat. Rec. *120*, 708 (1954).

[7] W. SCHULER und R. MEIER, Arch. exp. Path. Pharmakol. *228*, 474 (1956).

[8] W. SCHULER und R. MEIER, Helv. physiol. Acta *15*, 279 (1957).